ASSOCIATION OF ENDOTHELIAL NITRIC OXIDE SYNTHASE (eNOS) GENE POLYMORPHISM WITH COMPLICATIONS OF HbSS IN GHANAIAN SICKLE CELL DISEASE PATIENTS

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DECLARATION

I, CHARLES ANTWI-BOASIAKO, author of this dissertation do hearby declare that, with the exception of references to other people's work which has been duly cited, this work has entirely resulted from my personal original research under the supervision of Dr. D. A. Antwi, Dr. B. Dzudzor and Dr. Ivy Ekem and has not been presented for another degree elsewhere.

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DEDICATION

This work is dedicated to my special wife Rev. Mrs. Kate Antwi-Boasiako and my wounderful children Anna and Aaron.

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ABSTRACT

Background

Nitric oxide (NO) is a potent vasodilator synthesized by endothelial nitric oxide synthase (eNOS) enzyme of the vascular endothelial cells and plays a significant role in the regulation of vascular homeostasis by attenuating leukocyte adhesion to the endothelium. Nitric oxide levels are found to be low in Sickle cell disease (SCD) patients. The eNOS gene polymorphism has been implicated. Moreover, very scanty data with conflicting findings exist in theliterature on the association between eNOS gene polymorphism and SCD; there is no data on this association between SCD complications such as vaso-occlusive crisis (VOC), leg ulcers and Priapism in Ghana.

Aim

The aim of this study was to determine the association between eNOS gene polymorphism, endothelial dysfunction, angiogenesis and the complications of HbSS in Ghanaian SCD patients.

Methodology

This was a case control study involving 694 subjects, haematological analyses was done for all the subjects. In addition to haematological analysis, vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), E-selectin, Angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), Vascular endothelial growth factor (VEGF), NO and genotyping were determined for 213 subjects which included 153 SCD patients with 135 HbSS (46 steady state, 57 VOC, 21 leg ulcers, 11 priapism) and 18 HbSC patients in VOC and 60 age -matched healthy controls (HbAA) who were voluntary blood donors recruited from the Center for Clinical Genetics and Accra Area

Blood Centre respectively all at the Korle-Bu Teaching Hospital, Accra. Genotypic analysis of two functionally significant eNOS variants (T786C) polymorphism in the promoter region and the variable number of tandem repeats (VNTR) in intron 4 were carried out with a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. Plasma concentrations of VCAM-1, ICAM-1, and E-selectin were measured using commercial enzyme immunoassay kits (GenWay, Califonia, USA). Ang-1, Ang-2, and VEGFwere measured by enzyme-linked immunosorbent assay (ELISA*)* (R&D Systems, UK) and NO using Griess reagent system by ELISA method (Promega, Madison, USA).

Results

The results showed that there were significantly lower plasma NO levels in the SCD complications as compared to SCD asymptomatic patients and control groups ($P<0.001$). The results showed that SCD patients had significantlyhigher frequencies of mutant alleles of the eNOS gene polymorphisms ($p<0.001$). Furthermore, there was a significantly strong positive association between both TCandCC genotypes (OR, 10.33; 95% CI, 1.24-86.06) and (OR, 10.38; 95%CI, 1.781-60.47) respectively of the T786C polymorphism of the eNOS gene in SCD patientswith leg ulcer. Ang-1, Ang-2 and VEGF levels were significantly elevated in SCD patients as compared with controls ($P<0.001$) and were much higher in patients with complications. Ang-2/Ang-1 ratio was high in SCD patients and still higherin SCD complications. The VCAM-1, ICAM-1, and E-selectin were significantly higher in SCD patients and much higher in those with complications.

Conclusion

This study confirms reduced plasma NO levels in SCD patients in general. This disorder is severe in the disease complications. The VNTR4a/b polymorphisms of the eNOS gene are associated with lower levels of NO production in SCD patients with and without complications. There was an association between eNOS gene polymorphism (T786C and VNTR) and SCD in Ghana. The T786C of the eNOS gene polymorphism is associated with leg ulcer a complication SCD.Therefore these polymorphisms may acts as genetic modifiers for complications in SCD.There is endothelial dysfunction in SCD patients as demonstrated by increased serum levels of adhesion molecules. This abnormality is severe in the disease complications.There are elevated angiogenic factors in SCD patients in general with a further increase in the complications.Polymorphism in the eNOS gene is linked to endothelial dysfunction and angiogenesis in Ghanaian SCD patients with and without complications.

LIST OF ABBREVIATIONS

ABC:	Avidin-Biotin peroxide complex
ACS:	Acute chest syndrome
Ang-1:	Angiopoietin-1
Ang-2:	Angiopoietin-2
ANOVA:	Analysis of variance
ARDS:	Acute respiratory distress syndrome
bFGF:	basic fibroblast growth factor
BSA:	Bovine Serum Albumin
cGMP:	cyclic guanosine monophosphate
CI:	Confidence interval
CVD:	Cardiovascular Disease
DNA:	Deoxyribonucleic acid
dNTP:	Deoxynucleotide triphosphate
EDTA:	ethylene-diamine-tetra-aceticacid.
ELISA:	enzyme linked immunosorbent assay
eNOS:	endothelial nitric oxide synthase
GLM:	General linear mode
Hb:	haemoglobin
HbAA:	Haemoglobin AA
HbAS:	Haemoglobin AS
HbF:	Haemoglobin F
HbSC:	Haemoglobin SC
HbSD:	Haemoglobin SD
HbSS:	Haemoglobin SS
$HbS\beta^+$:	Haemoglobin $S\beta^+$ thalasaemia
HCT:	Haematocrit
ICAM-1:	Intercellular cell adhesion molecule-1
iNOS:	Inducible nitric oxide synthase
KO:	knockout
MCH:	Mean Cell Haemoglobin
MCHC:	Mean Cell Haemoglobin Concentration
MCV:	Mean cell volume

$MgCl_2$:	magnesium chloride
MPV:	Mean platelet volume
mRNA:	messenger ribonucleic acid
NaEDTA:	Sodium ethylene-diamine-tetra-acetic *acid.*
NED:	N-1-naphthylethylenediamine dihydrochloride
NGS:	Normal goat serum
NMS:	Normal mouse serum
nNOS:	Neuronal nitric oxide synthase
NO:	Nitric oxide
NOS:	Nitric oxide synthase
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PCT:	Platelet crit
PCV:	Packed cell volume
PDE:	Phosphodiesterase
PDW:	Platelet distribution width
PIGF:	Plancental growth factor
PLT:	Platelet count
RBC:	Red blood cell
RDW:	Red cell distribution width
RFLP:	Restriction Fragment Length Polymorphism
RPM:	Revolutions per minute
SCA:	Sickle cell anaemia
SCD:	Sickle Cell Disease
sFIt:	Soluble fms-like tyrosine kinase-1
SNP:	Single nucleotide polymorphism
TRJ:	Tricuspid regurgitant jet
VCAM-1:	Vascular cell adhesion molecule-1
VEGF:	Vascular endothelial growth factor
VNTR:	Variable Number ofTandem Repeat
VOC:	vaso-occlusive crisis
VWF:	Von Willebrand Factor
WBC:	White Blood Cell

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CHAPTER ONE
INTRODUCTION

1.1 Background

Sickle cell disease is a co - dominant genetic disorder characterized by a point mutation at codon6 of the globin gene $\beta^{(GAG \rightarrow GTG)}$(Steinberg, 2008). The resulting haemoglobin S from this mutation causes polymerization of the sickle haemoglobin and red blood cell sickling on exposure to low oxygen tension that leads to red cell rigidity (Natarajan*et al.*, 2010). That causes poor microvascular blood flow, with consequent tissue ischaemia and infarction (Kato *et al.*, 2009). There are four common expressions of SCD namely: HbSS, HbSC, HbSβ^{+}thal and HbSD (Konotey-Ahulu, 1991) with varying degrees of disease severity and with HbSS being the severest form.

All across sub-Saharan Africa and regions where SCD exists, differences in disease severity have been established (Driss*et al.*, 2009; Weatherall, 2010). Worldwide, distribution of SCD constitutes 1% of the global population; over 75% of this percentage is found in Sub-Saharan Africa (WHO, 2006; Diallo & Tchernia 2002). It has been estimated that SCD results in the annual loss of several millions of disability-adjusted life years, particularly in the developing world (Weatherall *et al.*, 2005). In Ghana, the prevalence rate is 2% of all births per year (Ohene – Frimpong, 2005). In addition, SCD presents a significantly high rate of morbidity and mortality in Ghana (Makani *et al.*, 2013).

The most frequently reported symptom at the instance of morbidity or mortality in hospitals is VOC being marked by severe pain in the bones and muscles (Platt *et al.*, 1994; Vichinsky *et al.*, 2000). For many decades, it was widely assumed that the pathophysiology of SCD was only caused by the rigid red cells due to the sickle

haemoglobin leading to tissue infarction and organ dysfunction in patients with SCD. Although these advances explain the dramatic impact of deoxygenation on the shape and rheology of sickle erythrocytes, they provide only a partial understanding of the *in vivo* pathogenesis of VOC, the hallmark or principal feature of HbSS SCD. A number of new evidence now support the hypothesis that vaso-occlusion and several of the complications of SCD arise also from adhesive interactions of sickle red blood cells (RBC), leukocytes (Hebbel *et al.,* 2009) , and the endothelium (Hebbel *et al.,* 2004; Marilyn, 2007).It has become increasingly apparent that inflammation plays a critical role in both the initiation and propagation of vaso-occlusion (Hebbel *et al.,* 2009; Natarajan *et al.,* 2010). Both expressions and genetic evidence provide support for the importance of these interactions.

Experimental data suggests that up to 50% of patients with SCD have endothelial dysfunction due to impaired bioavailability of endogenous NO. This is believed to be caused by scavenging of NO by cell-free plasma haemoglobin and consumption of L-arginine amino acid by cell-free arginase enzyme released from haemolysed RBCs (Wood*et al.,* 2008; Nishank*et al.,* 2013). Reduced endothelial NO bioavailability in SCD leads to activation of endothelial cell adhesion molecules such as VCAM-1, ICAM-1, and E-selectin besides platelet activation, which in turn results in vascular occlusion and vasoconstriction (Kato, 2005). The impaired NO bioavailability due to significant intravascular haemolysis may lead to many complications such as VOC, priapism and leg ulcers in SCD patients (Mack, 2006).

Nitric oxide is a very potent vasodilator synthesized from its precursor L-arginine by three isoforms of the nitric oxide synthase (NOS) namely constitutive neuronal NOS,

inducible NOS and endothelial NOS (Thomas *et al.,* 2013). Endothelial nitric oxide synthase derived from vascular endothelium is the most dominant form of these isoforms (Rafikov *et al.,* 2011). Nitric oxide plays a pivotal role in the regulation of cardiovascular homeostasis. Nitric oxide is highly reactive, it facilitates and maintains basal vasodilator tone, and it inhibits platelet aggregation and attenuates leukocyte adhesion to the endothelium.

Endogenous NO synthesized by the eNOS enzyme, has been shown to have an important role in the pathogenesis of VOCin sickle cell transgenic mice (Pritchard *et al.,* 2004). These studies assign eNOS gene to be a critical regulator of vasodilation and inhibitor of cell adhesion and aggregation, protecting humans and the animal from vaso-occlusion (Nishank *et al.,* 2013). In normal healthy individuals, the level of NO in the plasma is found to be linked to haplotypic variation of the eNOS gene (Tsukada *et al.,* 1998). Even though several polymorphic forms of this gene have been found, the most important polymorphic variants of the eNOS gene which is of clinical importance and are responsible for bringing about variation in NO levels are the T786C variant in the promoter region, the Glu298Asp variant in exon 7, and the variable number of tandem repeats (VNTR) in intron 4 (Pritchard, 2004). These eNOS variants have been found to be associated with several vascular disorders such as myocardial infarction (Kunnas *et al.,* 2002), atherothrombosis (Voetsch, 2004), erectile dysfunction (Sinici, 2009), stroke (Endres, 2004) and renal disease (Wang&wang, 2000) in normal general population of Asian and European origin. As compared with these reports, data on eNOS gene polymorphism in SCD has been scanty worldwide and absent in Ghanaian SCD population where this disease is highly prevalent with its complications.

Soluble endothelial cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin) play a vital physiological role in the recruitment and binding of inflammatory cells to vascular endothelium, particularly in venules (Alon & Feigelson, 2002). The expressions of these molecules are found to be modulated by endothelial NO produced from eNOS. Nitric oxide is known to suppress the expression of these adhesion molecules (Kato *et al.*, 2005). Since NO bioavailability is found to be impaired in patients and mice with SCD (Nath *et al*, 2000;Belhassen *et al*, 2001; Gladwin *et al*, 2003), increased levels of soluble endothelial cell adhesion molecules are associated with decreased NO bioavailability in patients with SCD (Kato *et al.*, 2005). Therefore, excessive endothelial activation and vaso-constriction due to impaired NO bioavailability may contribute to vascular instability in patients with SCD. These observations suggest that soluble adhesion molecules are potential markers of endothelial dysfunction, characterised by endothelial activation and insufficient bioavailability of endothelium-derived NO.

Vascular endothelial growth factor (VEGF) and angiopoietin (Ang)-1 and Ang-2 are mediators of angiogenesis (Lim *et al.*, 2004). Current data suggest that the balance between these factors may affect the integrity of the vascular endothelium because Ang-1 promotes endothelial cell survival, stabilizes endothelial interactions with supporting cells, and limits the permeability-inducing effects of VEGF (Koblizek, 1998; Joussen *et al.*, 2002; Pizurki, 2003). Where as Ang-2 has been proposed as a natural antagonist of Ang-1, promoting vessel regression in the absence of VEGF (Lobov, 2002). Because SCD is closely associated with endothelial perturbation, the study of plasma levels of these angiogenic growth factors in patients with SCD; their relationship with endothelial damage or dysfunction; and the effect on the disease complications is essential.

The eNOS gene variants have been studied in SCD population of India without reference to specific SCD haplotype (Nishank *et al.*, 2013). Another study has also been donerecently by Thakur *et al.* (2014) in a specific SCD haplotype (HbSS) in Malians with a conflicting result from the study in India about the association of eNOS gene polymorphism in SCD. In addition to the fact that these studies have conflicting findings, they did not investigate eNOS gene polymorphism in association with SCD complications, angiogenesis and endothelial dysfunction. Moreover, thereare no data on angiogenesis and endothelial dysfunction in SCD and its complications such as VOC, leg ulcer and priapismin Ghana and Africa. There is no report on the role of eNOS polymorphism, angiogenesis and endothelial dysfunction in the pathogenesis of SCD in Ghana. Therefore, the present study was designed to explore the possible association of eNOS gene polymorphism as a potential genetic modifier of SCD severity inpatients in Ghana

1.2 Problem statement

Sickle cell disease is a major genetic disease associated with an increased mortality in Ghana (Danquah *et al.*, 2010). The homozygous SCD (HbSS) has a wide variety of clinical manifestations, which vary from an almost asymptomatic condition to severe illness even though all subjects with this disease have the same base change in their DNA (Chies & Hutz, 2003). The source of this variation is partly environmental, but a large part of this variability derives from the presence of genetic modulators linked or unlinked to the ß-globin gene cluster which are not fully understood (Chies & Hutz, 2003).

Work done to date have related and underpinned NO bioavailability to SCD clinical complications (Gladwin & Aschdev, 2012). Lack of NO production is observed in

vascular diseases including SCD. Hence it is believed thata healthy endothelium is necessary for the production of NO. The levels of NO are significantly low in HbSS patients. It is firmly established that the HbSS patients develop progressive vasculopathy with age leading to dysregulation of the endothelial NO signaling system resulting in impaired NO bioavailability in HbSS patients. Abnormalities in vasodilation in HbSS patients have also been reported but the causes are uncertain.

Low levels of NO have been linked to HbSS complications such as VOC, priapism and leg ulcers. Polymorphism of eNOS genes, whose products modify the pathophysiology initiated by the sickle haemoglobin mutation, has been proposed to be responsible for these complications. Therefore, the influence of eNOS gene polymorphisms on HbSS complications needs extensive investigation in Ghana.

The NO synthesized by the eNOS affects the level of soluble endothelial cell adhesion molecules: ICAM-1, VCAM-1 and E-selectinby suppressing its expression. Since there is an impairment of NO bioavailability in SCD eNOS polymorphism leading to reduced NO bioavailability may contribute to vascular instability in patients with SCD and may result in endothelial activation. Hence the measurement of these soluble adhesion molecules as markers of endothelial dysfunction is imperative.

Vascular endothelial growth factor, Ang-1 and Ang-2 regulate angiogenesis under specific circumstances, such as hypoxia and inflammation (Duits *et al.*, 2006), which frequently occurs in SCD. Therefore Hypoxia-induced angiogenesis may play an important role in the pathophysiology of sickle cell disease (SCD). Plasma levels of angiopoietin Ang-1, Ang-2, determination are also very important.

In Ghana, considerable work have been done on the epidemiology of SCD and its complications (Edwin *et al.*, 2001); however, the data linking these complications with eNOS gene polymorphisms, angiogenesis and endothelial dysfunction that may specify differences in the disease presentation are missing. Data is therefore needed to be collated in that regard, hence the focus of the current study.

1.3 Justification/Relevance

Studies on the association between eNOS gene polymorphisms, angiogenesis and endothelial dysfuntion related to the sickle cell gene may provide a strong correlation with clinical presentation in Africa. However, till date, a genetic study on these in Ghanaian HbSS patients had not yet been done. This made it imperative for data to be collated for the different complications such as VOC, priapism and leg ulcers of HbSS genotype in Ghanaian SCD for a better understanding of the pathophysiology of the disease and disease management; hence, the focus of the current study. The eNOS, a key factor in endothelial function needs to be investigated as well as endothelial dysfunction and angiogenesis since these are interrelated (Gopi *et al.*, 2012).

1.4 Aim

To determine the association between eNOS gene polymorphism, endothelial dysfunction and angiogenesis in the complications of HbSS in Ghanaian SCD patients.

1.5 Specific objectives

To determine:

- To determine the mean Plasma NO levels in SCD patients in steady state, complications and controls.
- To determine the association between eNOS gene polymorphism and SCD in Ghana
- To determine the association between eNOS gene polymorphism and HbSS SCD Complications.
- To determine Plasma levels of ICAM-1, VCAM-1 and E-selectin as markers of endothelial dysfunction in SCD patients in steady state, complications and controls.
- To determine plasma levels of Ang-1, Ang-2 and VEGF as angiogenic factors in SCD patients in steady state, complications and controls

1.6 Null Hypotheses

- There no association between eNOS gene Polymorphism and SCD with its complications in Ghana
- There is no difference in angiogenesis among controls, SCD asymptomatic state and complications.
- There is no difference in endothelial dysfunction among controls, SCD asymptomatic state and complications

CHAPTER TWO

LITERATURE REVIEW

2.1 The history of sickle cell disease (SCD)

Traditionally, SCD has been known to the people of Africa for hundreds of years, long before it was recognized in the western hemisphere (Edelstein, 1981). The inhabitants of western Africa gave the disease-specific names that evoke acute, painful episodes or death or refer to children destined to die and to be reborn as their own siblings (Onwubalili, 1983; Nzewi 2001).

A Physician named James Herrick was the first to identify SCD in a medical student from Grenada (Herrick, 1910). The disease was named "sickle cell anaemia" by Vernon Mason in 1922 (Huck, 1923). It was the discovery by Emmel in 1917 of the sickling phenomenon, *in vitro*, in members of a family, which first suggested the genetic basis for SCD (Emmel, 1917). So it was discovered to be an inherited condition. Later on it was explained that the sickling phenomenon, *in vitro*, was due to deprivation of oxygen (Hahn & Gillespie, 1927). Huck, who did the detailed analysis of the pedigree of his patients, concluded that the sickle cell phenomenon was inherited as a Mendelian autosomal recessive characteristic (Huck, 1923).

Demonstration of the abnormal electrophoretic mobility of the sickle haemoglobin in an affected individual was done by Linus Pauling (Pauling *et al.*, 1949). Vernon Ingram discovered that the defect of the disease was a single amino acid substitution in the haemoglobin molecule of sickle cells (HbS) (Ingram, 1957); the structure of haemoglobin was worked out by Max Perutz to explain the molecular basis of its function (Perutz et *al.*, 1960). In addition, Janet Watson discovered that the symptoms of

SCD appeared in infants only after concentrations of foetal haemoglobin (HbF) had fallen, hence, established the notion of the beneficial effect of HbF on disease manifestations (Watson *et al.*, 1948).

Two separate studies that were carried out by Neel and Beet established that the heterozygous state for sickle gene is without significant symptoms and homozygous state for sickle is symptomatic in individuals (Beet, 1949; Neel *et al.*, 1951). The abnormally slow rate of migration of sickle haemoglobin on electrophoresis was found in 1949 (Pauling *et al.,* 1949).Symptoms of SCD could be traced back to the year 1670 in one Ghanaian family (Konotey-Ahulu, 1973). Since then there has been a rapid expansion of information about SCD.

2.2 The global epidemiology of SCD

Approximately, 300,000 children are born every year with SCD world wide. This constitutes 1% of the global population of SCD, of which75% of these cases are found in Sub-Saharan Africa (Diallo & Tchernia, 2002; WHO, 2006). It has been estimated that SCD results in the annual loss of several millions of disability-adjusted life years, particularly in the developing world (Weatherall *et al.*, 2005).

Although there is general acceptance that African populations are mostly affected estimates of the disease incidence and prevalence varies greatly among different African populations. The distribution of the sickle cell gene in the world is extensive, covering predominantly Tropical Africa and ranging from 10% to 25% in West Africa. In Mediterranean countries it is up to 30% in some areas of Greece and 7%-10% among American Negroes (Konotey-Ahulu, 1991). Sickle cell haemoglobinopathy poses a

health problem to many other ethnic groups, including populations native to Italy, Greece, Turkey, Saudi Arabia, India, Pakistan, Bangladesh, China, and Cyprus (Nietert *et al.,* 2002).

Sickle cell disease is very common in the United States where 1 out of every 10 to 12 people of African descent has the sickle cell trait and about 1 of every 400 African American newborns and 1 in 1000 individuals of Hispanic descent has sickle cell anaemia (Bunn, 1997). Caucasians, particularly those of Mediterranean origin, are occasionally affected. Individuals of Middle Eastern origin are also affected, although the phenotypic expressions are often less severe (Serjeant, 1975).

Additionally, the HbS gene is prevalent in *Plasmodium falciparum* endemic region as in Equatorial Africa (10 – 30%), central India (20 – 30%), Saudi Arabia (up to 25% in the eastern parts) but also in areas around the Mediterranean (North Africa, Italy, Greece, Turkey) (Ashley-Koch *et al.,* 2000). The occurrence of the HbS gene and subsequent gene selection by falciparum malaria are the causes of this distribution (Ashley-Koch *et al.,* 2000). Relative resistance to malaria in people with sickle cell trait (HbAS) increases their chance of survival and thus passing on the HbS gene. Through slave trade and migration, SCD is also prevalent in the Americas and Northern Europe (Ashley-Koch *et al.,* 2000).

In Ghana, the prevalence rate of SCD is 2% of all births per year (Ohene – Frempong *et al.,* 2005). Sickle cell disease placed 37th and 36th positions in the years 2002 and 2003 respectively on the outpatient morbidity reports compiled by the Ghana Health Service.

2.3 The burden and disease pattern in Ghana

Sickle cell disease presents a major medical problem in Ghana. It affects work capacity and its responsible for significantly high rate of morbidity and mortality in Ghana. In the tropics, the incidence of sickle cell crisis is highest during the cooler rainy seasons (Addae, 1971). Patients with SCD incur large numbers of hospital admissions, emergency department visits, and outpatient visits due to crisis, often at substantial costs. This imposes a financial burden on individual families and the national health budget. The labor hours lost due to frequent crisis is also a cost to the nation (Nietert, 2002).

2.4 Pathophysiology of SCD

The formation of polymers of the sickle haemoglobin (HbS) is responsible for the characteristic sickled-shape of the RBC in SCD (upon deoxygenation). Recent research has made it very clear that, the etiology of the sickle cell disease is as a result of a very complexinterplay between the endothelial activation, cytokine release, intercellular adhesion, vasoconstriction and coagulation activation (Hebbel, 2008; Kaul *et al.*, 2009; Manwani & Frenette, 2013).

2.4.1 Polymerization of HbS

During the deoxygenation of the Hb in RBC which follows its passage in the microcirculation the normal haemoglobin (HbA) of the RBCs maintain their shape and solubility, regardless of the degree of deoxygenation (Konotey-Ahulu, 1991). However, the HbS molecule undergoes a conformational change and forms insoluble and viscous polymers. The deoxygenated HbS polymerizes by sticking to each other to form long helical strands, which stretches the membrane of the red blood cell distorting the cell into a sickle shape which is referred to as sickling (Zhi *et al.*, 2001).

Polymerization of deoxygenated sickle haemoglobin leads to decreased deformability of RBCs (Eaton & Hofrichter, 1990; Maier-Redelsperger *et al.*, 1994; Babra, 2006; Marie-Hélène*et al.*, 2011; Manwani *et al.*, 20013). This sickling process needs a certain time to be primed, referred to as delay time, and also inversely proportional to the intracellular concentration of HbS. The formation of these long polymer fibres triggers a cascade of several other cellular abnormalities, which changes the shape of the RBCs.

This change in molecular structure, stability and solubility in the HbS is due to a genetic mutation substituting thymine for adenine in the sixth codon of the β globulin-gene (GAG→GTG), thereby encoding valine instead of glutamic acid in the sixth position of the β- chain (David *et al.*, 2010). This mutation produces a hydrophobic motif in the deoxygenated HbS tetramer that results in binding between β1 and β2 chains of two haemoglobin molecules.

This crystallisation produces a polymer nucleus, which grows and fills the RBC, disrupting its structural design and flexibility and promoting cellular dehydration. The rate and extent of HbS polymerization is proportional to the extent and duration of haemoglobin deoxygenation, the intracellular HbS concentration (about 34^{th} power), and the presence of foetal haemoglobin in the erythrocyte (David *et al.*, 2010).

The polymerization of haemoglobins deforms the RBCs. The problem of the polymerization however, is not only about the distortion of the RBCs but also, their membranes become rigid, due to repeated episodes of haemoglobin polymerization and depolymerization. These rigid cells are unable to move through the microvasculature

and thereby block or reduce local blood flow to the tissues (Gladwin *et al.*, 2003). The recurrence of such episodes leads to tissue hypoxia resulting in pain, and often damage to organs. The damage to the RBC membrane contributes to many of the complications of sickle cell disease (Belcher *et al.*, 2000).

The main determinant of the severity of SCD 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, such as the protective effects of co-inherited α-thalassaemia or hereditary persistence of foetal haemoglobin (David *et al.*, 2010).

2.4.2 Endothelial activation and increased adhesion

Recent investigation has revealed that the pathophysiology of SCD is not limited to the HbS polymerization but includes the RBCs, white blood cells, and platelets adhesiveness to the vascular endothelium in SCD patients. Manwani *et al.*,(2013) and colleagues had suggested earlier that increased adherence of sickled RBCs to vascular endothelium play a role in vasocclusive events of SCD and therefore proposed that increased adherence of sickle RBCs to vascular endothelium may initiate vaso-occlusive events in SCD **(**Hebbel *et al.*, 1981).

This was demonstrated in a study that used *in vitro* adhesion assays or a rat mesocecum *ex vivo* perfusion model, which also revealed the role of sickled RBCs in initiating and propagating the vasocclusive events through adhesive interactions with the vascular endothelium (Kaul, 2009). The adhesion of low-density sickled RBCs and reticulocytes

in the immediate post capillary venules leads to trapping of the older, denser, and deformed RBCs resulting in reduced blood flow.

Occasionally, a few of the dense sickled RBCs randomly obstruct the pre-capillary vessels and also contribute to VOC (Kaul, 2009).Other data indicate that other blood cell elements that are not directly affected by the sickle cell mutation play a direct role in VOC. Therefore, a new model has been proposed in which the process is viewed as multistep and multicellular cascade driven by inflammatory stimuli and the adherence of leukocytes providing a paradigm shift of sickle cell VOC (Manwani & Paul, 2013).

Chronic inflammatory reaction, which is characterized by cytokine production, endothelial activation and coagulation activation, constitute a very important pathophysiological mechanism in SCD (Lim *et al.,* 2013). This is because under normal conditions the duration of the exposure of erythrocytes to hypoxic and acidic conditions in the microvasculature is too short to induce local polymerization in HbS. However due to local adhesive interaction of erythrocytes, leukocytes and endothelial cells the blood flow velocity in the microvasculature decreases. Consequently local polymerization of HbS and formation of sickled cells occur, which ultimately leadto VOC.

The resulting ischaemia is responsible for the subsequent inflammatory reaction that causes further activation of the endothelium. Thus VOC in SCD seems to start with adhesion of RBCs and leukocytes to adhesion molecule on activated endothelial cells predominantly in the post capillary venules (Deepa *et al.,* 2013). Both during VOC and asymptomatic periods, SCD patients have a high number of circulating white blood cells with increased expression of adhesion molecules such as ICAM 1, VCAM-1 and E-

selectin, reflecting ongoing endothelial activation and damage (Kuryliszyn *et al.*, 2005). Through a complexinterplay of adhesive events among blood cells, the altered sickled RBC can obstruct the vasculature, producing episodes of pain, haemolyticanaemia, organ injury, and early mortality (Manwani *et al.*, 20013).

2.4.3 Vasoconstriction

Nitric oxide produced by the endothelial cells, is a very potent vasodilator and has blood flow regulatory effect. Due to chronic haemolysis in SCD a high concentration of extra-cellular haemoglobin can be found in circulation. This free haemoglobin scavange NO resulting in a compensatory increased production of NO, and subsequently a lower concentration of L-arginine, the substrate for NO synthesis (Claudia, 2008).The increased production of the NO during low substrate availability results in the production of peroxide instead of NO. This results in less NO availability and more oxidative stress and endothelial damage caused by the superoxides (Claudia, 2008). Decreased bioavailability of NO will result in VOC partly due to an increase in the production of the vasoconstrictor peptide endothelin-1. Haemolysis induced scavenging of NO is an important etiological mechanism in the development of SCD related complication like pulmonary hypertention (Ergul *et al.*, 2004).

Both components of the blood vessel wall: the vascular endothelium and vascular smooth muscle develop pathology in SCD that may contribute to vasculopathy orcoagulopathy. Patients with SCD have blunted vasodilatory responses to NO, the critical mediator of vascular homeostasis (Kato, 2012). Similarly blunted responses to NO areseen in the SCD mouse (Kaul *et al.*, 2000; Nath *et al.*, 2000). Patients with SCD

express high levels of endothelial activation markers (Kato *et al.*, 2005; Duits *et al.*,2003; Conran *et al.*, 2004) and haemostatic activation of platelets and coagulation factors.

2.4.4 Coagulation activation

Sickle cell disease patients have chronically activated coagulation factors (Ataga & Orringer, 2003) promoting this hyper coagulable state due to the increased expression of tissue factor on circulating monocytes and endothelial cells and increased expression of procoagulant phospholipids such as phosphotidyleserine on erythrocytes (Sparkenbaugh & Pawlinski, 2013). Coagulation activation may affect VOC in SCD by numerous mechanisms. Importantly, thrombin is one of the strongest activators of endothelial cells and is responsible for the increased expression of P-selectin of endotheilial cells and monocytes in SCD thereby promoting cellular adhesion.

2.5 Endothelial Dysfunction

Endothelial cell dysfunction is a broad term which implies dysregulation of endothelial cell functions. This include impairment of the barrier functions of endothelial cells, vasodilation, and disturbances in proliferative capacities, migratory as well as tube formation properties, angiogenic properties, attenuation of synthetic function, and deterrence of white blood cells from adhesion and diapedesis (Goligorsky, 2005).

Also, it refers to an imbalance between vasodilating and vasoconstricting substances produced by the vascular endothelium cells or overall functions of the endothelium (Deanfield *et al.*, 2005). The normal functions of vascular endothelium include the production of NO, regulation of platelet adhesion, coagulation, and immune function. Endothelial dysfunction is primarily due to reduction in NO bioavailabilty which is a

marker for vascular health (Gopi *et al.,* 2012). Endothelial dysfunction can result from and could also contribute to several disease processes, as occurs in diabetes mellitus, hypertension and SCD (Cai & Harrison, 2000).

Endothelial dysfunction is associated specifically, with decreased NO production, anticoagulant properties, increased platelet aggregation, increased expression of vascular adhesion molecules (VCAM-1, ICAM-1 and E-selectin), increased expression of chemokines and cytokines and increased reactive oxygen species production from the endothelium (Al-Isa *et al.,* 2010). These all play important roles in the development of SCD complications including VOC, leg ulcers and priapism. Endothelial dysfunction has been shown to be of prognostic importance in predicting vascular events (Perticone *et al.,* 2001; Corrado *et al.,* 2008), consequently endothelial function testing may potentiate the detection of vascular diseases such as myocardial infarction, ischemic stroke (Bucala *et al.,* 1991; Perticone *et al.,* 2001) and SCD complications.

An important characteristic of endothelial dysfunction is the inability of arteries and arterioles to optimally dilate in response to an appropriate stimulus by vasodilators acting on the endothelium (Gopi *et al.,* 2012). This endothelial dysfunction is strongly associated with decreased NO bioavailability, which is due to impaired NO production by the endothelium and increased inactivation of NO by reactive oxygen species (Witting *et al.,* 2007; Kaneto *et al.,* 2010). Reduced NO bioavailability decreases the ability of endothelial cells to execute their functions in regulating vascular tone and homeostasis.

2.6 Physiology of NO

As the vascular endothelial cells produce the endothelial derived NO, it acts on the smooth muscle cells as a vasodilator. Nitric oxide diffuses through the cell membrane and binds to the haem moiety of guanylate cyclase. This leads to cGMP production, which decreases smooth muscle cell contractility by reducing the intracellular free calcium concentration (Rongen *et al.,* 1994). Nitroc oxide reacts with oxyhaemoglobin to form nitrate and methaemoglobin. Nitrate can be reduced to nitrite, which is a known storage pool for NO. Nitric oxide can be generated from nitrite in a NOS-independent manner, and this has been shown to have potential therapeutic importance (Gladwin *et al.,* 2005).

2.7 The eNOS, an enzyme of importance in the endothelial cells

There are three NOS isoforms: the neuronal isoform (nNOS) found in neuronal tissue, the inducible isoform (iNOS) found in cells and tissues, and the endothelial isofrm (eNOS) found in vascular endothelial cells (Wood *et al.,* 2006). The eNOS, derived from vascular endothelium, is the most dominant form of these isoforms, (Akinsheye & Klings, 2010; Rafikov *et al.,* 2011; Thomas *et al.,* 2013). Other sources are cardiac myocytes, blood platelets and the brain (Landmesser *et al.,* 2004).

Variants of eNOS gene contribute to endothelial dysfunction and attenuate the NO production (Hingorani, 2000). Dysfunctional eNOS may play a critical role in the pathogenetic pathway, leading to vascular disease such as SCD. The eNOS gene has been extensively screened for variations. Currently, over 40 publications have described eNOS polymorphisms and their potential associations with disease including cardiovascular and SCD. Variants detected include numerous single nucleotide

polymorphisms (SNPs) (Hingorani *et al.*, 1999, Nakayama*et al.*, 2000, Seattle, 2005) and a variable-number of tandem repeat in the intron 4 (Wang *et al.*, 1996). The common variation identified that leads to an amino acid substitution in the mature protein is the G894T or Glu298Asp (rs1799983) variant, in which a guanine/thymine substitution at exon 7 leads to a glutamate/aspartate substitution at position 298 (Hingorani *et al.*, 1999). Several promoter SNPs have been identified.

Several specific allelic variations of the gene have been identified and evaluated for possible links to SCD as well as cardiovascular disease Fig.2.1. In general, three classes of variations have been identified: those in intron regions, those in 5′-flanking DNA promoter and those within the open reading frame. The evidence suggest that NO may inhibit several key steps in the pathophysiology of SCD and that an alteration in NO production within the vascular endothelium could contribute to pathogenesis of complications in SCD such as VOC, ACS, Leg ulcers and priapism.

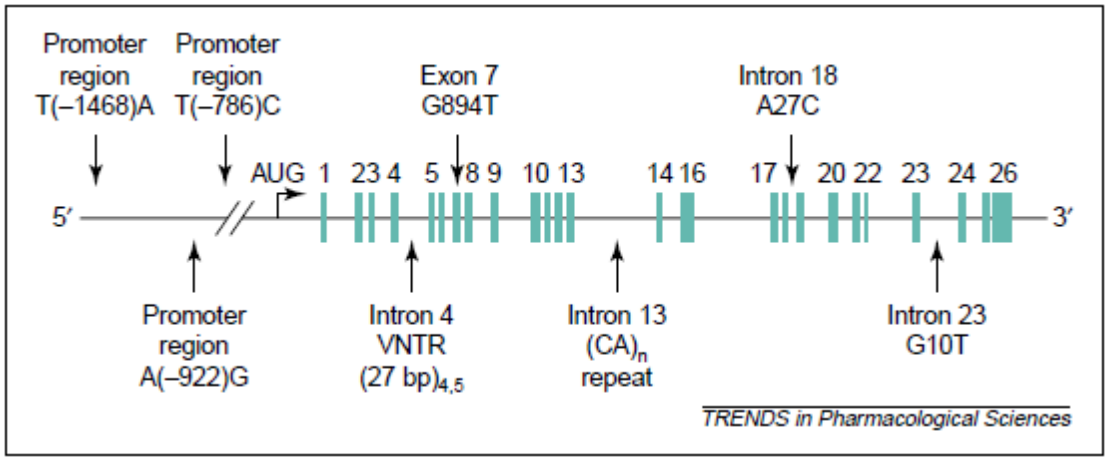

Figure 2.1 Schematic of the gene encoding endothelial eNOS.
The human eNOS gene (located at 7q35–36) contains 26 exons that span 21 kb. Exons are described by number. AUG denotes transcription start site. Specific polymorphisms in the NOS3 gene are marked by arrows. The G894T single nucleotide polymorphism in exon 7 results in an altered eNOS protein sequence (Glu298Asp). Abbreviation: VNTR, variable number of tandem repeats. (Searles, 2006).

The eNOS gene has a common polymorphism at position 298 (Glu298Asp) which has been associated with both altered NO production (Wang *et al.,* 1997) and with vascular disorders including hypertension (Benjafield & Morris, 2000), myocardial infarction (Hibi *et al.,* 1998), coronary artery spasm (Yoshimura *et al.,* 2000), stroke (Elbaz *et al.,* 2000), and renal disease (Noiri *et al.,* 2002). Another polymorphism in exon 7 that codes for a glu–asp substitution has been demonstrated in some studies to impair NO bioavailability. Reduced eNOS mRNA has also been reported in individuals homozygous for the T to C polymorphism in the promoter region (Tesauro *et al.,* 2000; Casas *et al.,* 2006; Searles, 2006).

2.8 eNOS gene polymorphism

Several eNOS polymorphisms have been reported, but three of these have been shown to have clinical relevance due to their association with disease pathophysiology (Thomas *et al.,* 2013). These are located in the promoter region (T-786C), exon7 (Glu298Asp), and a 27- base pair (bp) variable number of tandem repeats (VNTR) in intron 4 (chromosome 7q35-q36) (Thomas *et al.,* 2013; Nishank *et al.,* 2013). Several reports have suggested the involvement of eNOS polymorphisms in the pathogenesis of SCD and its complications such as acute chest syndrome (ACS), VOC (Graido-Gonzalez *et al.,* 1998; Hammerman *et al.,* 1999; Hingorani, 2001; Chaar *et al.,* 2006) in humans, leg ulcers (Paul *et al.,* 1999) and priapism (Trinity, 2007) both in mice. There is no literature investigating eNOS polymorphism in SCD associated leg ulcers and priapism in man and even the ones demonstrating this in animals is very scanty. The VNTR4a/b have been associated with the mean plasma NO level (Hayashi *et al.,* 2010; Xu *et al.,* 2010; Luo *et al.,* 2012; Morando *et al.,* 2013; Nishank *et al.,* 2013), while the eNOS T-786C mutant reduces eNOS gene promoter activity (Nakayama *et al.,* 1999) and is a

genetic risk factor for ACS in adult female SCD patients (Sharan *et al.,* 2004; Chaar*et al.,* 2006).

Considering the several complications and their diversity of disease pathophysiology in SCD and the wide range of differences in clinical presentation among African and African American patients, it is important to determine whether eNOS polymorphisms play any role in this diversity. Thomas *et al.* (2013) studied the ethnogenomic diversity of eNOS polymorphic forms from three clearly distinct and well-defined ethnic groups (Africans from Mali, African Americans and Caucasians from United States) and found that the T786C variant was more common in Caucasians than in Africans and African Americans, the Asp-298 variant had the highest frequency in Caucasians followed by African Americans, but was completely absent in Africans.

Also, the very rare intron 4 allele, eNOS 4c, was found in some Africans and African Americans, but not in Caucasians. The eNOS 4d allele was present in 2 Africans (Thomas *et al.,* 2013). These findings suggest a consistent and widespread genomic diversity in the distribution of eNOS gene variants in Africans, comparative to African Americans and Caucasians. Previous studies have examined Caucasians versus African Americans in the United States, (Tanus-Santos *et al.,* 2001), black and white Brazilians (Marroni *et al.,* 2005) and white, black and mixed populations from Colombia (Serrano *et al.,* 2010). Ethnogenotypic diversity studies of eNOS polymorphisms have become very important because of the possible clinical and pathophysiological implications of certain diseases, as well as potential deconvolution of ethnic-based responses and observations to certain chemotherapeutic agents. Tanus-Santos *et al.* (2001) have reported on the potential possibility that these polymorphisms may predispose African

Americans in the United States to coronary artery disease and hypertension (Tanus-Santos *et al.*, 2001; Stein *et al.*, 2001; Tsujita*et al.*, 2001). In addition, the ethnic distribution of these polymorphisms and pathophysiological implications might carry a geographical import, considering results from a Japanese cohort (Li *et al.*, 2004).

Thomas *et al.*,(2013) found very extensive and clearly marked differences in the interethnic distribution and haplotype frequency of these eNOS variants, within and between the populations studied supporting the growing consensus that eNOS variants play more significant roles than previously thought in disease pathophysiology (Casas *et al.*, 2004; Kalinowski *et al.*, 2004; Serrano *et al.*, 2004). This was consistent with the result obtained in other population studies that also showed marked interethnic differences in the distribution of eNOS polymorphisms between Caucasians and African-Americans (Tanus-Santos *et al.*, 2001) with a similar finding reported between black and white Brazilians (Marroni *et al.*, 205) and white, black and mixed populations from Colombia (Serrano *et al.*, 2010). Inter-estingly, an Italian group (Bolli *et al.*, 2007) reported a novel allele of the eNOS gene in an Italian population that had not been previously reported. These findings may explain the ethnic differences in cardiovascular outcome, response to and bioavailability of certain medications.

A closer examination of the T786C polymorphism (located in the promoter region) revealed that the homozygous CC variant was commonly encountered in the Caucasian population (14.5%), compared to its frequency among “blacks” (3.2% and 1.8% in Africans and African Americans respectively), and further confirms observations made elsewhere (Tanus-Santos *et al.*, 2001; Serrano *et al.*, 2004). On the other hand, this observation clearly contradicts the report in a Brazilian “white” cohort (Marroni *et al.*,’

2005). It could be that the differing frequency of this variant is, individually or in combination, population and geographic-dependent (Tanus-Santos *et al.,* 2001 Serrano *et al.,* 2004; Marroni *et al.,* 2005).

2.9 eNOS polymorphism and SCD

Published results have suggested roles for several eNOS SNPs, as risks for stroke, leg ulcers, pulmonary hypertension, priapism and osteonecrosis in SCD patients in the promoter region T786C; another in exon 7 G894T, and the variable number of tandem repeats (VNTR) in intron 4. (Graido-Gonzalez *et al.,* 1998; Hammerman *et al.,* 1999; Morris *et al.,* 2000; Hingorani 2001; Sharan *et al.,* 2004;Chaar *et al.,* 2006) of the eNOS gene.

Recently published reports revealed higher prevalence of mutant alleles and genotypes of all three eNOS single nucleotide polymorphisms in SCD individuals, implying an association between eNOS gene polymorphisms and SCD in India (Nishank *et al.,* 2013). The finding of higher incidence of mutant alleles and genotypes among the SCD severe group in contrast to higher incidence of wild alleles and genotypes among the SCD mild group of patients indicate the eNOS gene probably acting as a genetic modifier of phenotypic variation among SCD patients in Indian (Nishank *et al.,* 2013). However, the result from a study by Thakur *et al.* (2014) report no differences at all levels in the percentile distribution or frequencies of eNOS polymorphisms between SCD patients and control groups.

Other studies have also shown that there is an extensive interethnic diversity in the distribution of eNOS variants, (Tanus-Santos *et al.,* 2001; Marroni *et al.,* 2005: Bolli *et*

al., 2007; Serrano *et al.,* 2010; Thomas *et al.,* 2013) and this difference could potentially clarify interethnic differences in nitric oxide bioavailability and SCD pathophysiology.

2.10 Role of eNOS in SCD patients with priapism

One of the common complications of SCD that can significantly affect quality of life of men with SCD is priapism. Priapism is a pathologic disorder of penile erection that persists beyond 4 hours in which is unrelated to sexual interest or stimulation (Montague *et al.,* 2003). Overall, erections lasting up to 4 hours are defined as prolonged and may occur at all ages. The incidence of priapism in the general population is as low as (0.5–0.9 cases per 100 000 person-years) (Eland *et al.,* 2001).

However, in SCD male patients the prevalence is as high as 3.6% in patients <18 years of age (Furtado *et al.,* 2012) increasing up to 42% in patients ≥18 years of age (Adeyoju *et al.,* 2002;Olujohungbe *et al.,* 2011;Lionnet *et al.,* 2012). Vascular smooth muscle cells in the penis are constantly subjected to the action of basally released NO from the vascular endothelium. This NO can regulate the vascular tone in the penis by controlling downstream targets of NO cyclic guanosine monophosphate (cGMP) andPhosphodiesterase 5(PDE5).

It has been shown recently that eNOS knockout (KO) and transgenic sickle cell mice have an exaggerated erectile response to cavernous nerve stimulation and have phenotypic changes in erectile function consistent with priapism (Champion *et al.,* 2005; Bivalacqua *et al.,* 2009; Bivalacqua *et al.,* 2010). Furthermore, in characterizing the molecular events associated with the priapism phenotype in the sickle and eNOS KO mice, it was established that Priapism arises from chronically impaired basal endothelial

NO bioavailability due to a defect in the NO signaling pathway in the penis (Champion *et al.*, 2005; Bivalacqua *et al.*, 2007; Bivalacqua *et al.*, 2009). Calcium-dependent NOS activity and cGMP production are basally down -regulated in the penis of sickle cell transgenic mice and mice lacking eNOS (Champion *et al.*, 2005; Bivalacqua *et al.*, 2007; Bivalacqua *et al.*, 2009).

A molecular basis for chronically reduced endothelium-derived NO bioavailability and eNOS functions in sickle cell disease-associated priapismare not defined. However, the cyclic nucleotide (cGMP)is produced is low, steady-state amounts of eNOS under the influence of priapism-related destruction of the vascular endothelium and chronic intravascular haemolysis which occurs (Kato *et al.*,2007; Kato*et al.*,2009).Thus, reduced endothelium-derived NO bioavailability occurs in the penile vasculature and this down regulatesthe set point of PDE5 function secondary to altered cGMPdependent feedback control mechanisms (Champion *et al.*, 2005; Bivalacqua *et al.*, 2007)**.**

Other studies involving transgenic and knockout mice provide additional evidence linking NO deficiency to Priapism. Mice that are genetically deficient in eNOS develop spontaneous priapic activity (Burnett *et al.*, 2002). The frequency of priapic activity is even greater in the mouse doubly deficient in both endothelial and neuronal NO synthase (Champion *et al.*, 2005).

Even though the prevalence of priapism is high in SCD, the possible influence of genetic risk factors on the incidence of priapism is not well understood. A study conducted by Laine *et al.* (2007) examined genetic polymorphisms in 199 unrelated, adult (>18 years), male patients with HbSS and Hbβ- thalassaemia, and found that 83 (42%) reported a

history of priapism. Candidate genes for association with priapism were identified based on their involvement in adhesion, coagulation, inflammation and cell signaling (Elliott *et al.*, 2007).

2.11 Leg ulcer and eNOS

Leg ulcer is defined as a defect in the skin below the level of knee and above the foot persisting for six or more weeks. Leg ulcers are the most common cutaneous complication and manifestation of SCD (Halabi-Tawil *et al.*, 2008; Bazuaye *et al.*, 2010). The cause of leg ulcers in SCD is not known but is thought to bedue to micro-thrombi in the small capillaries of the legs resulting in ischaemia (Bauer, 1940, Gabuzda 1975; Eckman, 1996). This obstruction is said to up regulate integrins which promoteplatelet aggregation and adherence to the endothelium with additional obstruction of the vessels.

The factors ensuring appropriate intercellular communication during wound repair are not completely understood. Several emerging evidence from both animal and human studies indicate that NO plays a key role in wound healing and repairs (Smith *et al.*, 1991, Schaffer *et al.*, 1996; Schaffer *et al.*, 1997; Frank*et al.*, 2002; Jian-dong &Alex, 2005). The beneficial effects of NO on wound repair may be attributed to its functional influences on angiogenesis, inflammation, cell proliferation, matrix deposition, and remodeling. The eNOS gene polymorphism has not been reported in leg ulcers in SCD. Till date there is no data on the eNOS polymorphism and leg ulcers in SCD.

The only data available is by Paul *et al.*, 1999where the role for NO in wound healing has been proposed and the absolute requirement of NO for wound healing *in vivo* and the

contribution of eNOS was determined experimentally using eNOS gene knockout (KO) mice. They determined the requirement for eNOS on wound closure and wound strength. They observed that incisional wound tensile strength demonstrated a 38% reduction in the eNOS KO mice.

2.12 Other additional haplotypes of significance (Haemoglobin haplotypes of significance)

The human haemoglobin is encoded by a cluster of genes located on chromosomes 11 and 16 that are expressed in a developmentally regulated manner (Makani *et al.*, 2013). They are tetramers of two pairs of α-like and β-like globin chains (Weatherall, 2001). Mutations in these globin chains do exist. One such mutation that has been widely studied is the βS (Antonarakis, 1984). There are known haplotypes that are associated with the βS mutation. They are named after the regions where they have the highest frequency: Benin, Senegal, Bantu (Central African Region) and Arab-Indian. The haplotypes are defined by restriction fragment length polymorphisms (RFLPs) in the β-globin locus. It is believed that the sickle cell mutation arose independently in these populations due to the population specificity of the haplotypes, and hasremained to this day (Kulozik, 1986).

Initially when the single mutation theory about sickle cell gene was postulated, it was suggestedthat a single mutation occurred in Neolithic times in the then fertile Arabian Peninsula (Lehmann, 1964).Then, the changing climatic conditions and the consequent conversion of this area to a desert caused the migration of people who could have carried the gene to India, parts of the Arabian Peninsula and down to Equatorial Africa.

It is now quite clear however that the sickle cell mutations occurred as several independent events (Wainscoat *et al.,* 1983). By using a series of different restriction endoucleases, different chromosome structures (haplotypes) are identified and HbS gene has been found to be linked to certain commonly occurring haplotypes which are generally different from those bearing the Hb A gene (Wainscoat *et al.,* 1983; Antonarakis *et al.,* 1984).

In Africa the HbS gene is associated with at least three haplotypes representing independent mutations (Pagnier *et al.,* 1984; Nagel & Fabry, 1985). These are the Benin haplotype, the Senegal haplotype and the Central African Republic or the Bantu haplotype found along the West African coast and Central Africa (Bantu speaking Africa). A fourth haplotype, the Asian haplotype is found in the eastern province of Saudi Arabia and central India.

It appears that mutation of the HbS gene has occurred on at least three occasions in the African continent and at least once in either the Arabian Peninsula or Central India and from the primary sites the migration to the other regions occurred. This can explain the observation made by many investigators that there is widespread chromosomal heterogeneity of HbS gene cluster haplotypes in United States as compared to the homozygous condition in Africa, Arabia or Asia (El Mouzan, 1989).

Slaves with sickle cell trait who were exported from various parts of Africa to United States had the specific HbS gene haplotype found in their region, but after arrival in US, Jamaica and Brazil, there has been considerable mixture of African ethnic groups over the years (Nagel &Fabry, 1985).Available calculations suggest that the sickle gene has

developed between 3000 and 6000 generations, approximately 70000-150000 years ago (Kurnit, 1979; Soloman and Bodmer, 1979).

The existence of haplotypes specific to certain regions of the world suggests that the mutant beta globin gene arose separately in these locations (Oner *et al.*, 1992). All the areas in question have been or are now endemic locations of malarial infestation. This observation is consistent with the idea that the high incidence of sickle mutation in these areas is derived from natural selection (Carlson *et al.*, 1994). People with one sickle haemoglobin gene and one normal haemoglobin gene (sickle cell trait) are somewhat more resistant to malariathan people with two normal haemoglobin genes (Gendrel *et al.*, 1991; Carlson *et al.*, 1994**).**

The widely accepted theory is that HbS offers selective protection against falciparum malaria probably because of induction of sickling even at physiological oxygen tension by Plasmodium falciparum followed by sequestration of parasitized red cells deep within reticulo-endothelial system where the microenvironment is hostile for growth of the parasite (Friedman 1978; Pasvol and Weatherall, 1979). Thus people with sickle cell trait would have a better chance of surviving an outbreak of malaria and passing their genes (sickle and normal haemoglobin) to the next generation when they have children (Allison, 1954; Luzzatto & Reddy, 1970).

2.13 Other variable clinical spectrum of SCD

Clinically, SCD is characterized by a chronic haemolyticanaemia (haemoglobin levels range between 6-10 mmol/L). Patients have baseline anaemia that varies widely. White blood cell counts are often elevated due to marrow hyperactivity. Reticulocyte counts are

elevated, reflecting increase in erythropoiesis replacing the rapidly cleared older cells and red blood cell life span is markedly reduced. Jaundice in SCD is normally due to frequent haemolysis (Chestnut, 1994).

2.14 Presentations in SCD

Steady state of SCD: Sickle cell disease patients who are not in crisis are said to be in steady state (Konotey- Ahulu1991). The steady state therefore refers to the period in the life of the SCD patient when he is able to go about his work.

Sickle Cell Crisis: This is defined as any sudden deviation for the worse that will not have happened had the patient been without the sickle cell gene (Diggs 1965; Konotey-Ahulu, 1991). Implicit in the definition are elements of unpredictability, suddenness and clinical worsening of the condition of the patient. Pathophysiologically, the crisis can be considered as the onset of acute symptoms due generally to sudden *in vivo* sickling. It is never without a precipitating cause (Konotey- Ahulu, 1991). There are four major types of sickle cell crisis some of which are painful, and these include; sequestration crisis, haemolytic crisis, aplastic crisis and VOC. (Konotey- Ahulu, 1991).

2.14.1 Vaso-occlusive crises (VOC)

Although clinical manifestation of SCD displays a wide array of symptoms, recurrent attacks of vaso-occlusive crisis are responsible for most of the morbidity and mortality in SCD (Palmer 1987; Bunn, 1997).Vaso-occlusive crisis are caused by sickled red blood cells which obstruct capillaries and restrict blood flow to an organ, resulting in ischaemia, pain, and organ damage.Vaso-occlusive crisis is the most frequent crisis that results in tissue ischaemia, organ failure, pain and, occasionally, death (Embury*et*

al.,1994; Robert, 1997). Because of its narrow vessels and function in clearing defective red blood cells, the spleen is frequently affected.

The sites of acute painful crises vary for each patient. Pain occurs commonly in the extremities, thorax, abdomen, and back. Pain tends to recur at the same site for a particular person. For each person, the quality of the pain is usually similar from one crisis to another (Ballas and Delengowski, 1993). Bone is also a common target of vasoocclusive damage, especially when the bone is weight-bearing. Such damage may result inavascular necrosis (especially of the femur) and bone deterioration (Hernigou *et al.*, 1993). The pain experienced by sickle-cell patients is partly due to bone ischaemia (Shapiro and Hayes, 1984).

2.14.2 Sequestration crisis

Splenic sequestration results from the acute entrapment of large amounts of blood in the spleen (Sears and Udden, 1985) with the haemoglobin level falling to and below 5g/dl (Lowe and Adams, 1945). The manifestations are upper quadrant pain, sudden enlargement of the spleen and/or liver due to sequestration of blood in these organs resulting in severe anaemia and hypotension. In children, a large fraction of the circulating blood volume is frequently sequestered (Walterspiel 1984). Splenic sequestration crisis is a medical emergency that demands prompt and appropriate treatment.

2.14.3 Aplastic Crisis.

Aplastic crisisis a potentially deadly complication of sickle cell disease, which develops when erythrocyte production temporarily drops due to maturation arrest, and suppression of bone marrow activity (Singer *et al.;* 1950). Infection with parvovirus B-19 frequently causes aplastic crises (Saarinen *et al.,* 1986). The virus has trophism for erythroid progenitor cells, and impairs cell division for a few days during the infection. A shut-down in erythroid production for a few days in these patients can lead to potentially fatal decline in Haematocrit (Mallouh & Qudah, 1993). Often, but not always, aplastic crises coincide with painful crises. It is characterized by severe anaemia and a decrease in haemoglobin and bile pigment in the plasma. In some patients there is pancytopenia accompanied by weakness, drowsiness and symptoms of cardiac failure (Konotey-Ahulu1991).

2.14.4 Haemolytic crisis.

This is caused by massive haemolysis usually by infections like malaria (Harris and Kellermeyer, 1970). It is characterized by severe anaemia, jaundice and associated by the passage of typical coca-cola colored urine due to the presence of haemoglobin.

2.15 Factors that precipitate crisis.

Predicting when a crisis may occur can be difficult. In many patients, the exact cause cannot be determined (Shapiro, 1989). A number of factors, however, are known to precipitate crises (Shapiro, 1989; Serjeant *et al.*, 1994). These factors include the following:

Infection: Most commonly malaria, but also common cold, sore throat, ear infection, urinary tract infections or infections in other areas of the body (Graham, 1924; Thompson, 1963; Diggs, 1965).

Dehydration: this is loss of fluid from the body. It makes the blood viscous and causes it to flow sluggishly and may provoke crisis. Causes of dehydration include excessive sweating as happens in hot weather and during feverish conditions, or from inadequate fluid intake to replace losses, for example when there is diarrhoea or vomiting (McCormick, 1961; Christian, 1967).

Exposure to cold: crises are common during the cold rainy season. Inadequate protection in cold weather, walking in the rain, bathing cold water, swimming in cold water can all precipitate VOC. This is because when the skin is chilled, blood flows more slowly due to increase in viscosity of the blood accompanied by reduced velocity leading to sickling (Edigton, 1953; Addae, 1971).

Stress: This may be in the form of physical stress, example from over exertion at work or strenuous exercise. Examination, unemployment, financial stress and bereavement are examples of emotional and mental stress that can also precipitate crises (Diggs, 1965).

2.16 Angiogenesis and Sickle Cell Disease

Angiogenesis is a global term which indicates the physiological process involving the growth of new blood vessels or neovascularization. This is a vital process for embryological growth, tissue development, and wound healing in damaged tissues (Gopi *et al.,* 2012). Neovascularization or angiogenesis has also been interchangeably

associated with vasculogenesis, which primarily refers to developmental formation of vascular structures from circulating or tissue-resident endothelial.

Sickle cell disease is characterized by micro-vascular occlusion with subsequent ischaemia–reperfusion injury resulting in vasculopathy, organ damage and a decreased life expectancy (Stuart and Nagel, 2004). The vaso-occlusion-induced tissue hypoxia may well lead to a potent angiogenic response (Duits *et al.*, 2006). As patients with SCD are characterized by continuous vaso-occlusion that results in significant ischaemic organ damage, angiogenesis is likely to be of importance in the disease-related pathophysiology (Stuart & Nagel, 2004). In infarcted tissue, the rate of tissue regeneration may be dependent on the integrity of angiogenesis and unbalanced angiogenesis could contribute to the development of pulmonary hypertension, proliferative retinopathy, moya–moya vasculopathy and leg ulcers (Duits *et al.*, 2006) which are all complications in SCD.

Haemoglobin polymerization causes RBCs to assume abnormal, often sickled shapes and the rigid, abnormally shaped RBCs are believed to cause vaso-occlusions in all organ systems of the sickle cell subject (Cao *et al.*, 1999). For example, in the sickle cell retina, vaso-occlusions occur at a younger age (Talbot *et al.*, 1988) and are first observed in the periphery, resulting in a non-perfused and presumably ischaemic peripheral retina (Cao *et al.*, 1999). New blood vessels develop at the border of perfused and non-perfused retina (Cao *et al.*, 1999). Cao *et al.* (1999) demonstrated for the first time that VEGF and basic fibroblast growth factor (bFGF) are associated with sea fan formations in sickle cell retinopathy.

High angiogenic level detected among patients with SCD may be negatively influenced by regular blood transfusion and hydroxyurea therapy, while; early onset of chelation therapy may decrease angiogenin level in β-thalasaemia (Matter *et al.*, 2014). Anaemia is a cause of tissue hypoxia and results in increased angiogenesis (Matter *et al.*, 2014). Matter *et al.* (2014) found that there were higher angiogenic levels among patients with SCD especially, in the severe form of the disease.

Increasing evidence suggests a role for VEGF in the pathophysiology of cardiovascular disease (CVD) (Felmeden *et al.*, 2003). Elevated plasma VEGF has been shown in patients with hypertension and diabetes (Blann *et al.*, 2002; Felmeden *et al.*, 2003), with levels correlating with measures of endothelial damage or dysfunction and overall cardiovascular risk in hypertensive patients (Felmeden *et al.*, 2003). Furthermore, VEGF has independent prognostic significance in patients with acute coronary syndromes. In similar fashion, increased exposure to angiogenic factors such as VEGF has been associated with altered cardiac function in sickle cell disease (Nius *et al.*, 2009). Elevated levels of VEGF have been implicated in the development of pulmonary hypertension in SCD patients (Nius *et al.*, 2009). Nius *et al.* (2009) reported that, there were elevated levels of VEGF in patients with SCD compared with healthy controls. It was postulated that altered levels of circulating angiogenic factors and pro-inflammatory markers in SCD children and adolescents with elevated tricuspid regurgitation velocity, may be at risk for developing worsening pulmonary hypertension.

Sickle cell disease is often characterized by marked inflammation, leukocytosis, leukocyte activation and potentially increased leukocyte homing and adhesion in the vasculature (Okpala, 2004). This leukocyte adhesion to the endothelium could itself

promote vaso-occlusion (Brittain & Parise, 2007). A host of speculative mechanisms for activating inflammation have been proposed, including spontaneous oxygen radical formation in the sickle red cell, reperfusion tissue injury through transient vaso-occlusion and reperfusion, and activation of leukocytes by red blood cells (Vercellotti, 2003). For example, sickle cell patients have elevated white blood cell counts, activated granulocytes, monocytes and endothelial cells, enhanced expression of endothelial cell adhesion molecules, elevated cytokine levels and elevated acute phase reactants (Vercellotti, 2003).

Hence, the marked inflammatory process correlates with vascular occlusion and angiogenesis. Several angiogenic growth factors have been found to be elevated in SCD (Ofori-Acquah *et al.*, 2012). Ang-2 and erythropoietin were higher in adults with HbSS compared to healthy controls and further elevated during acute painful crisis (Duits *et al.*, 2006).

2.16.1 Vascular endothelial growth factor (VEGF) in SCD

Vascular endothelial growth factor was found to be increased in subjects with Haemoglobin SS compared to controls in some studies (Solovey *et al.*, 1999; Niu *et al.*, 2009). When present, higher VEGF levels were found to be associated with reduced odds of elevated tricuspid valve regurgitant velocity by echocardiography in children with SCD, a noninvasive measure suggesting pulmonary artery hypertension (Niu *et al.*, 2009).

Vascular endothelial growth factor was one of the first cytokines identified as potentially elevated in SCD (Brittain & Parise, 2007). Its level has been found elevated during the

steady state in SCD (Solovey *et al.*, 1999; Perelman *et al.*, 2003). However, plasma VEGF levels may almost triple during a VOC in some patients (Gurkan *et al.*, 2005). VEGF is released by a host of inflamed cells during vascular injury or hypoxia and is a multifunctional growth factor (Yla-Herttuala *et al.*, 2007).

High levels of VEGF promote the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell-adhesion molecule-1 (VCAM-1) and E-selectin on vascular endothelial cells and activates nuclear factor-KB, a known mediator of vascular inflammation (Kim *et al.*, 2001). Thus, increased levels of VEGF present during steady state or crisis could be predicted in vivo to recruit adhesive red blood cells derived from SCD patients RBCs and leukocytes to inflamed endothelium, impede blood flow and promote hypoxia and vascular inflammation (Brittain & Parise, 2007).

Vascular endothelial growth factor functions as one of the most potent activators of endothelial cells that increases expression of intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and is elevated in the plasma of patients with SCD (Sultana *et al.*, 1998). The activation of endothelial cells in SCD contributes to vascular occlusions (Sultana *et al.*, 1998). Vascular endothelial growth factor is also considered a hypoxia inducible factor that activates potent angiogenic signals that under ideal conditions promotes vascular remodeling, sets vascular tone and induces collateral formation in chronically obstructed blood vessels. However, the correct balance of angiogenic signals in SCD is not maintained (Duits *et al.*, 2006).

One of the primary antagonists and regulators of VEGF function and signaling *in vivo* is soluble fms-like tyrosine kinase-1 (sFlt-1), the soluble VEGF receptor (Goldman *et al.*,

1998). This molecule binds to and prevents VEGF from interacting with its receptors, thus controlling VEGF's angiogenic potential. As VEGF levels rise, levels of sFlt-1 rise concomitantly, tightly regulating the angiogenic process (Mohan *et al.*, 2005). This increase in sFLt-1 is not seen in SCD, which may shift the balance of angiogenesis toward aberrant blood-vessel development (Brittain & Parise, 2007).

Placental growth factor (PlGF) acts as a potent stimulant for VEGF secretion by monocytes via activation of flt-1, which results in activation of PI3 kinase/AKT and ERK-1/2 pathways (Bottomley *et al.*, 2000; Vercellotti, 2003). PlGF, an angiogenic factor belonging to the VEGF family postulated to be the tie that binds enhanced erythropoiesis, inflammation and angiogenesis together in sickle cell disease (Vercellotti, 2003). Mohan*et al.*,(2005) reported elevated VEGF plasma levels in clinically asymptomatic SCD patients. Placental growth factor is produced not only by placental trophoblasts and umbilical vein endothelial cells during pregnancy; but also maturing erythroblasts (Tordjman *et al.*, 2001). Plasma levels of PlGF are higher in patients with SCD than in healthy control subjects and have been reported to correlate with the frequency of acute pain episodes (Perelman *et al.*, 2003). Placental growth factor is related to VEGF and its blood concentrations are high in steady-state SCD and increase further during acute pain.

The PlGF is angiogenic and also activates monocytes and is potentially important in the complications associated with pregnancy in SCD (Brittain & Parise, 2007). PlGF levels were found to correlate with many other parameters in adults with SCD, including total haemoglobin and tricuspid regurgitant jet (TRJ) (Sundaram *et al.*, 2010).

Levels of VEGF are elevated in SCD and correlate with many other biomarkers, including those indicating inflammation, haemolysis and elevated TRJ velocity (Niu *et al.*, 2009). Therefore, pathological elaborations of unbalanced angiogenesis are found in SCD and that, sickle retinopathy; pulmonary hypertension and moya moya vasculopathy can each be attributed to unchecked VEGF and its pro-angiogenic function (Brittain and Parise, 2007). Thus, in addition to promoting endothelial cell activation and sickle RBC and leukocyte adhesion, VEGF may also contribute to the vascular malformations seen in SCD (Brittain & Parise, 2007).

2.16.2 Angiopoietin-1 (ANG-1) and Angiopoietin-2 (ANG-2) in SCD

Marked endothelial damage or dysfunction is associated with SCD (Blann *et al.*, 2003) and endothelial proliferation as a means of effecting endothelial repair may be a mechanism for attempting to preserve endothelial homeostasis (Mohan *et al.*, 2005). Ang-1 has been shown to have anti-apoptotic effects on endothelial cells. Mohan *et al.*, (2005) reported increased Ang-1 levels in clinically asymptomatic SCD patients and explained that because of the endothelial damage in SCD, Ang-1 levels raised in order to provide vascular support. In contrast, Duits *et al.*, (2006) reported elevated levels of Ang-2; but not Ang-1 in HbSS and HbSC patients in the asymptomatic and during painful crises states and concluded that, SCD patients were in pro-angiogenic state mainly because of elevated Ang-2 levels.

The Ang-2 induces proliferation and migration of endothelial cells and stimulates sprouting of new blood vessels when VEGF is present, whereas it promotes endothelial cell death and vessel regression when the activity of endogenous VEGF is inhibited (Lobov *et al.*, 2002). High Ang-2 relative to Ang-1 and VEGF levels favoured

angiogenesis and responsiveness to hypoxic and inflammatory stimuli as demonstrated in neoplasms (Zhang *et al.,* 2003). It was found that there were elevated Ang-2 levels (with further increments during painful crises) in SCD patients suggesting enhanced angiogenesis (Duits *et al.,* 2005).

In one study, it was concluded that systemic profiles of angiogenic growth factors indicate a pro-angiogenic tone in adults with SCD and this was mainly because of Ang-2 increments that are probably caused by tissue hypoxia as a result of the ongoing vaso-occlusive process characteristic of SCD (Duits *et al.,* 2005). The enhanced Ang-2 serum levels may, therefore, reflect rapid Ang-2 release from endothelial Weibel Palade bodies in response to endothelial damage in SCD (Duits *et al.,* 2005). Mohan*et al.* (2005) reported elevated Ang-2, VEGF and Ang-1 plasma levels in clinically asymptomatic SCD patients. Ang-2 in SCD alongside raised VEGF, are consistent with the concept of increased angiogenic activity in SCD generally.

2.16.3 Ang-2 to Ang-1 ratio

Both Ang-1 and Ang-2 bind to the endothelial receptor tunica intima endothelial kinase-2 (Tie-2) a tyrosine kinase that contains immunoglobulin-like loops and epidermal growth factor-like domains) with the relative Ang-1/Ang-2 levels in relation to VEGF levels determining the balance of vessel quiescence as opposed to angiogenesis (Fam *et al.,* 2003). Angiogenesis is influenced by the relative levels of Ang-1, Ang-2 and VEGF (Fam *et al.,* 2003).

High Ang-1 relative to Ang-2 and VEGF levels favoured sustained vessel integrity and quiescence whereas high Ang-2 relative to Ang-1 and VEGF levels favoured

angiogenesis and responsiveness to hypoxic and inflammatory stimuli as demonstrated in neoplasms (Zhang *et al.,* 2003). Furthermore, Ang-1 and VEGF promote angiogenesis and vessel maturation, whereas Ang-2 serves to antagonize the mural cell contact induced by Ang-1 (Metheny-Barlow & Lu, 2003) but in the presence of VEGF, angiogenesis ensues while in the absence of VEGF, vessels regress with Ang-2 (Maisonpierre *et al.,* 1997).

Higher levels of Ang-2 and Ang-2 to Ang-1 ratios have been reported in some disease severity and complications. For instance, clinical studies have reported that higher plasma Ang‑2 levels are predictive of myocardial infarction and stroke recurrence and are independent of traditional risk factors and that the extent of myocardial damage may correlate with serum Ang‑2 and Ang‑2 to Ang‑1, indicating that Ang‑2 and Ang‑2 to Ang‑1 ratio are potential biomarkers of the severity of the disease (Chen *et al.,* 2013). In sepsis, Ang-2 and/or the Ang-2 to Ang-1 ratio have been found to be markers of disease severity as well as in acute lung injury or acute respiratory distress syndrome (ARDS), febrile neutropenia, and malaria (Davis *et al.*, 1996; Parikh *et al.*, 2006).

Again, in healthy individuals, serum levels of Ang-1 exceed those of Ang-2; in contrast, serum and plasma levels of septic individuals contains high levels of Ang-2 and can disrupt normal endothelial barrier function when added to human microvascular cell in vitro (Davis *et al.,* 1996). Moreover, in patients with severe sepsis, circulating Ang‑2 levels correlate with markers of endothelial cell activation and 28‑day mortality (Patel *et al.,* 2011). In acute lung injury patients, Ang‑2 to Ang‑1 ratio is an independent predictor of mortality (Ong *et al.*, 2010). Lim *et al.*, (2004) found that, plasma Ang-2 and VEGF levels were selectively elevated in patients with diabetes and were associated

with indexes of endothelial damage/dysfunction, regardless of vascular disease. Luz *et al.*(2013) found a higher Ang-2/Ang-1 ratio as a biomarker of septic shock development in patients with cancer and chemotherapy-related febrile neutropenia.

Lower ratios of the angiopoietins in SCD compared to normal subjects as well as the trend of lower Ang-2/VEGF from ‘‘no retinopathy’’ has been observed (Mohan *et al.*, 2005). Previous studies suggested that the angiopoietins may also be involved in the regulation of endothelial integrity and inflammation (Tsigkos *et al.*, 2003), with Ang-1 having anti-inflammatory and potentially atheroprotective properties (Nykanen *et al.*, 2003). Hence, selective increase in plasma VEGF and Ang-2, but not Ang-1, may favor aberrant neovascularization and endothelial abnormalities. Circulating Ang‑2 levels and the Ang‑2 to Ang‑1 ratio may be suitable biomarkers of inflammatory diseases (Nykanen *et al.*, 2003). Ang‑1 and Ang‑2 are well known to be associated with several forms of cardiovascular (Patel *et al.*, 2008) and inflammatory diseases (Ricciuto *et al.*, 2011).

In SCD, the strong inter-correlations among the growth factors, suggest a high degree of coordinated and complementary angiogenic activity and perhaps a role for these factors in abnormal angiogenesis in SCD (Mohan *et al.*, 2005). Vessels induced in the presence of Ang-1 are not leaky and resist leakage induced by inflammatory agents and part of the means by which this resistance to leakiness occurs may be attributed to markedly enhanced pericyte coverage of the nascent vessels (Gamble *et al.*, 2000). Maisonpierre*et al.* (1997) described a close relationship between Ang-1, Ang-2 and VEGF in angiogenesis.

This was predicted by analysing Ang-1, Ang-2 and VEGF expression in cyclical rat ovary angiogenesis. Ang-2 and VEGF mRNA were co-expressed at the front of invading sprouts during active angiogenesis. Up-regulation of Ang-2 with down-regulation of VEGF led to vessel regression. However, Ang-1 mRNA expression was relatively suitable throughout the process. Also, in one study, Ang-2 to Ang-1 ratio was drastically elevated during corpus luteum vessel regression compared to angiogenesis during corpus luteum formation (Goede *et al.,* 1998).

When a sustained NO-releasing compound was administered to SCD patients, it reversed dysregulated NO signal transduction in priapism (Lagoda *et al.*, 2014) meaning there is a need to understand how eNOS and polymorphisms contribute to NO bioavailability. Furthermore, there is a paucity of data on how this observation plays within and between genetically homogeneous SCD groups as Ghanaians. To my understanding, this will be the first report to investigate the role of eNOS gene polymorphisms in a population with SCD complications.

Previous studies in SCD in general were carried out in genetically heterogeneous populations in India and homogenous population for HbSS SCD patients in Mali. To this end, investigating the role of eNOS gene polymorphisms, endothelial damage and angiogenic balance in HbSS Ghanaians in relation to complications of the disease is very important. The present study examined the genomic diversity and the association of functionally significant eNOS gene polymorphisms in Ghanaian individuals with SCD complication.

CHAPTER THREE

RESEARCH METHODOLOGY

3.1 Study design

This study was a case-control study, which involved recruitment of SCD patients from the Center for clinical Genetics and control group from Accra Area Blood Centre for National blood donation, the biggest tertiary sickle cell clinic, and blood donation centers in Ghana respectively.

3.2 Study sites

The study was conducted at the Korle-Bu Teaching Hospital. Both male and femaleSCD patients and HbAA controls (blood donors) participants were recruited. The Korle-Bu Teaching Hospital situated in the nation's capital, Accra, Ghana, is the leading tertiary hospital and the major referral centre in the country. The hospital has expanded in phases and now has 1600 beds with three centres of excellence, the National Cardiothoracic Centre, the National Plastic and Reconstructive Surgery and the Radiotherapy Centre.

It also serves as the teaching hospital for the University of Ghana Medical School, Accra.Most of the sickle cell patients within the nation's capital Accra receive their treatment at the Center for Clinical Genetics.The population within Accra originates from different social and ethnic groups as well as Geographical distinct areas. Therefore the demographics of the study participant who were enrolled in this study were not limited to a specific social group.

3.3 Subjects

In order to conduct preliminary haematological assessment on this study population, a total of six hundred and ninety-four (694) subjects were sampled in this study. This was made up of two hundred and eight (208) HbSS SCD patients in steady state (110 males, 98 females), eighty two (82) HbSC SCD patients in steady state (30 males, 52 females), one hundred and fifty-six (156) HbSS SCD patients in VOC (64 males, 92 females), thirty- four (34) HbSC SCD patients in VOC (12 males, 22 females), thirty- four (34) HbSS SCD patients in post VOC (15 males, 19 females), twenty one (21) HbSS SCD patients with leg ulcer (14 males, 7females), eight (11) HbSS SCD patients with priapism. The control group was made up of one hundred and forty-eight (148) HbAA blood donors (93 males, 55 females) from the Korle-Bu blood bank. Subjects' recruitment was done with the approval of the Ethical and Protocol Review Committee of the University of Ghana Medical School with protocol identification number MS-Et/M.11-P5.7/2012-13 and the participants' consented.

Out of the total of the 694 subjects stated above for the preliminary haematological assessment, genotyping and chemistry were performed on 153 SCD patients with 135 HbSS (46 steady state, 57 VOC, 21 leg ulcers, 11 Priapism) and 18 HbSC patients in VOC with age -matched 60 healthy controls (HbAA blood donors) according to the minimum sample size calculation and high cost of assay kits and reagents for analysis.

3.4 Definitions of terminologies used in describing the subjects.

Steady state was clinically defined as a patient who has been well and has not been in crisis for at least 2 weeks and he/she is able to go about his work.

Vaso-occlusive crisis was clinically defined as pains in the bones, muscles and joints not attributable to any other cause and requiring parenteral analgesia and detention in the Centre for some hours.

Post-Crisis was clinically defined as a period when patient no longer experiences the VOC pains 3 days after the parenteral analgesia and is certified by a doctor as healthy.

Leg ulcer is defined as a defect in the skin below the level of knee and above the foot persisting for sixor more weeks.

Priapism was defined as a purposeless, persistent penile erection not accompanied by sexual desire or stimulation, lasting more than 6 hours (Broderick *et al.*, 2010).

3.5 Inclusion criteria

SCD patients with HbSS and HbSC genotype as well as healthy blood donors aged 15 years and above were included.

3.6 Exclusion criteria

SCD patients with genotype other than HbSS and HbSC.To successfully match participants, all those with ages below15 yearswere excluded from the study.

Patients with renal failure (serum creatinine >2 µmol/dL), pulmonary edema, cardiogenic shock, history of myocardial infarction in the last 6 months, diabetes mellitus, congestive heart failure, a cerebrovascular accident in the last 6 months, acute asthma, or angina pectoris were excluded.

3.7 Minimum Sample size determination

The minimum sample size was determined by the use of software from:

http://sampsize.sourceforge.net/iface/s3.

With the following values:

Minimum Odds Ratio to detect = 2

Percentage exposed among controls=40%

Power=80

Number of controls per case=1

Alpha risk=5%

1:1 matched study design

The minimum sample size for SCD cases was 105. The SCDcategories of interest were HbSS steady state, HbSSVOC, HbSS Leg ulcersand HbSS Priapism. A few of HbSC were included for comparison. Based on the preliminary data available from the Center for Clinical Genetics, the incident ratio of HbSS steady state: HbSS VOC: HbSC VOC HbSS Leg ulcers: HbSSPriapismwas 12:5:2:1:1 respectively. Therefore the Samples sizes for VOC: Priapism: leg ulcers were60:25: 10:5:5patients according to the ratio. These were age matched with 60 HbAA controls. The same group of control was compared with all the SCD categories.

3.8 Demographics

The weight, sex, height, age and occupation of all the participants were obtained through the administration of questionnaires. The questionnaire for patient baseline demographics and clinical information was pretested with participants (20 SCD and 20 controls) at the sickle cell clinic and blood bank, Korle-Bu, Accra.

3.9 Blood collection

The method as described in the Standard Operating Procedure for performing venepuncture in the National Blood Bank (Korle-bu) by Acquaye (1991) was used. Venous blood samples were collected from the patients into labeled sodium EDTA tubes. Rubber tourniquet was tied to the biceps about 8cm above the elbow joint for less than one minute and the site to be punctured cleansed with methylated spirit. Then 5ml of blood was drawn from the brachial vein with a 19G hypodermic needle fixed on 5mls syringe. All aseptic conditions were adhered to. The blood was immediately divided into two NaEDTA tubes (2.5ml each) and the blood was mixed in the bottles to prevent clotting by gently inverting the tubes four times manually.

Aliquots of 2.5mls of each blood sample was transferred into labeled test tubes (13x100mm) and spun immediately after collection in a centrifuge at a speed of 2x000 RPM. The plasma was drawn from the test tube with a micropipette into labeled eppendorf tubes and stored at -80^0C. The buffy coat was carefully separated into an eppendorf tube, labeled appropriately and stored in a refrigerator at -80 ^{o}C till the time for the DNA extraction.

3.10 Measurement of Haematological profile

The full blood count was done on the same day of collection of the blood sample using labsystem Multiskan MS (manufactured by Amisham Bioscience LTD, UK), a three-part auto analyzer able to run 19parameters per sample including haemoglobin concentration, packed cell volume, red blood cell concentration, mean corpuscular haemoglobin, mean cell volume, mean corpuscular haemoglobin concentration, white bloodcells and platelet parameters.

Letting the equipment-sampling probe into the blood sample and then pressing the start button aspirated the well-mixed blood sample. The auto analyzer aspirated approximately 20µl of blood. Result of analysis is displayed after about 30seconds.A printout copy of result was released on thermal printing paper.

3.11 Measurement of Nitric oxide

The amount of NO in each sample was measured using the Griess reagent system (Promega, Madison, USA). The assay relies on a diazotization reaction that was originally described by Griess in 1879 (Griess, 1879). The Griess Reagent System is based on the chemical reaction, which uses sulfanilamide and *N*-1-napthylethylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions. This system detects Nitrite in a variety of biological and expressions liquid matrices such as plasma, serum, urine and tissue culture medium.

3.11.1 Preparation of a Nitrite Standard Reference Curve

A Nitrite Standard reference curve was prepared in distilled water for each assayfor accurate quantification of nitrite levels in the study samples. A volume of 1ml of a 100µM nitrite solution was prepared by diluting the provided 0.1M Nitrite Standard (0.1M sodium nitrite in water) 1:1,000 in distilled water. Three columns (24 wells) in the 96-well plate were designated and labeled appropriately with a blue marker for the Nitrite Standard reference curve. A 50µlvolume of the distilled water was dispensed into the wells in rows with a micropipette. A100µl of the 100µM nitrite solution was added to the remaining 3 wells in row. Serial twofold dilutions (50µl/well) were performed in triplicate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13

and 1.56) µM by plotting the average absorbance value of each concentration of the Nitrite Standard on the X-axis and the nitrite concentration on the Y-axis (Figure 3.1).

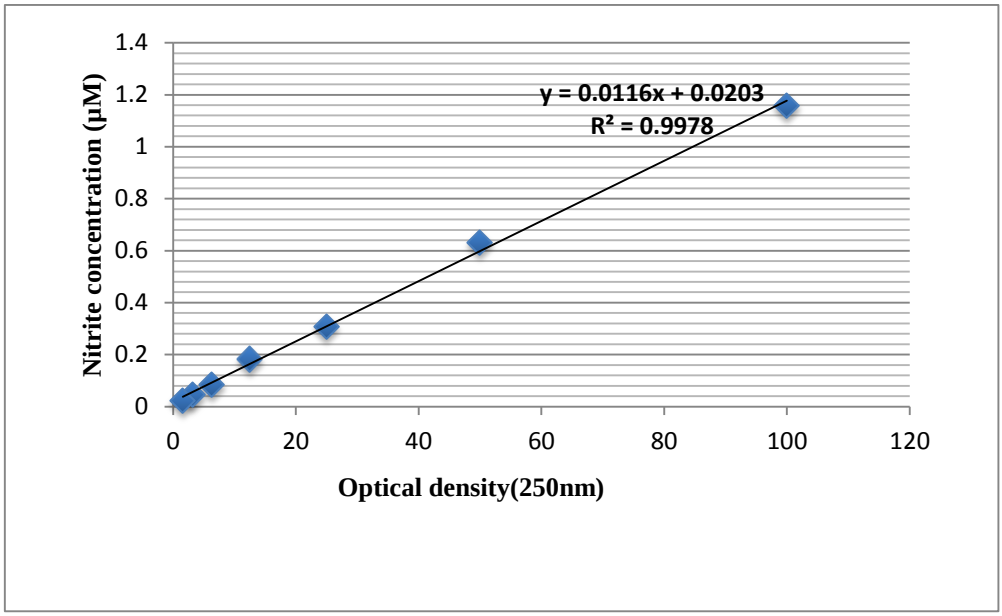

Figure 3.1 Nitrite Standard Reference Curve

From this graph the formula Y = 0.011X + 0. 020was derived and was used to calculate the nitrite concentration. Values on the X-axis are the average absorbance (520nm) and values on the Y-axis are thenitrite concentration (µM).

3.11.2 Nitrite Measurement as an index for NO level (Griess Reaction)

The Sulfanilamide Solution (1% sulfanilamide in 5% Phosphoric acid) and NED Solution (0.1% N-1-napthylethylenediamine dihydrochloride in water) were allowed to equilibrate to room temperature (30 minutes). A volume of 50µl of plasma was added to the wells in triplicate and labeled with a marker. Using a multichannel pipette, 50µl of the Sulfanilamide solution was dispensed to all the plasma in the wells and the wells containing the diluted series of the Nitrite Standard reference curve.

It was then incubated for 10 minutes at room temperature and protected from light by keeping it in a dark box. Using a multichannel pipette, 50µl of the NED solution was dispensed to all wells. It was incubated again for 10 minutes at room temperature, still keeping it in a dark box.

3.11.3 Determination of Nitrite Concentrations in Expressions Samples

The average absorbance value of the triplicates of each sample was determined. The average absorbance value of each concentration of the nitrite standard as a function of "Y" was plotted against nitrite concentration as a function of "X". Theconcentrations of NO in the samples were determined by comparison to the Nitrite standard reference curve.

In order to generate a nitrite standard reference curve, the average absorbance value of each concentration of the nitrite standard "Y" was plotted against nitrite concentration "X". The average absorbance values for the samples were calculated and their respective concentrations were determined by comparison to the nitrite standard curve.

3.12 Measurement of vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1) and E-Selectin by ELISA method

3.12.1 Methodology for VCAM-1

The VCAM-1 concentrations were estimated using the Human VCAM-1 ELISA kit (GenWay, Califonia, USA). The manufacturer's protocol was followed as described below;

Procedure

Standard human VCAM-1 solutions of concentrations 10,000pg/mL, 5000pg/mL, 2500pg/mL, 1250pg/mL, 625pg/mL, 313pg/mL and 156pg/mlwere prepared from the 10ng stock using the sample diluent buffer provided. Aliquots of 0.1mL of each standard were added to the 96-well precoated plate in duplicate and 0.1mL of the sample diluent buffer into the control well. A volume of 0.1mL of the sample plasma (diluted 1:100 with sample diluent buffer) was added into the remaining wells in duplicate. The plate was sealed with cellophane and incubated at 37°C for 90 min. After the incubation period, the plate content was discarded and the plate blotted witha paper towel.

Aliquots of 0.1mL biotinylated anti-human VCAM-1 antibody working solution were added into each well and the plate incubated at 37°C for 60 min. The plate was then washed 3 times with 0.01M phosphate buffered saline (PBS) using an automated plate washer. The washing buffer was then discarded and the plate blotted dry on paper towels. A volume of 0.1mL of prepared Avidin-Biotin-Peroxidase Complex(ABC) working solution was added into each well and the plate incubatedat 37°C for 30 min. The plate was then washed 5 times with 0.01M PBS and blotted onto paper towels. After this, 90µl of prepared TMB color developing agent was added into each well and the plate incubated at 37°C in the dark for 25 min. 0.1ml of prepared TMB stop solution was then added into each well. The optical density absorbance was then read at 450nm in a microplate reader (Amersham Bioscience Limited, UK). Refer to Fig. 3.2.

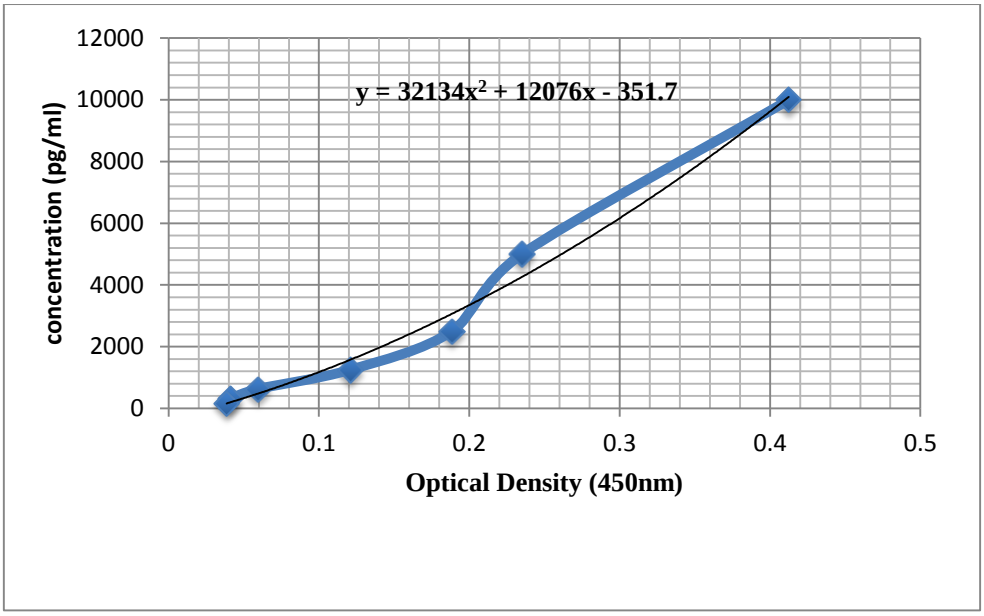

Figure 3.2 Standard reference curve for V-CAM

3.12.2 Methodology for ICAM-1

ICAM-1 concentrations were estimated using the GenWay Human ICAM-1 ELISA kit (GenWay, Califonia, USA). The manufacturer's protocol was followed as described below:

Procedure

The procedure is the same as described in the protocol for VCAM-1 insection 3.12.1 above. Refer to Fig. 3.3 for the standard curve.

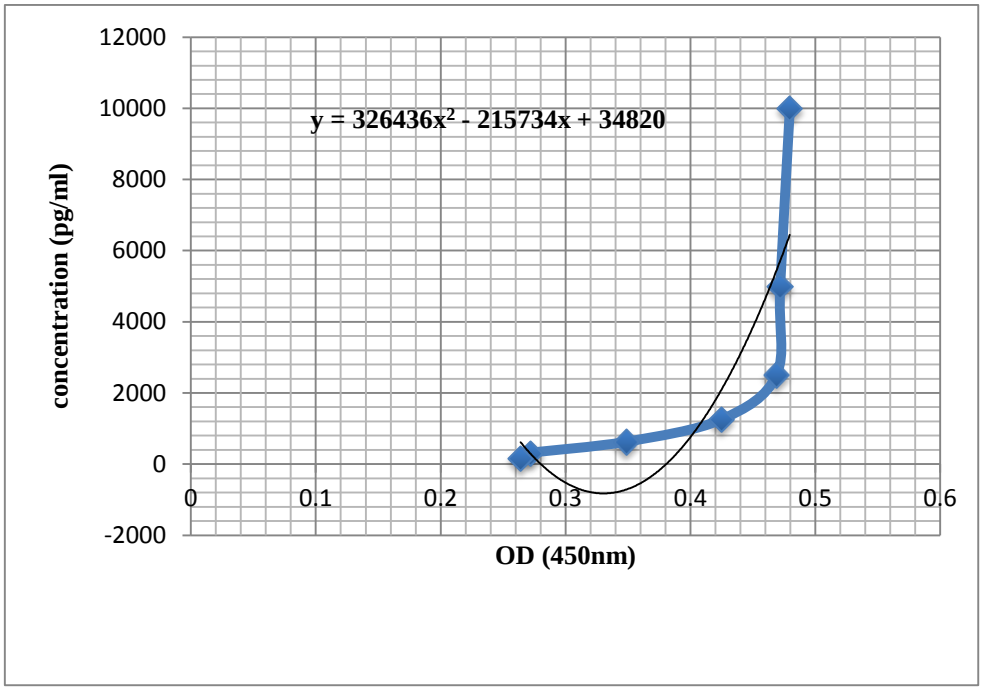

Figure 3.3 Standard reference curve for ICAM-1

3.12.3 Methodology for Human E-Selectin

E-Selectin concentrations were estimated using the GenWay Human E-Selectin ELISA kit (GenWay, Califonia, USA). The manufacturer's protocol was followed as described below;

Procedure

Standard human E-Selectin solutions of concentrations 10000pg/mL, 8000pg/mL, 4000pg/mL, 2000pg/mL, 1000pg/mL, 500pg/mL, 250pg/mL and 125pg/mL were prepared from the 10ng stock using the sample diluent buffer provided. Aliquots of 0.1mL of each standard were added to the 96-well precoated plate in duplicate and 0.1mL of the sample diluent buffer into the control well. A volume of 0.1mL of the

sample plasma (diluted 1:100 with sample diluent buffer) was added into the remaining wells in duplicate.

The plate was sealed and incubated at 37°C for 90 min. After the incubation period, the plate content was discarded and the plate blotted onto paper towels. Aliquots of 0.1ml biotinylated anti-human E-Selectin antibody working solution were added into each well and the plate incubated at 37°C for 60 min. The plate was then washed 3 times with 0.01M PBS using an automated plate washer. The washing buffer was then discarded and plate blotted dry on paper towels. A volume of 0.1ml of the prepared ABC working solution was added into each well and the plate incubated at 37°C for 30 min.

The plate was then washed 5 times with 0.01M PBS and blotted onto paper towels. After this, 90µl of prepared TMB color developing agent was added into each well and the plate incubated at 37°C in dark for 25 min. A volume of 0.1ml of prepared TMB stop solution was then added into each well. The optical density absorbance was then read at 450nm in a microplate reader (Amersham Bioscience Limited, UK). Refer to Fig. 3.4.

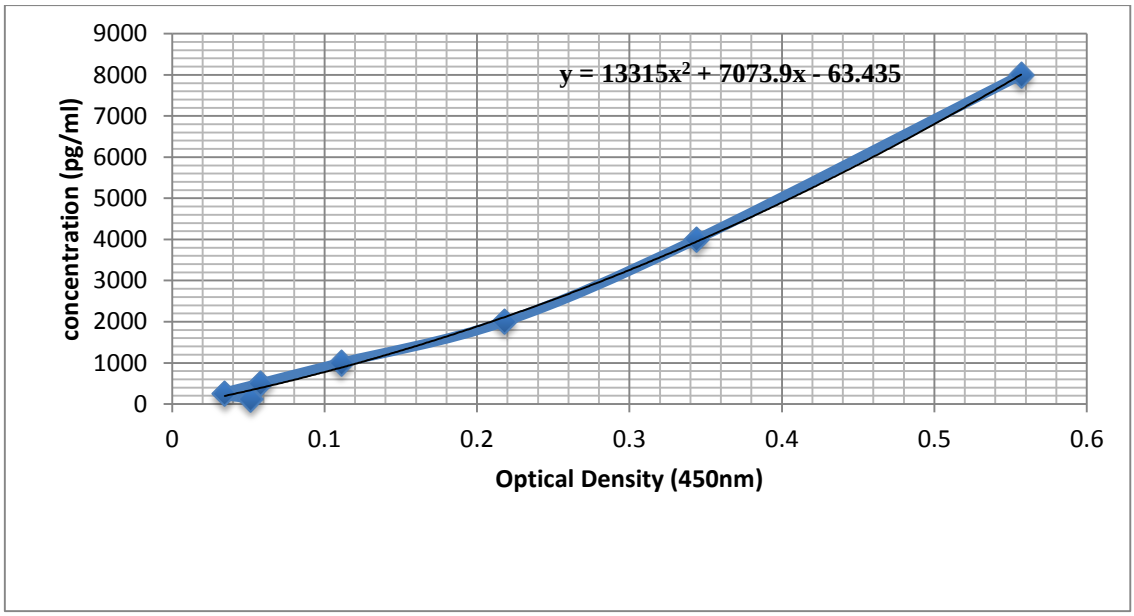

Figure 3.4 Standard reference curve for E-selectin

3.13 Measurement of Angiopoietin-1, Angiopoietin-2and vascular growth factor (VEGF) by ELISA method

3.13.1 ELISA for the measurement of Angiopoietin-1

The levels of Angiopoietin- 1 in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit). According to manufacturer's instructions, 96-well polystyrene microtitre plates (R&D) were coated with 100 µL of mouse anti-human Ang-1 antibody (capture antigen) per well and incubated overnight at room temperature.

The plate wells were aspirated and washed three times using 400 µL of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent (1% Bovine Serum Albumin (BSA) in PBS) and incubated for an hour at room temperature. The plates were then aspirated and washed three times as mentioned above. A 100 µL of plasma samples (20 fold dilution in PBS) were added per well. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. Each well was aspirated and washed three times by filling with wash buffer (400 µL) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. A volume 100 µL of detection antibody diluted in reagent diluent with normal goat serum (NGS) were added to each well.

The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as

described above. A volume of 100 µL of working dilution of Streptavidin –HRP was added to each plate well. The plates were covered with an adhesive strip and incubated for 20 minutes in the dark at room temperature. The plates were aspirated and washed three times as described above. A volume 100 µL of Substrate Solution was added to each well for colour development and incubated for 20 minutes at room temperature in the dark. A 50 µL volume of Stop Solution was added to each well to arrest the colour change. The optical density was determined within 30 minutes, using a microplate reader set to 450 nm (Amersham Bioscience Limited, UK).

3.13.2 ELISA for the measurement of Angiopoietin-2

The levels of Angiopoietin- 2 in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit) according to manufacturer's instructions. Ninty six (96-well) polystyrene microtitre plates (R&D) were coated with 100 µL per well of mouse anti-human Ang-2 antibody (capture antigen) per well and incubated overnight at room temperature. The plate wells were aspirated and washed three times using 400 µL of wash buffer (0.05% Tween 20 in Phosphate Buffered Saline (PBS) and blotted against clean paper towel after the third wash to adequately remove excess unbound capture antigen. The plate wells were then blocked with 300µl per well of reagent diluent (1% Bovine Serum Albumin (BSA) in PBS and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

A volume of 100 µL of plasma samples (20 fold dilution in PBS) was added per well. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm.

Each well was aspirated and washed three times by filling with wash buffer (400 µL) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. A 100 µL volume of detection antibody diluted in reagent diluent with normal mouse serum (NMS) was added to each well. The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above.A 100 µL volume of working dilution of Streptavidin –HRP was added to each plate well.

The plates were covered with an adhesive strip and incubated for 20 minutes in the dark at room temperature. The plates were aspirated and washed three times as described above. A 100 µL volume of Substrate Solution was added to each well for colour development and incubated for 20 minutes at room temperature in the dark. A 50 µL volume of Stop Solution was added to each well to arrest the colour change. The optical density was determined within 30 minutes, using a microplate reader set to 450 nm (Amersham Bioscience Limited, UK).

3.13.3 ELISA for the measurement of VEGF

The levels of VEGF in the plasma of patients were determined by performing a double sandwich ELISA technique (R&D DuoSet ELISA Development kit) according to manufacturer's instructions. A 96-well polystyrene microtitre plates (R&D) were coated with 100 µl per well of mouse anti-human VEGF antibody (capture antigen) and incubated overnight at room temperature. The plate wells were aspirated and washed thrice using 400 µl of wash buffer [0.05% Tween 20 in Phosphate Buffered Saline (PBS)] and blotted against clean paper towel after the third wash to adequately remove

excess unbound captured antigen. The plate wells were then blocked with 300µl per well of reagent diluent [1% Bovine Serum Albumin (BSA) in PBS] and incubated for an hour at room temperature. The plates were then aspirated and washed thrice as mentioned above.

A 100 µl volume of plasma samples (20 fold dilution in PBS) were added per well. The plates were covered with an adhesive strip and incubated for 2 hours at room temperature on a horizontal orbital microplate shaker (0.12" orbit) set at 500 ± 50 rpm. Each well was aspirated and washed three times by filling with wash buffer (400 µl) using a manifold dispenser. The plates were blotted against clean paper towels at the end of the wash to completely remove liquid. About 100 µl of detection antibody diluted in reagent diluent with normal goat serum (NGS) were added to each well. The plates were covered with a different adhesive strip and incubated for 2 hours at room temperature on a shaker after which the plates were aspirated and washed three times as described above. About 100 µl of working dilution of Streptavidin-HRP was added to each plate well. The plates were covered with an adhesive strip and incubated for 20 minutes in the dark at room temperature. The plates were aspirated and washed three times as described above. About 100 µl of Substrate Solution was added to each well for colour development and incubated for 20 min at room temperature in the dark. Then 50 µl of Stop Solution was added to each well to halt the colour change. The optical density was determined within 30 min, using the EL808 BioTekmicroplate reader company set to 450 nm.

3.14 Genotyping

3.14.1 DNA Extraction

DNA was extracted from leukocytes in the buffy coat samples using the Quick-gDNA™ Blood MiniPrep DNA extraction kit (Epigenetics Company, USA). The concentration and purity of the extracted DNA samples were determined using the NanoDrop 2000/2000C (Thermo Scientific, USA).The DNA samples were stored at -80°C until needed for further analysis.

3.14.2 Polymerase Chain Reaction (PCR)

3.14.2.1 Endothelial nitric oxide synthase (eNOS) genotyping

3.14.2.1.1 The variable number of tandem repeats (VNTR) polymorphism in intron 4

The extracted DNA samples were amplified for polymorphic VNTR in intron 4 by PCR with a standard protocol (Serrano *et a.l*, 2010) and previously published primers (Thomas *et al.*, 2013). The primer sequences were:

5′ CTATGGTAGTGCCTTGGCTGGAGG-3′ (forward) and

5′ ACCGCCCAGGGAACTCCGCT-3′ (reverse),

with conditions altered to 25 µL final volumes. The PCR was performed in heat block PCR tubes to ensure that the mixture did not evaporate in the course of the reaction.

The constituents of each PCR tube and their respective quantities are shown below (Table 3.1). Positive and negative controls were set up. The tubes for the two controls had the same materials as the test PCR tubes, but the 2µL of DNA was replaced with 2µL nuclease free water in the negative control tube. The reaction was performed for 35 cycles in a Gene Pro thermal cycler (HanghouBioer Technology Co. Ltd., China). The conditions for the PCR were as follows: initial denaturation was done at 94 °C for 4 min,

denaturation at 94 °C for 1 min, annealing at 63 °C for 30 sec, extension at 72 °C for 5 min and final extension at 72 °C for 5 min The thermal cycler was set to hold the products at 4 °C until they were retrieved at the end of the 35 cycles (Table 3.2). The products were then resolved on 3% agarose gel.

Table 3.1: Constituents of the PCR Mixture

ORDER	ITEM	VOLUME (µL)
1	PCR water	12.16
2	Q solution	5
3	PCR coral load	2.5
4	$MgCl_2$	3
5	dNTP	1
6	Forward primer	0.125
7	Reverse primer	0.125
8	DNA	1
9	Taq DNA polymerase	0.09
	TOTAL VOLUME	**25**

The PCR mixrsture constituents were added in the order which it is presented in the Table

Table 3.2 PCR conditions VNTR in intron 4 by PCR

ACTIVITY	TEMPERATURE (°C)	TIME
Initial denaturation	94	4 minutes
Denaturation	94	1 minute*
Annealing	63	30 seconds*
Extension	72	5 minutes*
Final extension	72	5 minutes
Holding	4	∞

*repeated for 35 cycles

The PCR conditions were as described by Thomas *et al.*, 2013.

3.14.3 The T786Cpolymorphism in the 5′-flanking region

To genotype for the T786C (rs2070744) variant in the promoter region. This polymorphism was genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), with the primer pairs as follows:

5′ TGGAGAGTGCTGGTGTACCCCA-3'' forward and

5′-GCCTCCACCCCCACCCTGTC-3′.Reverse

Was done for 35 cycles in a volume of 25 µL. The PCR was performed in heat block PCR tubes to ensure that the mixture did not evaporate in the course of the reaction. The constituents of each PCR tube and their respective quantities are shown above (Table 3.1).

Positive and negative controls were set up. The tubes for the two controls had the same materials as the test PCR tubes, but the 2µL of DNA was replaced with 2µL nuclease free water in the negative control tube. The reaction was performed for 35 cycles in a

Gene Pro thermal cycler (HanghouBioer Technology Co. Ltd., China). The conditions for the PCR were as follows:

The PCR mixtures were heated to 94 °C for 4 min for denaturation and underwent 35 cycles at 94 °C for 30 s for denaturation, 65 °C for 30 s for annealing, and 72 °C for 1 min for extension. Finally, extension was conducted at 72 °C for 5 min (Table 3.3).

Table 3.3 PCR Conditions for T786Cpolymorphism in the 5′-Flanking Region

ACTIVITY	TEMPERATURE (°C)	TIME
Initial denaturation	94	4 minutes
Denaturation	94	1 minute*
Amplification	61	30 seconds*
Annealing	72	1 minute*
Final extension	72	5 minutes
Holding	4	∞

*repeated for 35 cycles

The PCR products were digested for 2 hour by Msp I restriction enzyme at 37 °C.

3.14.3.1 Restriction Fragment Length Polymorphism (RFLP) digestion with MspI

Restriction fragment length polymorphism (RFLP) analysis was done on DNA samples of SCD patients and controls. To 5 µL of the PCR product in an eppendorf tube, 9 µL of nuclease-free water, 1 µL of 10x buffer Tango (Thermo Fisher Scientific Inc.) and 1 µL of MspI restriction enzyme (Thermo Fisher Scientific Inc.) were added. The mixture was tapped gently and spun down for 5 seconds. It was then incubated for 2hrs at 37°C in a water bath (U Clear, England).

The RFLP products obtained after the restriction enzyme digestion were resolved on 3% agarose gel. The eNOS polymorphism contains a unique MspIrestriction site, which was expected to give fragment sizes producing fragments of 140 and 40 base pairs for the wild-type (TT) allele, or 90, 50, and 40 base pairs for polymorphic variants (CC).

3.15 Agarose Gel Electrophoresis

The PCR and RFLP products were separated on 3% agarose gel (Thermo Fisher Scientific Inc.) which was prepared with 1X tris acetate diaminoethanetetra- acetic acid (TAE) buffer (AppendixII) the gel was poured and allowed to set (see Appendix I for the preparation of 50X TAE buffer). It was placed in an electrophoretic chamber (Owl Separation System Inc., USA) filled with 750 ml of 1X TAE buffer containing 20 µL of 10 mg/mL ethidium bromide solution (Sigma Chemical, USA; Appendix III). An O'Gene ruler 50 bp DNA ladder (Thermo Fisher Scientific Inc.) was ran alongside the PCR products in the first well. The electrophoresis was performed using 56 mAmp electric current at a potential difference of 80 volts for 90 min. The gel was visualized with an ultraviolet transilluminator (Uvitec, Cambridge, UK) and photographed with a photoman (Uvitec, Cambridge, UK). The images were printed with a video copy processor (Mitsubishi, Malaysia).

3.16 Statistical analysis

All values expressed as mean (M) ± standard deviation (SD) [M±SD]. The differences between the two groups of subjects were analyzed statistically using the Student t-test for unpaired data. Analysis of variance (ANOVA) was used to compare the difference between more than two means of groups of subjects for normally distributed data and Kruskal Wallis for Skewed data. Chi square goodness of fit was used to verify the

agreement of the observed genotype frequencies with those of the expected. Odds ratio [95%confidence interval (CI)] was calculated as an index of association of the eNOS genotypes with HbSS complication. Statistical significance was defined as $p<0.05$. Data was analyzed using SPSS version 20.0 software.

CHAPTER FOUR

RESULTS

4.1 General characteristics

A total of 694 subjects were sampled. This was made up of 148 controls HbAA (93 males, 55 females) with mean age of (31.9± 10.0) years, 208 HbSS SCD patients in steady state (110 males, 98 females) withmean age of (25.5± 9.7) years , 82 HbSC SCD patients in steady state (30 males, 52 females) withmean age of (34.9± 14.1) years, 156 HbSS SCD patients in VOC (64 males, 92 females)with mean age of (26.2 ± 9.4) years, 34 HbSC SCD patients in VOC (12 males, 22 females)with mean age of (32.4± 12.0) years,34 HbSS SCD patients in post VOC (15 males, 19 females) withmean age of (26.3± 9.5) years, 21 HbSS SCD patients with leg ulcer (14 males, 7 females) withmean age of (27.9± 5.6) years, 11 HbSS SCD patients with Priapism mean age of (30.9± 13.0) years.

4.2. Plasma NO levels in controls and SCD patients with and without complications.

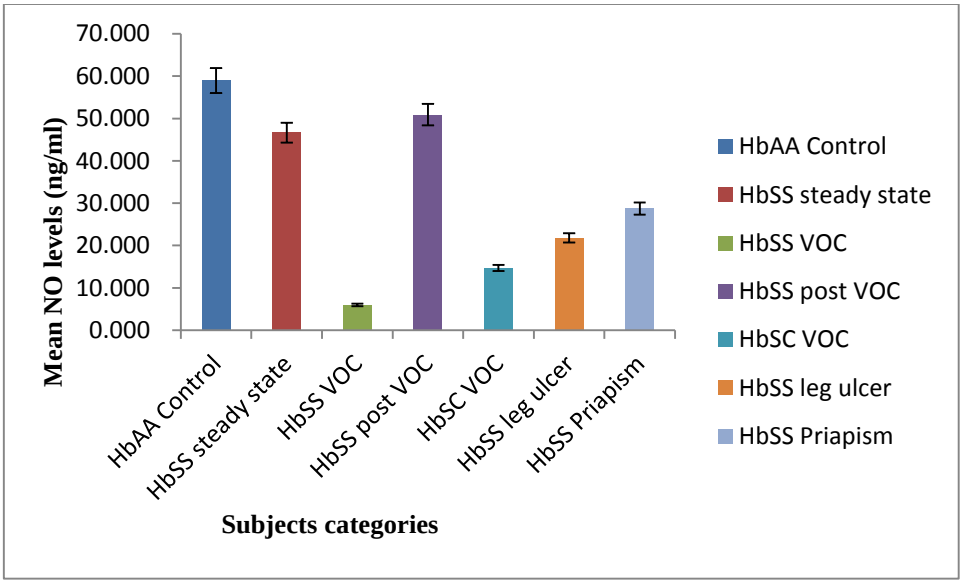

Figure 4.1 Plasma NO levels in controls and SCD categories.

Plasma NO level is significantly low in the SCD complications as compared to the controls, steady state and post-crisis (Fig 4.1)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

Group difference that were not significant are not included

I: NO

HbSC VOC vrs HbSS VOC;	p-value<0.001
HbSS Leg ulcer vrs HbSS VOC;	p-value <0.001
HbSS Leg ulcer vrs HbSC VOC;	p-value =0.0044
HbSS Priapism vrs HbSS VOC;	p-value<0.001
HbSS Priapism vrsHbSC VOC;	p-value=0.0104
HbSS steady state vrs HbSS VOC;	p-value <0.001
HbSS steady state vrs HbSC VOC;	p-value <0.001
HbSS steady state vrs HbSS Leg ulcer;	p-value<0.001
HbSS steady state vrs HbSS priapism;	p-value<0.001
HbSS post VOC vrs HbSS VOC;	p-value <0.001
HbSS post VOC vrs HbSC VOC;	p-value <0.001
HbSS post VOC vrs HbSS Leg ulcer;	p-value<0.001
HbSS post VOC vrs HbSS priapism;	p-value<0.001
HbSS post VOC vrs HbSS steady state;	p-value=0.0216
HbAA control vrs HbSS VOC;	p-value <0.001
HbAA control vrs HbSC VOC;	p-value <0.001
HbAA control vrs HbSS Leg ulcer;	p-value<0.001
HbAA control vrs HbSS priapism;	p-value<0.001
HbAA control vrs HbSS steady state;	p-value<0.001
HbAA control vrs HbSS post VOC;	p-value<0.001

4.3 Haematological parameters for male and female controls HbAA

The mean values of the haematological parameters for male and female controls are presented in Table 4.1 below.

There were significant differences between the mean cell haemoglobin concentration (MCHC) p <0.001; red cell distribution width (RDW) p <0.001; mean platelet volume (MPV) p<0.001; platelets count (PLT) p = 0.025; platelets distribution width (PDW) p = 0.0003; white blood cells (WBC) p= 0.019.

Table 4.2 Haematological comparison between male and female Controls (HbAA)

Parameters	Male (n=93) Mean±SD	Female (n=55) Mean±SD	P-value
Hb (g/dL)	15.38 ± 3.68	13.19 ± 3.32	0.234
HCT (%)	42.20 ± 6.51	40.91 ± 10.94	0.348
RBC (10^6/mm^3)	5.14 ± 0.82	4.97 ± 1.26	0.289
MCV (µm^3)	82.62 ± 6.09	82.62 ± 5.29	0.929
MCH (pg)	27.44± 2.67	26.68 ± 2.05	0.0576
MCHC (g/dL)	33.18±1.36	32.29 ± 0.90	<0.001*
RDW (%)	33.713 ± 13.251	18.587 ± 13.251	<0.001*
MPV (µm$^{3)}$	9.090 ± 1.383	8.153 ± 1.082	<0.001*
PDW (%)	13.761 ± 1.919	14.909 ± 1.970	0.0003*
Plt (10^3/mm^3)	224.22 ± 61.17	250.82 ± 80.77	0.025
PCT (%)	0.18 ± 0.07	0.19 ± 0.06	0.4837
WBC (10^3/mm^3)	5.309±1.107	5.793 ± 1.282	0.019*

SD; Standard Deviation
Hb, haemoglobin; HCT, Haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; **Plt** platelets count, PCT plateletcrit. *Significant at p≤ 0.05

4.3.1 Haematological parameters for male and female HbSC patients in steady state

The Table 4.2 below shows the haematological parameters of male and female HbSC patients. From this table, there were significant differences in the Hb; (p=<0.001), HCT; (p<0.001), RBC; (p=0.0001), PDW; (p=0.0117) and WBC; (p=0.0219) between both sexes.

Table 4.3 Haematological comparison between male and female HbSC patients in steady state

Parameters	Male (n=30) Mean±SD	Female (n=52) Mean±SD	P-value
Hb (g/dL)	12.51 ± 1.33	10.74 ± 1.73	<0.001*
HCT (%)	39.83 ± 4.63	33.02 ± 7.08	<0.001*
RBC (10^6/mm^3)	4.82 ± 0.49	4.04 ± 0.94	0.0001*
MCV (μm^3)	82.90 ± 5.15	81.96 ± 8.53	0.5860
MCH (pg)	26.02 ± 1.83	26.61±2.26	0.4225
MCHC (g/dL)	30.02 ± 1.831	31.86 ± 1.15	0.308
RDW (%)	14.97 ± 1.35	15.01 ± 2.58	0.9367
MPV (μm^3)	8. 32 ± 1.01	8. 23 ± 1.01	0.9657
PDW (%)	14.23 ± 2.23	12.66 ± 2.89	0.0117*
PLT (10^3/mm^3)	289.64 ± 18.31	322.87 ± 20.70	0.2492
PCT (%)	0.23 ± 0.08	0.26 ± 0.09	0.1980
WBC (10^3/mm^3)	6.87± 1.77	9.32 ± 2.56	0.0219*

SD; Standard Deviation
Hb, haemoglobin; HCT;haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW;red cell distribution width, MPV; mean platelet volume, PLT; platelets count, PCT; plateletcrit.PDW platelets distribution width; WBC white blood cells.
*Significant at p≤ 0.05

4.3.2 Haematological parameters for male and female HbSC patients in VOC

A comparison of the Haematological parameters of male and female HbSC patients in VOC showed no significant differences in all the haematological parameters (Table 4.3)

Table 4.4 Haematological comparison between male and female HbSC patients in VOC

Parameters	Male (n=12) Mean±SD	Female (n=22) Mean±SD	P-value
Hb (g/dL)	12.10 ± 1.63	11.26 ±1.50	0.0505
HCT (%)	38.345 ± 4.37	35.209 ± 4.66	0.0725
RBC (10^6/mm^3)	4.45 ± 0.53	4.28 ± 0.56	0.4006
MCV (µm^3)	84.42 ± 6.20	82.50 ± 7.61	0.4611
MCH (pg)	27.200 ± 2.04	26.427 ± 2.87	0.4161
MCHC (g/dL)	32.24 ± 0.58	31.96 ± 1.03	0.4061
RDW (%)	15.43 ± 1.16	14.86 ± 1.16	0.2683
MPV (µm^3)	7.875 ±0.766	7.936 ± 0.781	0.8270
PDW (%)	13.42 ± 2.22	12.79 ± 2.22	0.5024
PLT (10^3/mm^3)	238.558± 109.941	319.00 ± 114.88	0.0563
PCT (%)	0.19 ± 0.07	0.25 ± 0.08	0.0997
WBC (10^3/mm^3)	9.78 ± 2.09	8.25 ± 1.52	0.2632

SD; Standard Deviation
Hb;haemoglobin; HCT;haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW; red cell distribution width, MPV mean platelet volume, PLT platelet count, PCT plateletcrit. PDW platelet distribution width; WBC white blood cells.

4.3.3 Haematological parameters for male and female HbSS patients in steady state

In comparing the Haematological parameters of male and female HbSS steady state patients, significant differences were observed in MCV; p= 0.0411, MCH; p= 0.0145, MCHC; p=0.0205 , RDW; p= 0.0431 and WBC; p = 0.0016 as in Table 4.4 below.

Table 4.5 Haematological comparison between male and female HbSS patients in steady state

Parameters	Male (n=110) Mean±SD	Female (n=98) Mean±SD	p-value
Hb (g/dL)	8.532 ± 1.610	8.308 ±1.570	0.3128
HCT (%)	25.82 ± 5.15	24.84 ± 5.18	0.1705
RBC (10^6/mm3)	3.06±0.71	2.89±0.77	0.0892
MCV (µm^3)	84.97 ± 8.47	87.44 ± 8.83	0.0411*
MCH (pg)	28.20 ± 3.51	29.42 ± 3.60	0.0145*
MCHC (g/dL)	33.12 ±1.46	33.59 ± 1.44	0.0205*
RDW (%)	18.56 ± 2.54	17.82 ± 2.68	0.0431*
MPV (µm^3)	7.466 ± 0.686	7.316 ± 0.697	0.1197
PDW (%)	13.915 ± 2.258	14.101 ± 1.956	0.5275
PLT (10^3/mm^3)	466.33 ± 121.39	461.95 ± 160.55	0.8236
PCT (%)	0.35 ±0.10	0.33 ±0.11	0.3045
WBC (10^3/mm^3)	12.18±3.56	10.69±3.11	0.0016*

SD; Standard Deviation
Hb;haemoglobin; HCT;haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW; red cell distribution width, MPV mean platelet volume, PLT platelet count, PCT plateletcrit. PDW platelet distribution width; WBC white blood cells.
* Significant at p≤ 0.05

4.3.4 Haematological parameters for male and female HbSS patients in VOC

In comparing the haematological parameters between male and female HbSS patients in VOC,There were significant difference in Hb; p= 0.0035, HCT; p= 0.0034, RBC; p=0.0015, MCV; p< 0.001, RDW; p=0.0048 and WBC; p= 0.0012 (Table 4.5).

Table 4.6 Haematological comparison between male and female HbSS patients in VOC

Parameters	Male (n=64) Mean±SD	Female (n=92) Mean±SD	p-value
Hb (g/dL)	9.24 ±1.67	8.42 ±1.68	0.0035*
HCT (%)	28.37 ±6.01	25.58 ±5.74	0.0034*
RBC (10^6/mm3)	3.50 ±0.89	3.05 ±0.87	0.0015*
MCV (µm^3)	82.59 ± 9.45	85.50 ± 9.45	<0.001*
MCH (pg)	27.50 ± 6.95	28.40 ±4.02	0.3078
MCHC (g/dL)	33.03 ±4.92	33.11 ±1.69	0.8848
RDW (%)	18.56 ±2.89	17.39 ±2.20	0.0048*
MPV (µm^3)	7.652 ±0.814	7.624 ± 0.704	0.8213
PDW (%)	13.194 ±3.006	13.529 ± 2.738	0.4707
PLT (10^3/mm^3)	439.34 ±158.77	459.09 ±193.64	0.5019
PCT (%)	0.34 ± 0.12	0.35 ±0.14	0.6394
WBC (10^3/mm^3)	16.24 ±7.37	13.01 ±4.83	0.0012*

SD; Standard Deviation
Hb;haemoglobin; HCT;haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW; red cell distribution width, MPV mean platelet volume, PLT platelet count, PCT plateletcrit.PDW platelet distribution width; WBC white blood cells.
*significant at p≤ 0.05

4.3.5 Haematological parameters for male and female HbSS patients in post VOC

A comparison of the Haematological parameters of male and female HbSS patients in post VOC showed no significant differences in all the Haematological parameters (Table 4.6)

Table 4.7 Haematological comparison between male and female HbSS patients in post VOC

Parameters	Male (n=15) Mean±SD	Female (n=19) Mean±SD	P-value
Hb (g/dL)	9.17 ±1.84	8.17 ±1.35	0.1162
HCT (%)	28.18 ±6.22	25.11 ± 4.79	0.1584
RBC (10^6/mm3)	3.42±0.72	3.02 ±0.70	0.2053
MCV (µm^3)	82.60 ±7.21	83.83 ±8.04	0.8067
MCH (pg)	26.96 ±3.02	27.59 ±3.66	0.7172
MCHC (g/dL)	32.59 ±1.27	32.86 ±2.11	0.6913
RDW (%)	17.77 ±2.80	17.90 ± 2.08	0.8810
MPV (µm^3)	7.653 ± 0.623	7.695 ± 0.690	0.8574
PDW (%)	14.607 ±2.187	15.084 ± 2.144	0.5272
PLT (10^3/mm^3)	443.00 ±136.11	453.47 ±222.51	0.8740
PCT (%)	0.34 ±0.09	0.34 ± 0.14	0.8814
WBC (10^3/mm^3)	13.45 ±6.37	12.02 ±4.40	0.4349

SD; Standard Deviation
Hb;haemoglobin; HCT;haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW; red cell distribution width, MPV mean platelet volume, PLT platelet count, PCT plateletcrit.PDW platelet distribution width; WBC white blood cells.

4.3.6 Haematological parameters for male and female HbSS leg ulcer patients

A comparison of the Haematological parameters of male and female HbSS leg ulcer patients showed significant differences in MPV; p=0.0088 and RDW; p=0.0080 between the sexes (Table 4.7).

Table 4.8 Haematological comparison between male and female HbSS leg ulcer patients.

Parameters	Male (n=14) Mean ±SD	Female (n=7) Mean ±SD	p-value
Hb (g/dL)	9.98 ±5.50	8.95 ±1.85	0.2986
HCT (%)	31.58 ±5.05	27.55 ±6.16	0.1744
RBC (10^6/mm3)	4.29 ±0.61	3.39±0.91	0.0500
MCV (µm^3)	82.40 ± 9.79	74.40 ± 12.97	0.2928
MCH (pg)	26.88 ±3.85	23.62 ±5.53	0.1238
MCHC (g/dL)	32.65 ±1.19	31.52 ±2.23	0.1369
RDW (%)	18.831 ±1.939	18.060 ±5.216	0.6107
MPV (µm^3)	8.056 ±0.807	5.800 ± 3.121	0.0088*
PDW (%)	14.439 ± 1.386	10.220 ± 5.865	0.0080*
PLT (10^3/mm^3)	428.56 ±193.94	385.20 ± 215.75	0.6698
PCT (%)	0.34 ±0.14	0.29 ±0.18	0.5189
WBC (10^3/mm^3)	10.35 ±4.03	6.96 ±3.41	0.1022

SD; Standard Deviation
Hb; haemoglobin; HCT; haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDW; red cell distribution width, MPV mean platelet volume, PLT platelet count, PCT plateletocrit.PDW platelet distribution width; WBC white blood cells.
*significant at p≤ 0.05

4.3.7 Haematological parameters among controls and sickle cell disease patients with different complications in males

Table 4.9 A comparison of the mean Haematological parameters among controls and sickle cell disease patients with different complications in males

Parameters	Control (n=93)	HbSS steady state (n=110)	HbSC steady state (n=30)	HbSS VOC (n=64)	HbSC VOC (n-12)	HbSS post VOC (n=15)	HbSS leg ulcer (n=14)	HbSS Priapism (n=11)	p-value
Hb (g/dL)	15.38± 3.68	8.532± 1.610	12.51 ± 1.33	9.24 ±1.67	12.10 ± 1.63	9.17 ±1.84	8.95 ±1.85	8.75± 2.12	<0.001
HCT (%)	42.20± 6.51	25.82± 5.15	39.83 ± 4.63	28.37± 6.01	38.345 ± 4.37	28.18 ±6.22	27.55 ± 6.16	26.30 ± 6.82	<0.001*
RBC (10^6/mm3)	5.14 ± 0.82	3.06± 0.71	4.82 ± 0.49	3.50±0.89	4.45 ± 0.53	3.42±0.72	3.39 ±0.91	3.055 ± 0.784	<0.001*
MCV (µm^3)	82.62± 6.09	84.97± 8.47	82.90 ± 5.15	82.59 ± 9.45	84.42 ± 6.20	82.60 ±7.21	82.40 ± 9.79	86.88 ± 4.94	0.185
MCH (pg)	27.44± 2.67	28.20± 3.51	26.02 ± 1.83	27.50 6.95	27.200 ± 2.04	26.96 ±3.02	26.88 ±3.85	28.96 ± 1.82	0.274
MCHC (g/dL)	33.18± 1.36	33.12± 1.46	30.02 ± 1.831	33.03 ±4.92	32.24 ± 0.58	32.59 ±1.27	32.65 ±1.19	33.46 ± 1.14	<0.001*
RDW (%)	33.713± 13.251	18.56± 2.54	14.97 ± 1.35	18.56 ±2.89	15.43 ± 1.16	17.77 ±2.80	18.831 ±1.939	19.40 ± 3.24	<0.001*
MPV (µm^3)	9.090± 1.383	7.466± 0.686	8. 32 ± 1.01	7.652 ±0.814	7.875 ±0.766	7.653 ± 0.623	8.056 ±0.807	8.30 ± 0.885	<0.001*
PDW (%)	13.761± 1.919	13.915± 2.258	14.23 ± 2.23	13.194 ±3.006	13.42 ± 2.22	14.607 ±2.187	14.439 ± 1.386	15.25 ± 1.052	0.0900
PLT (10^3/mm^3)	224.22± 61.17	466.33± 121.39	289.64 ± 18.31	439.34 ±158.77	238.558 ± 109.941	443.00 ±136.11	428.56 ±193.94	381.6 ± 185.19	<0.001*
PCT (%)	0.18 ± 0.07	0.35 ± 0.10	0.23 ± 0.08	0.34 ± 0.12	0.19 ± 0.07	0.34 ±0.09	0.34 ±0.14	0.3061 ± 0.13183	<0.001*
WBC (10^3/mm^3)	5.309± 1.107	12.18± 3.56	6.87± 1.77	16.24 ±7.37	9.78 ± 2.09	13.45 ±6.37	10.35 ±4.03	10.95 ± 2.213	<0.001*

Mean ± SD. MCV, mean cell volume; MCH, mean cell haemoglobin; RDWRed cell distribution width, PDWplatelets distribution width.

*significant at p≤ 0.05

The Haematological profiles in males such as haemoglobin (Hb), Haematocrit (HCT), red blood cells (RBC), mean corpuscular haemoglobin concentration (MCHC), Red cell distribution width (RDW), mean platelet volume (MPV),platelets count(Plt),platelet crit(Pct) and white blood cells (WBC) is statistically significant across board (Table 4.8)

Pair wise analysis: Student's t-test analyses of group difference were as follows:
Group difference that were not significant are not included

I: Hb

a. Control vrsHbSS steady state; p-value = <0.001
b. Control vrsHbSS VOC; p-value = <0.001
c. Control vrsHbSS post VOC; p-value = 0.0022
d. Control vrsHbSSleg ulcer; p-value = 0.0006
e. Control vrspriapism; p-value = 0.0132
f. HbSS steady statevrsHbSC steady state; p-value = 0.0079
g. HbSS VOCvrsHbSC steady state; p-value = 0.0418

For the Pair wise analysis: Student's t-test analyses of group difference for HCT, RBC, MCHC, RDW, PLT, PCT and WBC refer to Appendix IV.

4.3.8 Haematological parameters among controls and sickle cell disease patients with different complications in females

Table 4.10 Comparison of Haematological parameters among controls and sickle cell disease patients with different complications females

Parameters	Control (n=55)	HbSS steady state (n=98)	HbSC steady state (n=52)	HbSS VOC (n=92)	HbSC VOC (n-22)	HbSS post VOC (n=19)	HbSS leg ulcer (n=7)	p-value
Hb (g/dL)	13.19± 3.32	8.308 ±1.570	10.74± 1.73	8.42 ±1.68	11.26 ±1.50	8.17 ±1.35	9.98 ±5.50	<0.001*
HCT (%)	40.91± 10.94	24.84± 5.18	33.02± 7.08	25.58 ±5.74	35.209 ± 4.66	25.11 ± 4.79	31.58 ±5.05	<0.001*
RBC (10^6/mm3)	4.97 ± 1.26	2.89±0.77	4.04 ± 0.94	3.05 ±0.87	4.28 ± 0.56	3.02 ±0.70	4.29 ±0.61	<0.001*
MCV (µm^3)	82.62± 5.29	87.44± 8.83	81.96± 8.53	85.50 ± 9.45	82.50 ± 7.61	83.83 ±8.04	74.40 ± 12.97	<0.001*
MCH (pg)	26.68± 2.05	29.42± 3.60	26.61±2.26	28.40 ±4.02	26.427 ± 2.87	27.59 ±3.66	23.62 ±5.53	0.5410
MCHC (g/dL)	32.29± 0.90	33.59± 1.44	31.86± 1.15	33.11 ±1.69	31.96 ± 1.03	32.86 ±2.11	31.52 ±2.23	0.4588
RDW (%)	18.587± 13.251	17.82± 2.68	15.01± 2.58	17.39 ±2.20	14.86 ± 1.16	17.90 ± 2.08	18.060 ±5.216	<0.001*
MPV (µm^3)	8.153± 1.082	7.316± 0.697	8. 23± 1.01	7.624 ± 0.704	7.936 ± 0.781	7.695 ± 0.690	5.800 ± 3.121	<0.001*
PDW (%)	14.909± 1.970	14.101± 1.956	12.66± 2.89	13.529 ± 2.738	12.79 ± 2.22	15.084 ± 2.144	10.220 ± 5.865	<0.001*
PLT (10^3/mm^3)	250.82± 80.77	461.95± 160.55	322.87± 20.70	459.09 ±193.64	319.00 ± 114.88	453.47 ±222.51	385.20 ± 215.75	<0.001*
PCT (%)	0.19 ± 0.06	0.33 ±0.11	0.26 ± 0.09	0.35 ±0.14	0.25 ± 0.08	0.34 ± 0.14	0.29 ±0.18	<0.001*
WBC (10^3/mm^3)	5.793 ± 1.282	10.69±3.11	9.32 ± 2.56	13.01 ±4.83	8.25 ± 1.52	12.02 ±4.40	6.96 ±3.41	<0.001*

Hb, haemoglobin; HCT, Haematocrit; RBC, red cell count; MCV, mean cell volume; MCH, mean cell haemoglobin; MCHC, mean cell haemoglobin concentration; RDWRed cell distribution width, MPV mean platelet volume, PLTplatelets count, PCTplatelet crit. PDW platelets distribution width of; WBC white blood cells.*significant at p≤ 0.05

The Haematological profiles in females is thus haemoglobin (Hb), Haematocrit (HCT), red blood cells (RBC), platelets distribution width(PDW) Red cell distribution width (RDW), mean platelet volume **(**MPV),platelets count(Plt),platelet crit(Pct) andwhite blood cells (WBC) is statistically significant across board(Table 4.9).

Pair wise analysis: Student's t-test analyses of group difference were as follows:

Group difference that were not significant are not included

I: Hb

a. HbSC steady state vrs HbSS post VOC; p-value=<0.001
b. HbSC VOC vrs HbSS post VOC; p-value=<0.001
c. Control vrs HbSS post VOC; p-value=<0.001
d. HbSS steady state vrs HbSS steady state; p-value=<0.001
e. HbSC VOC vrs HbSS steady state; p-value=<0.001
f. Control vrs HbSS steady state; p-value=<0.001
g. HbSC steady state vrs HbSS VOC; p-value=<0.001
h. HbSC VOC vrs HbSS VOC; p-value=<0.001
i. Control vrs HbSS VOC; p-value=<0.001
j. Control vrs leg ulcer; p-value=<0.001
k. Control vrs HbSC steady state; p-value=<0.001
l. Control vrs HbSC VOC; p-value=<0.001

For the Pair wise analysis: Student's t-test analyses of group difference for HCT, RBC, RDW, MCV,PLT, PCT and WBC refer to Appendix V.

4.4. Plasma ICAM-1, VCAM-1 and E-selectin levels in controls and SCD patients with and without complications.

The Plasma levels ofICAM-1, VCAM-1 and E-selectin were elevated in SCD patients p< 0.001for all three. Furthermore, these levels were still higher in the SCD complication ICAM-1; 62.42 ± 26.09, VCAM-1; 634.99 ± 324.31 and E-selectin 236.77 ± 114.40 as shown in Table 4.10

Table 4.11 Plasma ICAM-1, VCAM-1 and E-selectin levels in controls and SCD patients with and without complications.

Parameter	HbAA Control (n=60)	HbSS Steady State (n=46)	HbSS VOC (n=57)	HbSC VOC (n=18)	HbSS leg ulcer (n=21)	HbSS Priapism (n=11)	p-value
ICAM-1 (ng/ml)	29.60 (12.03-40.32)	48.09 (24.72-70.14)	62.42 (26.09-62.42)	31.67 (13.56-68.94)	45.00 (17.42-95.06)	61.13 (34.46-81.18)	<0.001
VCAM-1 (ng/ml)	286.10 (179.36-356.21)	490.10 (314.45-980.15)	634.99 (324.31-934.69)	540.32 (258.73-876.37)	430.74 (143.48-739.75)	455.56 (374.24-852.49)	<0.001
E-selectin (ng/ml)	157.49 (138.96-543.53)	227.87 (119.68-624.46)	236.77 (114.40-632.50)	219.44 (92.51-829.49)	228.94 (94.83-721.96)	193.12 (70.24-562.18)	<0.001

Median (interquatile range)

Pair wise analysis: analyses of group difference were as follows:

Group difference that were not significant are not included

ICAM-1

a. HbSC VOC vrs HbSS VOC; p<0.001
b. HbSC VOC vrs HbSS steady state; p =0.009
c. HbSC VOC vrspriapism; p =0.002
d. HbSS VOC vrs HbSC VOC; p<0.001
e. HbSS VOC vrs HbSS steady state; p<0.001
f. HbSS VOC vrs leg ulcer; p=0.006

g. HbSS VOC vrs controls; p<0.001

h. HbSS steady state vrs HbSC VOC; p=0.009

i. HbSS steady state vrs HbSS VOC; p<0.001

j. HbSS steady state vrs controls; p<0.001

k. Priapismvrs HbSC VOC; p=0.0012

l. Priapismvrs controls; p<0.001

m. leg ulcer vrs HbSS VOC ; p =0.006

n. leg ulcer vrs controls; p =0.023

o. control vrs HbSS VOC ; p<0.001

p. control vrs HbSS steady state; p<0.001

q. control vrsPriapism; p=0.023

VCAM

a. HbSC VOC vrs controls; p =0.005

b. HbSS VOC vrs HbSS steady state; p =0.020

c. HbSS VOC vrs leg ulcer; p =0.022

d. HbSS VOC vrs controls; p<0.001

e. HbSS steady state vrs HbSS VOC ; p=0.020

f. HbSS steady state vrs controls; p=0.004

g. Leg ulcer vrs HbSS VOC ; p=0.022

h. Control vrs HbSC VOC ; p=0.005

i. Control vrs HbSS VOC ; p<0.001

j. Control vrs HbSS steady state; p=0.004

E-selectin

a. HbSC VOC vrs Control; p=0.023

b. HbSS VOC vrs Control; p<0.001

c. HbSS steady state vrs Control; p<0.001

d. Priapismvrs leg ulcer; p=0.036

e. leg ulcer vrsPriapism; p=0.036

f. leg ulcer vrs Control; p<0.001

g. Control vrs HbSC VOC ; p=0.023

h. Control vrs HbSS VOC ; p<0.001

i. Control vrs HbSS steady state; p=0.001

j. Control vrs leg ulcer; p<0.001

4.5 Plasma Ang-1, Ang-2 and VEGF levels in controls and SCD patients with and without complications.

There were raised plasma levels of Ang-1, Ang-2, VEGF and Ang-1/Ang-2 ratioin SCD patients, p<0.001 in all.SCD leg ulcer patients recorded the highest ratio for Ang-2/Ang-1 with 0.35 and the lowest Ang-1/Ang-2 ratio Tables 4.11. However, HbAA controls recorded the lowest Ang-2/Ang-1 ratio and highest Ang-1/Ang-2 ratio.

Table 4.12 Plasma Ang-1, Ang-2 and VEGF levels in controls and SCD patients with and without complications.

Parameter	HbAA Control n=60	HbSS Steady State n=46	HbSS VOC n=57	HbSC VOC n=18	HbSS leg ulcer n=21	HbSS Priapism n=11	p -value
Ang1 (pg/ml)	9,140.32 (6,360.4-21,722.175)	10,569.13 (5,206.56-18,569.13)	22,695.69 (15,770.71-46640.89)	20,833.96 (10,054.20-33,004.62	15,664.92 (6,848.75-27,351.24)	15,598.21 (1,828.61-20,137.51)	< 0.001
Ang-2 (pg/ml)	843.47 (551.3-898.9)	2,187.34 (1,830.28-4,051.51)	6,058.18 (2,525.35-9,058.18)	4,095.17 (1,400.04-9,095.17)	5,368.34 (1,892.73-7384.16)	4,218.51(1,123.32-6,214.37)	< 0.001
VEGF (pg/ml)	36.55 (23.82 - 52.15)	45.15 (20.01 - 60.15)	70.88 (22.64 - 90.71)	56.03 (13.05-79.08)	61.51 (44.36-86.97	54.85 (37.74-73.81)	< 0.001
Ang-2/ Ang-1 ratio	0.09	0.21	0.30	0.20	0.35	0.27	
Ang-1/ Ang-2 ratio	10.83	4.84	3.33	4.89	2.83	3.70	

Median (interquatile range)

Pair wise analysis: analyses of group difference were as follows:

Group difference that were not significant are not included

Ang-1

HbAA Controls vrs HbSS VOC	= 0.001
HbAA Controls vrs HbSC VOC	= 0.05
HbSS steady state vrs HbSS VOC	= 0.003
HbSS steady state vrs HbSC VOC	= 0.05

Ang-2

HbAA Controls vrs HbSS VOC	= 0.001
HbAA Controls vrs HbSC VOC	= 0.05
HbAA Controls vrs HbSS steady state	= 0.0062
HbAA Controls vrs HbSS leg ulcer	= < 0.05
HbAA Controls vrs HbSS Priapism	= 0.001
HbSS steady state vrs HbSS VOC	= < 0.001
HbSS steady state vrs HbSC VOC	= 0.001
HbSS steady state vrs HbSS leg ulcer	= <0.05
HbSS steady state vrs HbSS Priapism	= 0.014

VEGF

HbAA Controls vrs HbSS VOC	= <0.05
HbAA Controls vrs HbSC VOC	= <0.05
HbAA Controls vrs HbSS steady state	= 0. 02
HbAA Controls vrs HbSS leg ulcer	= < 0.05
HbAA Controls vrs HbSS Priapism	= 0.01
HbSS steady state vrs HbSS VOC	= < 0.001
HbSS steady state vrs HbSC VOC	= 0.0164
HbSS steady state vrs HbSS leg ulcer	= <0.05

4.6 Haematology, angiogenic factors and endothelial markers in controls and SCD patients with and without complications

Table 4.13 Relationship between haematology, angiogenic factors and endothelial markers in controls and SCD patients with and without complications.

Parameter	HbAA Control (n=60)	HbSS Steady State (n=46)	HbSS VOC (n=57)	HbSC VOC (n=18)	HbSS leg ulcer (n=21)	HbSS Priapism (n=11)	p-value
Hb (g/dL)	15.38 ± 3.68	8.532 ± 1.610	9.24 ±1.67	12.10 ± 1.63	8.95 ±1.85	8.75± 2.12	<0.001*
HCT (%)	39.31 ± 4.04	25.36 ± 4.46	27.63 ± 5.10	36.42 ± 5.52	27.02 ± 3.64	27.05 ± 8.08	<0.001*
RBC (10^6/mm3)	5.46 ± 1.21	12.02 ± 3.16	13.66 ± 4.66	8.05 ± 3.46	11.03 ± 1.35	8.96 ± 4.64	<0.001*
PLT (10^3/mm^3)	228.21 ± 52.17	452.23 ± 120.37	286.64 ± 13.34	439.34 ±148.77	238.55 ± 109.941	443.00 ±136.11	<0.001*
PCT (%)	0.16 ± 0.03	0.34 ± 0.10	0.22 ± 0.04	0.32± 0.12	0.19 ± 0.07	0.32 ±0.09	<0.001*
WBC (10^3/mm^3)	47.32 ± 18.83	61.24 ± 47.80	47.02 ± 25.03	72.69 ± 57.33	48.39 ± 29.82	62.35 ± 20.51	0.057
	36.53 ± 27.89	30.45 ± 14.05	8.69 ± 17.45	10.93 ± 14.79	15.10 ± 8.16	20.56 ± 55.35	<0.001*
ICAM-1 (ng/ml)	29.60 ± 12.03	48.09 ± 24.72	62.42 ± 26.09	31.67 ± 13.56	45.00 ± 17.42	61.13 ± 34.46	<0.001
VCAM-1 (ng/ml)	286.10 ± 279.36	490.10 ± 314.45	634.99 ± 324.31	540.32 ± 258.73	430.74 ± 343.48	455.56 ± 374.24	<0.001
E-selectin (ng/ml)	157.49 ± 38.96	227.87 ± 89.68	236.77 ± 114.40	219.44 ± 104.51	277.94 ± 94.83	193.12 ± 70.24	<0.001
Ang- 1 (pg/ml)	9,140.32 ± 780.63	10,569.13 ± 1,206.56	22,695.69 ± 5,786.64	20,833.96± 4,853.09	15,664.92 ± 6,848.75	15,598.21± 1,828.61	< 0.05
Ang-2 (pg/ml)	843.47 ± 188.52	2,187.34 ± 1,830.28	6,058.18 ± 2,525.35	4,095.17± 1,400.04	5,368.34 ± 1,892.73	4,218.51± 1,123.32	< 0.05
VEGF (pg/ml)	36.55 ± 8.67	45.15 ± 6.01	70.88 ± 22.64	56.03 ± 13.05	61.51 ± 11.25	54.85 ± 8.74	< 0.05

Pair wise analysis: Student's t-test analyses of group difference were as follows:
Group difference that were not significant are not included

I: Hb

a. Control vrsHbSS steady state; p-value = <0.03
b. Control vrsHbSS VOC; p-value = <0.001
c. Control vrsHbSS post VOC; p-value = 0.002
d. Control vrsHbSS leg ulcer; p-value = 0.001
e. Control vrspriapism; p-value = 0.011
f. HbSS steady statevrsHbSC steady state; p-value = 0.006
g. HbSS VOCvrsHbSC steady state; p-value = 0.044

For the Pair wise analysis: Student's t-test analyses of group difference for HCT, RBC, RDW, PLT, PCT and WBC, ICAM-1, VCAM-1, E-selectin, Angiopoitin 1, Angiopoitin 2, VEGF and NO refer to Appendix VI.

4.7 Electrophoresis

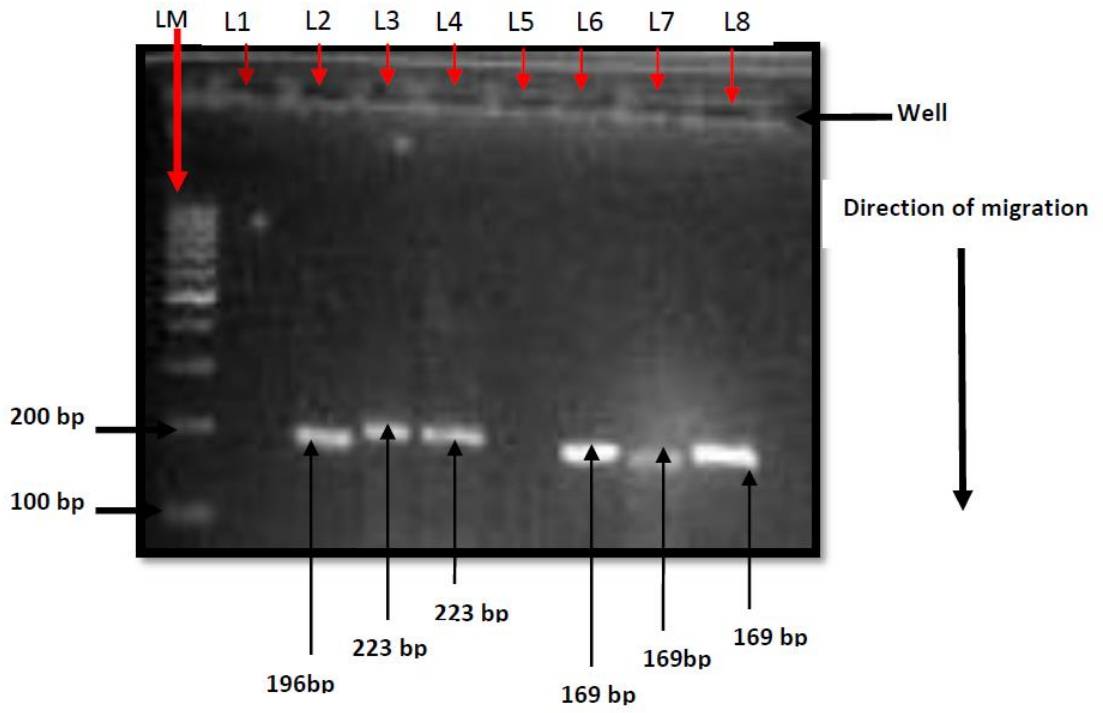

Figure 4.2A: sample of Agarose gel electrophoresis showing PCR products of the VNTRs of eNOS gene polymorphism. LM contains a standard size DNA ladder. L1 and L5 contain no DNA samples. L2, L3, L4,L6, L7, L8 contain PCR product of 196,223 and 223, 169,169 and 169 respectively. bp=base pairs

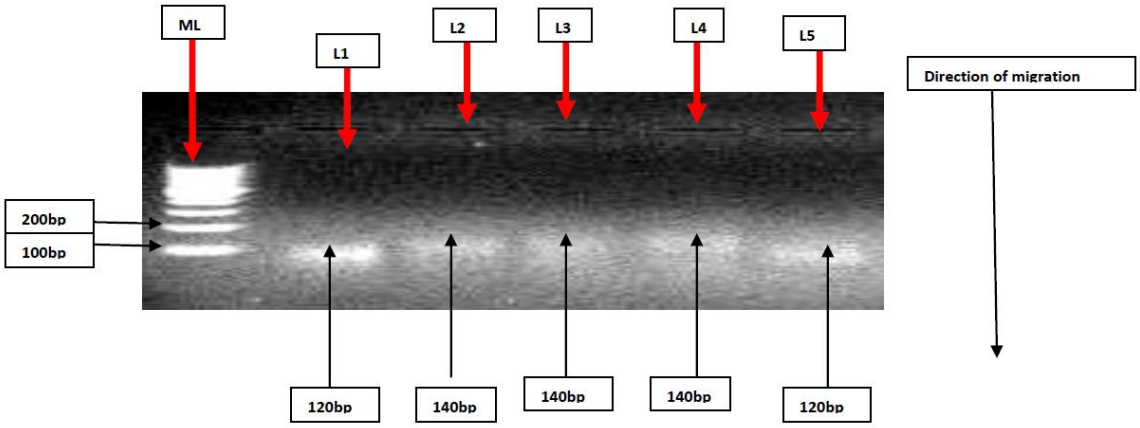

Figure 4.2B: Sample of Agarose gel electrophoresis showing variants of the T786C of the eNOS gene polymorphism. LM contains a standard size DNA ladder. L1, L2, L3, L4 and L5 contain amplicon of 120bp, 140bp, 140bp, 140bp and 120bp respectively. to L5 contain no DNA samples. L2 to L4 contains undigested PCR product of the T786C polymorphism of the eNOS gene with expected molecular size of 180bp. L6 to L8 indicated the MspI digested product of molecular size around 140bp but the 40bp has run out of the gel. bp=base pairs

4.8 Genotypic frequencies of the T786C and the VNTRs of the eNOS polymorphism in the study population

The genotypic frequencies of the study population is presented in Table 4.13 below

The TC haplotypes recorded high frequencies in SCD but low in controls. The CC haplotypes also presented high frequencies in all SCD patients categories except HbSS steady state and controls.

Table 4.14 Genotypic frequencies of study population for T786C polymorphism of the eNOS gene

Haplotype	**Control n=60(%)**	**HbSS steady state n=46(%)**	**HbSS VOC** n=57(%)	**HbSC VOC** n=18(%)	**HbSS leg ulcer** n=21(%)	**HbSS Priapism** n=11(%)
TT	**46 (77)**	**5(11)**	**4 (7)**	**2(11)**	**1(5)**	**2 (18)**
TC	**5 (8)**	**12(26)**	**20(35)**	**3 (17)**	**11(52)**	**4 (36)**
CC	**9 (15)**	**28(61)**	**31(54)**	**9(50)**	**9(43)**	**4 (36)**
T_{NEW}	**0 (0)**	**1(2)**	**2(4)**	**0 (0)**	**0(0)**	**1(9)**

Number (%)

The TT corresponds to the fragments length of 140bp of the T786C mutant gene.A new mutant length was found in the T786C mutant genewhich was neither TT nor CC asreported in literature (Thomaset al., 2013;Thakuret al., 2014) with fragments length of about 120bp it was therefore refered to as T_{NEW} in this study.

Priapism which recorded a frequency of 36% VNTR 4a and VNTR4b showed high frequencies in HbSS leg ulcers (48%) and HbAA controls (40%) respectively. VNTR 4c low in all disease categories except HbSS leg ulcers where it is absent. VNTR 4d was also very low 2% and 17% in HbSS VOC and HbSC respectively and absent in most categories. The differences in the proportions were significant by the chi square test p= 0.037. Refer to table 4.14

Table 4.15 Genotypic frequencies of study population for VNTR polymorphism of the eNOS gene

Haplotype	**Control n=60(%)**	**HbSS steady state n=46(%)**	**HbSS VOC** n=57(%)	**HbSC VOC** n=18(%)	**HbSS leg ulcer** n=21(%)	**HbSS Priapism** n=11(%)
VNTR 4a	**24 (40)**	**21(46)**	**28 (49)**	**8 (44)**	**9(43)**	**4(36)**
VNTR 4b	**20 (33)**	**17 (37)**	**21 (37)**	**6 (33)**	**10 (48)**	**6(55)**
VNTR 4c	**16 (27)**	**8 (17)**	**6 (11)**	**3(17)**	**1 (10)**	**1 (9)**
VNTR 4d	**0(0)**	**0 (0)**	**2 (3)**	**1 (6)**	**0 (0)**	**0 (0)**

Number (%)

VNTR4a = variable number of tandem repeats allele 4a is defined by the presence of 4 repeats of 27bp corresponding to a molecular length of 169 bp.
VNTR4b= variable number of tandem repeats allele 4b is defined by the presence of 5 repeats of 27 bp corresponding to a molecular length of 196 bp.
VNTR4c= variable number of tandem repeats allele 4c is defined by the presence of 6 repeats of 27 bp corresponding to a molecular length of 223 bp.
VNTR4d= variable number of tandem repeats allele 4d is defined by the presence of 3 repeats of 27 bp corresponding to a molecular length of 142 bp.
(Thomaset al., 2013;Thakuret al., 2014)

4.9 Comparison of the observed haplotypic frequencies with the expected Hardy-Weinberg frequencies (equilibrium).

From the analysis the observed haplotypic frequencies for both the TT and the TC polymorphisms were consistent with Hardy-Weinberg equilibrium (Table 4.14).

Table 4.16 Haplotypic frequencies for various eNOS polymorphisms TT and TC in the SCD categories and controls.

	TT allele	TC allele	
Disease category	**Observed (Expected≠)**	**Observed (Expected≠)**	**p-value**
CONTROL	27 (27.4)	7(4.3)	0.50
HbSS steady state	37 (37.3)	7(6.4)	0.57
HbSS VOC	39(39.6)	11(9.8)	0.38
HbSC VOC	9(9.4)	5(4.1)	0.42
HbSS leg ulcer	10(10.4)	5(4.2)	0.44
HbSS Priapism	5 (5.3)	3(2.4)	0.51

≠ Expected Hardy-Weinberg frequency, Observed =observed frequency

4.10 Association between eNOS gene polymorphism and complications of SCD

To investigate the associate of eNOS gene polymorphism with SCD complication, a multinomial logistical analysis was performed. The results are shown in Table 4.15. The odds of an SCD patient with any of the haplotypes or polymorphisms are displayed.

For instance SCD patients with TC and CC polymorphism were highly at risk of developing leg ulcers (OR, 10.33; 95% CI, 1.24-86.06) and (OR, 10.38; 95%CI, 1.781-60.47) respectively. There was a positive association for the entire haplotypes as shown in Table 4.15 with SCD complications but they were not significantly different.

Table 4.17 Association between eNOS gene polymorphism complications of SCD

Haplotype	**HbSS steady state n=46 OR (CI)**	**HbSS VOC** n=57 OR (CI)	**HbSC VOC** n=18 OR (CI)	**HbSS leg ulcer** n=21 OR (CI)	**HbSS Priapism** n=11 OR (CI)
TT	2.38 (0.35-46.17)	1.12 (0.03-18.21)	1.13 (0.64-27.12)	1.34 (2.34-57.02)	0.60(.03-10.18)
TC	3.22 (0.39-26.07)	2.13 (0.33-13.97)	3.64 (0.45-29.51)	10.33 (1.24-86.06)*	0.30 0(.01-11.13)
CC	1.35 (0.237-7.72)	1.82 (0.35-9.38)	6.00(0.99-36.07)	10.38 (1.781-60.47)*	0.79 (.03-26.69)
VNTR 4a	2.19 (<0.001->10)	0.46 (<0.001->10)	0.002 (<0.001 >10)	5.611 (<0.001->10)	1.49 (<0.001->10)
VNTR 4b	1.02 (<0.001->10)	0.36 (<0.001->10)	0.002 (<0.001->10)	1.059 (<0.001->10)	4.15 (<0.001->10)
VNTR 4c	0.29 (<0.001->10)	0.10 (<0.001->10)	0.001 (<0.001->10)	0.119 (<0.001->10)	0.22 (<0.001->10)
VNTR 4d	_-	-	-	-	-

***significant, OR= Odds Ratio, CI= 95% confidence intervals**

4.11 Comparisons of angiogenic factors, endothelial markers and haematological indices in each polymorphism across the disease categories

In order to compare the mean Haematological indices, angiogenic factors and endothelial markers in the different disease categories for each eNOS polymorphism, a one-way ANOVA was done and the results are displayed Tables 4.16, 4.17, 4.18, 4.19 and 4.20 below.

The results below revealed that among the subjects with the TT polymorphism there were significant differences between the mean values of HCT, Hb, WBCs, RBCs, MCV, ICAM-1, VCAM-1, E-selectin, Ang-1,Ang-2 and NO. Refer to Table 4.16 below.

From Table 4.17.belowit is shown that among the subjects with the TC polymorphism, there were significant differences between the mean values of HCT, Hb, WBCs, RBCs, MCV and NO

Table 4.18 Comparisons of angiogenic factors, endothelial markers and haematological indices in TT polymorphism across the disease categories

TT Polymorphism	Control (n=26)	HbSS steady state (n=37)	HbSS VOC (n=39)	HbSC VOC (n=9)	HbSSleg ulcer (n=8)	HbSS Priapism (n=5)	p-value
AGE (yrs)	32.23 ± 9.87	26.00 ± 8.67	25.64 ± 10.12	31.78 ± 12.46	29.25 ± 8.33	31.80 ± 14.04	0.066
HCT (%)	39.43 ± 3.88	25.10 ± 4.58	28.10 ± 5.20	35.62 ± 5.57	27.21 ± 5.16	23.86 ± 8.96	<0.001*
Hb (g/dL)	13.11 ± 1.24	8.47 ± 1.45	9.21 ± 1.61	11.26 ± 1.86	8.58 ± 0.95	10.64 ± 2.70	<0.001*
WBC (10^3/mm^3)	5.33 ± 1.35	12.02 ± 3.43	13.10 ± 3.86	9.63 ± 4.26	11.33 ± 1.13	10.76 ± 5.07	<0.001*
RBC (10^6/mm^3)	4.90 ± 0.65	2.88 ± 0.63	3.46 ± 0.76	4.51 ± 0.61	3.28 ± 0.62	2.68 ± 0.92	<0.001*
PLT (10^3/mm^3)	231.33 ± 59.79	478.73 ± 122.04	455.23 ± 140.48	294.11 ±89.52	514.12 ± 122.92	349.60 ±112.70	<0.001*
MCV (µm^3)	81.00 ± 6.89	80.41 ± 4.44	82.15 ± 8.44	79.11 ± 4.40	82.00 ± 10.42	84.40 ± 3.21	<0.001*
ICAM-1 (ng/ml)	31.77 ± 12.48	48.81 ± 25.05	64.85 ± 23.98	27.33 ± 12.91	49.38 ± 14.00	63.80 ± 39.05	<0.001*
VCAM-1 (ng/ml)	281.43 ± 275.76	493.78 ± 328.16	635.81 ± 341.29	623.42 ± 260.01	389.44 ± 225.73	554.40 ± 449.47	0.001*
E-selectin (ng/ml)	161.65 ± 38.80	228.16 ± 86.16	231.95 ± 112.87	224.44 ± 125.81	291.75 ± 99.26	210.40 ± 76.62	0.009*
Ang-1 (pg/ml)	9703.80 ± 9694.17	10503.00 ± 8537.37	8454.80 ± 8049.90	18378.00 ± 13229.88	15607.00 ± 10451.90	27591.00 ± 17738.24	<0.001*
Ang-2 (pg/ml)	632.29 ± 436.29	1098.60 ± 601.37	1115.80 ± 858.33	969.08 ± 456.69	870.89 ± 596.52	728.91 ± 834.18	0.066
VEGF (pg/ml)	47.10 ± 20.45	64.30 ± 48.37	43.23 ± 23.75	69.48 ± 57.81	47.83 ± 31.41	53.96 ± 17.62	0.106
NO (ng/ml)	58.90 ± 9.00	46.97 ± 8.62	10.97 ± 2.97	22.22 ± 9.96	32.14 ± 4.87	34.56 ± 10.47	<0.001*

*Significant at p≤ 0.05

Refer to Appendix VII for the post hoc analysis.

Table 4.19 Comparisons of angiogenic factors, endothelial markers and haematological indices in TC polymorphism across the disease categories

Haplotype TC	Control (n=7)	HbSS steady state (n=4)	HbSS VOC (n=5)	HbSC VOC (n=2)	HbSS leg ulcer (n=3)	Priapism (n=3)	p-value
AGE (yrs)	**34.57 ± 12.99**	**23.00 ± 7.62**	**22.00 ± 7.62**	**20.00 ± 7.62**	**27.00 ± 5.00**	**34.00 ± 11.53**	0.212
HCT (%)	38.64 ± 4.38	29.08 ± 2.99	26.39 ± 5.20	36.74 ± 2.46	26.73 ± 0.91	32.39 ± 0.68	<0.001*
Hb (g/dL)	12.74 ± 1.60	9.58 ± 0.96	8.63 ± 1.60	11.88 ± 0.74	8.83 ± 0.06	11.20 ± 0.62	<0.001*
WBC ($10^3/mm^3$)	5.86 ± 0.65	12.18 ± 1.64	17.92 ± 5.80	7.40 ± 0.83	9.83 ± 0.75	5.97 ± 1.53	<0.001*
RBC ($10^6/mm^3$)	4.99 ± 0.65	3.35 ± 0.32	3.24 ± 0.67	4.21 ± 0.36	3.87 ± 0.70	4.30 ± 0.70	<0.001*
PLT ($10^3/mm^3$)	226.29 ± 51.77	448.25 ± 36.89	443.27 ± 112.89	323.40 ± 140.63	512.67 ± 46.76	163.33 ± 51.47	<0.001*
MCV (μm^3)	77.90 ± 8.10	80.08 ± 2.99	82.09 ± 9.29	87.40 ± 7.40	81.33 ± 8.02	83.00 ± 4.36	<0.001*
ICAM-1 (ng/ml)	25.71 ± 7.32	33.75 ± 14.93	47.18 ± 31.45	39.80 ± 10.85	33.33 ± 20.82	56.67 ± 32.53	0.349
VCAM-1 (ng/ml)	336.79 ± 34093	468.00 ± 214.23	665.61 ± 251.26	461.32 ± 209.55	337.73 ± 400.38	290.83 ± 145.03	0.134
E-selectin (ng/ml)	155.00 ± 39.44	215.00 ± 144.57	289.09 ± 137.15	252.00 ± 99.35	202.67 ± 31.01	164.33 ± 59.47	0.174
Angio-1 (pg/ml)	7353.40 ± 7946.76	16684.00 ± 16739.57	10137.00 ± 7853.45	8420.00 ± 4871.80	17797.00 ± 5966.00	18278.00 ± 19547.32	0.394
Angio2 (pg/ml)	418.37 ± 233.94	814.68 ± 238.97	1322.20 ± 1148.87	1309.10 ± 570.48	636.14 ± 230.41	334.50 ± 203.51	0.112
VEGF (pg/ml)	53.54 ± 8.53	70.31 ± 66.82	45.86 ± 23.26	60.14 ± 54.69	35.11 ± 13.24	76.34 ± 19.54	0.587
NO (ng/ml)	55.42 ± 5.39	47.69 ± 8.31	9.17 ± 3.65	21.44 ± 7.36	30.32 ± 2.78	33.97 ± 10.29	<0.001*

*Significant

Refer to Appendix VIII for the post hoc analysis.

Table 4.18 shows that there were significant differences between the mean values of HCT, Hb, WBCs, RBCs, MCV, Ang-1 and NO among subjects with VNTR 4a polymorphism.

Table 4.20 Comparisons of angiogenic factors, endothelial markers and haematological indices in VNTR 4apolymorphism across the disease categories

VNTR 4a Polymorphism	Control (n=3)	HbSS steady state (n=15)	HbSS VOC (n=13)	HbSC VOC (n=2)	leg ulcer (n=9)	p-value
AGE (yrs)	29.67 ± 3.79	24.67 ± 8.23	25.62 ± 10.48	29.50 ± 6.36	30.00 ± 7.91	0.063
HCT (%)	35.10 ± 3.04	24.89 ± 5.09	29.14 ± 3.18	38.55 ± 0.92	27.53 ±4.56	<0.001
Hb (g/dL)	11.40 ± 0.95	8.55 ± 1.55	9.46 ± 0.98	12.35 ± 0.64	8.48 ± 0.97	<0.001
WBC (10^3/mm^3)	5.33 ± 1.61	11.66 ± 3.80	14.58 ± 4.28	7.65 ± 0.35	11.19 ± 1.49	0.003
RBC (10^6/mm^3)	4.66 ± 0.58	2.78 ± 0.70	3.46 ± 0.66	4.53 ± 0.13	3.46 ± 0.72	0.000
PLT (10^3/mm^3)	268.33 ± 49.50	452.07 ± 134.71	468.38 ± 140.94	214.50 ± 67.18	549.67 ± 153.15	0.016
MCV (µm^3)	75.93 ± 8.88	81.65 ± 4.76	85.62 ± 2.58	85.00 ± 4.24	81.22 ± 10.51	0.000
ICAM-1 (ng/ml)	25.00 ± 0.00	38.73 ± 19.25	50.53 ± 26.17	27.50 ± 10.61	44.44 ± 18.28	0.190
VCAM-1 (ng/ml)	417.83 ± 493	495.53 ± 357.65	716.38 ± 406.36	421.60 ± 305.33	339.17 ± 211.67	0.192
E-selectin (ng/ml	165.33 ± 63.57	221.60 ± 78.86	273.85 ± 133.39	255.00 ± 91.92	260.56 ± 105.06	<0.001
Angio-1 (pg/ml)	4678.80 ± 4148.40	6338.70 ± 5091.49	15531.00 ± 7325.57	16394.00 ± 19678.87	19292.00 ± 10833.64	0.004
Angio2 (pg/ml)	500.90 ± 366.49	1077.30 ± 680.96	1642.30 ± 1194.23	960.59 ± 1064.27	800.69 ± 272.14	0.169
VEGF (pg/ml)	48.00 ± 3.61	77.45 ± 52.98	49.24 ± 23.36	29.43 ± 5.57	49.25 ± 29.04	0.280
NO (ng/ml)	55.33 ± 8.06	46.81 ±7.57	9.93 ± 2.05	21.29 ± 14.82	32.50 ± 4.20	<0.001

*Significant

Refer to Appendix IX for the post hoc analysis.

The result from Table 4.19 below revealedthat among the subjects with the VNTR4b polymorphism there were significant differences between the mean values of HCT, Hb, WBCs, RBCs, MCV, Ang-2 and NO.

Table 4.21 Comparisons of angiogenic factors, endothelial markers and haematological indices in VNTR 4bpolymorphism across the disease categories

Haplotype VNTR 4b	Control (n=10)	HbSS steady state (n=17)	HbSS VOC (n=24)	HbSC VOC (n=5)	HbSSleg ulcer (n=7)	HbSSPriapism (n=6)	p-value
AGE (yrs)	30.50 ± 11.44	28.82 ± 9.27	26.29 ± 10.46	29.80 ± 14.54	29.14 ± 8.47	30.83 ± 8.23	0.859
HCT (%)	49.66 ± 4.17	24.74 ± 4.56	27.11 ± 5.63	36.46 ± 6.72	26.36 ± 2.11	30.17 ± 6.67	<0.001*
Hb (g/dL)	13.53 ± 1.27	8.27 ± 1.48	8.96 ± 1.84	11.40 ± 2.16	8.74 ± 0.92	11.30 ± 0.87	<0.001*
WBC (10^3/ mm^3)	5.86 ± 1.40	12.31 ± 3.18	13.75 ± 5.52	8.72 ± 4.79	10.81 ± 1.24	7.56 ± 4.21	<0.001*
RBC (10^6/ mm^3)	5.16 ± 0.58	2.88 ± 0.62	3.38 ± 0.72	4.54 ± 0.54	3.46 ± 0.54	3.67 ± 1.07	<0.001*
PLT (10^3/mm^3)	229.20 ± 77.48	509.94 ± 112.72	418.38 ± 122.56	299.40 ± 84.56	538.86 ± 108.94	243.67 ± 125.90	<0.001*
MCV (μm^3)	79.00 ± 5.96	80.74 ± 4.56	81.00 ± 8.14	80.20 ±7.05	81.43 ± 8.22	84.00 ± 3.41	<0.001*
ICAM-1 (ng/ml)	27.00 ± 10.18	60.88 ± 25.47	58.92 ± 25.24	25.60 ± 9.96	45.71 ± 17.66	63.33 ± 29.10	0.001*
VCAM-1 (ng/ml)	143.90 ± 23.15	532.38 ± 323.19	645.70 ± 288.68	467.18 ± 269.79	548.49 ± 454.37	516.25 ± 413.26	0.004
E-selectin (ng/ml)	160.20 ± 40.49	252.35 ± 105.03	233.58 ± 111.05	186.00 ± 50.30	300.29 ± 82.02	205.83 ± 76.69	0.048
Angio-1 (pg/ml)	10746.00 ± 9675.04	12292.00 ± 9755.59	68562.00 ± 6966.50	19024.00 ± 16967.69	15616.00 ± 7551.84	22940.00 ± 19170.25	0.013
Angio2 (pg/ml)	888.35 ± 470.88	1069.40 ± 526.10	908.96 ± 756.32	1017.70 ± 453.34	802.46 ± 675.91	595.63 ± 767.83	0.728
VEGF (pg/ml)	41.96 ± 12.41	34.98 ± 9.07	40.61 ± 23.02	62.89 ± 58.94	47.28 ± 33.11	62.11 ± 24.21	0.123
NO (ng/ml)	57.83 ± 10.85	47.32 ± 10.03	6.75 ± 2.50	20.90 ± 5.55	29.58 ± 2.77	33.62 ± 9.76	<0.001*

*SignificantRefer to AppendixX for the post hoc analysis.

From Table 4.20 below that there were significant differences between the mean values of HCT, Hb, WBCs, RBCs, MCV, Ang-1, Ang-2, VEGF and NO among the subjects with VNTR 4c polymorphism

Table 4.22 Comparisons of angiogenic factors, endothelial markers and haematological indices in VNTR4cpolymorphism across the disease categories

Haplotype VNTR 4c	Control (n=22)	HbSS steady state (n=14)	HbSS VOC (n=18)	HbSC VOC (n=18)	p-value
AGE (yrs)	33.55 ± 10.39	24.79 ± 8.75	28.17 ± 11.41	36.13 ± 10.96	0.034*
HCT (%)	39.27 ± 3.84	26.60 ± 3.58	27.32 ± 5.57	36.39 ± 6.55	<0.001*
Hb (g/dL)	13.03 ± 1.24	8.86 ± 1.14	8.91 ± 1.63	11.70 ± 2.01	<0.001*
WBC (10^3/mm^3)	5.30 ± 1.09	12.06 ± 2.52	12.89 ± 3.94	8.11 ± 3.81	<0.001*
RBC (10^6/mm^3)	4.87 ± 0.70	3.09 ± 0.48	3.47 ± 0.87	4.42 ± 0.66	<0.001*
PLT (10^3/mm^3)	217.50 ± 49.94	475.00 ± 79.58	445.06 ± 152.37	250.25 ± 84.18	<0.001*
MCV (µm^3)	81.45 ± 7.17	81.60 ± 3.58	80.11 ± 9.90	82.13 ± 5.79	<0.001*
ICAM-1 (ng/ml)	31.41 ± 13.43	42.57 ± 23.88	72.50 ± 23.76	35.88 ± 17.02	<0.001*
VCAM-1(ng/ml)	332.78 ± 297.53	432.93 ± 264.70	561.63 ± 323.10	638.65 ±254.16	0.033*
E-selectin (ng/ml)	155.18 ± 36.77	204.86 ± 78.51	212.22 ± 96.44	236.25 ± 137.42	0.056
Angio 1 (pg/ml)	8736.00 ± 9338.88	13263.00 ± 11036.21	9412.00 ± 8435.93	8215.20 ± 4987.41	0.0464*
Angio2 (pg/ml)	514.21 ± 374.22	1027.00 ± 463.25	1197.50 ± 932.66	933.06 ± 216.99	0.005*
VEGF (pg/ml)	49.77 ± 22.23	75.75 ± 57.92	53.18 ± 27.30	84.30 ± 60.78	0.087
NO (ng/ml)	58.11 ± 8.04	45.70 ± 8.29	7.78 ± 1.76	24.80 ± 8.73	<0.001*

*Significant

Refer to AppendixXI for the post hoc analysis.

4.12 The main effect and interaction effect between VNTR of the eNOS polymorphism and the disease categories on NO

General Linear Model (GLM) was used to establish the main effects between the VNTR eNOS gene polymorphism and the SCD categories on NO. This is to determine whether the VNTR polymorphism alone (main effect) has any effect on NO or it has to exist with a complication or a disease category to have an effect (interaction effects).

The result were as follows:

There was significant main effect by VNTR4a ($p < 0.001$) and interaction effects between VNTR4a and disease category ($p = 0.038$).

There was significant main effect by VNTR4b ($p< 0.006$) and interaction effects between VNTR4b and disease category ($p< 0.001$).

There was no significant main effect by VNTR4c ($p =0.074$), but there was a interaction effects between VNTR4c and disease category ($p < 0.001$).

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

Sickle cell disease is a major genetic disease with a wide variety of clinical manifestations. The study was performed to determine the interaction analysis of some clinical outcomes (complications) and eNOS gene polymorphism in the severe genotype of SCD (HbSS) in Ghana. It was further aimed at elucidating the clinical correlation of eNOS gene polymorphisms in SCD pathophysiology, which until this report was unknown in this population.

The study involved SCD HbSS patients with age matched healthy controls of HbAA from a genetically homogenous population.Few SCD of HbSC genotype subjects were included in the study for comparison. Considering the degree of chronic haemolysis and higher risk of infections in SCD patients, results in Tables 4.1 to 4.10 were to be expected. The red cellindices such as Hb, MCV, MCH, and MCHCwere generally lower in HbSS SCD patients than in controls (HbAA).However, thewhite blood cell and platelet counts were higher in HbSS SCD patients than in control participants. Further more, these red cell indiceswere higher in males than females inboth cases and controls. This results was consistent with the results obtained in previous studies(Omoti, 2005; Ehsan*et al.,* 2010, Okocha *et al.,* 2011; Olurotimi, 2012; Akinsegun *et al.,* 2012). Blood viscosity increases with increase in PCV, therefore most SCD patients have adapted to low red cell indices in view of the fact that raising the PCV to over 30% could increase the viscosityof the blood (Kaul *et al.,* 1983) that could precipitate VOC.

Due to re-distribution of the WBC between the marginal and circulating pools, pain, nausea and vomiting and anxiety have been reported to cause leucocytosis in the absence of infection (Milhorat *et al.*, 1942). Epidemiologic data also indicate that higher WBC count is associated with poor prognosis (Ohene-frempong *et al.*, 1997; Powers, 2000; Okpala, 2004)while reduced neutrophil count is associated with good prognosis. Elevated WBC count in SCD patients may also be due to autosplenectomy resulting from recurrent splenic vessels occlusion, which makes patients more susceptible to overwhelming infections like *Streptococcus pneumonia* and *Haemophilus influenza*. Increased erythropoesis due toandrogens in males, and iron loss or blood loss infemales during menstruation may be responsible forhigher levels of the red cell indices in males compared to femles (Kato *et al.*, 2009).

Nitric oxide is a major endothelial-derived relaxing factor in normal physiology of the vasculature and plays a central role in vascular homeostasis in maintaining basal and stimulated vasomotor tone, limiting platelet aggregation, ischaemia-reperfusion injury, and modulating endothelial proliferation (Reiter *et al.*, 2003). Its role in the SCD complication is very crucial. Haemolysis-associated endothelial dysfunction is implication in SCD, The process of haemolysis initiates a large-scale attack on the L-arginine-NO pathway. This is becauseunder normal conditions, hemoglobin is safely packaged within the RBC plasma membrane; however, during haemolysis it is decompartmentalized and released into plasma, where it rapidly reacts with and destroysNO. This results in abnormally high NO consumption and the formation of reactive oxygen species, ultimately inhibiting vasodilation. NO destruction by haemoglobin can also cause further impairment in vascular endothelial function via transcriptional activation of adhesion molecules, including VCAM-1 and E-selectin, and

potent vasoconstrictors such as endothelin-1. The simultaneous release of erythrocyte arginase during hemolysis will limit the availability of arginine to NOS, contributing to a deficiencyof NO.

It was observed in this study that plasma NO levels were significantly low in the SCD patients with the complications; HbSS VOC, HbSC VOC, HbSS leg ulcers and HbSS Priapism in increasing order as compared to SCD patients in asymptomatic states (HbSS steady state and HbSS post crisis state) and controls. The trend in NO levels was also observed with significantly high levels of NO in controls and in SCD patients in steady state, the level reduced significantly during VOC and the NO re-bounces in the immediate post crisis state in SCD patients (Fig. 4.1). This result is consistent with other reports (Enwonwu *et al.*, 1990; Lopez *et al.*, 1996; Stuart *et al.*, 1999; Claudia *et al.*, 2000; Morris *et al.*, 2000, Antwi-Boasiako *et al.*, 2015).

These authors had shown that elevated NO levels were associated with low pain scores whilst lower NO levels were associated with high pain scores. These results suggest that the severity of SCD complications may be related to the degree of depletion of NO as a contribution to the clinical complications suffered by patients with SCD. Claudia *et al.* (2000) reported on NO patternsduring hospitalization in SCD patients. Their results showed that initial NO levels were low on admission in patients with VOC, decreased further during hospitalization and on recovery the NO levels increased to steady state level at the time of discharge.

Sickle cell disease is characterized by chronic inflammation (Belcher *et al.*, 2000) and ischaemia-reperfusion injury (Kaul & Hebbel, 2000; Osarogiagbon *et al.*, 2000), which

result from the repeated occlusion of the microvasculature by erythrocytes made rigid by intracellular polymerization of deoxyhaemoglobin S. It is clear that NO participates in the compensatory response to chronic vascular injury in patients with SCD. During VOC it is expected that NO will play its compensatory role in maintaining vascular function by vasodilating to dislodge the occlusion. Although lack of NO may not be the primary cause of VOC, its presence could ameliorate the VOC if not prevent it entirely. Therefore failure of NO compensatory or signalling system to operate and its reduced bioavailability during VOC, enhance the severity of the disease as observed in this study.

During the process of VOC the balance of local vasoconstrictors and vasodilators is altered in favour of vasoconstriction. This may account for the vaso constriction that enhances VOC caused by the sickled erythrocyte. Lower levels of NO and vasoconstriction may also result from conditions that interfere with NO bioavailability (Marin and Rodriguez-Martinez, 1997) such as elevated levels of cell-free haemoglobin in plasma resulting from intravascular haemolysis and chronic anaemia which are common features of SCD (Gladwin & Kato, 2005). The cell free haemoglobin in plasma rapidly destroys the NO (Kaul and Hebbel, 2000; Reiter *et al.,* 2003) thereby limiting NO bioavailability (Reiter *et al.,* 2002).

Intravascular haemolysis also produces a state of endothelial dysfunction characterized by reduced NO bioavailability in patients with SCD. This leads to dysregulation of the balance between endothelium-derived vasodilator and vasoconstrictor system resulting in acute vasoconstriction and chronic proliferative vasculopathy. This vasculopathy has been proposed to characterize SCD into clinical subphenotype of cutaneous leg ulcers, priapism, VOC among others (Gladwin & Kato, 2005). Therefore new therapies

targeting this vasculopathy and aiming at normalizing the vasodilator- vasoconstrictor balances have been proposed.Furthermore, recent data suggest that chronic intravascular haemolysis is associated with a state of endothelial dysfunction characterized by reduced NO bioavailability, pro-oxidant and pro-inflammatory stress and coagulopathy, leading to vasomotor instability and ultimately producing a proliferative vasculopathy, a hallmark of which is the development of SCD complications. (Reiter *et al.*, 2002; Kaul*et al.*, 2004; Rother *et al.*, 2005) as obswerved in this study.

Endothelial cells also play a key role in regulating the local vascular function in part by secreting vasodilator NO and vasoconstrictor endothelin-1 (ET-1) during VOC (Haynes & Webb, 1998). Interaction between sickled erythrocytes and endothelium may contribute to the vascular occlusion in SCD via several mechanisms including further erythrocyte sickling and local vascular instability precipitated by the imbalance of local vasoconstrictors and vasodilators produced by endothelial cells in response to interaction with sickled erythrocytes (Kaul *et al.*, 2000; Belhassen *et al.*, 2001).

Studies suggest that patients with sickle cell disease suffer from decreased NO reserves (Gladwin *et al.*, 2003). Blood plasma levels of L-arginine are depressed in patients with sickle cell disease, particularly during VOC and the ACS, and these levels vary inversely with pain symptoms (Enwonwu *et al.*, 1990; Morris *et al.*, 2000). Also, NO-dependent blood flow is impaired in SCD patients (Belhassen *et al.*, 2001; Eberhardt *et al.*, 2003; Gladwin *et al.*, 2003). Although reports are varied, some investigators have found low levels of NOx (nitrate and nitrite) levels in patients with SCD and these reduced levels are consistent with impaired endothelial generation of NO, and its subsequent reactions with haemoglobin and oxygen. These mechanisms of impaired NO bioavailability

appear to be due to decreased plasma L-arginine and consumption of NO by cell-free plasma haemoglobin and by reactive oxygen species (Kato & Mack, 2008).

Nitric oxide is proposed to alleviate pain in acute crisis in several ways. With lower levels of NO, there is increased inflammation and decreased vasodilation, which are promoting factors in cell aggregation. Notric oxide has the natural effects of vasodilation on smooth muscle vasculature, anti-inflammatory effects, and also inhibits platelet aggregation (Natarajan *et al.*, 2010).

The cell aggregation, results in blockage of blood flow, which causes pain in these patients and can lead to potentially fatal events. Low levels of NO are shown to be related to increased pain levels experienced by patients during acute VOC (Morris *et al.*, 2000). Pain levels correspond to increased risk for death and, since levels of NO correspond with pain levels, it is therefore suggested that levels of NO in complications may correspond to risk of death. A previous study by Head *et al.* (1997) showed that low supplemental levels of NO increase the affinity of oxygen for sickle haemoglobin, without tovrsic levels of methaemoglobin, which is NOxious when produced in high concentration. This suggests a possible therapeutic approach for the treatment of Sickle Cell disease.

It was observed from this study as shown in Table 4.12 that SCD patients had abnormally increased plasma levels of adhesion molecules: VCAM-1, ICAM-1, and E-selectin the levels were even higher in patients with complications. Theseresultsreveal that SCD involves an abnormally activated, pro-adhesive endothelial cell state and demonstrate increased expression of the adhesion molecules confirming the result of

some prevous studies (Duits *et al.*, 1996; Alex *et al.*, 2001; Nicola *et al.*, 2004). These higher levelsare coupled with significantly low plasma NO levels. This could be derived from the stimulating action of various biological modifiers such as hypoxia, thrombin, and cytokines on the normally quiescent endothelium (Alex *et al.*, 2001) as well as from the pro-inflammatory effects of reperfusion-injury physiology (Kaul &Hebbel, 2000; Osarogiagbon *et al.*, 2000). This view of SCD as a state of abnormal endothelial activation presents a potential opportunity for novel therapeutic approaches in that pharmacologic inhibition of endothelial cell activation might be clinically beneficial. This is because VCAM-1, ICAM-1, and E-selectin are all markers of endothelial dysfunction, and have been implicated in the pathology of SCD (Belcher *et al.*, 2006; Aslan & Freeman, 2007).

The VCAM-1 and ICAM-1 are normally expressed at low levels on the luminal surface of endothelial cells. Their expression is induced by a variety of biological stimuli, particularly by inflammation. The VCAM-1 and ICAM-1 provide an adhesive surface for specific ligands present on the surface of leucocytes physiologically recruited as part of the inflammatory programme. In contrast, as part of the biochemical program that facilitates blood flow and maintains vascular homeostasis, NO normally suppresses the expression of VCAM-1, ICAM-1 and E-selectin (Spiecker *et al*, 1998; Lee *et al*, 2002). Endothelial dysfunction or impairment of NO bioavailability results in pathological activation of endothelial cells to express these adhesion molecules. Subsequent shedding of soluble adhesion molecules into blood plasma therefore can serve as markers either of endothelial dysfunction or of inflammation with endothelial activation.

The complex pathophysiology of SCD is influenced by the many physiologic functions of the vascular wall endothelium (Hebbel & Vercellotti, 1997). Also the characteristic development of acute vascular occlusion due to red cell sickling may be triggered by proximate adhesion of red cells to endothelial cells (Alex *et al.,* 2001). This abnormal cell to cell interaction uses various mechanisms, many of which involve adhesion receptors that can be expressed on vessel wall endothelial cells. In the same way, other processes that involve the vascular endothelium, such as thrombosis or white cell adhesion, may play a critical role in the vascular occlusion. Through these mechanisms, it is likely that function or dysfunction of the vascular endothelium could contribute to the overall vascular pathobiology of SCD, which includes recurrent VOC, leg ulcers and priapism.

Endothelial dysfunction generally leads to vascular remodelling and potential changes in mechanical vascular properties (Stoyanova *et al.,* 2012).Endothelial cell proliferation plays a role in vascular injury repair and blood vessels formations. The growth of new blood vessels (angiogenesis) is important for wound healing and for restoring blood flow to tissues after injury or damage (Sameh *et al.,* 2013). In normal vascular physiology, inhibitors and angiogenic growth factors, such as VEGF, regulate angiogenesis. When regulation fails, blood vessels are formed excessively or insufficiently (Birk *et al.,* 2009).

To characterize angiogenesis in SCD and its complications, plasma levels of important angiogenic growth factors Ang-1, Ang-2 and VEGF were determined in the different categories of SCD patients and controls (Table 4.12). Generally, Ang-1, Ang-2 and VEGF levels were significantly elevated in SCD patients as compared to controls and were even higher in the patients with complications. Furthermore, Ang-2/Ang-1 ratio

was high in SCD patients and also higher in SCD complications with the HbSS leg ulcer patients recording the highest ratio.The result from this study was consistent with thosefrom other studies (Mohan *et al.*, 2005; Duits *et al.*, 2006). Duits *et al.* (2006) demonstrated this in painful crisis and Mohan *et al.* (2005) reported elevated Ang-2, VEGF and Ang-1 plasma levels in clinically asymptomatic SCD patients.

Sickle cell disease patientsexperience continuous vasoocclusion that results in significant ischaemic organ damage. Angiogenesis is likely to be of importance in the pathophysiology of the disease (Stuart & Nagel, 2004). The rate of tissue regeneration in infarcted tissue may be dependent on the integrity of angiogenesis. Angiopoietin dysregulation (decreased Ang-1 and increase Ang-2 culminating in unbalanced angiogenesis could contribute to the development of VOC, priapism and leg ulcers in SCD. Angiogenesis is influenced by the relative levels of Ang-1, Ang-2 and VEGF in the plasma (Fam *et al.*, 2003). Relatively Higher levels of Ang-1 and lower levels of Ang-2 and VEGF favors sustained vessel integrity and quiescence (Duits *et al.*, 2006). However, High Ang-2 relative to Ang-1 and VEGF level favors angiogenesis and responsiveness to hypoxic and inflammatory stimuli as demonstrated in neoplasms (Zhang *et al.*, 2003).

In relation to the observed VEGF and Ang-1 levels, the elevated Ang-2, with further increments in HbSS leg ulcers patients suggests enhanced angiogenesis, which is an attempt to repair the damaged tissues at the site of the wound. This agrees with an *in vitro* data in which hypoxia induced the expression of Ang-2, but not Ang-1 in microvascular endothelial cells (Oh *et al.*, 1999).

Ang-2 has been demonstrated to be located in endothelial Weibel Palade bodies, co-localised with von Willebrand factor (VWF) and interleukin-8 (IL-8) and is rapidly released upon endothelial activation (Fiedler *et al.,* 2004).Plasma VWF antigen levels are increased in asymptomatic SCD patients, with a further increment during painful crisis (Stuart & Nagel, 2004). Serum levels of IL-8 are also elevated during painful SCD (Duits *et al.,* 1998). The enhanced Ang- 2 serum levels may, therefore, reflect rapid Ang-2 release from endothelial Weibel Palade bodies in response to endothelial damage in SCD as was observed in this study.

The result from this study shows that NO levels were signgnificantly low in HbSS patients with Priapism. Although autonomic factors and a low blood flow state contribute to making erectile tissue prone to sickle cell-induced priapism, abnormal regulation of NO pathway and its signaling may be key in the development of priapism as complications in SCD (Crane *et al.,* 2011). Nitric oxide being a potent vasodilator is key to tumescence as free NO is readily scavenged in the blood by cell free haemoglobin (Reiter, 2002) which does not occur in the HbAA since in this case the ability of haemoglobin to scavenge NO is normally dramatically reduced by its compartmentalization within red blood cells, which creates a cell-free and therefore haemoglobin-free zone adjacent to the vessel endothelium due to laminar flow.

In addition, the presence of the cell membrane, cytoskeleton and other factors increase the diffusion distance between NO and haemoglobin. Haemoglobin contained in red cells has less than 0.2% of the scavenging capacity of free haemoglobin (Liao, 2002). The chronic intravascular haemolysis in SCD changes this tight regulation of NO. SCDpatientshaveelevatedfreehaemoglobinconcentrationscompared to normal volunteers

(Reiter, 2002). Further haemolysis andNO scavenging may exacerbate the severity of the complication.

This study shows that plasma NO levels are significantly reduced in SCD leg ulcer patients. Recent literature has emerged suggesting a more prominent role of NO deficiency in the pathophysiology of ulceration. Nitric oxide is an important regulator of vascular tone, cell adhesion, and blood flow. The production of NO in SCD patients is dramatically reduced, as its bioavailability, through the breakdown of Arginine, its substrate, increases in free radical production, and uncoupling of endothelial nitric oxidesynthase (eNOS). Cell free haemoglobin released during haemolysis affects the bioavailability of NO asdeoxy-HbS and oxy-HbS react with NO forming a stable Fe^{2+} Hb-NO complex, methaemoglobin, and nitrate (Chirico and Pialoux, 2008).During haemolysis there is the release from the damaged erythrocytes the enzymeArginase.

Arginase competes with eNOS to metabolize arginine, the precursor to NO formation, into ornithine and urea. As L-arginine is the rate limiting substance in the NOS pathway, its absence disrupts electron transport through eNOS and diminishes the amount of NO that is produced. This consumption of NO and its precursor causes such impaired vascular smooth muscle relaxation, increased adhesion molecule and endothelin-1 expression, and platelet activation and aggregation (Wood *et al.*, 2008). The resulting vasculopathy skews the normal balance between vasoconstriction and vasodilatation toward vasoconstriction, endothelial activation, and proliferation (Jones *et al.*, 2013). Eventually, the resulting deficiency of NO and abnormal vascular activity leads to tissue ischaemia and ulcer formation. Chronic oxidative stress caused by repeated episodes of HbS de-oxygenation and re-oxygenation has also been implicated in the pathogenesis of

leg ulceration (Chirico & Pialoux, 2008). Re-oxygenation of haemoglobin is a major source of free radical formation generated byan electron transfer between haem iron and oxygen (Chirico & Pialoux, 2008).

All polymorphisms studied (the 27 VNTR and the T786C) were equally distributed, and followed the Hardy-Weinberg equilibrium (no deviations) in both sickle cell disease and control groups, showing this observation is not due to any selection pressure, attributable to disease.

This study also revealed a significantly higher percentage (Table 4.12) of mutant alleles and mutant genotypes of the T786C polymorphism of the eNOS gene (TC and CC) in all SCD categories as compared with controls indicating that T786C polymorphism is associated with SCD patients in Ghana. The few available results supporting this evidence include a recent study conducted by Nishank *et al.*(2013) in Indians. The findings of this study are consistent with their result. Two separate studies conducted by Chaar *et al.*(2006) and Sharan *et al.*(2005) in SCD children and SCD female subjects respectively, found significant association of T786C with ACS, a complication of SCD, where females having eNOS variant were found to be more susceptible to ACS (Chaar *et al.,* 2006; Sharan *et al.,* 2004). However, other studies reported lack of association of all the three clinically relevant SNPs of the eNOS gene with SCD patients in Brazil (Vargas *et al.,* 2005) and in Africans with SCD in Mali (Thakur *et al.,* 2014).

The results from these latter studies do not agree with the observation made in this study, but potentially confirms previous reports of extensive inter-ethnic diversity of eNOS gene polymorphisms. This genetic diversity could be a contributory factor in the current

observation (Tanus-Santos *et al.*, 2001; Serrano*et al.*, 2010; Thomas *et al.*, 2013) as well as environmental influence on genetics. Additionally, the frequencies of the TC and CC genotypes of T786C were higher in SCD patients with complications as compared to patients without complications. This result is consistent with that reported by Nishank*et al.* (2013). The significantly higher percentage of the T786C polymorphism among the SCD complications categories compared to the uncomplicated HbSS patients, the milder form of the SCD (HbSC) patients and controls (HbAA) indicates that the eNOS gene probably acts as a genetic modifier of the different complications among SCD patients in Ghana.

Furthermore, there was a significantly strong positive association between both TC and CC haplotypes of the T786C polymorphism of the eNOS gene with SCD leg ulcers. To the best of my knowledge this is the first study to test for such an association. This result agrees with the studies conducted by Paul *et al.* (1999) in non-SCD mice.

The result from this study demonstrates that eNOS gene is required for effective wound healing and repairs. These are physiological process known to depend on intact angiogenesis stimulated by growth factors. This is supported *in vivo* by the findings that both excisional wound closure and incisional wound strength were impaired in eNOS KO mice (Paul *et al.*, 1999). This study demonstrated that eNOS indeed plays a significant role in facilitating growth factor-stimulated angiogenesis (Table 4.12).

Angiogenesis, the process of forming new micro vessels, is an important component of normalwound repair. NO plays a central role in this process (Donnini & Ziche 2002) as it has been found to increase angiogenesis in ischemic murine tissues (Murohara *et al.*,

1998). Conversely, eNOS inhibitors impair angiogenesis in granulation tissue during gastric ulcer healing (Konturek *et al.,* 1993) and suppress capillary organization *in vitro* (Papapetropoulos *et al.,* 1997). NO is also vital to the activity of pro-angiogenic cytokines. Vascular endothelial growth factor is a potent angiogenic factor, which involves the modulation of NO generation (Zhang *et al.*, 2003). VEGF increases NO production via upregulation of eNOS (Gelinas *et al.,* 2002). Conversely, the angiogenic effect of VEGF also depends on NO as pharmacological blockade of eNOS prevent both VEGF-induced endothelial cell proliferation and mitogen-activated protein kinase (Ziche *et al.,* 1997). VEGF-stimulated endothelial cell migration, decreased adhesion, and organization are also dependent on NO (Schwentker *et al.*, 2002).

Recently, several studies revealed that there are various polymorphisms on *eNOS* gene and these mutations might be a risk factor for several diseases including Coronary artery disease. The polymorphisms differ largely among races. In this study, a strong association was found between T-786C mutation of eNOS gene and HbSS leg ulcers specifically in this population. The association between this polymorphism and SCD leg ulcer hadnot been studied in this population or elswhere. The results demonstrated an association between both TC and CC alleles with HbSS leg ulcers in the Ghanain population.

This polymorphism of eNOSgene was also investigated by several studies for an association with hypertension (Hyndman *et al.,* 2002), the presence and severity of Coronary artery disease (Rossi *et al.,* 2003) and myocardial infarction (Nakayama *et al.,* 2000). Rossi *et al.,* (2003) reported a significant association between the T-786C mutation and Coronary artery disease. Colombo *et al.* (2003) showed a positive association between the polymorphisms and the extent of Coronary artery disease in the Italian population. The T-786C polymorphism was also reported as being related to coronary spasm in the Japanese by

Nakayama *et al.* (2000). In this study high frequency of TC and CC genotype of T-786C was noticed in the SCD complication especially HbSS leg ulcers patients. The results of this study are in agreement with those studies.

The T-786C mutation in the promoter region of eNOS gene is associated with the reduction of eNOS promoter transcription rate and significantly reduced promoter activity (Nakayama *et al.,* 1999), leading to endothelial dysfunction and reduced NO production in the vascular endothelium (Kim *et al.,*2007). The reduced endothelial production of NO in the vascular endothelium predisposes carriers of the TC and CC allele to vascular occlusion. The occlusion might be more severe and prolonged in TC and CC homozygotes, increasing the time for the healing of the leg ulcer since blood supply to the wound area will be impaired therefore depriving the damaged tissue nutrients and oxygen for the wound repair. Therefore the presence of the eNOS mutant allele led to the reduced endothelial NO production which might have predisposed the patients carrying the mutant allele to leg ulcers. There were also a positive association between the TC and CC alleles of the T786C polymorphism for priapism and VOC but the difference was not significant.

In addition, this study showed that VNTR4a/b polymorphisms of the eNOS gene have main and interaction effect on the NO levels in the study subjects (result; section 4.12). Meaning VNTR 4a/b has a consequence and significance on the plasma levels of the NO observed in the subject categories. This result then explains why patients suffering from SCD complications like leg ulcers; VOC and priapism have significantly low plasma NO levels. This observation made in this study agrees with some previous studies (Hayashi *et al.,* 2010; Xu *et al.,* 2010; Luo *et al.,* 2012; Morando *et al.,* 2013). These studies reported that eNOS VNTR4a/bare associated with low plasma nitrite and nitrate concentration in healthy normal population refere to Tables 4.18.4.19 and 4.20.

The low level of plasma NO along with significant association of mutant variants of the eNOS gene observed among the SCD patients with complications in contrast to controls and asymptomatic SCD patients in this study indicates that the eNOS gene and the eNOS-derived NO may be involved in differentiation of clinical manifestations between complications and asymptomatic group of sickle of SCD patients.

It has previously been observed that plasma NO levels reduce significantly during VOC in SCD patients (Lopez *et al.,* 1996) however; there is lack of functional studies on the association between eNOS gene polymorphism and plasma NO metabolites (NOx) levels in SCD patients with complications such as leg ulcers and priapism. On the other hand, functional association between the eNOS gene and plasma NOx levels have been studied in other conditions not related to SCD, mostly patients suffering from osteonecrosis, hypertension, carotid atherosclerosis and coronary disease (Nishank *et al.,* 2013). These studies have, however, produced contradictory results. For instance, among the patients with coronary artery disease, eNOS gene polymorphisms were found to be associated with either high plasma NOx levels (Yoon*et al.,* 2000) or no effects on NOx level (Afrasyap & Ozturk, 2004).

However, several workers from different parts of the world have found low plasma NOx level associated with mutant variants of three SNPs of eNOS gene polymorphism, which includes two of the polymorphisms studied in this work (T786C and VNTR) among these patients (Wang &Wang*et al.,* 2000; Hingorani *et al.,* 2003; Fatini *et al.,* 2004; Glueck *et al.,* 2009; Sinici*et al.,* 2009; Gururajan *et al.,* 2010; De Marco *et al.,* 2011). The findings of low level of plasma NOx associated with mutant variants of the eNOS gene in this study are supported by these group of studies as well.

In the same way, in the present study, the lack of difference in plasma NO levels between different eNOS gene polymorphisms in control group is supported by an earlier observation made among normal population of South India by Angeline *et al.* (2010) as well as the findings of Nagasaki *et al.* (2005) observed among Japanese healthy individuals. The higher incidence of specific mutant haplotype VNTR4a/b associated with low level of median plasma NO2 among the SCD patients with complications indicates that this haplotype may have pharmacogenetic implications, perhaps therapeutic implication of genetic variations in the eNOS gene. This study is the first report of an association between VNTR4a/b eNOS gene polymorphism and HbSS SCD complications in the world at the time of this report.

This study presents the first report of an association between angiopoietin dysregulation and SCD complications.

5.2 Conclusion

This study confirms reduced plasma NO levels in SCD patients in general. This disorder is severe in the disease complications. The VNTR4a/b polymorphisms of the eNOS gene are associated with lower levels of NO production in SCD patients with and without complications. There was an association between eNOS gene polymorphism (T786C and VNTR) and SCD in Ghana. The T786C of the eNOS gene polymorphism is associated with leg ulcer a complication SCD. Therefore these polymorphisms may acts as genetic modifiers for complications in SCD. There is endothelial dysfunction in SCD patients as demonstrated by increased serum levels of adhesion molecules. This abnormality is severe in the disease complications. There are elevated angiogenic factors in SCD patients in general with a further increase in the complications. This study has

provided data linking eNOS gene polymorphisms, endothelial dysfunction and angiogenesis in Ghanaian SCD patients with and without complications.

5.3 Recommendations

Further studies are needed for confirmation the association. In view of lack of sufficient studies in different ethnic population of other countries on eNOS gene polymorphism in SCD patients, further studies with increased number of individuals carrying these polymorphisms may be planned to extend the results of this study. Sequencing should be done for the new mutant fragment length that was found in the T786C mutant gene that was referred to as "New" in this study to ascertain its sequence.

5.4 Limitations

Sequencing could not be done for the new mutant length that was found in the T786C mutant gene in this study which has not been reported in literature but was referd to in this work as TC haplotype due to financial constraint.This study could not be extended to other sickle cell complications like ACS.Genotyping and chemistries could not be done for all the samples due to financial constaraints.

5.5 Clinical implications

The application of pharmacologic strategies designed to increase NO levels and decrease endothelial adherence will show clinical promise in decreasing the morbidity and mortality of patients with SCD.This gives a target for gene therapy. The transfer of eNOS gene to the host tissue to intervene in a disease process, with resultant alleviation of the symptoms.

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APPENDICES

Appendix I: Solution Preparation

(Preparation of 50XTris acetate diaminoethanetetra- acetic acid (TAE) buffer)

Ethylenediaminetetra acetic acid (EDTA) solution of concentration 0.5M was prepared by weighing 29.23 g of the EDTA powder (FW= 292.25) and dissolving in 200 ml distilled water. The pH of this solution was adjusted to 8.03 using concentrated NaOH solution.

TAE buffer (50x) was prepared by dissolving 12.11 g of Trizma base (FW = 121.14) in 37.5 ml distilled water, 5 mL of 0.5 M EDTA (pH 8.03) and 2.9 mL glacial acetic acid (99+%, FW = 60.05) solutions were added and made up to the 50 mL mark with distilled water.

To prepare the working solution of 1X TAE buffer, 20 ml of the 50xTAE buffer was measured and diluted with distilled water to a total volume of 1 L.

Appendix II: Preparation of Agarose gel (3%)

To prepare 3% agarose solution, 6 g of agarose was weighed and dissolved in 200mL 1xTAE buffer.

Appendix III: Ethidium bromide solution (10 mg/mL)

To make 10 mg/mL ethidium bromide solution, 0.1 g ethidium bromide tablet was weighed and dissolved in 10mL 1X TAE buffer.

Appendix IV: A comparison of the mean Haematological parameters among controls and sickle cell disease patients with different complications in males (Table 4.8)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

II: HCT

a. Control vrs HbSS steady state; p-value =<0.001
b. Control vrspriapism; p-value=<0.001
c. Control vrs leg ulcer; p-value=<0.001
d. Control vrs HbSS post VOC p-value=<0.001
e. Control vrs HbSS VOC; p-value=<0.001
f. Control vrs HbSC VOC; p-value =0.0369
g. HbSC steady state vrs HbSS steady state; p-value=<0.001
h. HbSC steady state vrspriapism; p-value =<0.001
i. HbSC steady state vrs leg ulcer; p-value =<0.001
j. HbSC steady state vrs HbSS post VOC; p-value=<0.001
k. HbSC steady state vrs HbSS VOC; p-value=<0.001
l. HbSC VOC vrs HbSS steady state; p-value=<0.001
m. HbSC VOC vrspriapism; p-value=<0.001
n. HbSC VOC vrs leg ulcer; p-value=<0.001
o. HbSC VOC vrs HbSS post VOC; p-value=<0.001
p. HbSC VOC vrs HbSS VOC; p-value =<0.001
q. HbSS VOC vrs HbSS steady state; p-value=0.0055

III: MCH

a. HbSS steady state vrs HbSC steady state; p-value=0.0115

IV: MCHC

a. HbSC VOC vrs HbSC steady state; p-value=<0.001
b. HbSS post VOC vrs HbSC steady state; p-value=<0.001
c. Leg ulcer vrs HbSC steady state; p-value=<0.001
d. HbSS VOC vrs HbSC steady state; p-value=<0.001

e. HbSS steady state vrs HbSC steady state; p-value=<0.001
f. Control vrs HbSC steady state; p-value=<0.001
g. Priapismvrs HbSC steady state; p-value=<0.001

V: RDW

a. HbSS VOC vrs HbSC steady state; p-value=0.0289
b. HbSS steady state vrs HbSC steady state; p-value=0.0188
c. Control vrs HbSS steady state; p-value=<0.001
d. Control vrs HbSC VOC; p-value=<0.001
e. Control vrs HbSS post VOC; p-value=<0.001
f. Control vrs HbSS VOC; p-value=<0.001
g. Control vrs HbSS steady state; p-value=<0.001
h. Control vrs leg ulcer p-value=<0.001
i. Control vrspriapism p-value=<0.001

VI: PLT

a. HbSC steady state vrs control; p-value=0.0145
b. Priapismvrs control; p-value=0.0006
c. Leg ulcer vrs control; p-value=<0.001
d. HbSS VOC vrs control; p-value=<0.001
e. HbSS post VOC vrs control; p-value=<0.001
f. HbSS steady state vrs control; p-value=<0.001
g. Priapismvrs HbSC VOC; p-value=0.0275
h. Leg ulcer vrs HbSC VOC; p-value=0.0002
i. HbSS VOC vrs HbSC VOC; p-value=<0.001
j. HbSS post VOC vrs HbSC VOC; p-value=0.0001
k. HbSS steady state vrs HbSS VOC; p-value=<0.001
l. Leg ulcer vrs HbSC steady state; p-value=0.0002
m. HbSS VOC vrs HbSC steady state; p-value=<0.001
n. HbSS post VOC vrs HbSC steady state; p-value=<0.001
o. HbSS steady state vrs HbSC steady state; p-value=<0.001

VII: MPV

a. HbSC steady state vrs HbSS steady state; p-value=0.0063
b. Leg ulcer vrs HbSS steady state; p-value=0.0176
c. Priapismvrs HbSS steady state; p-value=0.0197
d. Control vrs HbSS steady state; p-value=<0.001
e. Control vrs HbSS VOC; p-value=<0.001
f. Control vrs HbSS post VOC; p-value=<0.001
g. Control vrs HbSC VOC; p-value=<0.001
h. Control vrs HbSC steady state; p-value=<0.001
i. Control vrs leg ulcer; p-value=<0.001
j. Control vrspriapism p-value=<0.001

VIII: PCT

a. Priapismvrs HbSC VOC; p-value=0.0342
b. HbSS VOC vrs HbSC VOC; p-value=0.0001
c. HbSS post VOC vrs HbSC VOC; p-value=0.0014
d. Leg ulcer vrs HbSC VOC; p-value=0.0005
e. Control vrs HbSC VOC; p-value=<0.001
f. HbSS steay state vrs HbSC VOC; p-value=<0.001
g. HbSS VOC vrs HbSC steady state; p-value=<0.001
h. HbSS post VOC vrs HbSC steady state; p-value=<0.0024
i. Leg ulcer vrs HbSC steady state; p-value=0.0007
j. Control vrs HbSC steady state; p-value=<0.001
k. HbSS steady state vrs HbSC steady state; p-value=<0.001

VIX: PDW

a. HbSS steady state vrs HbSS VOC; p-value=0.0454
b. HbSC steady state vrs HbSS VOC; p-value=0.0455
c. Leg ulcer vrs HbSS VOC; p-value=0.0417
d. HbSS post VOC vrs HbSS VOC; p-value=0.0317
e. Priapismvrs HbSS VOC; p-value=0.0168

Appendix V: A comparison of the mean Haematological parameters among controls and sickle cell disease patients with different complications in females (Table 4.9)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I: WBC

a. HbSC VOC vrs control; p-value=0.0141
b. HbSC steady state vrs control; p-value=<0.001
c. HbSS steady state vrs control; p-value=<0.001
d. HbSS post VOC vrs control; p-value=<0.001
e. HbSS VOC vrs control; p-value=<0.001
f. HbSS steady state vrs leg ulcer; p-value=0.0420
g. HbSS VOC vrs Leg ulcer; p-value=0.0011
h. HbSS post VOC vrs leg ulcer; p-value=0.0124
i. HbSS steady state vrs HbSC VOC; p-value=0.0101
j. HbSS post VOC vrs HbSC VOC; p-value=0.0029
k. HbSS VOC vrs HbSC VOC; p-value=<0.001
l. HbSS steady state vrs HbSC steady state; p-value=0.0451
m. HbSS post VOC vrs HbSC steady state; p-value=0.0126
n. HbSS VOC vrs HbSC steady state; p-value=<0.001
o. HbSS VOC vrs HbSS steady state; p-value=<0.001

II: RBC

a. HbSC steady state vrs HbSS steady state; p-value=<0.001
b. HbSC VOC vrs HbSS steady state; p-value=<0.001
c. Leg ulcer vrs HbSS steady state; p-value =<0.001
d. Control vrs HbSS steady state; p-value=<0.001
e. HbSC steady state vrs HbSS VOC; p-value=<0.001
f. HbSC VOC vrs HbSS VOC; p-value=<0.001
g. Leg ulcer vrs HbSS VOC; p-value=0.0029
h. Control vrs HbSS VOC; p-value=<0.001
i. HbSC steady state vrs HbSS post VOC; p-value=<0.001
j. HbSC VOC vrs HbSS post VOC; p-value=<0.001
k. Leg ulcer vrs HbSS post VOC; p-value=0.0085

l. Control vrs HbSS post VOC; p-value=<0.001
m. Control vrs HbSC steady state; p-value=<0.001
n. Control vrs HbSC VOC; p-value=0.0031

IV: HCT

a. HbSC steady state vrs HbSS steady state; p-value=<0.001
b. HbSC VOC vrs HbSS steady state; p-value=<0.001
c. Control vrs HbSS steady state; p-value=<0.001
d. HbSC steady state vrs HbSS post VOC; p-value=<0.001
e. HbSC VOC vrs HbSS post VOC; p-value=<0.001
f. Control vrs HbSS post VOC; p-value=<0.001
g. HbSC steady state vrs HbSS VOC; p-value=<0.001
h. HbSC VOC vrs HbSS VOC; p-value=<0.001
i. Control vrs HbSS VOC; p-value=<0.001
j. Control vrs leg ulcer; p-value=0.0036
k. Control vrs HbSC steady state; p-value=<0.001
l. Control vrs HbSC VOC; p-value=0.0010

V: MCV

a. Control vrs leg ulcer; p-value=0.0386
b. HbSS post VOC vrs leg ulcer; p-value=0.0381
c. HbSS VOC vrs leg ulcer; p-value=0.0046
d. HbSS steady state vrs leg ulcer; p-value=0.0009
e. HbSS VOC vrs HbSC steady state; p-value=0.0166
f. HbSS steady state vrs HbSC steady state; p-value=<0.001
g. HbSS steady state vrs HbSC VOC; p-value=0.0139
h. HbSS steady state vrs control; p-value=<0.001

VI: MCHC

a. HbSC steady state vrs HbSS VOC; p-value=0.0390
b. HbSC steady state vrs HbSS steady state; p-value=0.0425

VII: RDW

a. HbSS VOC vrs HbSC VOC; p-value=0.0196
b. HbSS steady state vrs HbSC VOC; p-value=0.0061

c. HbSS post VOC vrs HbSC VOC; p-value=0.0341
d. Control vrs HbSC VOC; p-value=0.0015
e. HbSS VOC vrs HbSC steady state; p-value=0.0038
f. HbSS steady state vrs HbSC steady state; p-value=<0.001
g. HbSS post VOC vrs HbSC steady state; p-value=0.0205
h. Control vrs HbSC steady state; p-value=<0.001

VIII: PLT

a. HbSC steady state vrs control; p-value=0.0193
b. HbSS post VOC vrs control; p-value=<0.001
c. HbSS VOC vrs control; p-value=<0.001
d. HbSS steady state vrs control; p-value=<0.001
e. HbSS post VOC vrs HbSC VOC; p-value=0.0069
f. HbSS VOC vrs HbSC VOC; p-value=<0.001
g. HbSS steady state vrs HbSC VOC; p-value=<0.001
h. HbSS post VOC vrs HbSC steady state; p-value=0.0026
i. HbSS VOC vrs HbSC steady state; p-value=<0.001
j. HbSS steady state vrs HbSC steady state; p-value=<0.001

VIX: MPV

a. HbSS steady state vrs leg ulcer; p-value=<0.001
b. HbSS VOC vrs leg ulcer; p-value=<0.001
c. HbSS post VOC vrs leg ulcer; p-value=<0.001
d. HbSC VOC vrs leg ulcer; p-value=<0.001
e. HbSC steady state vrs leg ulcer; p-value=<0.001
f. Control vrs leg ulcer; p-value=<0.001
g. HbSS VOC vrs HbSS steady state; p-value=0.0133
h. HbSC VOC vrs HbSS steady state; p-value=0.0022
i. HbSC steady state vrs HbSS steady state; p-value=<0.001
j. Control vrs HbSS steady state; p-value=<0.001
k. HbSC steady state vrs HbSS VOC; p-value=0.0093
l. Control vrs HbSS VOC; p-value=<0.001
m. Control vrs HbSS post VOC; p-value=0.0401

X: PCT

a. HbSCsteady state vrs control; p-value=0.0134
b. HbSS steady state vrs control; p-value=<0.001
c. HbSS post VOC vrs control; p-value=<0.001
d. HbSS VOC vrs control; p-value=<0.001
e. HbSS steady state vrs HbSC VOC; p-value=0.0015
f. HbSS post VOC vrs HbSC VOC; p-value=0.0088
g. HbSS VOC vrs HbSC VOC; p-value=<0.001
h. HbSS steady state vrs HbSC steady state; p-value=<0.001
i. HbSS post VOC vrs HbSC steady state; p-value=0.0053
j. HbSS VOC vrs HbSC steady state; p-value=<0.001

XI: PDW

a. HbSC VOC vrs leg ulcer; p-value=0.0406
b. HbSS VOC vrs leg ulcer; p-value=0.0046
c. HbSS steady state vrs leg ulcer; p-value=<0.001
d. Control vrs leg ulcer; p-value=<0.001
e. HbSS post VOC vrs leg ulcer; p-value=<0.001
f. HbSS VOC vrs HbSC steady state; p-value=0.0185
g. HbSS steady state vrs HbSC steady state; p-value=<0.001
h. Control vrs HbSC steady state; p-value=<0.001
i. HbSS post VOC vrs HbSC steady state; p-value=<0.001
j. HbSS steady state vrs HbSC VOC; p-value=0.0285
k. Control vrs HbSC VOC; p-value=<0.001
l. HbSS post VOC vrs HbSC VOC; p-value=0.004
m. Control vrs HbSS VOC; p-value=0.0014
n. HbSS post VOC vrs HbSS VOC; p-value=0.0151

Appendix VI:

Relationship between haematology, angiogenic factors and endothelial markers in controls and SCD patients with and without complications (Table 4.12)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

Group difference that were not significant are not included

I. ANG1

a. HbSC VOC vrspriapism; p-value=0.005
b. HbSS VOC vrspriapism; p-value=0.000
c. HbSS VOC vrs leg ulcer; p-value=0.003
d. HbSS steady state vrspriapism; p-value=0.000
e. HbSS steady state vrs leg ulcer; p-value=0.013
f. Priapismvrs HbSC VOC; p-value=0.005
g. Priapismvrs HbSSVOC; p-value=0.000
h. Priapismvrs HbSS steady state; p-value=0.000
i. Priapismvrs control; p-value =0.000
j. Leg ulcer vrs HbSS VOC; p-value=0.003
k. Leg ulcer vrs HbSS steady state; p-value=0.013
l. Leg ulcer vrs control; p-value=0.003
m. Control vrspriapism; p-value=0.000
n. Control vrs leg ulcer; p-value=0.003

II. ANG 2

a. HbSC VOC vrs control; p-value=0.022
b. HbSS VOC vrspriapism; p-value=0.02
c. HbSS VOC vrs leg ulcer; p-value=0.047
d. HbSS VOC vrs control; p-value=0.000
e. HbSS steady state vrs control; p-value=0.000
f. Priapismvrs HbSS VOC; p-value=0.020
g. Leg ulcer vrs HbSS VOC; p-value=0.047
h. Control vrs HbSC VOC; p-value=0.022
i. Control vrs HbSS VOC; p-value=0.000
j. Control vrs HbSS steady state; p-value=0.005

III. VEGF

a. HbSC VOC vrs HbSS VOC; p-value=0.009
b. HbSC VOC vrs control; p-value=0.016
c. HbSS VOC vrs HbSC VOC; p-value=0.009
d. HbSS VOC vrs HbSS steady state; p-value=0.047
e. HbSS steady state vrs HbSS VOC; p-value=0.0047
f. Control vrs HbSS VOC; p-value=0.016

IV. VCAM

a. HbSC VOC vrs leg ulcer; p-value=0.005
b. HbSS VOC vrs leg ulcer; p-value=0.001
c. HbSS steady state vrs leg ulcer; p-value=0.001
d. Priapismvrs leg ulcer; p-value=0.023
e. Leg ulcer vrs HbSC VOC; p-value=0.005
f. Leg ulcer vrs HbSS VOC; p-value=0.001
g. Leg ulcer vrs HbSS steady state; p-value = 0.001
h. Leg ulcer vrspriapism; p-value=0.023
i. Leg ulcer vrs control; p-value=0.001
j. Control vrs leg ulcer; p-value=0.001

V. **E- SELECTIN**

a. HbSC VOC vrs Leg ulcer; p-value=0.002
b. HbSS VOC vrspriapism; p-value=0.033
c. HbSS VOC vrs leg ulcer; p-value=0.000
d. HbSS VOC vrs control; p-value=0.019
e. HbSS steady state vrs leg ulcer; p-value=0.001
f. Priapismvrs HbSS VOC; p-value=0.033
g. Leg ulcer vrs HbSC VOC; p-value=0.002
h. Leg ulcer vrs HbSS VOC; p-value=0.000
i. Leg ulcer vrs HbSS steady state; p-value=0.001
j. Leg ulcer vrs control; p-value=0.006

VI. HCT

a. HbSC VOC vrs HbSS VOC; p-value=0.000
b. HbSC VOC vrs HbSS steady state; p-value=0.000
c. HbSC VOC vrspriapism; p-value=0.000
d. HbSC VOC vrs leg ulcer; p-value=0.000
e. HbSC VOC vrs control; p-value=0.041
f. HbSS VOC vrs HbSC VOC; p-value=0.000
g. HbSS VOC vrs steady state; p-value=0.019
h. HbSS VOC vrs control; p-value=0.000
i. HbSS steady state vrs HbSC VOC; p-value=0.000
j. HbSS steady state vrs HbSS VOC; p-value=0.019
k. HbSS steady state vrs control; p-value=0.000
l. Priapismvrs HbSC VOC; p-value=0.000
m. Priapismvrs control; p-value=0.000
n. Leg ulcer vrs HbSC VOC; p-value=0.000
o. Leg ulcer vrs control; p-value=0.000
p. Control vrs HbSC VOC; p-value=0.041
q. Control vrs HbSS VOC; p-value=0.000
r. Control vrs HbSS steady state; p-value=0.000
s. Control vrspriapism; p-value=0.000
t. Control vrs leg ulcer; p-value=0.000

VII. RBC

a. HbSC VOC vrs HbSS VOC; p-value=0.000
b. HbSC VOC vrs HbSS steady state; p-value=0.000
c. HbSC VOC vrspriapism; p-value=0.000
d. HbSC VOC vrs leg ulcer; p-value=0.000
e. HbSC VOC vrs control; p-value=0.009
f. HbSS VOC vrs HbSC VOC; p-value=0.000
g. HbSS VOC vrs HbSS steady state; p-value=0.000
h. HbSS VOC vrs control; p-value=0.00
i. HbSS steady state vrs HbSC VOC; p-value=0.000
j. HbSS steady state vrs HbSS VOC; p-value=0.00

k. HbSS steady state vrs leg ulcer; p-value=0.007
l. HbSS steady state vrs control; p-value=0.000
m. Priapismvrs HbSC VOC; p-value=0.000
n. Priapismvrs control; p-value=0.000
o. Leg ulcer vrs HbSC VOC; p-value=0.000
p. Leg ulcer vrs HbSS steady state; p-value=0.007
q. Leg ulcer vrs control; p-value=0.000
r. Control vrs HbSC; p-value=0.009
s. Control vrs HbSS VOC; p-value=0.000
t. Control vrs HbSS steady state; p-value=0.000
u. Control vrspriapism; p-value=0.000
v. Control vrs Leg ulcer; p-value=0.000

VIII. WBC

a. HbSC VOC vrs HbSS VOC; p-value=0.000
b. HbSC VOC vrs HbSS steady state; p-value=0.000
c. HbSC VOC vrs leg ulcer, p-value=0.013
d. HbSC VOC vrs control; p-value=0.011
e. HbSS VOC vrs HbSC VOC; p-value=0.000
f. HbSS VOC vrs HbSS steady state; p-value=0.018
g. HbSS VOC vrspriapism; p-value=0.000
h. HbSS VOC vrs leg ulcer, p-value=0.008
i. HbSS VOC vrs control; p-value=0.000
j. HbSS steady state vrs HbSC VOC; p-value=0.000
k. HbSS steady state vrs HbSS VOC; p-value=0.018
l. HbSS steady state vrspriapism; p-value=0.023
m. HbSS steady state vrs control; p-value=0.000
n. Priapismvrs HbSS VOC; p-value=0.000
o. Priapismvrs HbSS steady state; p-value=0.023
p. Priapismvrs control; p-value=0.011
q. Leg ulcer vrs HbSC VOC; p-value=0.013
r. Leg ulcer vrs HbSS VOC; p-value=0.008
s. Leg ulcer vrs control; p-value=0.000

t. Control vrs HbSC VOC; p-value=0.011
u. Control vrs HbSS VOC; p-value=0.000
v. Control vrs HbSS steady state; p-value=0.000
w. Control vrspriapism; p-value=0.011
x. Control vrs leg ulcer; p-value=0.000

IX. PLT

a. HbSC VOC vrs HbSS VOC; p-value=0.000
b. HbSC VOC vrs HbSS steady state; p-value=0.00
c. HbSC VOC vrs leg ulcer; p-value=0.000
d. HbSS VOC vrs HbSC VOC; p-value=0.000
e. HbSS VOC vrspriapism; p-value=0.000
f. HbSS VOC vrs leg ulcer; p-value=0.002
g. HbSS VOC vrs control; p-value=0.000
h. HbSS steady state vrs HbSC VOC; p-value=0.000
i. HbSS steady state vrspriapism; p-value=0.000
j. HbSS steady state vrs control; p-value=0.000
k. Priapismvrs HbSS VOC; p-value=0.000
l. Priapismvrs HbSS steady state; p-value=0.000
m. Priapismvrs leg ulcer; p-value=0.000
n. leg ulcer vrs HbSC VOC; p-value=0.000
o. leg ulcer vrs HbSS VOC; p-value=0.002
p. leg ulcer vrsPriapism; p-value=0.000
q. leg ulcer vrs control; p-value=0.000
r. control vrs HbSS VOC; p-value=0.000
s. control vrs HbSS steady state; p-value=0.000
t. control vrs leg ulcer; p-value=0.000

X. MCV

a. HbSC VOC vrs HbSS steady state; p-value=0.000
b. HbSS VOC vrs HbSS steady state; p-value=0.000
c. HbSS steady state vrs HbSC VOC; p-value=0.000
d. HbSS steady state vrs HbSS VOC; p-value=0.000
e. HbSS steady state vrspriapism; p-value=0.000
f. HbSS steady state vrs leg ulcer; p-value=0.000
g. HbSS steady state vrs control; p-value=0.000
h. Priapismvrs HbSS steady state; p-value=0.000
i. Leg ulcer vrs HbSS steady state; p-value=0.000
j. Control vrs HbSS steady state; p-value=0.000

Appendix VII: Comparisons of angiogenic factors, endothelial markers and haematological indices TT polymorphism across the disease categories(Table 4.23)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I: HB

a. Control vrs HbSS steady state; p-value < 0.001
b. Control vrs Leg ulcer; p-value < 0.001
c. Control vrs HbSS VOC ; p-value < 0.001

II: HCT

a. HbSS VOC vrs HbSS steady state; p-value = 0.0263
b. Control vrs HbSS steady state; p-value < 0.001
c. Control vrs Leg ulcer; p-value < 0.001
d. Control vrs HbSS VOC ; p-value < 0.001

III: RBC

a. Leg ulcer vrs HbSS steady state; p-value = 0.0378
b. HbSS VOC vrs HbSS steady state; p-value = 0.0022
c. Control vrs HbSS steady state; p-value < 0.001
d. Control vrs Leg ulcer; p-value < 0.001
e. Control vrs HbSS VOC ; p-value < 0.001

IV: Hb*

a. Control vrs HbSS steady state; p-value < 0.001
b. Control vrs Leg ulcer; p-value < 0.001
c. Control vrs HbSS VOC; p-value < 0.001

V: WBC

a. Leg ulcer vrs Controls; p-value < 0.001
b. HbSS steady state vrs Controls; p-value < 0.001
c. HbSS VOC vrs Controls; p-value < 0.001

VI: Ptl

a. HbSS steady state vrs Controls; p-value < 0.001
b. HbSS VOC vrs Controls; p-value < 0.001
c. Leg ulcer vrs Controls; p-value < 0.001

VII: MCV

a. Leg ulcer vrs HbSS steady state; p-value < 0.001
b. Control vrs HbSS steady state; p-value < 0.001
c. HbSS VOC vrs HbSS steady state; p-value < 0.001

VIII: VCAM

a. Leg ulcer vrs Controls; p-value = 0.0263
b. Leg ulcer vrs Steady state; p-value = 0.0191
c. Leg ulcer vrs HbSS VOC; p-value = 0.0415

IX: VGEF

a. HbSS steady statevrs HbSS VOC; p-value = 0.0022
b. HbSS steady state vrs Controls; p-value = 0.0052
c. HbSS steady state vrs Leg ulcer ; p-value = 0.0098

X: ANGIO 1

a. HbSS VOC vrs HbSS steady state; p-value = 0.0196
b. Leg ulcer vrs HbSS steady state; p-value < 0.001
c. Leg ulcer vrs Controls; p-value = 0.0047
d. Leg ulcer vrs HbSS VOC; p-value = 0.0245

XI: NO

a. HbSS steady state vrs Controls; p-value = 0.0390
b. HbSS VOC vrs Controls; p-value = 0.0460

AppendixVIII: Comparisons of angiogenic factors, endothelial markers and haematological indices TC polymorphism across the disease categories(Table 4.24)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I.Hb

a. Priapismvrs HbSS steady state; p-value = 0.0031

b. Control vrs HbSS steady state; p-value < 0.001

c. Priapismvrs HbSS VOC; p-value < 0.001

d. Control vrs HbSS VOC; p-value < 0.001

II.HCT

a. HbSC VOC vrs HbSS steady state; p-value = 0.0035
b. Control vrs HbSS steady state; p-value < 0.001
c. HbSC VOC vrs HbSC VOC; p-value = 0.0352
d. Control vrs HbSC VOC; p-value < 0.001
e. Control vrsPriapism; p-value = 0.0048

III.RBC

a. HbSS VOC vrs HbSS steady state; p-value = 0.354
b. HbSC VOC vrs HbSS steady state; p-value < 0.001
c. Control vrs HbSS steady state; p-value < 0.001
d. HbSC VOC vrsPriapism; p-value = 0.103
e. Control vrsPriapism; p-value < 0.001
f. Control vrs HbSS VOC; p-value < 0.001

IV.WBC

a. HbSC VOC vrs Control; p-value = 0.0496
b. HbSS VOC vrs Control; p-value < 0.0007
c. HbSS steady state vrs Control; p-value < 0.0006

V.Plt

a. HbSS VOC vrs Control; p-value = 0.0045

b. HbSS steady state vrs Control; p-value < 0.001

c. HbSS steady state vrs HbSC VOC; p-value = 0.0168

d. HbSS steady state vrsPriapism; p-value = 0.173

c. HbSS steady state vrs IbSS VOC; p-value = 0.292

VI.MCV

a. HbSS VOC vrs HbSS steady state; p-value < 0.001
b. Control vrs HbSS steady state; p-value < 0.001
c. HbSS VOC vrs HbSS steady state; p-value < 0.001
d. Priapismvrs HbSS steady state; p-value < 0.001

VII. ANGIO 1

a. Priapismvrs HbSC VOC; p-value = 0.00233
b. Priapismvrs HbSS VOC; p-value < 0.001
c. Priapismvrs Controls; p-value = 0.0169
d. Priapismvrs HbSS steady state; p-value = 0.0148

VIII. NO

a. Control vrsPriapism; p-value = 0.389
b. HbSC VOC vrsPriapism; p-value = 0.0267
c. HbSS steady state vrsPriapism; p-value < 0.001
d. HbSS steady state vrs Control; p-value = 0.0720

Appendix IX: Comparisons of angiogenic factors, endothelial markers and haematological indices VNTR4a polymorphism across the disease categories(Table 4.25)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I.HCT

a. HbSC VOC vrs Leg ulcer; p-value < 0.001

b. Control vrs Leg ulcer; p-value < 0.001

c. HbSC VOC vrs HbSS steady state; p-value < 0.001

d. Control vrs HbSS steady state; p-value < 0.001

e. HbSC VOC vrs HbSS VOC; p-value = 0.0096

f. Control vrs HbSS VOC; p-value < 0.001

II.RBC

a. HbSS VOC vrs HbSS steady state; p-value = 0.0164

b. Control vrs HbSS steady state; p-value < 0.001

c. Control vrs Leg ulcer; p-value < 0.001

d. Control vrs HbSS VOC; p-value < 0.001

III.WBC

a. HbSC VOC vrs Control; p-value = 0.0029

b. Leg ulcer vrs Control; p-value < 0.001

c. HbSS steady state vrs Control; p-value < 0.001

d. HbSS VOC vrs Control; p-value < 0.001

IV. Ptl

a. HbSS VRS VOC vrs Control; p-value < 0.001

b. HbSS steady state vrs Control; p-value < 0.001

c. Leg ulcer vrs Control; p-value < 0.001

d. HbSS VOC vrs HbSC VOC; p-value = 0.0142

e. HbSS steady state vrs HbSC VOC; p-value = 0.0059

f. Leg ulcer vrs HbSC VOC; p-value = 0.0071

V. MCV

a. HbSS VOC vrs HbSS steady state; p-value < 0.001

b. Control vrs HbSS steady state; p-value < 0.001

c.Leg ulcer vrs HbSS steady state; p-value < 0.001

VI. VCAM

a. HbSS VOC vrs Control; p-value = 0.0022

b. HbSS steady state vrs Control; p-value = 0.0051

c. HbSC VOC vrs Control; p-value = 0.0268

VII. VGEF

a. HbSS steady state vrs Leg ulcer; p-value = 0.0444

b. HbSS steady state vrs Control; p-value = 0.0078

c. HbSS steady state vrs IbSS VOC; p-value = 0.0291

VIII. ANGIO 1

a. Leg ulcer vrs HbSS VOC; p-value = 0.0312

b. Leg ulcer vrs Control; p-value = 0.0465

IX. E-SECT

a. Control vrs HbSS VOC; p-value = 0..0053

b. Leg ulcer vrs HbSS VOC; p-value < 0.001

c. Leg ulcer vrs HbSC VOC; p-value < 0.001

d. Control vrs HbSS steady state; p-value = 0.0423

e. Leg ulcer vrs Control; p-value = 0.0126

VRS. ANGIO 2

a. HbSS steady state vrs Control; p-value = 0.0315

b.HbSS VOC vrs Control; p-value = 0.0015

c.HbSC VOC vrs Control; p-value = 0.0268

Appendix X: Comparisons of angiogenic factors, endothelial markers and haematological indices VNTR 4b polymorphism across the disease categories(Table 4.26)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I. Hb

a. Control vrsPriapism; p-value < 0.001

b.Control vrs HbSS VOC; p-value = 0.0142

II.HCT

a. Control vrs Leg ulcer; p-value = 0.0020

III. RBC

a. Control vrs HbSS VOC; p-value = 0.0185

b. Control vrs Leg ulcer; p-value = 0.0077

IV.WBC

a. Leg ulcer vrs Control; p-value = 0.0028

b. HbSS VOC vrs Control; p-value < 0.001

c. HbSS VOC vrs Leg ulcer; p-value < 0.001

V. Ptl

a. Leg ulcer vrs Control; p-value = 0.0161

VI. VEGF

a. HbSS VOC vrs Leg ulcer; p-value < 0.001

VII. ANGIO 2

a. HbSS vrs Control; p-value = 0.0078

b. HbSS VOC vrs Control; p-value < 0.001

VIII. NO

a. Leg ulcer vrs Control; p-value = 0.0446

Appendix XI: Comparisons of angiogenic factors, endothelial markers and haematological indices VNTR4c polymorphism across the disease categories(Table 4.20)

Pair wise analysis: Student's t-test analyses of group difference were as follows:

I.Hb

a. Priapismvrs HbSS VOC; p-value = 0.0206

b. Control vrs HbSS VOC; p-value < 0.001

c.Control vrs Leg ulcer; p-value = 0.0029

d. Control vrsPriapism; p-value = 0.0376

II.HCT

a. Priapismvrs HbSS VOC; p-value = 0.0485
b. Control vrs HbSS VOC; p-value < 0.001
c. Control vrs Leg ulcer; p-value = 0.0049
d. Control vrsPriapism; p-value = 0.0299

III. RBC

a. Priapismvrs HbSS VOC; p-value = 0.0381
b. Control vrs HbSS VOC; p-value = 0.0012
c. Control vrs Leg ulcer; p-vaalue = 0.0087
d. Control vrsPriapism p-value = 0.0613

IV.WBC

a. HbSS VOC vrs Control; p-value = 0.0223
b. HbSS VOC vrsPriapism; p-value = 0.0109

V.Ptl

a. HbSS VOC vrsPriapism; p-value = 0.0048

b. Leg ulcer vrsPriapism; p-value = 0.0052

c. HbSS VOC vrs Control; p-value = 0.0287

d. Leg ulcer vrs Control; p-value = 0.0185

VI. ICAM

a. Control vrs Leg ulcer; p-value < 0.001
b. Control vrs HbSS VOC; p- value < 0.002

VII.VCAM

a. HbSS VOC vrsPriapism; p-value = 0.0049
b. HbSS VOC vrs Leg ulcer; p-value = 0.0364

VIII. VGEF

a. Priapismvrs HbSS VOC; p-value = 0.0178

IX. ANGIO 1

a. Priapismvrs Control; p-value = 0.0474
b. Priapismvrs HbSS VOC; p-value = 0.0297