Immunophenotyping Markers and Cell Adhesion Molecules in Sickle Cell Anaemia Patients in Saudi Arabia

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DECLARATION

I declare that this work has not been accepted for any degree before and is not currently being submitted in candidature for any degree other than the degree of Doctor of Philosophy of the Manchester Metropolitan University.

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LIST OF ABBREVIATIONS

ACS	Acute Chest Syndrome
ALT	Alanine Transferase
APC	Antigen Presenting Cell
AST	Aspartate Transaminase
BCAM	Basal Cell Adhesion Molecules
BMP	Bone Morphogenic Protein
CAMs	Cellular Adhesion Molecules
CAR	Central African Republic
CD	Cluster of Differentiation
cDNA	Complementary Deoxy Ribonucleic Acid
CR3	Control Register number 3
DNA	Deoxy ribonucleic Acid
Da	Dalton
EDTA	Ethylene Diamine Tetra Acetic Acid
EGF-like	Epidermal Growth Factor-like
ELISA	Enzyme- Linked Immunosorbent Assay
ELAMA-1	Endothelium Leukocyte Adhesion Molecule A-1
ESL-1	Endothelium Selectin Specific Ligand-1
E-selectin	Endothelium selectin

FITC	Fluorescein-Isothiocyanate
FcR	Fragment crystallizable Receptor
GMP	Guanosine-5-Monophosphate
GMP-140	Granular Membrane Protein-140
GTP	Guanosine-5-Triphosphate
HbAA	Haemoglobin AA
HbSS	Sicled heamoglobin
Hib	*Haemophilus influenzae* type b
HLA- II	Human Leukocyte Antigen class II
ICAM-1	Intracellular Adhesion Molecule-1
ICAM-2	Intracellular Adhesion Molecule-2
IGF1-R	Insulin-Like Growth Factor 1- Receptor
IPD	Invasive Pneumococcal Disease
KDa	KiloDalton
LAM	Laminin
LDH	Lactate Dehydrogenase
Le CAM-1	Leukocyte Cell Adhesion Molecule-1
Le CAM-3	Leukocyte Cell Adhesion Molecule-3
LFA-1	Leukocyte Factor Activation-1
L-selectin	Leukocyte selectin

L-selectin (CD62L)	Leukocyte selectin (Cluster of Differentiation 62 leukocyte)
LU (BCAM)	Lutheran Basel Cell Adhesion molecule
LW	Lewis Antigen
Mad CAM-1	Mucosal Adhesion Cell Molecule-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NK cell	Natural Killer cell
NO	Nitric Oxide
NF - Kb	Nuclear Factor kappa-b
$ONOO^-$	Peroxy nitrite
PAF	Platelet activating factor
PADGEM	Platelet Activation Dependent Granule to External Membrane
PCV	Polysaccharide-protein Conjugate Vaccine
PE	Phycoerythrin
Per-CP	Peridinin-chlorophyll-protein-complex
PMNS	Polymorphonuclear leukocytes
PGE2	Prostaglandin E2
P-selectin	Platelet selectin
P-selectin (CD62P)	Platelet selectin (Cluster of Differentiation 62 Platelet)
P-SGL-1	Platelet Selectin Glycoprotein Ligand-1
RBC	Red Blood Cell

RPM	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
PSGL-1	P-Selectin Glycoprotein Ligand-1
SCA	Sickle Cell Anaemia
SCD	Sickle Cell Disease
Sle	Sialyl Lewis
sE-selectin	Soluble E-selectin
sP-selectin	Soluble P- selectin
TNF	Tumour Necrosis Factor
TGFb	Transforming Growth Factor b
TSP	Thrombospondin
USA	United States of America
VCAM-1	Vascular Cellular Adhesion Molecule-1
VLA-4	Very Late Antigen-4
VOC	Vaso Occlusive Crises
vWF	von Willebrand Factor
WBC	White Blood Cell
WPB	Weibel-Palade Bodies

ABSTRACT

Sickle cell anaemia (SCA) is an autosomal recessive inherited haemoglobinopathy, characterized by chronic haemolysis and inflammation, frequent infections and recurrent vaso-occlusive crisis (VOC). The hallmark of VOC is severe acute pain due to ischaemic tissue injury. The resultant end organ damage contributes to the significant morbidity and mortality observed in this disease. Genetic and environmental interactions result in a variable phenotype.

This study investigates the most common complications in SCA, including VOC and infections. Specifically, phagocytic activity of neutrophils and monocytes in the pathogenesis of SCA, and the most frequent bacterial infection. Furthermore, the study investigates changes in leukocytes subsets and the relationship between cell adhesion molecules expression and disease manifestations in patients during steady state and acute VOC.

The results indicate that *staphylococcus aureus* is the most common organism isolated in SCA related infections, followed by *Escherichia coli* and *Pseudomonas aeruginosa*.

There was no significant difference in phagocytic activity between both patient groups and control subjects. Lymphocyte subsets showed high percentages of total T lymphocytes, T helper and suppressor lymphocytes, B lymphocytes as well as NK cells in patients with SCA during steady state, while B lymphocytes and NK cells were significantly higher during acute VOC crisis, in comparison to control group.

One of most important and novel findings of the present study in Saudi Arabia was the presence of high levels immunophenotypic expression of L-selectin on neutrophils, monocytes and lymphocytes during acute VOC in comparison to normal control subjects. Accordingly, as markers for leukocytes activation, L- selectin can be

used as a predictor for VOC, which of value in the early diagnosis and intervention of these painful episodes.

In addition another anoval finding, is the high levels of both soluble E- selectin (sE-selectin), soluble P- selectin (sP- selectin) markers were demonstrated in the serum of patients with SCA during both steady state and acute VOC. Levels of selectins were significantly higher in acute VOC.

These findings suggest that patients with SCA have intact phagocytic activity and increased expression of adhesion molecules; L- selectin, E-selectin and P-selectin, which play an important role in the pathogensis of VOC.

While sickle cell disease is a well-recognized state of chronic inflammation, the role of specific adhesion molecules should be further elucidated. Studies are needed to investigate the potential role of selectin antagonists, for prevention and reversal of acute vascular occlusions in SCA patients.

CHAPTER 1

CHAPTER 1: GENERAL INTRODUCTION

Sickle cell anaemia (SCA) is a common inherited haemolytic disorder characterised by chronic haemolysis, frequent infections and recurrent vascular occlusion of small and large blood vessels. Recurrent vaso-occlusive crises (VOC) results in chronic organ damage and ultimately organ failure, and can lead to morbidity and mortality. Moreover, the chronic inflammation and infection increase cell adhesion molecule expression, which precipitate vaso-occlusive crisis (Ataga & Key, 2007; Makis *et al.*, 2000; Matsui *et al.*, 2002).

Sickle cell anaemia is caused by a mutation of the gene encoding the β-chain of haemoglobin. This mutation leads to the production of sickle haemoglobin, which has an abnormal tendency to polymerise. Haemoglobin S polymerisation and denaturation results in oxidant damage to the red blood cell membrane, with subsequently disturbed homeostasis which in turn, can result in dehydrated dense cells and irreversibly sickled cells. Red cell abnormalities lead to either haemolysis or vaso-occlusion. Vaso-occlusion results from interaction between red cells, leukocytes and endothelial cell inflammation, endothelial injury, leukocytes adhesion and activation of coagulation pathways contribute to the pathophysiology of vaso-occlusive crisis. The clinical hallmark of SCA is the excruciating pain, associated with sickle cell crisis. Such crisis occurs with variable frequency and duration, and they commonly require hospitalisation. Frequent attacks of painful crisis result in serious morbidity and hamper quality of life (Mousa *et al.*, 2010).

Sickle cell anaemia is frequently seen in sub-Saharan and Western regions of Africa, where 15-30% of the population carry the abnormal sickle cell gene (World Health

Organization, 2006; Weatherall & Clegg, 2001). Each year, between 300,000 and 400,000 infants are born with major haemoglobin disorders including more than 200,000 cases of sickle cell anaemia in Africa, with 150,000 in Nigeria alone (Banerjee *et al.*, 2001).

The wide distribution of the sickle cell gene is the result of survival advantage, which provides protection of the heterozygote against malaria infection. The prevalence of carriers in the Middle East and some parts of India ranges from 5 to 40% or more of the population (Hoffbrand *et al.*, 2006; Kumar & Clark, 1994) (Figure 1.1).

Sickle cell anaemia is also found in Arab and Mediterranean countries, the India subcontinent, the Caribbean and the Southern United States (Hoffbrand *et al.*, 2005; Hoffbrand *et al.*, 2006; Williams *et al.*, 2001). The epidemiology of SCA in Arab region and the high prevalence of the mutation are well documented. A study conducted in the populations of Bahrain and Oman, reported a prevalence of sickle cell trait of 11.2% and 6% respectively (Al-Hamdan *et al.*, 2007; Mohammed *et al.*, 1992; Mousa *et al.*, 2010).

In Saudi Arabia, the distribution of SCA is primarily in the Eastern, Western, and South Western provinces. As part of the Saudi National Premarital Screening Program, the prevalence of beta-thalassaemia and sickle cell disorders have been determined in the adult Saudi population. Notably, out of 488,315 individuals screened, 4.2% had sickle cell trait whilst 0.26% had sickle cell disease (Al-Hamdan *et al.*, 2007).

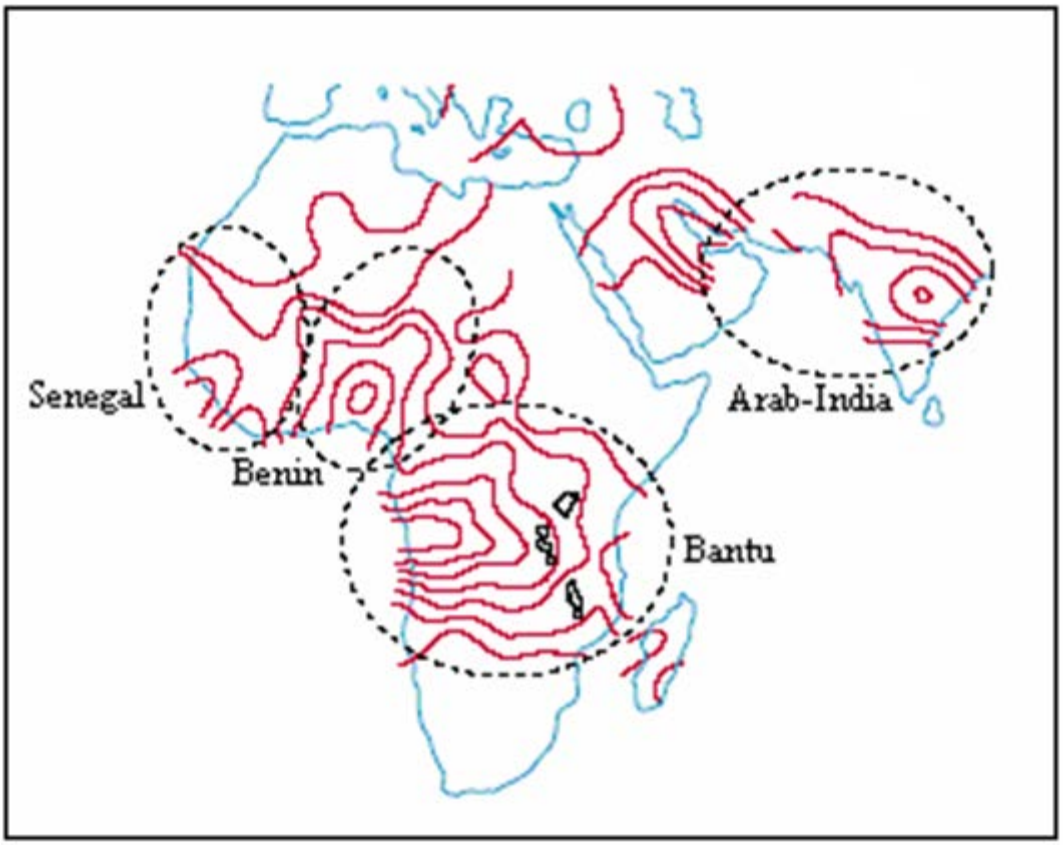

Figure 1.1 Geographical distribution and schematic representation of the sickle cell gene. The map identifies the three distinct areas in Africa and one in the Arab-India region where the sickle cell gene is present (dotted lines). The number of individuals with sickle-cell disease (red lines) in Senegal, Benin and Bantu are higher near the coast, and falls concentrically inland. Adapted from (Stuart & Nagel, 2004).

Extensive studies, conducted in different provinces of Saudi Arabia, have shown that a wide distribution pattern of haemoglobin S gene is present in different provinces. The overall prevalence of haemoglobin S carriers varies from 7% in the Western, 12% in the Southern, and approximately 25% in some parts of Eastern region (EI-Hazmi & Warsy, 1999; El-Hazmi & Warsy, 1994)

Perrine *et al*. (1978) established that serious complications including jaundice, spleen damage and haematuria occurred in Saudi patients less frequently than that reported in North American Blacks (6% Saudi versus 25% American Blacks) (Perrine *et al.*, 1978).

Between 1995 and 1999, about 256 patients were diagnosed with SCA at King Abdulaziz University Hospital (KAUH), either through our haematology clinic or the emergency department. There is a large body of evidence that the pathogenisis of SCA, the different clinical presentations, and complications are influenced by several variables, including the epistatic factors, adhesion mechanisms, coagulation activation, and leukocyte functions. Consequently, the effects of these variables were studied independently, and new approaches in the management of VOC were explored.

1.1 History

The first age of the haemoglobinopathies commenced in 1904, when Horrick first discovered sickle cell anaemia and continued with the identification in 1949, Pauling and Itano, of the presence of an electrophoretically abnormal component in the haemoglobin of patients with sickle cell anaemia (Stuart & Nagel, 2004). The identification of the cause of this change in mobility had to await the development of peptide mapping by Ingram in 1956, in 1963; Ingram confirmed that all patients with

sickle cell anaemia had the same substitution of valine for the glutamate in position 6 of the beta globin chain. This defect was subsequently identified as a single nucleotide mutation in the gene, which results in easy polymerisation of the abnormal haemoglobin (Matsui *et al.*, 2002).

1.2 Epidemiology

Worldwide, there is limited information on the exact number of individuals affected with homozygous sickle cell anaemia (SCA). The World Health Organization's global prevalence map of SCA suggests that approximately 20–25 million individuals worldwide are homozygous for HbS; with 12–15 million in sub-Saharan Africa, 5–10 million in India and approximately 3 million distributed across different parts of the world (Serjeant, 2006). In the United States of America (USA) approximately 70,000 patients with SCA have been registered. The life expectancy has improved amongst patients living in the USA and Western Europe, which means that organ complications associated with older age are now being observed with increasing frequency in adults. Furthermore, pulmonary hypertension, avascular necrosis of the femoral head, nephropathy, cholelithiasis, retinopathy, cardiomyopathy, and delayed growth and sexual maturation are frequent long term complications associated with increased morbidity and mortality (Aliyu *et al.*, 2008).

In many places, despite more than a decade of work, epidemiological data are still fragmentary. Precise population data and accurate gene frequency estimation are needed in order to define the magnitude of the problem in a given country. Furthermore, a phenotype screening for HbS usually provides epidemiological data (Labie & Elion, 2002; Williams *et al.*, 2001). With this in mind, sickle cell screening at birth is the most effective method and it is a common practice in the USA. This type of screening has also been implemented in European countries, owing to

increasing immigration from Africa or Western India. Moreover, cost efficiency of screening has been demonstrated using isoelectric focusing performed from dried blood spots on filter paper disks (Ryan *et al.*, 2010). In African countries, screening at birth may be problematic owing to the fact that the majority of deliveries take place at home and not in hospital (Labie & Elion, 2002).

1.3 Pathophysiology

Sickle cell anaemia is as an autosomal recessive trait, caused by a single-base mutation of adenine to thymine in the gene encoding the beta globin (β-globin) chain on chromosome 11. This modification leads to substitution of one glutamic acid residue by valine in the 6 position of the same chain (Hoffbrand *et al.*, 2006; Williams *et al.*, 2001)

In the homozygous state, both β- globin genes are abnormal (HbSS); however, as the synthesis of foetal haemoglobin (HbF) is normal, the anaemia usually does not manifest itself until the HbF production decreases to adult levels, at approximately 6 months of age (Hoffbrand *et al.*, 2005; Hoffbrand *et al.*, 2006; Williams *et al.*, 2001).

Vaso-occlusion results from a combination of abnormalities in the haemoglobin structure and function, a loss of red blood cell membrane integrity, the promotion of inflammatory mediators, activation of endothelial cell adhesion, disrupted vascular smooth muscle function, and enhanced coagulation (Aliyu *et al.*, 2008; Chiang & Frenette, 2005).

The clinical situations in which the red cell undergoes low oxygen tension, deoxygenation, will induce potassium efflux and increased red cell density, as well as

the tendency of haemoglobin S to polymerise. These cells then line up to form crystals known as tactoids (Horiuchi *et al.*, 1990). As a result, the intracellular contents of abnormal haemoglobin become more viscous and the flexibility of the red blood cells is decreased, so that they become rigid and form the sickle shape during their passage through the microcirculation. The process is initially reversible, but with repeated sickling, the cells eventually lose their membrane flexibility and remain in the irreversible sickle form (Williams *et al.*, 2001) (Figure 1.2).

Haemolysis contributes to dysregulation of nitric oxide. Nitric oxide (NO), produced by endothelial NO synthase, reacts with free haemoglobin to produce met-haemoglobin and nitrate which leads to NO depletion. Nitric oxide has a major role in maintaining the integrity of vascular homeostasis, whereby it regulates and mediates smooth muscle relaxation and vasomotor tone; it down-regulates endothelial adhesion molecule expression and inhibits platelet activation and aggregation (Mack & Kato, 2006; Reiter *et al.*, 2002; Rother *et al.*, 2005) (Figure 1.3).

The abnormal adhesiveness of sickle red blood cells to endothelial cells slows the transit of blood cells through small vessels and precipitates in the occurrence of VOC (Hoffbrand *et al.*, 2005; Odièvre *et al.*, 2008). Notably, the adherence of sickle red cells to endothelial cells is a complex process requiring interactions between multiple adhesion molecules. Cell adhesion molecules (CAM) on the red blood cell include integrin α4b1(VLA-4), α5b3 integrin and CD36 (thrombospondin receptor) and are expressed on young reticulocytes (Telen, 2001, 2005), whilst endothelial adhesion molecules include endothelin-1, vascular cellular adhesion molecule-1 (VCAM-1), intracellular cell adhesion molecule-1 (ICAM-1), basal cell adhesion molecule (BCAM), platelets selectin (P-selectin), endothelial selectin (E-selectin) and fibronectin (Okpala, 2004b, 2006).

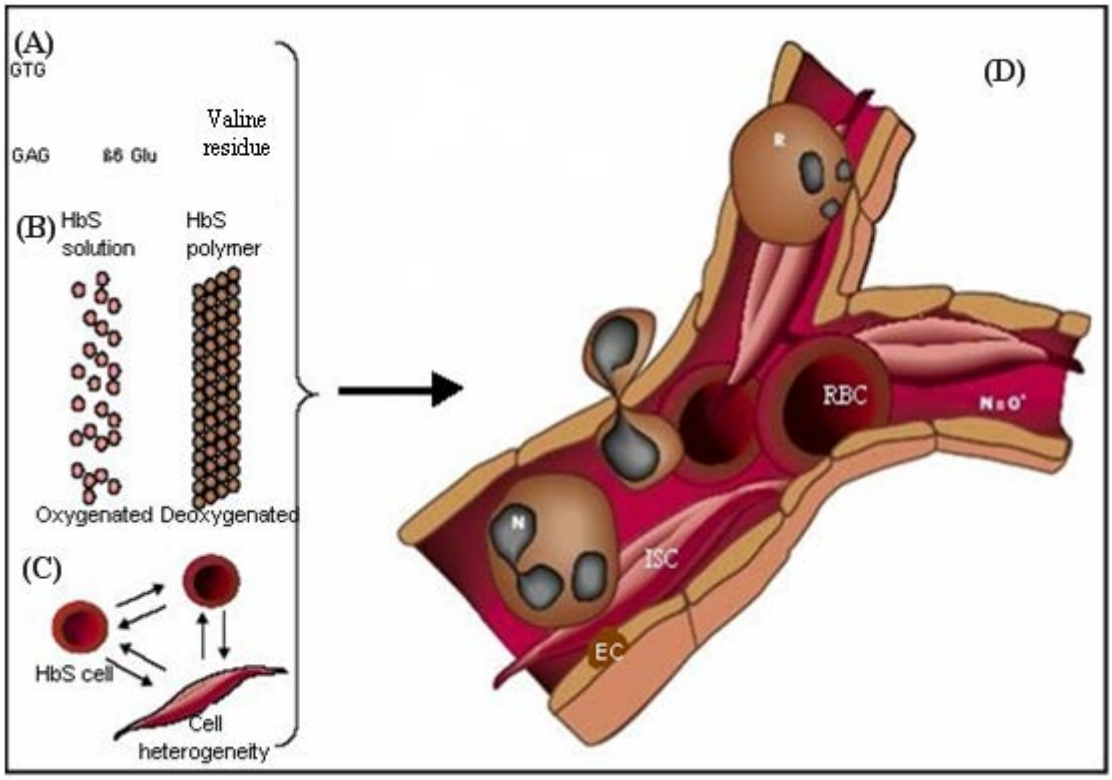

Figure 1.2 The schematic representation of the molecular basis of the pathophysiology of vaso-occlusion interactions. (A) Single nucleotide substitution (GTG for GAG). (B) HbS polymerisation. (C) Cell shape changes of HbS-polymer-containing erythrocyte. (D) Cross-section of microvascular bifurcation. EC=endothelium. R=reticulocyte. ISC=irreversibly sickled cell. N=leukocyte. N:O =NO bioavailability. RBC=red blood cell. Luminal obstruction has been initiated by the attachment of proadhesive reticulocyte to endothelium with secondary trapping of irreversible sickled cells. Leukocytes participate in formation of heterocellular aggregates, and NO bioavailability, crucial to vasodilation, is impaired. Adapted from (Stuart & Nagel, 2004).

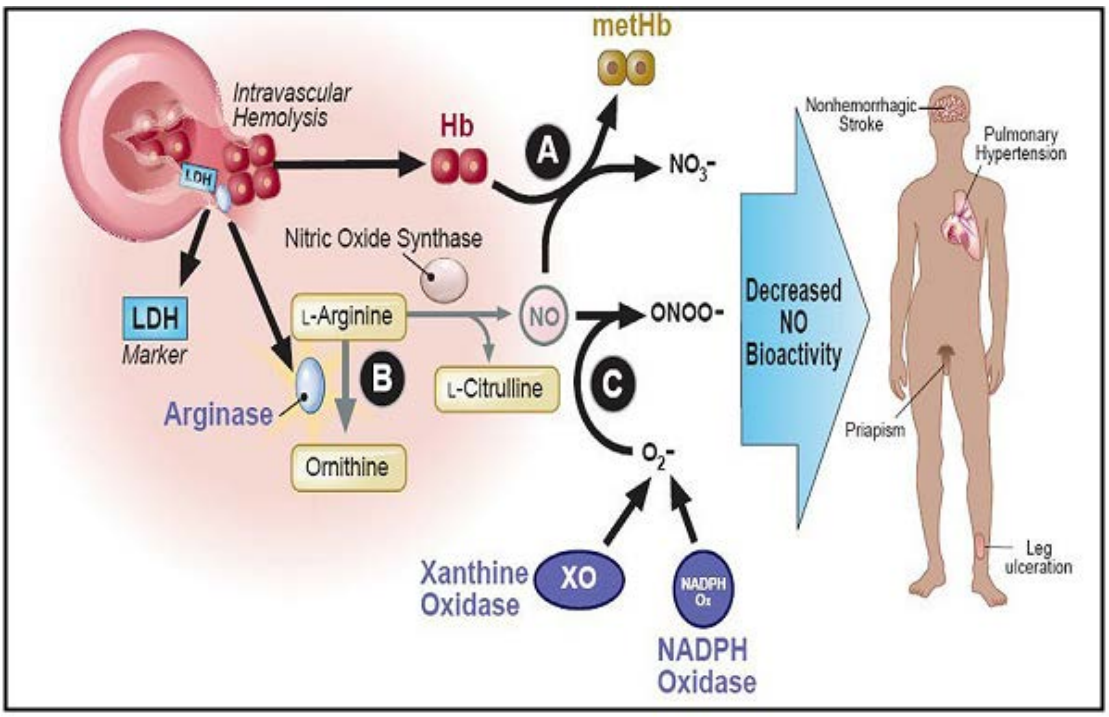

Figure 1.3 Intravascular haemolysis reduces (NO) bioactivity. Nitric oxide is produced by isoforms of nitric oxide synthase, using the substrate L-arginine. Intravascular haemolysis simultaneously releases haemoglobin, arginase, and lactate dehydrogenase (LDH) from red cells into the blood plasma. Cell-free plasma haemoglobin stochiometrically inactivates NO, generating methaemoglobin and inert nitrate (A). Plasma L-arginase subsequently consumes plasma L-arginine to ornithine, thereby depleting its availability for NO production (B). LDH also released from the red cell into blood serum serves as a surrogate marker for the magnitude of haemoglobin and arginase release. NO is also consumed by reactions with reactive oxygen species (e.g., O_2^-) produced by the high levels of xanthine oxidase activity and NADPH oxidase activity seen in sickle cell disease, producing oxygen radicals like peroxynitrite ($ONOO^-$)(C). These radicals also decrease NO bioactivity. Adapted from (Kato *et al.*, 2007).

Subendothelial matrix components and plasma proteins such as thrombin, interleukin-1 (IL-1) and tumours necrosis factors form bridges between the red cells, endothelial cells and leukocytes, and also play a major role in the adhesion process (Steinberg & Rodgers, 2001).

High granulocyte count is a risk factor for death in sickle cell disease. Granulocytes interact with sickle cells and endothelial cells to release injurious cytokines such as IL-1, β and tumour necrosis factor-α (TNFα). These will induce the expression of adhesion molecules on the vascular endothelium, and can also cause exposure of the underlying extracellular matrix components to which RBCs attach (Madigan & Malik, 2006). Furthermore, on the vascular side, the activated platelets release thrombospondin, which induces the expression of adhesion molecules on the endothelium, and can similarly cause the exposure of the underlying extracellular matrix components, to which RBCs attach (Madigan & Malik, 2006)).

The sickling process takes time owing to the fact that deoxy-HbS must assemble before polymerization can occur. Usually, transit through the microcirculation is completed before this 'delay time' elapses, and so sickling does not take place; however, with prolonged transit time of RBCs, low O_2 tension leads to increased deoxy-HbS concentrations and hence sickling will occur (Stuart & Nagel, 2004).

Role of Anti Oxidants and Free Radicals

Molecular oxygen has the capacity to form highly reactive metabolites such as superoxide anion radical $O_2^{\cdot-}$, hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH). These reduced metabolites of oxygen are referred to as reactive oxygen species (ROS). Reactive oxygen species can induce oxidative damage to the cell and form a very stable structure by extracting electrons from other sources. Superoxide

dismulated into H_2O_2 and oxygen. Hydrogen peroxide has the ability to form the more damaging ·OH, through acombination of the Fenton reaction.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^-$$

The inhibition of the mitochondrial electron transport chain activity lead to ROS generation, by inducing a leak of electrons from complex I. Generation of ROS result from the activation of enzymes such as xanthine oxidase (XO), NADPH oxidase, nitric oxide synthase (NOS), cytochrome P450, cyclo-oxygenase, and lipoxygenase. All these enzymes can be activated during the repeated cycle of hypoxia/reoxygenation or ischemia/reperfusion (Chirico & Pialoux, 2012).

Major ROS defense mechanisms include enzymatic and nonenzymatic systems. These protective mechanisms include the enzymatic antioxidants: superoxide dismutase (SOD), catalase, glutathione peroxidase (GPX), and nitric oxide (NO) and the nonenzymatic antioxidants: tocopherols, reduced glutathione(GSH), ascorbic acid (A-HOOH), lipoic acid selenium, riboflavin, zinc, carotenoids, and uric acid (Amer *et al.*, 2006).

Unneutralized ROS can target biological molecules such as DNA, lipids, proteins and carbohydrates, which result in cell dysfunction or cell death. Although there is elevated oxidative stress in SCA, it is effect can be affected by other factors such as diet, physical activity, environment, and other comorbid diseases associated with SCA (Wood *et al.*, 2008).

The intracellular polymerization of HbS, during deoxygenation, is the primary pathogenetic event in SCA. Polymerization can transform a normal red blood cell (RBC) into a dense, inflexible blood cell. The rate of polymerization has been shown

in vitro to be correlated with the concentration of HbS and with the cell-free haeme released after auto-oxidation (Chirico & Pialoux, 2012).

The RBC reoxygenation phase is a major source of free radical production in SCA. Normal RBCs generate a significant amount of superoxide due to an electron transfer between the haeme iron and oxygen. In the presence of oxygen, haeme auto-oxidizes inducing methemoglobin and superoxide formation. Although both haemoglobin A and HbS blood have a tendency to auto-oxidize into methemoglobin and superoxide, haemoglobin A can counter this reaction by forming harmless byproducts; HbS can become overwhelmed by the continual source of superoxide and, via its dismutation, H_2O_2. The formation of H_2O_2, when exposed to methemoglobin, decomposes hemoglobin and releases iron. This iron then react with remaining H_2O_2 to further produce ·OH, the most reactive and harmful of the reactive species.

Haemoglobin Haplotype and Sickle Cell Phenotype

Sickle cell disease haplotypes are described as polymorphic restriction of endonuclease sites in and around the mutant β-globin gene (Powars & Hiti, 1993). Although all the haplotypes have numeric identifiers, they are nevertheless frequently designated by the geographic areas in which they were first identified: Senegal, Benin, Central African Republic (CAR) or Bantu, Cameroon and Arab-India (or Asian) (Alsultan *et al.*, 2014).

The Senegal haplotype is represented in Senegal and in the Western province of Africa above the Niger River, whilst the Benin haplotype were found in Nigeria, Benin, and countries in the Bight of Benin. Furthermore, the Central African Republic or Bantu haplotype encompasses those haplotypes discovered in the Central African Republic and countries in south central Africa. In contrast, the Cameroon haplotype

has been found in only one ethnic group in the Cameroons. The Arab-India haplotype frequently refers to those haplotypes found in the Persian Gulf and India (Alsultan *et al.*, 2014).

The existence of haplotypes specific to certain regions of the world suggests that the mutant β-globin gene arose separately in these locations (Oner *et al.*, 1992). All of these areas have been endemic locations of malaria infestation, thus, the high incidence of the sickle mutation in these areas is most likely derived from natural selection (Carlson *et al.*, 1994). Moreover the mutation that produces sickle haemoglobin occurs spontaneously at a low rate. Notably, people with sickle cell trait are more resistant to malaria than those with normal haemoglobin. Therefore they have a better chance of passing the sickle gene to the next generation.

The haplotype of the sickle mutation has certain effects on the clinical presentation. The three common haplotypes in the USA are Senegal, Benin, and Central African Republic or Bantu (Hattoni *et al.*, 1989; Powars *et al.*, 1994). Each of these is associated with various different degrees of disease severity. For instance, people with the Senegal haplotype have a mild clinical course, whilst those with the CAR/Bantu haplotype usually have the more severe form of the disease. People with the Benin haplotype have an intermediate severity (Powars *et al.*, 1994). In India and the Persian Gulf Province, SCA essentially follows a more benign course than it does in Africa and USA. The mechanism by which haplotypes influence sickle cell disease severity remains unidentified. Individual haplotypes retain varying levels of foetal haemoglobin. Patients with the Senegal haplotype, often preserve foetal haemoglobin levels of 20% or more. In contrast, patient with the Bantu/CAR haplotype generally express the lowest foetal haemoglobin levels and therefore, patients tend to run more severe clinical courses. Importantly the Benin haplotype is associated with intermediate foetal haemoglobin levels (Powars *et al.*, 1994), which corresponds to its intermediate clinical course. With this in mind, there is a contradictory view that the

5' promotor region of the, ‘G-gamma’ globin influences foetal haemoglobin level (Economou *et al.*, 1991; Powars *et al.*, 1994). Irrespective of the cause, however, the observation remains that higher levels of HbF are seen in patients with the Senegal haplotype, with clinically milder disease.

Sickle cell anaemia genes occur in two major areas in Eastern and Southwest Saudi Arabia. The gene frequency in the Eastern province of Saudi Arabia varies from 0 - 0.145 %. In the Western Province the β^s-globin gene is linked to the Benin haplotype of African origin, whilst the Arab-India haplotype is linked to the Eastern province (Daar *et al.*, 2000; Serjeant *et al.*, 2001). The Arab- India haplotype is predominant in the Eastern province whilst the Benin haplotype imported from Africa is common in the Southwest (Serjeant *et al.*, 2001). The Arab-India haplotype most likely originated in the Indus valley Harappa culture and by gene flow was distributed to Saudi Arabia particularly to Eastern Province (Stuart & Nagel, 2004).

Other genetic factors influencing disease severity include α- globin gene deletion and the level of haemoglobin F. The α-thalassaemia disease occurs in 50% of the population in both areas. Furthermore, high levels of haemoglobin F, which is a characteristic of Arab- India haplotype, could explain the milder clinical course in the Eastern Province compared to the Southwestern (Serjeant *et al.*, 2001).

Sickle cell anaemia leads to clinical manifestation of chronic haemolysis and its complications, vascular occlusion, tissue ischemia, bone marrow fat embolisation syndromes, susceptibility to infections and end-organ damage. The clinical manifestations comprise stroke, retinopathy, a vascular necrosis of bone, priapism, splenic infarction or sequestration, renal and, hepatic complications and secondary pulmonary hypertension (Steinberg, 1999). Moreover organ damage frequently results

from a combination of progressive vasculopathy and acute infarction (Chiang & Frenette, 2005).

The clinical presentation of sickle cell disease is dominated by either haemolysis or vaso-occlusion. Furthermore, pulmonary hypertension, priapism, leg ulceration, and non-haemorrhagic stroke are all related to the degree of haemolysis by a similar pathophysiology, which is seen in other chronic intravascular haemolytic anaemias (Kato *et al.*, 2007) (Figure 1.3).

Haemolysis, in conjunction with endothelial dysfunction, causes proliferative vasculopathy and the deregulation of vasomotor function. High levels of haemolytic markers such as reticulocyte count, serum lactate dehydrogenase, plasma haemoglobin and arginase, indicate a state of impaired NO bioavailability under conditions of low haemoglobin (Madigan & Malik, 2006).

However, haemolysis plays an insignificant role in the vaso-occlusive complications of the disease, including the acute painful episodes, osteonecrosis of bone or the acute chest syndrome. Moreover, those patients with SCA with higher haemoglobin levels tend to have a higher frequency of vaso-occlusive hyperviscosity related complications, linked with the polymerisation of sickle haemoglobin, thereby resulting in erythrocyte sickling and adhesion. The prevalence and severity of each of these subphenotypes overlap with each other (Alexander *et al.*, 2004).

1.4 Clinical Features

The clinical features of SCA are severe haemolytic anaemia punctuated by crisis. The symptoms of anaemia are often mild in relation to the measured haemoglobin levels.

The clinical expression of HbSS is variable: some patients have an almost normal life, free from crisis whilst others develop severe and frequent crisis.

Types of Crisis

Painful Vaso-Occlusive Crisis

Sickle cell disease is a chronic disorder, characterised by acute events that result in a shortening of life expectancy. Vaso-occlusion is responsible for the majority of the severe complications of SCA. The chief presentations amongst the clinical features of SCA are acute episodes of severe pain in the chest, back and abdomen, or extremities, lasting for days or weeks. Multiple areas are often involved simultaneously, and symmetric involvement of extremities is common. The most serious vaso-occlusive crisis are those which affect the brain (strokes occur in 7% of all patients) or spinal cord. The painful dactylitis caused by infarcts of small bones of the hands and feet, known as 'hand-foot' syndrome, may also occur (Hoffbrand *et al.*, 2005).

Visceral Sequestration Crisis

This type of crisis is caused by sickling within organs and pooling of blood, often associated with severe anaemia. The acute chest syndrome is a frequent and sometimes fatal complication affecting approximately 40% of all people with SCA. The clinical syndrome is less severe in children. Chest syndrome can occur postoperatively and, when recurrent, can lead to chronic respiratory insufficiency. Its cardinal features are fever, pleuritic chest pain, referred abdominal pain, cough, lung infiltrates and hypoxia. Other sequestration crisis can occur in the liver and the spleen, which is typically seen in infants and presents with splenomegaly, falling haemoglobin levels and abdominal pain (Telen, 2001).

Aplastic Crisis

Aplastic crisis may be precipitated by parvovirus infection or folate deficiency and are characterised by a sudden fall in haemoglobin level and reticulocytes, as the bone marrow ceases to respond to anaemia (Telen, 2001).

Haemolytic Crisis

This is characterised by an increased rate of haemolysis, which results in a fall in haemoglobin level, but a rise in reticulocytes in response to anaemia.

Hepatic Manifestations in Sickle Cell Anaemia

Sickle cell hepatopathy encompasses a wide range of hepatic pathologies arising from a variety of insults to the liver. The direct manifestations of SCA in the liver relate predominantly to vascular occlusion, the complications of multiple transfusions and chronic haemolysis. Vascular hepatic occlusion is caused by acute ischaemia, sequestration, and cholestasis. The main hepatic complications of multiple transfusions include infection with hepatitis B and C and iron overload. The potential consequence of chronic haemolysis is the development of pigment stones, with consequent cholecystitis and acute and chronic biliary obstruction (Banerjee *et al.*, 2001).

Clinical Syndromes

***Acute Sickle hepatic Crisis*:** Acute sickle hepatic crisis occurs in approximately 10% of patients with SCA. Patients commonly present with acute right upper quadrant pain, nausea, low-grade fever, tender hepatomegaly, and jaundice. Plasma aspartate

transaminase (AST) and alanine transferase levels are usually raised (Oner *et al.*, 1992). Liver biopsy confirms the diagnosis (Banerjee *et al.*, 2001).

***Hepatic Sequestration Crisis*:** Hepatic sequestration result in right upper quadrant pain, thereby increasing hepatomegaly and a falling haematocrit (Ebert *et al.*, 2010) described two cases of hepatic sequestration with little alteration in the liver function tests. Exchange transfusion is preferable to partial exchange or additive transfusion in such patients.

***Sickle Cell Intrahepatic Cholestasis*:** This is a disastrous and often fatal condition representing a severe variant of sickle hepatic crisis. It occurs owing to widespread sickling cells within sinusoids, with hepatic ischemia. Notably, damage in this regard is caused by hypoxia of the hepatocytes, with resultant intracanalicular cholestasis. The presentation is similar to sickle hepatic crisis, although leukocytosis, striking jaundice, bleeding diathesis, and increasing encephalopathy are major differentiating features. Plasma alanine, AST, alkaline phosphatase and lactic dehydrogenase levels are often markedly elevated (Hoffbrand *et al.*, 2005). The characteristic finding is that of very high plasma bilirubin concentrations which is caused by a combination of on-going haemolysis and intrahepatic cholestasis. In most cases, the conjugated fraction exceeds 50% of the total bilirubin. Bilirubin levels correlate with lactic dehydrogenase levels. Moreover, the prolongation of the prothrombin time and partial thromboplastin time are common. Hypofibrinogenaemia, thrombocytopenia and lactic acidosis may accompany this type of liver failure. Elevations in blood urea, creatinine and ammonia are also observed. Importantly, the reversal of this process could be achieved through vigorous exchange transfusions and correction of coagulopathy with fresh frozen plasma (Banerjee *et al.*, 2001).

Cholelithiasis in Sickle Cell Anaemia Patients

Cholelithiasis is extremely common in patients with SCA, with gall stones being present in up to 58% of patients aged between 10 and 65 years. With this in mind, elective laparoscopic cholecystectomy should be considered in patients with symptomatic gall stones (Al-Mulhim *et al.*, 2002).

Hepatic Iron Overload in Sickle Cell Patients

Serum ferritin levels correlate with the number of blood transfusion. High serum ferritin occurs during painful vaso-occlusive sickle crisis, and hence steady-state ferritin levels provide a better estimate of the degree of iron overload. In this regard, iron chelation therapy, with intravenous or subcutaneous desferrioxamine, results in increased urinary and biliary excretion of iron and is associated with a significant fall in serum ferritin level (Telen, 2001).

Viral Hepatitis in Sickle Cell Anaemia Patients

The prevalence of chronic Hepatitis B infection in SCA patients is low (Al-Fawaz & Ramia, 1993; Ocak *et al.*, 2006). A relatively high prevalence of Hepatitis C infection has been reported (Ocak *et al.*, 2006). The risk of infection has, however, been related to the number of units of blood transfused. Patients with acute Hepatitis B have a high level of bilirubin, AST and ALT levels (Ocak *et al.*, 2006). Sickle cell anaemia patients should receive the Hepatitis B vaccine.

Renal Complications

Sickle cell anaemia is associated with numerous renal complications, ranging from hyposthenuria to end-stage renal failure. In this regard, the hypoxic, acidotic and hypertonic microenvironment of the renal medulla causes the sickling of red blood

cells in the *vasa recta*, consequently leading to infarction of the renal medulla, hyposthenuria, haematuria and enuresis. Repeated episodes of hyperchloraemic acidosis and enuresis occur in children with papillary necrosis. Renal impairment in SCA causes poor urinary concentrating ability, and so plasma osmolarity can arise, thereby promoting RBC dehydration, which will further worsen the sickling process.

Urinary tract infections are usually caused by *Escherichia coli* and are more common in females than in males. Frequent urinary tract infection results in renal infarction.

Electrolytes imbalance including, impaired potassium excretion, hyperchloraemic acidosis and hyperuricaemia have been reported. Hyperuricaemia owing to increased bone marrow activity, with consequent enhanced purine metabolism appears to cause an acquired defect in the renal tubules. Resultant gout has also been described in a few patients (Ballas, 2001; Johnson, 1999).

Nephrotic syndrome occurs infrequently, either with or without hypertension. Microscopic haematuria, proteinuria and hypertension are markers of incipient end stage renal failure, the pathologic finding of which is usually glomerulosclerosis. Once chronic renal failure becomes apparent, patients require chronic haemodialysis and are candidates for kidney transplantation (Ballas, 2001; Johnson, 1999).

1.5 Pathogenesis of Infections in Sickle Cell Anaemia

The sickle gene indirectly confers an increased susceptibility to infection, especially to certain microbial agents, and at the same time, infection provokes a cascade of SCA-specific pathophysiological changes (Booth *et al.*, 2010). Infection is a significant contributor to morbidity and mortality in SCA.

Impaired Splenic Function

Splenic dysfunction has a key role to play in the increased susceptibility to certain bacterial infections seen in SCA. Normal spleen functions act as a phagocytic filter, removing old and damaged cells and blood-borne microorganisms, and accordingly producing antibodies. Blood from the splenic artery first traverses the white pulp, which contains collections of B and T lymphocytes in follicles and periarteriolar lymphatic sheaths. Moreover, the activation of these cells by antigenic material enables the initiation and expansion of a specific acquired immune response. Subsequently, blood then enters the splenic cords of the red pulp, where cells flow over a fine reticular meshwork and pass through fenestrated epithelium to enter the venous sinuses. This creates a slow flow, enabling splenic macrophages to remove defective RBCs and bacteria and presenting antigen to lymphocytes (Bohnsack & Brown, 1986). Some bacteria can be recognized directly by macrophages, but many require opsonisation. Opsonisation is the coating of the microbial surface by complement components (especially C3b) or other molecules, which subsequently interact with receptors on phagocytic cells (Janeway & Travers, 2001). Opsonised bacteria are removed efficiently by macrophages in the spleen or liver, whilst poorly opsonised bacteria are only cleared by the spleen. Such pathogens include encapsulated bacteria in particular *Streptococcus pneumoniae* and *Haemophilus influenzae*. Their polysaccharide capsule impedes binding complement or prevents complement assembled on the cell wall from interacting with macrophage receptors (Bohnsack & Brown, 1986). Notably, the clearance of these bacteria requires anti-polysaccharide IgM antibodies, which facilitate phagocytosis either directly or via complement deposition. IgM lies on the surface of memory B cells, which reside in the marginal zone of the spleen. These cells persist after an initial infection and rapidly produce antibody on subsequent exposures (Bohnsack & Brown, 1986). The spleen is the site of synthesis of tuftsin, an immunostimulatory peptide, and properdin, which participates in complement activation (William & Corazza, 2007). Individuals

with SCA typically suffer from functional hyposplenism or asplenism. The sluggish circulation through the spleen, high rates of O_2 extraction and local acidosis cause deoxygenation of HbS, promote sickling, which leads to the congestion of the sinusoids with sickled cells; this can subsequently cause diversion of blood via intrasplenic shunts by passing the normal filtering mechanisms. In this regard, macrophages engulfing the abnormally shaped cells may become 'blocked', thereby impairing their phagocytic function. Together these effects produce a hyposplenic state which is initially reversible (William & Corazza, 2007). However, over time, repeated episodes of sickling and ischaemic damage with progressive sclerosis of arterioles lead to multiple infarcts of spleen tissue. Unable to regenerate, the spleen becomes scarred and atrophied; culminating in 'autosplenectomy', where the organ shrinks to a small remnant and the individual is rendered effectively asplenic (Lucas, 2004). In HbSS, this sequence develops from the age of 6 months through to 3 years (William *et al.*, 2007). Hyposplenic and asplenic individuals lack memory B cells, thereby suggesting a role for the spleen in their generation or function; as a result, they cannot mount a rapid specific response to encapsulated organisms. Overwhelming sepsis is the ultimate result of the spread of local infections and the loss of the spleen's filtering function. The main pathogen of concern is Streptococcus pneumoniae, although severe and systemic infections with *Haemophilus influenzae*, *Neisseria meningitidis*, and *salmonellae* also occur. Prior to preventive measures being implemented, children with SCA were 30-600 times more likely to develop overwhelming sepsis. Overwhelming sepsis can develop rapidly with no obvious primary source of infection, and can consequently result in invasive pneumococcal disease (IPD), including pneumonia, meningitis, and septicaemias (Halasa *et al.*, 2007). Shock, disseminated intravascular coagulation, adrenal haemorrhage and also death can occur within 24-48 hours (William *et al.*, 2007). Mortality rate reaches 10% from meningitis and up to between 35% and 50% from septicaemias. The risk is more common in children, with a reported incidence of 5.8 per 100 in children aged less

than 3 years, 1.1 per 100 in those aged 5-9 years, and 0.6 per 100 in those aged over 10 years, all during the pre-treatment period (Overturf, 2003).

Defects in Complement Activation

Despite the modern prophylactic measures, major infections occur mostly in early infancy, when the spleen is still partially functional; which suggests additional immune deficits (Tamouza *et al.*, 2002). Patients are commonly predisposed to other infections, including *Escherichia coli* urinary tract infection, *Mycoplasma pneumoniae* (*M. pneumoniae*) or *Chlamydophila pneumoniae* (*C. pneumoniae*) respiratory infections, and dental infections and cholecystitis caused by anaerobes. The complement system involves a large number of plasma proteins which are cleaved sequentially by protease enzymes to generate active fragments. These function as opsonins or chemo-attractants, and the terminal components can kill various pathogens directly through creating pores in their membranes. The cascade can then be activated either via the classical pathway, following binding of IgM or IgG to surface antigens, or via the alternative pathway, in which C3b interacts directly with the pathogen cell surface, subsequently recruiting further downstream components (Janeway & Travers, 2001).

Deficiencies in Micronutrients

Zinc is known to be important for immune function. Zinc deficiency is associated with lymphopenia, possibly owing to activation of the hypothalamic-pituitary-adrenocortical axis. Notably, this will subsequently cause chronic glucocorticoid production, which stimulates apoptosis of B and T cells in bone marrow and the thymus (Fraker *et al.*, 2000). Moreover zinc deficiency has also been linked with reduced production of interleukin 2 (IL) a cytokine needed for expansion and maintenance of thymocytes and peripheral T cells, reduced natural killer (NK) cell lytic activity, thymulin activity, CD4:CD8 ratio, and impaired T helper cell function (Prasad *et al.*, 1999). In this regards high protein turnover increases zinc requirements, whilst released zinc from haemolysed RBCs is lost via the kidneys owing to renal tubular damage which impairs re-absorption. At the same time, poor diet and inadequate intestinal absorption could further reduce its level. Zinc deficiency which affects 60-70% of patients with SCA, is a contributory factor in susceptibility to infection (Prasad, 2002). A study in 21 zinc deficient children from Detroit Medical Center suggests that giving supplements increased IL-2 levels, reduced the incidence of bacterial infections and further cut hospital admissions (Prasad *et al.*, 1999); however, this was not a fully controlled trial, although, if such a simple measure as a mineral supplement could improve quality of life, the issue may accordingly warrant further exploration. Similar studies have been reported amongst Saudi Patients with SCA whereby a significant decrease in serum levels of zinc was found in patients compared with controls. In addition zinc supplementation ameliorated some of the SCA symptoms and improves the overall quality of life (Hasanato, 2006).

Genetic Factors

Despite sharing the same underlying genetic mutation, the range of severity in the phenotype of SCA is striking, with some patients disabled by frequent crisis and long-term complications whilst others live virtually normal lives. Individuals are also differently predisposed to particular pathological manifestations of the disease, which suggests that the phenotype is multigenic. Moreover many unlinked genes are involved in the underlying pathological processes and variation in alleles at multiple loci may modify outcome (Stuart & Nagel, 2004).

In this regard, polymorphisms in a number of genes involved in the immune response have been suggested as a contributing factor to increased susceptibility to infection in SCA. Although unlinked to the sickle gene, these variants may coincidentally occur with increased frequency in SCA population. Particular HLA-II subtypes have been shown to influence infectious complications (Tamouza *et al.*, 2002).

Certain polymorphisms of the fragment crytallizable receptor (FcR), mannose-binding lectin, insulin-like growth factor 1 receptor (IGF1-R), and genes of the transforming growth factor β (TGF β)/bone morphogenic protein (BMP) pathway have been associated with an increased risk of bacteraemia (Adewoye *et al.*, 2006). In this regard, studies also highlight the need for caution in generalising, in terms of immune function in SCA; for example, complement activation or neutrophil action, based on experiments using small numbers of subjects in localised geographical areas. Importantly, observed differences may increase individual risk, but may not be a universal feature (Wong *et al.*, 1992).

Mechanical Factors

The pathological effects of SCA can create an environment which supports infection. Bone marrow space expands in such a way so as to accommodate the increased haematopoiesis to compensate for chronic haemolysis. At the same time, circulation is sluggish. Notably, these factors render bone vulnerable to vaso-occlusive episodes and infarction. Areas of necrotic bone act as foci for bacterial infection established via haematogenous spread (Atkins *et al.,* 1997); therefore, children with SCA are predisposed to osteomyelitis. *Salmonella species* is the commonest agent in SCA osteomyelitis, followed by *Staphylococcus aureus* then Gram-negative enteric bacteria (Antonio & Irene, 2005). *Edwardsiella tarda* is another enterobacterium which has been reported with increased incidence in SCA owing to increased gut permeability and biliary sludging (Wang *et al.,* 2005). In contrast, *Staphylococcus aureus* is the predominant pathogen for osteomyelitis in children unaffected by SCA.

Another consequence of microvascular disease is the association with acute chest syndrome. With this in mind, several reports in the literature document an association between respiratory infections and acute chest syndrome (Neumayr *et al.*, 2003; Vichinsky *et al.,* 2000). Sickle cell anaemia carries an increased risk of prolonged and severe respiratory infections owing to *Mycoplasma*, *Chlamydia* and other pathogens. This is particularly true in the case of children with microvascular sequestration, such as those with SC haemoglobinopathy. Further evidence is provided by patients with SCA, who may be predisposed to certain iatrogenic infections because of therapeutic interventions.

Transfusion Transmitted Infections

Blood transfusion is commonly used to treat complications, particularly aplastic crisis or splenic sequestration, acute chest syndrome, priapism, or strokes. In the instance of stroke, exchange transfusion is used to reduce the proportion of HbS (Win, 2004). Increasingly, chronic transfusion therapy is being applied in children in order to prevent strokes by keeping HbS levels below 30%. In developed countries, 5-10% of children may be involved in a chronic transfusion programme at some point in their lives (Dick, 2007). In general, such programmes are potentially associated with an increased risk of blood-borne infections, particularly Hepatitis B and C and the human immunodeficiency virus (HIV). Although all blood products in developed countries are screened for these viruses, standards in other countries may not be so exacting, and so early Hepatitis B immunisation is recommended as a preventive measure. Other viruses, including cytomegalovirus (CMV) and parvovirus B19, can also be transmitted. Although not a problem in immune competent individuals, CMV is a significant pathogen in the immune compromised, and so children who are potential candidates for bone marrow transplant should receive CMV seronegative blood (Win, 2004). With this in mind, it is also noteworthy to highlight that patients are also at risk of catheter-related infections, particularly those on prolonged courses of parenteral antibiotics or with indwelling vascular devices for chronic blood transfusion; this has been reported in adults, and seems to be particularly associated with bone infections (Zarrouk *et al.*, 2006).

1.6 The Effect of Infection on Sickle Cell Anaemia

Infection has long been recognised as one of the most common precipitants of crisis in SCA. During infection with any pathogen, changes occur at a cellular level, which predispose to crisis. Levels of circulating leukocytes and inflammatory cytokines

increase, with an elevated expression of adhesion molecules occurring on both the vascular endothelium and leukocytes. Vaso-occlusion, initially assumed to be due to passive mechanical blockage by sickled RBCs, is in fact, a complex, dynamic process involving active interaction between cell adhesion molecules. Notably, this could contribute directly to occlusion, but also acts via slowing transit of RBCs through the microvasculature.

Leukocyte adhesion may ultimately be the initiating event in vaso-occlusive episodes. Leukocyte attachment occurs in post-capillary venules the site of leukocyte passage into the extra-cellular space. Moreover, patients with severe SCA have increased expression of leukocyte adhesion molecules such as Mb2 integrin, L-selectin, and CD18 during steady state (Frenette & Atweh, 2007). Leukocytes produce cytokines TNF- α and IL-1β induce the expression of VCAM and can cause exposure of the underlying extracellular matrix components which attaches to RBCs (Madigan & Malik, 2006). In addition, narrowing of the vessel lumen by attached leukocytes may enable the accumulation of RBCs, platelets, and further leukocytes, with increasing occlusion. Resultant local hypoxia promotes RBC sickling and propagation of the blockage, culminating in a crisis.

In addition, neutrophils, basophils, and monocytes increased during inflammation produce cytotoxic proteins such as proteases, collagenase, and elastase and generate reactive O_2 radicals, which cause oxidative damage. This notably promotes further endothelial activation and cell adhesion (Frenette & Atweh, 2007). Importantly, the Lutheran blood group glycoprotein (Lu), also known as basal cell adhesion molecule (B-CAM) is an adhesion glycoprotein involved in RBC adhesion to endothelium in SCA. This cell adhesion to laminin, a major component of the subendothelium, is stimulated by epinephrine. Importantly, stress mediates the release of epinephrine, which is believed to contribute to vascular occlusion in sickle cell disease (Stuart & Nagel, 2004). Furthermore, at inflammatory sites, the sluggish circulation, hypoxia,

local acidosis and hypercapnia shift the Hb-oxygen dissociation curve to the right, thereby promoting unloading of oxygen from Hb and thus increasing sickling process.

The Ca^{2+} sensitive K^{+} efflux (Gardos channel) is constitutively over expressed in SCA, but is further activated by low pH. The resultant K^{+} and water efflux will lead to RBC dehydration. Gardos channel, which is activated by cytokines, chemokines, and prostaglandin E2 (PGE2), enhances sickling during infection (Madigan & Malik, 2006; Stuart & Nagel, 2004). Furthermore, steady state neutrophil count correlates with the severity of SCA, and treatment with hydroxyurea, which lowers neutrophil numbers, reducing the frequency of painful crisis and hospital admissions (Okpala, 2004a). In addition, infections can have non-specific effects on the host physiological environment, which increase the risk of sickling. Fever with water loss owing to sweating, anorexia, and nausea with reduced oral fluid intake, diarrhoea, and vomiting all contribute to dehydration.

Effect of Specific Infections on SCA

Parvovirus B19

Parvovirus B19 is a single-stranded DNA virus transmitted by respiratory droplets, occurring in outbreaks particularly in late winter and early spring (Servey *et al.*, 2007). It is a common childhood infection with an incidence of 11.3 per 100 SCA patient-years (Servey *et al.*, 2007).

Moreover, approximately 26% of children are seropositive by age 5, increasing to 47% aged 10, 64% aged 15, and 73% in adulthood (Smith-Whitley *et al.*, 2004). In normal individuals infection is often asymptomatic or otherwise gives mild flu-like symptoms, or even may cause erythema infectiosum, characterised by fever, malaise

and ‘slapped cheek’ rash on the face, progressing to a generalised maculopapular eruption on the trunk and limbs (Servey *et al.*, 2007). Its significance in SCA and other haemolytic conditions is that it commonly causes aplastic crisis. Parvovirus B19 specifically infects erythroid progenitor cells in bone marrow and peripheral blood, using surface P-antigen as a receptor, this results in temporary arrest of erythropoiesis, lasting 7-10 days.

In healthy individuals, the normal lifespan of a RBC is 120 days. In the absence of erythroid precursors in the bone marrow, erythropoietin production will increase, promoting energetic haematopoiesis once bone marrow recovers. However, in SCA, a shortened RBC lifespan in addition to transient lack of RBC production, results in a severe anaemia (Chisaka *et al.*, 2003). Transient aplastic crisis occurs in 65-80% of parvovirus B19 infections. Aplastic crisis is uncommon after the age of 15 years (Serjeant *et al.*, 2001). Although most children recover within two weeks, the majority require blood transfusion. In this regard, neutropenia has been reported in 18% of children and thrombocytopenia in 26.5% with other complications including acute splenic or hepatic sequestration at a rate of 19%, acute chest syndrome at 11.8%, painful crisis, stroke, nephrotic syndrome, and meningoencephalitis.

Notably, a similar report on the incidence of aplastic crisis from the Eastern province of Saudi Arabia included forty-six patients with SCA all of whom were hospitalised at Saudi Aramco-Dhahran Health Center. Evidence of recent human parvovirus infection was present in 91% of the cases. Leukopenia was present in 21%, neutropenia in 27% and thrombocytopenia in 42% (Mallouh & Qudah, 1993).

Acute infection can be confirmed by measuring parvovirus B19-specific IgM (89% sensitive and 99% specific) or otherwise utilising PCR to amplify viral DNA. This is important as the virus is highly contagious, with a secondary attack rate of over 50%

amongst other household members (Servey *et al.*, 2007). Siblings with SCA should be tested for infection and monitored for the development of aplastic crisis. Following infection immunity is life long, thereby leading to calls for the development of a vaccine.

Atypical Bacteria and Acute Chest Syndrome

The acute chest syndrome (ACS) in SCA is defined as the combination of chest pain, dyspnoea, fever, and pulmonary infiltrates on chest X-ray (Dick, 2007). It affects 15-43% of patients, particularly in early childhood. Moreover, acute chest syndrome is responsible for 25% of deaths and is the second most common cause of hospital admission (Castro *et al.*, 1994; Platt *et al.*, 1994). Various processes can precipitate the condition, including infection, pulmonary fat embolism and hypoventilation, all of which could arise from thoracic bony crisis or post surgically or secondary to excessive narcotic analgesia. The unifying end result is sickling and vaso-occlusion in the small vessels of the lung, causing local ischaemia and sometimes infarction. The ventilation-perfusion mismatch causes systemic hypoxia, predisposing to further sickling and vascular occlusion in a vicious cycle ending by right heart failure (Siddiqui & Ahmed, 2003). In a large multicentre study, the cause of ACS was found in 38% of cases. Moreover, infection was identified in a third of patients. The most prevalent infectious agents were *Chlamydia pneumoniae* (14% of all patients) and *Mycoplasma pneumoniae* (9%), the latter of which was identified more in younger patients (Vichinsky *et al.*, 2000). Although isolation does not confirm causation, the inflammatory response to lung infection would nevertheless seem likely to provoke leukocyte and RBC adhesion and intravascular sickling. Patients with SCA in the Eastern Province of Saudi Arabia suffers less severe acute chest syndrome than those with African haplotypes (Alabdulaali, 2007).

Malaria

The relationship between malaria and SCA is an interesting one. The persistence of the sickle mutation at such high frequency in African populations in spite of the severity of SCA has been attributed to the fact that heterozygous sickle trait confers protection against severe and life threatening malaria particularly cerebral malaria caused by *Plasmodium falciparum*. The presence of HbS is associated with reduced parasitic invasion of erythrocytes, impaired multiplication, and accelerated clearance of parasites by the spleen; however infected RBC becomes hypoxic, thereby provoking sickling and splenic filtration of infected cells (Makani *et al.*, 2007).

A similar situation is recognized in Saudi Arabia, where both malaria and SCA could coexist. Abudulhadi, 2007 confirms that the heterozygous sickle trait as being associated with reduced parasitic erythrocytes invasion and a mild clinical course of malaria infection; this occurs owing to alterations in binding of the sickled erythrocyte to the peripheral blood mononuclear cells and cytoadherence.

It might be assumed that homozygous SCA would confer greater resistance to malaria; however, the co-existence of the two is associated with increased mortality and morbidity, with the malaria being the most common precipitating cause of crisis in endemic countries (Oniyangi & Omari, 2006). Among children with malarial and SCA, 8.7% die and 66% develop severe anaemia. (Ambe *et al.*, 2001).

In normal individuals, infected red cells adhere to the capillary endothelium causing obstruction. The metabolic activity of parasites within RBCs causes hypoxia, acidosis, and hence sickling (Onwubalili, 1983). In SCA patients, the deleterious effects of this are magnified; the spleen plays an important role in the control of malaria, thereby removing damaged and parasitised RBCs from the circulation, ‘pitting’ infected cells,

and generating specific B and T cell responses. Splenectomised individuals with *Plasmodium falciparum* have reduced clearance of parasitised RBCs, but it remains unclear as to whether or not they suffer more severe malarial symptoms (Engwerda *et al.*, 2005).

Malaria causes anaemia via a number of mechanisms (Menendez *et al.*, 2000). Accordingly haemolysis of infected RBCs occur as merozoites emerge after multiplying, whilst haemolysis of non-infected cells occurs owing to the production of auto-antibodies against RBC surface molecules. Macrophages phagocytose both infected and noninfected cells worsening the anaemia. Malaria can also cause dyserythropoiesis and splenic sequestration of RBCs particularly in young children who have not undergone autosplenectomy is a contributing factors. Furthermore, recurrent haemolysis can produce folate deficiency anaemia. In one Nigerian centre, hyperhaemolytic crisis was found to be the most common cause of severe anaemia in children (Juwah *et al.*, 2004).

Malaria is therefore a significant pathogen in SCA, and long-term prophylaxis has been highlighted as lowering the incidence of severe anaemia and the number of hospital admissions and crisis, as well as reducing overall mortality (Oniyangi & Omari, 2006). With this in mind, Bashawri *et al.*, (2001) report in a study in the South Western part of Saudi Arabia that *Plasmodium falciparum* is the most common species amongst Saudis (83%) (Bashawri *et al.*, 2001). The frequent clinical signs are splenomegaly and jaundice; anaemia and thrombocytopenia are the common haematological laboratory findings.

Human Immunodeficiency Virus and Sickle Cell Anaemia

Trivial information is available regarding the impact of coexistent HIV infection and SCA. These two conditions can present unique challenge particularly in Africa where the incidence of both conditions is highest and resources are scarce. In a retrospective study in the USA, hospital discharges of children with both HIV and SCA over a 10-year period (1994-2003) were analysed (Kourtis *et al.*, 2007). Accordingly, it was found that children with both conditions are at increased risk of bacterial infection and sepsis and the average hospital stay is longer. Pneumococcal infection risk is high amongst HIV-infected adults with SCA (Godeau *et al.*, 1992). Moreover, a high frequency of long-term non-progressor patients with HIV and SCA may suggest that SCA attenuates the clinical progression of the HIV virus (Bagasra *et al.*, 1998). In Saudi Arabia, a study conducted by Madani *et al.*, (2004) notes that the number of HIV cases is limited, with heterosexual contact being the main mode of transmission (Madani *et al.*, 2004). Preventive strategies include prevention of non-marital sex and intravenous drug use.

Prevention of Infection in SCA

Infection can lead to a range of complications in SCA which are not readily reversed simply by treating the infection. For this particular reason, prevention is the key strategy in the management. Interventions during the last 20 years have dramatically reduced mortality, especially in children, and the recommendations continue to evolve (Booth *et al.*, 2010).

Simple general measures are important when reducing the risk of infection, though the aim is to ensure as normal a lifestyle as possible (Atkins *et al.*, 1997).

Nutritional supplementation with zinc has been reported to reduce infection risk (Prasad *et al.*, 1999), improve growth rates in children with SCA in Nigeria, and improve skeletal and sexual maturation (Zemel *et al.*, 2002). Parents are therefore encouraged to monitor their children closely at home and to accordingly seek advice if they have a fever or respiratory symptoms, whilst maintaining good hydration. Notably, there should be a low threshold for the use of antibiotics in ill children with SCA, particularly in the presence of chest signs or symptoms, which may herald ACS.

Antibiotic Prophylaxis

Pneumococcal infection is a major threat to patients with SCA. The first presentation of the infection may be with sudden death owing to overwhelming sepsis. The first major pneumococcal infection breakthrough came in 1986 with the pivotal PROPS trial, which showed that prophylactic oral penicillin reduced the risk of IPD incidence from 9.8/100 patient-years, to 1.2/100 in children aged less than 3 years (Gaston *et al.*, 1986). This inexpensive, simple and safe intervention was rapidly implemented.

Current recommendations state that oral penicillin V should be commenced at the age of 6 months, when protective levels of foetal haemoglobin start to decline and splenic hypo function begins to develop. The duration of the prophylaxis remains controversial. Since the risk of IPD declines markedly with age, it may be possible to modify or stop prophylaxis without compromising the outcome. Importantly, the PROPSII trial evaluated the consequences of discontinuing penicillin at 5 years and found no significant difference in the incidence of infection between the penicillin and control groups, thereby suggesting no added advantage (Falletta *et al.*, 1995). However, most of guidelines for asplenic individuals still recommend that penicillin prophylaxis be continued lifelong (Davies *et al.*, 2002).

Long-term penicillin prophylaxis is not without hazards. Prolonged antibiotic use can promote the development of resistant strains, and so the continuation with lower pneumococcal infection must be balanced against the danger that resistant organisms pose to the whole population (Hirst & Owusu-Ofori, 2002). With this in mind, studies by Reynolds *et al*., (2004) and Alexander *et al*., (2002) state that 9% of *Streptococcus pneumoniae* isolates from the general population were resistant to penicillin as compared with 25% of isolates from children with SCA (Alexander *et al.*, 2002; Reynolds *et al.*, 2004).

Compliance is a major issue in any long-term therapy. One study of children in New York found that patient reporting compliance with penicillin prophylaxis was 67.5%, but when compliance was measured objectively with a urine assay, the figure was 43.1%. In this regard, the subgroup of less than 5 years, in whom prophylaxis is most important, shows better adherence (61%) (Teach *et al.*, 1998). Moreover similar results have been found in British children (Elliott *et al.*, 2001).

Vaccination

Vaccination is a basic medical strategy in the prevention of infection. There are ninety serotypes of pneumococcal bacteria, which vary in the molecular composition of their capsular polysaccharide. The first pneumococcal vaccine produced immunity via a T cell independent effect, thereby triggering B cells in the splenic marginal zone to secrete antibodies (William *et al.*, 2007).

Early uncontrolled studies used a vaccine containing 14 antigens and produced a 50% reduction in IPD (Overturf, 2003), therefore, the vaccine became widely accepted. The current vaccine (Pneumovax, PPS-23) comprises 23 purified antigens, which should protect against 75% of IPD, with an added 14% prevented via cross-protection

(Overturf, 2002). Adamkiewicz *et al.*, (2003) reported a fifty percent reduction in IPD when PPS-23 with penicillin. Since the effectiveness of the vaccine diminishes over time, a booster dose is required every 5 years (Adamkiewicz *et al.*, 2003).

Unfortunately, however, many polysaccharides, especially those from the strains causing infections, are either not immunogenic in children under 2 years or otherwise produce only a minimal antibody response with a lack of immunological memory (Overturf, 2002). This has prompted the search for an alternative.

The polysaccharide-protein conjugate vaccine (PCV) binds capsular polysaccharides to protein in order to increase their immunogenicity. This does induce an effective response in infants under 2 years (Overturf, 2002). Moreover prevenar vaccine, a 7-valent PCV, is licensed for use in Europe and the USA and includes serotypes responsible for approximately 70% of the infections. Importantly, studies suggest that this vaccine would also cover 77% of isolates of resistant strains, which would avert prophylaxis failure and may ultimately help to reduce the frequency of such strains (Adamkiewicz *et al.*, 2003).

The routine immunisation of children with PCV-7 began in the USA in 2000 and in the UK in 2006. Multiple doses are required for the most effective response, which could be a limiting factor for the full vaccination protocol. The limited supply problem was defeated in the USA by 2005 (Nuorti *et al.*, 2008). With this in mind early analysis of the effects in patients with SCA have demonstrated very encouraging results. In Tennessee, for example, rates of IPD fell by 90.8% in children under 2 years and 93.4% in children aged less than 5 years. This brought the incidence to only 6.5 times higher than in unaffected children (Halasa *et al.*, 2007). In the UK, uptake in the first year was 6%- still somewhat lower than that for other routine childhood immunisations (Salisbury *et al.*, 2006). Children were advised to receive the PPS-23

vaccine every 2 years owing to the fact that they remain at high risk for developing IPD (Overturf, 2002). Penicillin prophylaxis is still required, in part because all trials included antibiotic prophylaxis; it also provides protection against non-PCV-7 pneumococcal strains. In Africa, pneumococcal prophylaxis programme is recommended, although the high cost remains an obstacle to the public health care planners (Kizito *et al.*, 2007).

Other important vaccines include *Haemophilus influenzae* (Hib), *Neisseria meningitidis*, hepatitis B, and influenza. Children travelling to endemic areas should be offered meningitis A and C vaccinations and malaria prophylaxis. Influenza infection can precipitate crisis and predisposes to bacterial pneumonia; therefore, vaccination is preferred (Dick, 2007; Salisbury *et al.*, 2006). Moreover, *Pneumococcal*, *Haemophilus influenza* and *meningococcal vaccinations* are effective at reducing infection rate. Hepatitis B vaccine should also be given (Castro, 1999).

In Saudi Arabia a study was conducted by El-Hazmi *et al.*, (1990) which concluded that pneumococcal vaccination and penicillin prophylaxis play a significant role in decreasing the morbidity and increasing the crisis- free interval in SCD patients (El-Hazmi *et al.*, 1990). Another contradictory report by Pejaver *et al.*, (1995) has shown that the *pneumococcal* vaccine and prophylactic oral penicillin had no effect on the frequency of hospital admissions rate of SCA patients per year (Pejaver *et al.*, 1995). Moreover, the *Pneumococcal* vaccination programme is still not part of the routine guidlines for managing patients with SCA; however, *Haemophilias influenzae* (Hib) vaccine is part of routine immunisation program (Al-Jam'a *et al.*, 2000).

1.7 The Role of Phagocytes in Sickle Cell Anaemia

Neutrophils and monocytes are the main phagocytic cells in the blood which are produced in the bone marrow and migrate to the blood stream particularly to the sites of infection (Hoffbrand *et al.*, 2006). Neutrophils make a one-way trip but monocytes differentiate into macrophages and may recirculate, to act as antigen-presenting cells (APCs). Importantly they interplay a regulatory role between the innate and adaptive immune system (Takeshi & Shin, 2003).

Importantly, the endothelium expresses adhesion molecules which are recognised by receptors on activated phagocytes which direct cell traffic to these areas. This process has been described in patients with SCA who have been found to exhibit interactions between sickle red blood cells, leukocytes and endothelial cells via adhesion molecules (AbouGhalia *et al.*, 2010; Makis *et al.*, 2000; Roitt *et al.*, 2002).

Inflammatory reactions have three major components, the increased blood supply and capillary permeability, followed by leukocytes migration. The local hyperaemia brings leukocytes and serum molecules (antibody and complement) to the affected site. Capillary permeability also increases allowing secretion of the serum proteins to control the infection. Finally, there is increased migration of leukocytes into the tissue.

Patterns of Leukocyte Migration

Leukocyte migration is a complex process which depends on the inflammatory cells involved, their state of activation and how they interact with endothelium in different vascular beds throughout the body (AbouGhalia *et al.*, 2010; Robinson & Babcock, 1998). Importantly, this is controlled by adhesion molecules, on the migrating cells

which interact with the endothelium, tissue cells, the extra cellular matrix, and soluble signalling molecules such as chemokines. Chemokines are small pro-inflammatory chemotactic cytokines which mediate inflammation by activation of integrin-mediated leukocyte adhesion to the vascular endothelium (Roitt *et al.*, 2002). In addition, the surface charge of the interacting cells, the haemodynamic shear force in the vascular bed and the expression of adhesion molecules on both the leukocytes and the endothelium regulate leukocytes migration. The venules provide the perfect environment for leukocytes migration, where the surface charge is lowest, haemodynamic shear is low and adhesion molecules are expressed (Roitt *et al.*, 2002) (Figure 1.4).

Notably, the first stage of leukocyte migration is the rolling of circulating leukocytes on the endothelial surface, the process is known as tethering. Subsequently, triggering occurs which indicates that the arrested leukocytes respond to cytokines and endothelial CAM. Once the binding between leukocytes and CAM takes place, the migration will start and this is known as latching and activation. Moreover, leukocytes move either between or through the endothelial cells towards the site of infection (Figure 1.5).

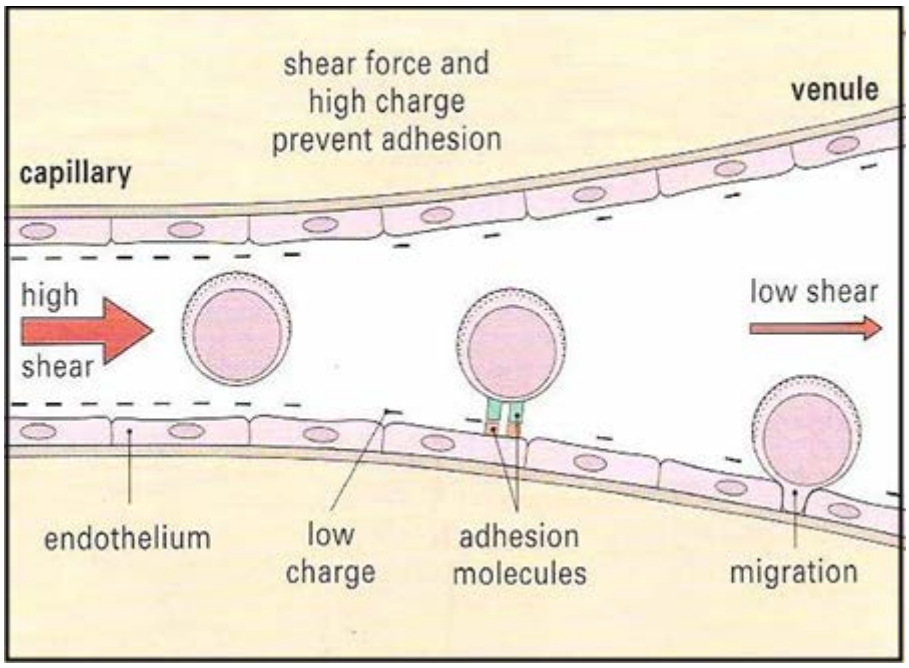

Figure 1.4 Leukocyte migration in the blood vesels from high to low shear force, with the expression of adhesion molecules. Adapted from (Roitt *et al.*, 2002).

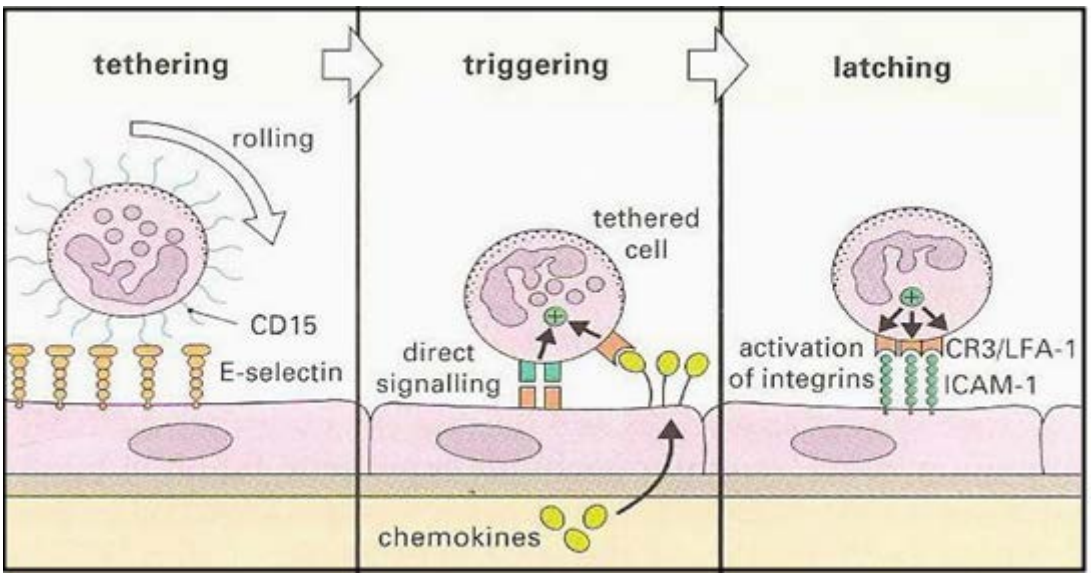

Figure 1.5 Three-step model of leukocytes adhesion. Tethering: binding and adhesion of leukocyte to aendothelium; Triggering: synthesis of adhesion molecules; Latching: by CR3/LFA-1 and ICAM-1. Adapted from (Roitt *et al.*, 2002).

1.8 Intercellular Adhesion Molecules

Intercellular adhesion molecules are membrane-bound proteins, which allow one cell to intermingle with another. These molecules are trans-membrane proteins which are connected to the cell cytoskeleton. As the cell moves, it uses them to gain traction on other cells, or on the extracellular matrix. Moreover cell adhesion molecules are expressed on both leukocytes and vascular endothelial cells. Adhesion molecules have the capability to bind more than one ligand by increasing the numbers of adhesion molecules on the surface or by otherwise altering their affinity. Importantly, the level of expression can be modified through increasing the synthesis and transporting of new molecules, or otherwise by directing the intracellular stores to the cell surface following cellular activation (Roitt *et al.*, 2002). Other mechanisms include increased affinity, such as leukocyte factor activation (LFA-1) following cell activation. Furthermore, reorganisation of adhesion molecules on the cell surface may result in the formation of high avidity patches (Figure 1.6).

The vascular endothelium expresses the immunologic supergene family comprising the cellular adhesion molecules ICAM-1, ICAM-2, VCAM -1 and MAdCAM-1 (mucosal adhesion molecules -1) (Figure 1.7).

There is a strong positive correlation between the role of cell adhesion molecules and pathophysiology of vascular occlusion which plays a major role in the disease severity in patients with SCA (Kutlar & Embury, 2014).

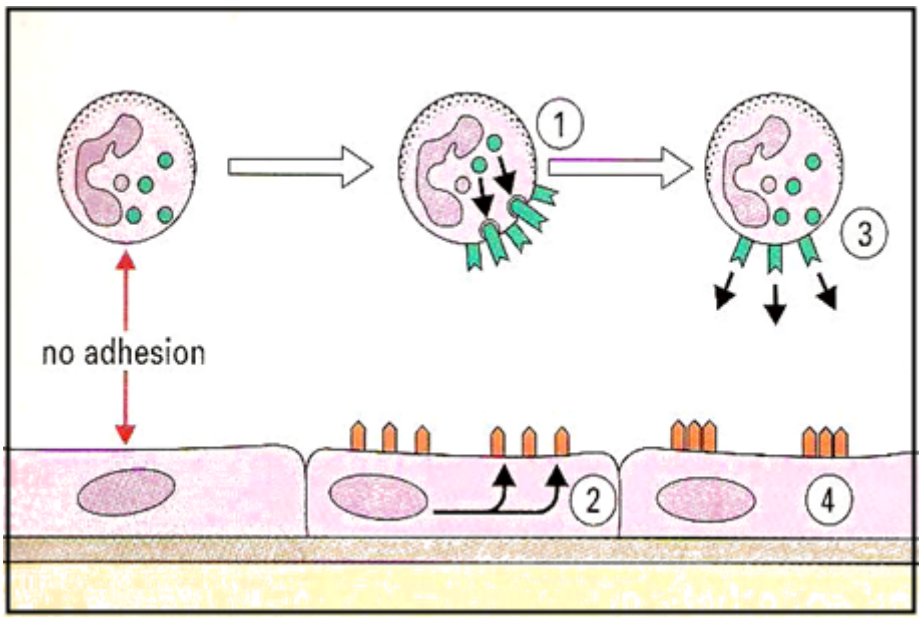

Figure 1.6 Modulation of leukocyte adhesion. Leukocyte binding to endothelium could become enhanced in four ways. (1). Many cells hold stores of adhesion molecules that can rapidly move to the cell surface. (2). Endothelial cells at the sites of inflammation may synthesize new adhesion molecules. (3) Molecules such as LFA-1 can increase their affinity following cell activation. (4). Reorganisation of adhesion molecules on the cell surface may result in the formation of high avidity patches. Adapted from (Roitt *et al.*, 2002).

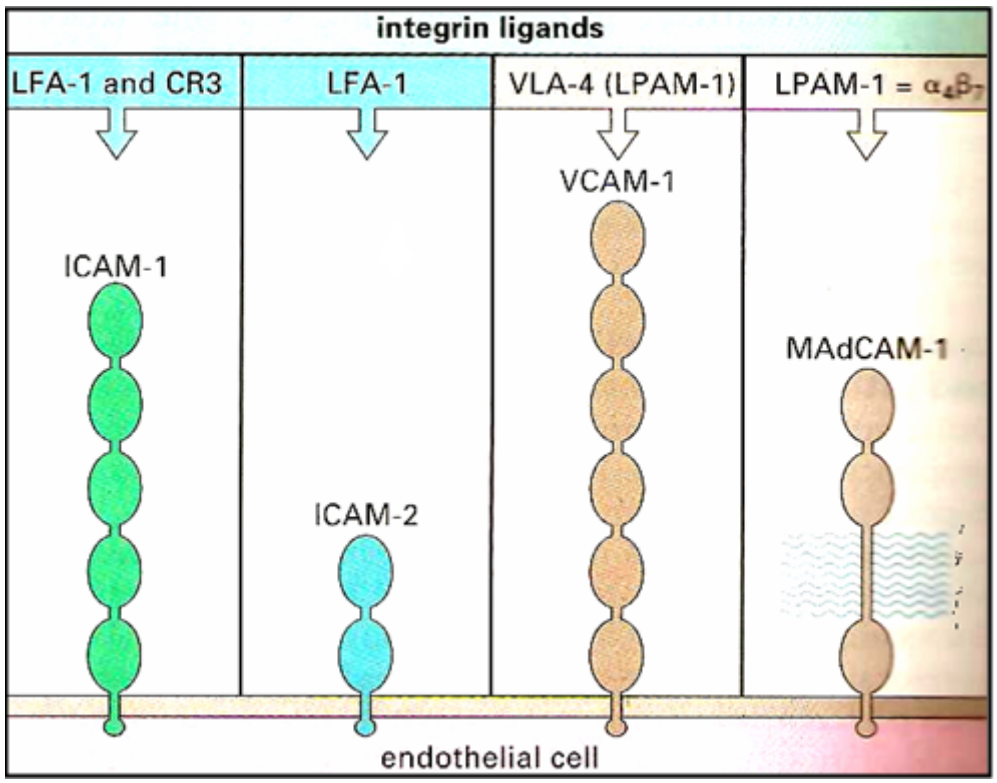

Figure 1.7 The endothelium cell expresses the adhesion molecules. LFA-1 and CR3: leukocyte factor activation – 1 and control register number 3; ICAM-1: intracellular adhesion molecule-1; ICAM-2: intracellular adhesion molecule-2; VLA-4 (LPAM-1): very late antigen-4; VCAM-1: vascular cellular adhesion molecule-1; Mad CAM-1: mucosal adhesion cell molecule-1. Adapted from (Roitt *et al.*, 2002).

Role of Integrins and Selectins in Leukocyte Interaction

Selectins and integrins are a group of transmembrane cell adhesion molecules present in many cells including leukocytes. Selectins consist of E-selectin, P-selectin and L-selectin (Figure 1.8). Furthermore integrins consist of two non-covalent bound polypeptides (α and β). They fall into three main subfamilies depending on whether they contain β_1, β_2 or β_3 chain. The β_1 integrins are involved in cell binding to the extracellular matrix, whilst the β_2 are implicated in leukocytes adhesion to endothelium, and β_3 integrins are involved in the interactions of platelets and neutrophils at inflammatory sites of vascular damage (Janeway & Travers, 2001; Roitt *et al.*, 2002).

Soluble E-selectin and P-selectin

The adhesion molecules are involved in the pathophysiology of SCA and can be measured in the peripheral blood during steady state and vaso-occlusive crisis. High levels of adhesion molecules such as soluble E-selectin and P-selectin (sE- selectin and sP-selectin) have been found in the sera of patients with SCA (Matsui *et al.*, 2002). Notably, various different levels of sE-selectin, and sP-selectin, have been reported in patients with SCA in different parts of the world; however, little information exists regarding Arab countries including Saudi Arabia.

E-selectin

E-selectin (Endothelial Leukocyte Adhesion Molecule-1), (ELAM-1), also known as CD62E, is an 115kDa, type I transmembrane glycoprotein expressed on endothelial cells following the activation by inflammatory cytokines (such as IL-1β or TNF-α) or endotoxins. Cell-surface E-selectin is a major extra- cellular adhesion molecule which

regulates binding and rolling of leukocytes to the endothelium. This is an essential step in the extravasation of leukocytes from the blood stream to the site of inflammation, thereby playing a key role in localised inflammatory response (AbouGhalia *et al.*, 2010; Simon, 2008).

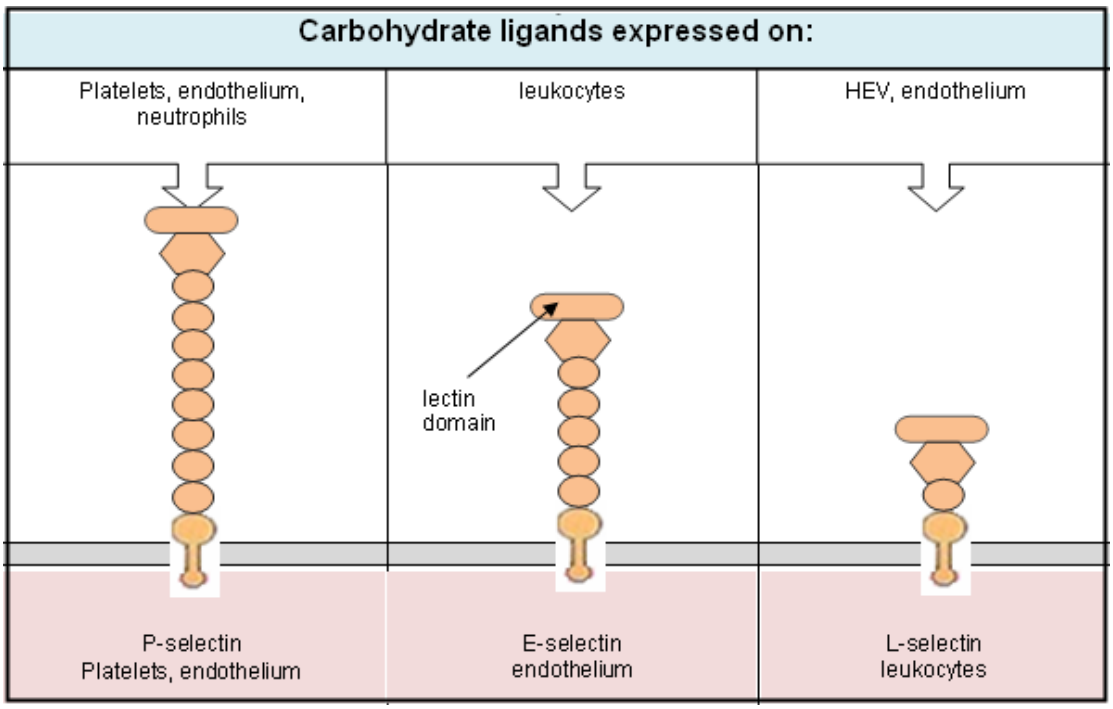

Figure 1.8 Structures of selectins. Adapted from (Roitt *et al.*, 2002).

The extra cellular part of E-selectin includes a calcium-dependent C2-type lectin domain, an epidermal growth factor (EGF) domain, and six repeats of a complement-regulatory-protein-like sequence (Figure 1.9). E-selectin binds sialyl Lewis X, a sialic acid-galactose-N-acetylglucosamine-fructose tetrasaccharide, although the actual recognition is considered to be for a specific presentation of those glycosyl units specific for glycoprotein (Varki *et al.*, 2008).

Soluble E-selectin (sE-selectin) is found in the blood of healthy individuals probably arising from the proteolytic cleavage of the surface-expressed molecule. Elevated levels of sE-selectin in serum have been reported in a variety of pathological conditions including SCA. Patients with SCA usually have high levels of circulating endothelial cells in the steady state condition, with further increments during vaso-occlusive crisis (Ataga & Key, 2007). High levels of circulating ICAM-1, VCAM-1, and E-selectin have been found in the blood of patients with acute painful crisis (Ataga & Key, 2007; Pathare *et al.*, 2003).

P-selectin

P-selectin (GMP-140, LECAM-3, PADGEM, CD62, CD62P) is a member of the selectin family of cell surface molecules. It comprises an NH2-terminal lectin type C domain, an EGF-like domain; nine complement control domains, a transmembrane domain, and a short cytoplasmic domain (Figure 1.9). P-selectin is found constitutively in a pre-formed state in the Weibel-Palade bodies of endothelial cells and also in the alpha granules of platelets. It is mobilised to the cells within minutes in response to a variety of inflammatory conditions or thrombogenic agents. The mobilised P-selectin is apparently present on the cell surface for few minutes, after which it is recycled to intracellular compartments. The molecular weight predicted from the cDNA for P-selectin is approximately 86,000Da.

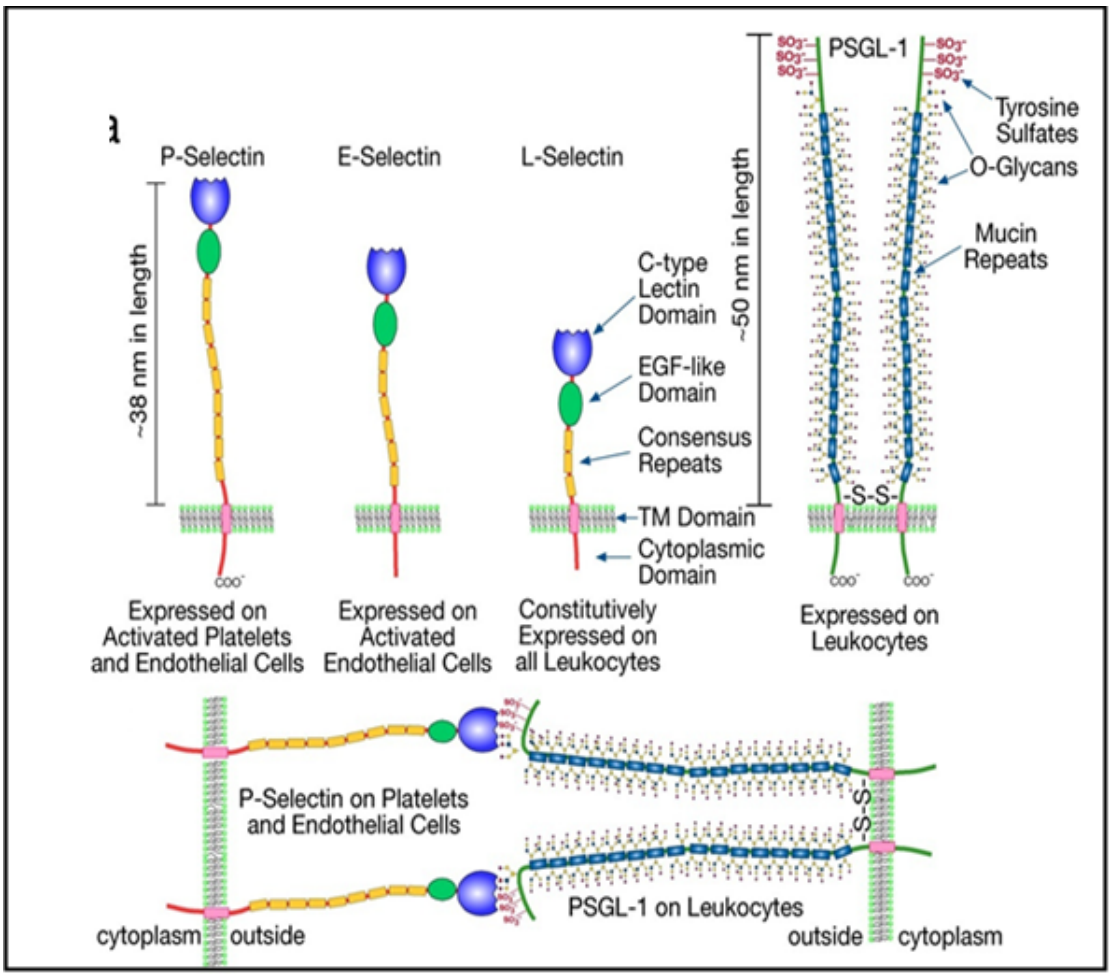

Figure 1.9 Overall domain structures of P-selectin, E-selectin, L-selectin and (P-selectin glycoprotein ligand 1(PSGL-1). Adapted from (Mei *et al.*, 1997).

The tetrasaccharide sialyl Lewis^x (sLW^x) has been identified as a ligand for both P- and E-selectin and can bind sLe^x and sLe^a under appropriate conditions (Figure 1.10).

P-selectin plays a critical role in the migration of lymphocytes into tissues; it also plays a role in the adhesion of myeloid cells, B cells and a subset of T cells to activated endothelium. It is also involved in the adhesion of platelets to monocytes and neutrophils, thereby playing a central role in the tethering, rolling, and firm adhesion of leukocytes to activated endothelial cells. The adhesion of leukocytes to the endothelium is initiated by weak interactions, which produce a characteristic 'rolling' motion of the leukocytes and neutrophils on the endothelial surface. P-selectin in cooperation with L-selectin mediates the initial interactions. Importantly, stronger interaction involving E-selectin follow the initial interactions, thereby eventually leading to extravasation through the blood vessel walls into lymphoid tissues and accordingly to sites of inflammation (Figure 1.11).

Soluble P-selectin has been found in the plasma of normal individuals at concentrations between 36 ng/ml and 250 ng/ml (Mei *et al.*, 1997). Blann *et al*., (2008) reported that sP-selectin level has been elevated in a variety of pathological conditions such as haemolytic- uremic syndrome, adult respiratory distress syndrome and disseminated intra vascular coagulation (Blann *et al.*, 2008).

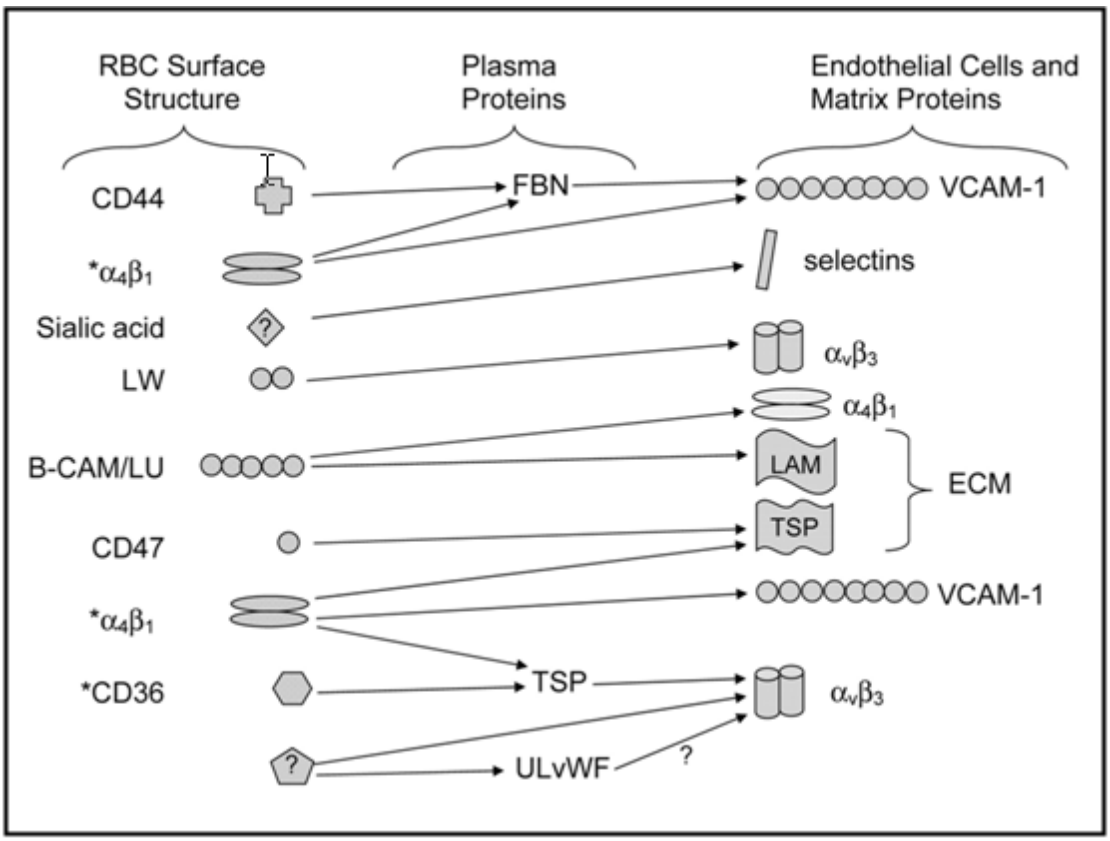

Figure 1.10 Adhesive interactions between SS red blood cells, plasma proteins, and components of the endothelium. FBN: fibronectin; TSP: thrombospondin; ULvWF: ultra-large von Willebrand factor; ECM: extracellular matrix; LAM: laminin. Adapted from (Telen, 2007).

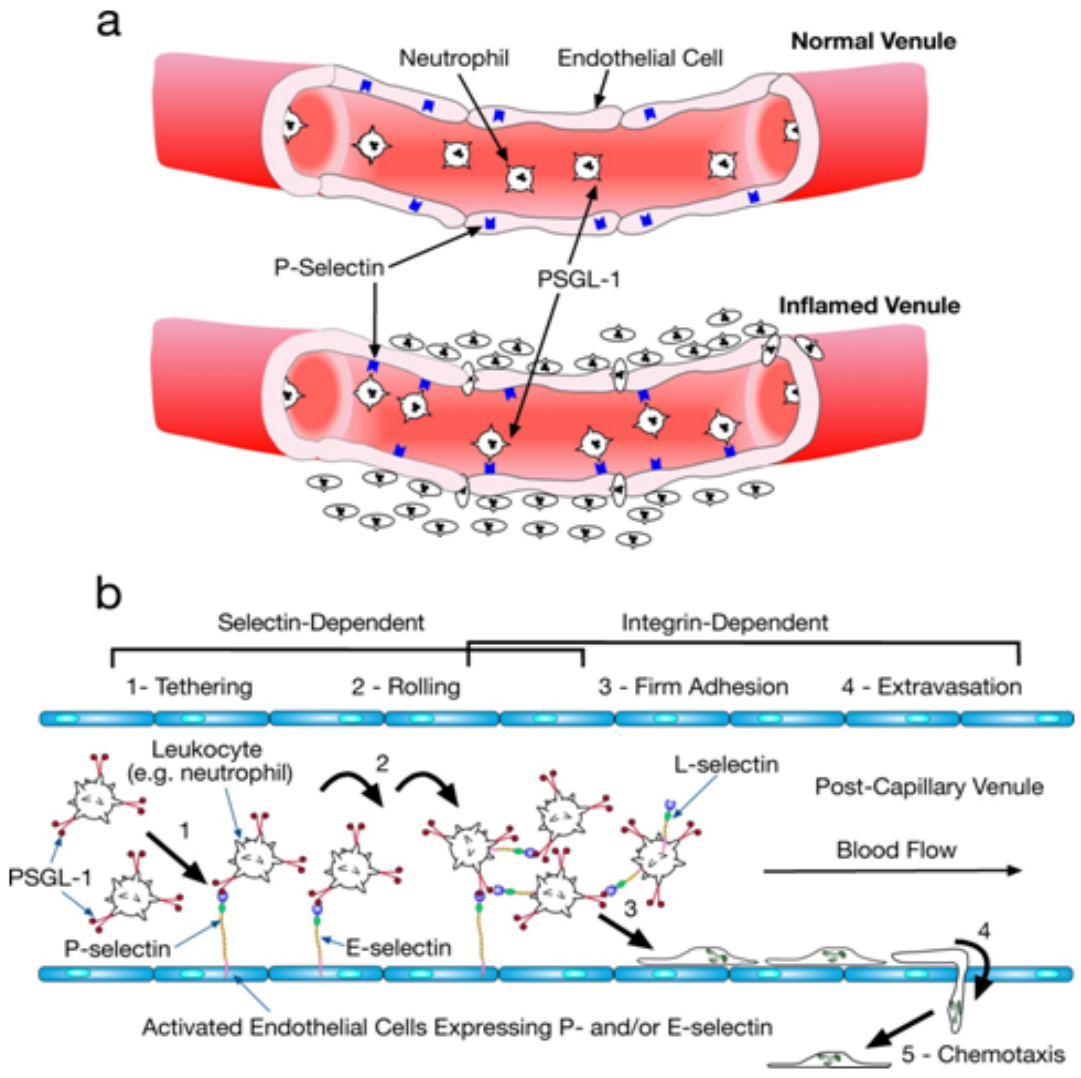

Figure 1.11 Tethering of circulating leukocytes to activated endothelium via interactions between selectins and their ligand. Adapted from (Spertini, 1997)).

Soluble P-selectin participates in the adhesion of sickle red blood cells to endothelial cells in the steady state condition and in acute painful crisis. At the site of vascular injury thrombin stimulates endothelial cells and P-selectin is then rapidly released from Weibel-Palade bodies to the luminal surface of the cells. Thrombin, histamine agonists, hypoxia and reperfusion have been proven to induce the expression of P-selectin on human endothelial cell surfaces (Matsui *et al.,* 2002). Patients with SCA during the acute painful crisis have abnormal presence of circulating endothelial cell adhesion molecules such as ICAM-1, VCAM-1, and sE-selectin in their plasma (Pathare *et al.,* 2003; Shiu *et al.,* 2000). In conclusion, sP-selectin is found to be proinflammatory and prothrombogenic mediator in patients with SCA (Wood *et al.,* 2004).

P-selectin also reportedly binds selectively to a 160 kDa glycoprotein present on human myeloid cells termed sP-selectin glycoprotein ligand-1 P-SGL-1. Soluble P-selectin mediated rolling of leukocytes can be completely inhibited by a monoclonal antibody specific for PSGL-1.

1.9 Treatment

Patients with SCA should avoid factors known to precipitate crisis, especially dehydration, hypoxia, infections and stasis of circulation. Good general nutrition, supplemented with folic acid and proper hygiene are all required.

Treatment of Painful Episodes

Painful episodes are sometimes triggered by infection, extreme temperatures or physical or emotional stress; more often, however, they are unprovoked and begin

with little warning. Patients with severe pain should be given an opiate parenterally at frequent, fixed intervals until the pain has diminished. Then the dose of opiate can be tapered and stopped and oral analgesic therapy instituted using paracetamol and/or non-steroidal anti-inflammatory drugs (Castro, 1999; Hoffbrand *et al.*, 2006).

Treatment of Anaemia

Blood transfusions are not needed for the usual anaemia or for painful episodes associated with SCA. Notably, the urgent replacement of blood is often required for sudden severe anaemia occurring in children, such as when blood is sequestered in an enlarged spleen or when parvovirus B19 infection triggers a transient aplastic crisis. Hypoxia accompanying the acute chest syndrome necessitates transfusion and oxygen treatment. Moreover, clinical trials have evaluated the efficacy of transfusion in stroke related to SCA. Repeated transfusion reduces the risk of recurrent stroke in children with sickle cell anaemia (Dacie & Lewis, 1991; Telen, 2001).

Notably, approximately 50% of children with SCA and stroke, who do not receive transfusions, experience recurrent stroke within three years, as compared with 10% of those who received transfusions (Adams *et al.*, 1998; Adams *et al.*, 1992). The aim of transfusion is to reduce the HbS concentration rapidly to less than 30% of total haemoglobin concentration and to accordingly maintain this percentage for 3-5 years. Exchange transfusion, either by manual or more often by an apheresis technique, is the treatment of choice in the acute phase (Telen, 2001). A two-year trial has investigated the use of prophylactic transfusion in SCA children from 2-16 years of age who were screened by transcranial Doppler ultrasonography (TCD) to measure cerebrovascular blood flow. Patients with persistently elevated TCD signals are at an ongoing stroke risk, which calls for blood transfusion (Kwiatkowski *et al.*, 2006; Lee *et al.*, 2006).

For patients undergoing general anaesthesia, preoperative top up transfusion, aiming at increasing the haematocrit to approximately 30%, prevents postoperative complications. This was associated with reduced transfusion-related complications by 50% (Adedeji *et al.*, 2001).

Hydroxyurea

Pharmacokinetics: Hydroxyurea is an analogue of urea which inhibits DNA synthesis in the S-phase through the inhibition of ribonucleotides reductase enzyme, resulting in depletion of deoxynucleoside triphosphate pool (Edward, 2004; Orah & Platt, 2008). The drug is specific for the S phase of the cell cycle and causes cells to arrest at the G1- S interface (Cababres & Chabner, 1996; Orah & Platt, 2008).

Hydroxyurea was found to cause leukopoenia, anaemia and megaloblastic changes in the bone marrow. It also exhibits antineoplastic activity against sarcoma. Previously it was thought that haemoglobin S polymerization helped in the entrapment of sickled, poorly deformable erythrocytes that mechanically blocked small calibre vessels. It was later recognized that these damaged erythrocytes alone are not sufficient to produce the vaso-occlusion in larger vessels. Therapeutic approaches to sickle cell anaemia focused on trials to reduce intracellular HbS polymerization by altering the haemoglobin molecules (Noguchi *et al.*, 1993).

Absorption and Excretion: In humans, hydroxyurea administered orally is absorbed from the gastrointestinal tract, and peak plasma concentrations are achieved 1-2 hours after a dose of 15-30 mg per kilogram of body weight. Notably, the plasma half-life is approximately 2 hours, and has almost 100% oral bioavailability. Moreover, hydroxyurea has the ability to cross the blood-brain barrier (Cababres & Chabner, 1996).

Approximately 80% of the drug is recovered in the urine within 12 hours following oral administration (Orah & Platt, 2008). Moreover, plasma and urine hydroxyurea levels can be measured using high performance liquid chromatography (HPLC) techniques (Bachir *et al.*, 2007).

Effect of Hydroxyurea therapy on cell adhesion molecules: Hydroxyurea has a potential effect on erythrocyte and leukocytes in patients with SCA. Moreover, it reduces the frequency and severity of acute vaso-occlusive crisis by decreasing erythrocyte cell adhesion receptors (Gambero *et al.*, 2007; Telen, 2007). Notably, sickle erythrocytes were shown to be abnormally adherents to extra cellular matrix proteins, thrombospondin (TSP), laminin, and fibronectin. Hydroxyurea was found to down regulate their interactions (Gambero *et al.*, 2007; Johnson & Telen, 2008; Telen, 2007). Moreover, Covas *et al.*, (2004) have shown that hydroxyurea decreases both erythrocyte HbS level as well as the reticulocyte adhesion receptor expression (Covas *et al.*, 2004). The sickled reticulocyte expression of VLA-4 and CD 36 was measured both before and following hydroxyurea treatment using flow cytometry. Notably, after hydroxyurea treatment both values were reduced, supporting a role for hydroxyurea in decreasing the frequency of acute VOC and eventually reducing frequent hospitalisation, morbidity and mortality (Telen, 2007; Wang, 2007).

Furthermore, the interactions between the red blood cells and leukocytes are inhibited by the effect of hydroxyurea; this reduces the leukocyte count and minimises the activation and adhesion of sickle leukocytes, particularly neutrophils and monocytes (Finnegan *et al.*, 2007). Hydroxyurea was also found to normalise L-selectin and H_2O_2 in patients with SCA (Benkerrou *et al.*, 2002). In addition, hydroxyurea provides a fundamental therapeutic approach to shift haemoglobin production from sickle to foetal haemoglobin (Orah & Platt, 2008), decreases haemoglobin polymerisation, sickling and haemolysis, accordingly reduces the need for blood transfusions (Gambero *et al.*, 2007; Johnson & Telen, 2008).

Finally, hydroxyurea metabolites restore NO bioavailability, thereby leading to normal vascular tone (Okpala, 2004a; Orah & Platt, 2008) and thus reducing the incidence and severity of the VOC.

With this in mind, the study will fucose on the role of the above mechanisms with specific emphasis on the Saudi Arabian population. The literature review has shown few studies which thus far have focused specifically on this aspect.

Aims

The overall aims of this study were:

- To determine the incidence and characteristics of infection in sickle cell anaemia patients in acute vaso-occlusive crisis.

- To evaluate leukocyte phagocytic function in neutrophils and monocytes, of Saudi and non-Saudi sickle cell anaemia patients, in steady state and comparing them with normal control subjects.

- To assess any gender related difference in leukocyte phagocytic function, in Saudi and non-Saudi sickle cell anaemia patients in steady state; and in normal control subjects.

- To evaluate characteristics of leukocytes by immunophenotyping, using flow cytometry in Saudi and non-Saudi sickle cell anaemia patients, both in steady state and in vaso-occlusive crisis. Findings were compared to normal control subjects.

- To determine the difference in the levels of the adhesion molecules, "sE-selectin and sP-selectin" between Saudi and non-Saudi sickle cell anaemia patients in both steady state and in acute vaso-occlusive crisis; and compared to normal control subjects.

CHAPTER 2

CHAPTER 2: MATERIALS AND METHODS

2.1 Selection of Subjects

The population study comprised of sickle cell anaemia patients (SCA) at both phases (the acute vaso-occlusive crisis and the steady state), and normal healthy control subjects with normal haemoglobin (HbAA). The status of selected patients and control subjects was confirmed through the utilizing of sickle solubility test and cellulose acetate electrophoresis. These tests were performed by technicians in the Department of Haematology at the King Abdulaziz University Hospital, (KAUH), Jeddah, Saudi Arabia.

Patients in Vaso-Occlusive Crisis

Subjects in this group were sickle cell anemia patient, with sickle haemoglobin (HbSS) and clinically diagnosed as having acute vaso-occlusive crisis (VOC). They were experiencing an episode of acute pain in the abdomen and/or the extremities with signs of increased haemolysis, usually presented to emergency room at KAUH.

The sickle cell anaemia patients with acute VOC aged 18-45 years old took part in this study. The age of SCA in steady state ranged between 15 and 45 years. The age of normal control subjects ranged between 18 and 45 years.

Patients in Steady State

Subjects in this group were HbSS patients, in haematology clinic at KAUH, who were clinically assessed and considered as steady state when they were free from pain, afebrile, had not been hospitalised and had not suffered a vaso-occlusive episode for at least 2 weeks prior to blood sampling. Female subjects were not pregnant. No patients were receiving hydroxyurea, anti-lipid therapy or had received a transfusion within the previous 8 weeks of the start of the study.

Control Subjects

The control population consisted of matched age and gender healthy staff members of KAUH and King Fahad Medical Research Center, Jeddah. In addition, healthy normal volunteer donors from KAUH blood bank, both Saudis and non-Saudi. All controls had their HbAA status confirmed, by sickling test, performed within our hospital laboratories. An inherent bias may be those blood donors who are of a super healthy class.

Principle of haemoglobin electrophoresis

Haemoglobin electrophoresis is used for separating and identifying the haemoglobin component in blood. Haemoglobin molecules in an alkaline solution have a net negative charge. This causes them to move towards the anode at a rate proportional to their negative charge. In addition quantitation of HbS and HbF values were assess to detect the severity of the disease.

Dissolved RBC (Hemolysate) was applied to cellulose acetate, at alkaline pH (8.6); electrical current was applied to separate normal and abnormal types of haemoglobin

in the blood. The haemoglobin proteins were identified by staining these bands and comparing their positions with normal controls (Bain *et al.*, 2011).

Selection criteria for screening of volunteer control subjects:

The control group used in the present study consisted primarily of volunteer blood donors. A questionnaire form was completed following interviewing each donor (Appendix 3). These subjects may not be representative of the general population of Saudi Arabia, as they have been selected for their high degree of health and well being. For example blood donations are only accepted from individual with a blood haemoglobin value of 12.5 gm/dL for women and 13.5 gm/dL for men. In addition blood donors are screened for a plethora of disorders, including HIV 1 and 2 antibodies, Hepatitis B (surface and core antigens and antibodies), Hepatitis C Virus RNA, Hepatitis B Virus RNA Syphilis antibodies. Some analyses require fresh blood samples, so colleagues and staff members, at KAUH, were a perfect source, their help is always appreciated.

The numbers of subjects in different studies were variable, depending on the flow of patients to emergency room and haematology clinic. But the statistical measure of power was established latter. This was convenient for establishing the statistical power of test.

Ethical Approval

Ethical approval was obtained from the Ethics Committee at King Abdulaziz University Hospital, Jeddah, Saudi Arabia (Appendix 1). Informed consent was obtained from each of the control subjects, from adult patients and also the parent or guardian of children, prior to participation in the study. A research questionnaire form

was developed (Appendix 2) and forms were completed during interviewing each participant. The questionnaire noted the date of birth, nationality, history of infection and other clinical and laboratory relevant information.

Study Population

Patients and control subjects were residents in the country of Saudi Arabia and were assigned to two groups, Saudi and non-Saudi. This was determined in accordance to their nationality in their official documents.

2.2 Study of Infection in Sickle Cell Anaemia Patients

Selection of Subjects

In this retrospective study, a review of medical records was carried out for known Saudi and non-Saudi homozygous sickle cell anaemia patients with acute vaso-occlusive crisis admitted to the Haematology Department at King Abdulaziz University Hospital, Jeddah, Saudi Arabia, from January 2006 through to December 2008. During the two years of the study, samples from febrile sickle cell patients in acute vaso-occlusive crisis were taken for bacterial culture.

The study involved 232 patients, all of whom were suffering from SCA in acute VOC. Sixty seven patients were deferred, as they were free from any signs of sepsis, or otherwise had no fever. Culture and sensitivity tests were negative in 113 patients and positive in 52 patients. The patients with documented infection totalled 29 males and 23 females, ranging in age from 18-46 years with a mean age of 29.6 years.

Importantly ethical approval to review the records of those patients was obtained from the Ethical Committee at King Abdulaziz University Hospital, Jeddah, Saudi Arabia (Appendix 1).

Sample Collection

Fifty two patient samples were taken from suspected infection sites for culture and sensitivity test by microbiological techniques as follows:

Skin swabs from 5 patients with skin infection, sputum culture from 11 patients with chest infection, blood culture from 19 patients with high fever, and urine culture from 17 patients with urinary symptoms.

Principle of Study

This study was conducted through the utilization of microbiological tests so as to identify the most common organisms in each group through culture and sensitivity tests.

2.3 Analysis of Leukocyte Phagocytic Function

Sample Collection

Blood samples were collected from homozygous asymptomatic SCA patients presented in a steady state without infection as defined by the fact that they were afebrile and had no other concurrent acute or chronic medical illness. From each patient 2 ml of blood was collected by venepuncture in to a heparinised tube, and

stored at room temperature. Samples were processed within 24 hours for flow cytometric analysis. The subjects were 17 female and 14 male patients, ranging in age from 14-43 years with a mean age of 22.5. The control population comprised twenty-five (25) age matched healthy volunteers (13 females, 12 males).

Principle

This study utilised flow cytometry to measure the capacity or activity of the phagocytic function of neutrophil and monocytes in each group. The technique required fresh heparinised whole blood, which was incubated with FITC-labeled E-coli bacteria at 37 ^{o}C and a negative control sample, which remained on ice. The phagocytosis was stopped by placing the sample on ice and adding quenching solution. Notably, this solution permitted discrimination between the attachment and internalisation of bacteria by quenching of FITC fluorescence of surface bound bacteria leaving the fluorescence of internalised (phagocytosed) particles unaltered.

The E-coli bacteria were opsonised with immunoglobulin and complement from pooled sera. Monocytes and neutrophils have receptors for a complement component (C3b) and for the constant part of the immunoglobulin molecule (Fc), mediating the adhesion of the bacteria to the cell surface. Through utilising the methodology of this commercially available kit, the capability of the phagocytic cells in SCA was determined.

Procedure

The phagotest kit was obtained from Becton Dickinson (USA), and used according to manufacturers' instructions. This kit quantified phagocytic activity in granulocytes and monocytes in whole blood.

Heparinised whole blood was mixed for three seconds on a vortex mixer and aliquoted into 100 µl per tube, using 5ml tubes. Care was taken to ensure that no blood remained on the sidewall of the tubes. The blood samples were incubated in an ice bath for 10 minutes. The pre-cooled bacteria were then mixed well (vortex mixer) and 20 µl were added per test to the whole blood. All test tubes were mixed once more and subsequently incubated for ten minutes at 37 ^{o}C in a water bath, whilst the control samples remained on ice. Following incubation, all samples were removed from the water bath simultaneously and subsequently placed on ice in order to prevent phagocytosis. Ice-cold quenching solution (100 µl) per test was added and the samples were mixed on the vortex mixer. A washing solution of 3ml was added to each tube and mixed. The tubes were then centrifuged for five minutes at 2100 rpm (250xg) at 4 ^{o}C. The supernatant was then discarded. The samples were washed with 3 ml of washing solution once again. The whole blood was lysed and fixed with 2 ml of lysing solution, diluted 1 in 10 and prewarmed to 23 ^{o}C. Samples were then mixed and incubated for 20 minutes at room temperature, and subsequently centrifuged at (250xg) for five minutes at 4 ^{o}C. The supernatant was discarded and the samples were washed once more with 3 ml of washing solution, with centrifugation at (250xg) for 5 minutes at 4 ^{o}C. Podium iodide 200 µl was added to each tube. The tubes were mixed and incubated on ice with light protection for 10 minutes. The cell suspension was analysed using the flow cytometric analysis scan (FACScan).

2.4 Flow Cytometric Analysis (FACScan)

Flow cytometric analysis was achieved through utilising the blue green excitation light (488 nm argon-ion laser). During data acquisition, a ‘live’ gate was set in the red fluorescence histogram on those events, which had at least the same DNA content as human diploid cells (i.e., excluding bacterial aggregates having the same light scattering properties as leukocytes). Between 10,000 and 15,000 cells per sample

were analysed. The percentages of neutrophil and monocyte cells that had completed phagocytosis were analysed. The number of ingested bacteria was indicated through utilizing of the mean fluorescence intensity.

The relevant leukocyte cluster was gated using the software program in the scatter diagram (linear Forward Scatter, FSC versus linear Side Scatter, SSC), and the green fluorescence histogram was analysed. The control sample was used to set a marker for fluorescence histogram so that less than 1% of the events were positive.

The percentage of phagocytosing cells in the test sample was then determined by counting the number of events, above this marker position. The mean fluorescence correlated with the number of bacteria per individual leukocytes (Ormerod, 2000; Stewart & Nicholson, 2000).

2.5 Immunophenotyping and Expression of Leukocyte Adhesion Molecules

Selection of Subjects

The study was carried out on 36 SCA patients in a steady state. There were 22 female and 14 male patients, the ages of whom ranged from 10–40 years with a mean age of 24.40 years. The control population consisted of 34 age matched healthy volunteers (17 females, 17 males) from a pool of normal blood donors.

Sample Preparation

Venous blood was obtained by venepuncture into tubes anticoagulated with EDTA from patients with SCA at both phases (the acute crisis and the steady state). Complete blood counts, including red blood cells (RBCs), leukocytes, platelets, and flow cytometry analysis, were performed on all samples. Complete blood counts were performed using a Coulter LH750 counter (Coulter, USA).

The calculation of the total leukocyte count permitted the determination of an absolute lymphocyte count, with the use of automated haematology analyser. From these values, the lymphocyte subsets were calculated through multiplying the percentages (acquired from the flow cytometry) by the absolute lymphocyte count (derived from the automated haematology analyser results).

Principle

Flow cytometry is a technique applied to make rapid measurements of individual particles or cells as they flow in a fluid stream through a sensing point. The method is based on the ability of laser and arc lamp-based flow cytometer to measure multiple cellular parameters, using light scatter and fluorescence (Salzman *et al.*, 1975). The applications of flow cytometry and cell sorting are numerous. Conjugation of fluorescent dyes to ligands and to polyclonal and monoclonal antibodies has enabled the study of the density and distribution of cell-surface and cytoplasmic determinants and receptors, as well as further permitting the identification of functional subpopulations of cells (Jackson, 1990).

Immunophenotyping Assay Procedures

Immunophenotyping was performed and analysed using the CellQuest software (Becton Dickinson, USA). An aliquot 100 µl of whole blood was added to 20 µl of the appropriate fluorochrome-conjugated monoclonal antibody (Becton Dickinson Tri test reagents, USA) and vortexed gently. The above mixture was incubated for 15-30 minutes in the dark at room temperature (20-25 ^{o}C), after which 2 ml of FACS lysing solution (Becton Dickinson, USA) was added. The mixture was vortexed gently and again incubated for 10 minutes in the dark. The sample was centrifuged at (500xg) for 5 minutes and the supernatant was then removed. Wash buffer (2-3 ml; phosphate buffered saline containing 0.1% sodium azide, filtered through 0.2 µm), was added to the mixture, and then centrifuged as above. The supernatant was removed and 0.5 ml of 1% paraformaldehyde was added and mixed thoroughly. The sample was then stored at 2-8 ^{o}C until analysed on the FACS Caliber flow cytometer (Becton Dickinson, USA).

Quality control procedures were performed with each batch of samples, according to manufacturer's instructions. IgG1 and IgG2a antibodies were used as negative controls.

The following monoclonal antibodies was obtained from (Becton Dickinson, USA), were used in steady state SCA patients.

1. $CD3^{+}$ (FITC) / $CD4^{+}$ (PE) / $CD45^{+}$ (PerCP) — T helper cells
2. $CD3^{+}$ (FITC) / $CD8^{+}$ (PE) / $CD45^{+}$ (PerCP) — T suppressor cells
3. $CD19^{+}$ (PE) / $CD45^{+}$ (PerCP) — B cells
4. $CD3^{+}$ (FITC) / $CD16{+}56^{+}$ (PE) / $CD45^{+}$ (PerCP) — NK cells
5. $CD33^{+}$ (FITC) /$CD13^{+}$ (PE) — Myelocyte

6. CD14+ (FITC) / $CD64^+$ (PE) Monocyte & neutrophil
7. IgG_1 (FITC)/IgG_2a (PE) Negative control

Additional monoclonal antibodies were used in patients with SCA in acute vaso-occlusive crisis:

8. CD 62L (APC) /$CD14^+$ (L selectin / monocytes)
9. CD 62L (APC)/$CD64^+$ (L selectin / granulocytes)
10. CD 62L (APC)/ $CD19^+$ (L selectin / lymphocytes)

Gating

The evaluation of different leukocytes is facilitated when they are separated graphically from one another. Satisfactory discrimination of leukocytes can be accomplished with the utilization of light scatter parameters. Forward Scatter (FSC), Side Scatter(SC), wich are functions of cell size and complexity respectively, are used to define the various leukocyte types: lymphocytes, monocytes and granulocytes (Marti *et al.*, 1986). Moreover, the process of gating is used to analyse a desired cell subpopulation out of the total population. During this process, analysis gate is set around cell populations in question (Kasschau *et al.*, 1996).

Multiset leukogate with multiset software were used to establish a lymphocyte analysis gate so as to permit automatic analysis of the gated cells. Setting manual gating was unnecessary for lymphocytes, although other leukocytes did require manual gating. In the manual procedure, arrow keys or the mouse were used to position the cursor once leukocyte subpopulations were located on the screen. The goal was to obtain a leukogate that included 95% or more of the normal mature leukocytes in the sample. The fluorescence display quadrants and the corresponding colours were indicated with the gate description shown on the screen.

Quality Control in Clinical Flow Cytometry

Successful flow cytometric analysis depends upon the sample preparation, fluorescent reagent, instrument calibration, and accurate sample and data analysis. Quality control procedures encompassed all of these aspects of flow cytometry, in order to determine the precision and accuracy of the procedure and to ensure optimal results (Marti *et al.*, 1986). The best results are obtained when flow cytometric analyses are performed with fresh samples, notably it is important to establish the appropriate conditions after

sample collection. A variety of factors can affect leukocyte constancy, including the storage temperature (Telen, 2001), anticoagulant and storage time (Nicholson *et al.*, 1984). Fluorescent reagent quality control includes the evaluation of the saturable binding concentrations of the reagents and the determination of specific binding to the cell of interest.

2.6 Determination of Soluble E-selectin and P-selectin

Sample Collection

Venous blood samples were collected from patients with SCA at both phases (the acute vaso-occlusive crisis and the steady state) and normal healthy control subjects. Blood from each subject (2.0 ml) was placed in a plain tube, which was permitted to clot at

room temperature for 30 minutes. Serum was separated via centrifugation of the collected blood for 15 minutes at a speed of (1000xg). All sera were aliquoted and stored at -20^{0}C until studied as one batch at the end of the study. The median age was 27 years, ranging from 15-45 years. Females were fifty five% (46) and forty five% (38) were males. Further samples were collected from normal healthy control subjects with no family history of SCA, forty eight% were female and fifty two% were male. The median age was 27 years, ranging from 18-45 years.

Principle

Soluble E-selectin and sP-selectin kits were obtained from R&W systems, (USA) and were used to estimate these two proteins, through quantitative sandwich enzyme

immunoassay (Bisset *et al.*, 2004) technique as described previously (Blum *et al.*, 2005). A monoclonal antibody specific for sE-selectin or sP-selectin was pre-coated onto a microplate. Standards, samples and controls were pipetted into the wells and the immobilised antibody bound any sE-selectin or sP-selectin present. After washing away any unbound substances, an enzyme-linked monoclonal antibody specific for sE-selectin or sP-selectin was added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was added to the wells and colour was developed in proportion to the amount of sE-selectin or sP-selectin bound in the initial step. The colour development was then stopped and the intensity measured.

Procedure

All reagents and samples assayed were brought to room temperature for analysis. Patients, controls and standards were assayed in duplicate.

Strips coated with a mouse monoclonal antibody against sE-selectin or sP-selectin were prepared. An aliquot (100 µl) of assay diluent RDIW (a buffered protein solution) was added to each well. An aliquot (100 µl) of standard patient serum control was then added and subsequently covered with the adhesion strip. Samples were incubated for 2 hours at room temperature. The content of each well was accordingly aspirated and the well was washed. The process was repeated three times in total and four washings were performed by filling each well with 400 µl wash buffer. Following the last wash, the remainder of the wash buffer was removed through decanting. The conjugate of sE-selectin or sP-selectin 200 µl was added to each well, covered with a new adhesive strip, and subsequently incubated for 2 hours at room temperature. Each well was then aspirated and washed using 400 µl of washing buffer. Moreover, a total of four washes were performed, then 200 µl of

substrate solution, was added to each well and then incubated for 30 minutes at room temperature in a dark room. Stop solution 50 µl (2N sulphuric acid) was added to each well. The colour in the wells changed from blue to yellow. The absorbance of each well was determined within 30 minutes using a microplate reader (Spectro Reader State Fax-2100, Awareness Technology Inc. Palm City, and Fl 34990) adjusted to 450 nm.

Calculation

The duplicate reading for each standard, control and samples was averaged and accordingly the zero standard absorbance was subtracted from the average.

A standard curve was created by plotting the mean absorbance for each standard on the Y-axis against the concentration on the X-axis and a curve was drawn through the points on the graph. The data was then linearalised by plotting the log of the sE-selectin and sP-selectin concentrations versus the absorbance. The concentration of selectin was read from the standard curve and multiplied by x10 (dilution factor) for sE- selectin and by x15 for sP–selectin. A standard curve was presented (Appendix4).

2.7 Statistical Analysis

Computer aided statistical analysis of data from the study was performed using the Student unpaired *t*-test. The *t*-test was used to establish statistical differences between sickle cell anaemia patients in steady state, and sickle cell anaemia patients in vaso-occlusive crisis compared to normal healthy control subjects. Student unpaired *t*-test was used to compare two independent sample means. Independence indicates that the values from both samples are numerically unrelated of each; there was no correlation

between corresponding values. The data populations follow the normal distribution. Results were then expressed as mean ± standard deviation (SD). Differences between groups were analysed with analysis of variance (ANOVA) for multiple comparisons. The limit of significant statistic differences was set at $p < 0.05$. All statistical analysis was performed using SPSS (version 15.0; SPSS Inc., Chicago) software package.

CHAPTER 3

CHAPTER 3: PATTERNS OF BACTERIAL INFECTION IN SICKLE CELL ANAEMIA

3.1 Introduction

Patients with SCA are prone to develop bacterial, viral and parasitic infections. Worldwide, infections are a major cause of mortality in SCA, particularly in children. Infections remain the leading cause of death, both in the developed and especially in the developing world. Infection was implicated in 20-50% of deaths in prospective cohort studies over a period of 20 years (Booth *et al.*, 2010). Indirectly the sickle gene confers an increased susceptibility to infection, particularly to certain bacterial pathogens, and at the same time, infection provokes a cascade of SCA specific pathophysiological changes. Infection is one of the major precipitating factors for VOC. Furthermore parvovirus infection is the underlying risk factor for aplastic crisis (Lowenthal *et al.*, 1996; Serjeant *et al.*, 1993).

Infection has long been identified as a cause of sickle sequestration crisis particularly acute chest syndrome (ACS). *Streptococcus pneumoniae* was considered to be the most common infectious agent implicated in chest infection. Penicillin prophylaxis and vaccination programs reduced the pneumococcal infection rate and reports showed that *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* are now becoming the most common infectious agents responsible for ACS (Bernard *et al.*, 2007; Vichinsky *et al.*, 2000). Furthermore, chest infection could mimic ACS secondary to sequestration crisis which require both treatment modalities; blood transfusion as well as antibiotic coverage (Vichinsky *et al.*, 2000). In the developed countries, measures to prevent infection including vaccination programmes, use of prophylactic

antibiotics, and successful treatment of infection have contributed to improvements in survival and quality of life of patients with SCA (Booth *et al.*, 2010; Kizito *et al.*, 2007).

Aims and Objectives

The aim of this study was to investigate the incidence of infection in SCA patients with acute vaso-occlusive crisis. The following objectives were pursued:

- To identify the incidence and site of infections in SCA patients with acute vaso-occlusive crisis.
- To identify the most common causative organisms in these cases of SCA patients.

3.2 Methods

The microbiological techniques applied in this study are described below.

Blood specimen collection and culture

Samples were collected from patients, with suspected sepsis or bacteremina for culture and sensitivity. At least two sets of aerobic and anaerobic bottles were collected from SCA patients. All blood culture bottles were incubated at 35 ^{o}C, in blood culture machine (Bact Alert Biomerieux, Bac T ALERT3D system (ORGANON TECNIKA) Microbial analysing system 43003-1) and were assessed by an automatic detection system (Bact Alert Biomerieux). While negative bottles were kept for 5 days, positive blood culture bottles were subjected to Gram and methylene

blue stains. In addition, positive samples were sub-cultured onto the plated media, including Chocolate, Mac Conkey's and Blood agar. Finally, based on the Gram stain results, *Gram-positive cocci, Gram-positive bacilli, Gram-negative cocci, Gram-negative bacilli* and yeast cells were identified.

Manually directed identification and antibiotic susceptibility testing were performed on the bottles containing positive samples, which were also subjected to direct VITEK II (Biomerieux) Microbial analysing system VTK2-2972, identification and susceptibility tests.

Sputum specimen collection and culture

Early morning sputum samples were obtained from the SCA patients, as these contain pooled overnight secretions, in which pathogenic bacteria are more likely to be concentrated. Sputum samples were processed promptly upon arrival at the Microbiology laboratory.

All sputum samples were Gram-stained to establish the specimen quality. Samples characterized by high levels of polymorph nuclear leukocytes and low number of epithelial cells were selected for culture. These samples were cultured on Blood, Chocolate and Mac Conkey's agar and were incubated for 48 hours at $35 - 37\ ^{o}C$. Blood and Chocolate agar cultures were incubated in a CO_2 incubator, as this environment supports the growth of pathogenic Capnophiles, such as *Hemophilus influenzae*, and *Streptococcus pneumonia*. On the other hand, MacConkey's agar cultures were incubated in an aerophilic incubator, which facilitated the growth of all facultative anaerobes, such as *Enterobacteria ceae*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, and *Enterococcus*.

Midstream urine specimen collection and culture

Midstream urine samples were collected using the clean-catch technique, whereby a sterile wide-mouthed container that can be covered with a tightly fitted lid ensured that any potential for sample contamination was eliminated. Based on the established values for urine colony counts, which should range between 10,000 and 100,000 colony forming unit (CFU/ml), it was expected that those pertaining to the infected patients would exceed 100,000 CFU/ml. To obtain this data and achieve accurate colony counts, all collected specimens were processed within two hours following collection.

Urine specimens were cultured on Biplates (Blood and MacConkey's agar) and subsequently incubated for 24 hours at 35 ^{o}C.

Skin lesions swab specimen collection and culture

As surface lesions are colonized by environmental bacteria, swabs were collected from infected skin surfaces after proper cleaning with an antiseptic. This protocol helped reduce the bacterial growth of skin contaminants, such as Coagulase negative, *Staphylococcus*. The collected skin swabs were cultured on Blood, Mac Conkey's and Sabouraud's dextrose agar, prior to 24-hour incubation at 35 – 37^{o}C. When the incubation period elapsed, the cultures were checked for the growth of *Staphylococcus*, *Streptococcus*, yeast, fungi and facultative anaerobic Gram-negative enterobacteriaceae. All skin lesion swabs were subsequently Gram-stained and assessed for high PMN'S count under high power field microscopy (Koneman, 2006).

3.3 Results

Table 3.1 displays the incidence of infections in sickle cell anaemia patients with vaso-occlusive crisis. There was a total of 232 SCA patients with VOC. Sixty seven of these (29%) had no signs of infection, and therefore were excluded (no culture was made), of the remaining 165 patients they were febrile. Full septic screen was done, 113 patients (49%) of total 232 came up with negative culture, and where as 52 patients (23%) of total 232 had positive bacterial cultures.

Table 3.2 shows the most common sites of infection and the percentage of positive culture samples of SCA patients with VOC. About one third of them (19) patients (37%) had a positive blood culture, whilst the second most common site of infection was the urinary tract as indicated by one third (17) patients (33%). Positive sputum samples were obtained from a further one fifth (11) patients (21%) while 5 patients (12%) gave positive cultures from skin and wound swabs.

Table 3.3 shows the numbers and percentages of isolated microbial organisms from patients with SCA in VOC. *Staphylococcus aureus* was the most frequently isolated organisms, it was found in about 29% of patients. *Escherichia coli*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa* had 10% occurrence. *Klebsiella pneumonia* was isolated from 8% of patients, while *Enterococcus faecalis*, *Acinetobacter baumannii* and *Staphylococcus hominis* were found in about 6% of patients. Less common organisms such as *Streptococcus pneumonia*, *Serratia marcescens* and *Salmonella species* which were isolated in 4% of patients. Finally, *Streptococcal agalactiae*, *Streptococcus Salivarius* and *Streptococcus haemolyticus* were reported in only 2% of patients.

Table 3.1 Incidence of infections in sickle cell anaemia patients with vaso-occlusive crisis

Studied groups	Number	Percentage
SCA patients without culture	67	28.8
SCA patients with negative culture	113	48.7
SCA patients with positive culture	52	22.4
Total SCA patients with Vaso-occlusive crisis	232	100

Table 3.2 Sites of infections and percentage of positive culture samples of sickle cell anaemia patients with vaso-occlusive crisis

Sample	Number	Percentage
Blood	19	36.54
Urine	17	32.69
Sputum	11	21.15
Pus (skin and subcutaneous infection)	5	9.62
Total SCA with Vaso-occlusive crisis	52	100.00

Table 3.3 Number and percentage of isolated microbial organisms from patients with sickle cell anaemia in vaso-occlusive crisis

Organisms isolated	Skin	Sputum	Urine	Blood	Total	Percentage
Staphylococcus aureus	2	5	1	7	15	28.8
Escherichia coli			3	2	5	9.6
Pseudomonas aeruginosa		1	3	1	5	9.6
Staphylococcus epidermidis		1	2	2	5	9.6
Klebsiella pneumonia		2	1	1	4	7.6
Enterococcus faecalis			3		3	5.7
Acinetobacter baumannii	2		1		3	5.7
Staphylococcus hominis				3	3	5.7
Streptococcus pneumonia		2			2	3.8
Serratia marcescens	1		1		2	3.8
Salmonella species				2	2	3.8
Streptococcal agalactiae			1		1	1.9
Streptococcus salivarius				1	1	1.9
Streptococcus haemolyticus			1		1	1.9
Total	5	11	17	19	52	100
Percentage	9.62	21.15	32.69	36.54	100.00	

3.4 Discussion

The present study has found that there was a significant increase in the incidence of bacterial infection (22.4%) compaired to other studies. The incidence of bacteraemia in 52 SCA patients, in acute VOC, was 22.4%. *Staphylococcus aureus* was the most prominent organism comprising 28.8% of infections.

Similar results were found in a study by Akuse (1996), in Nigeria, who reported the presence of bacterial infections in 304 children with SCA in acute VOC. Sixty per cent of the patients had positive bacterial cultures, with Gram negative organisms accounting for 55 %, but the single most predominant organism isolated was *Staphylococcus aureus* (Akuse, 1996). The low isolation rate of *Streptococcus pneumoniae, Haemophilus influenzae* and *Neisseria meningitidis* is due to implication of vaccination program in SCA and initial antibiotic treatment.

In Uganda, East Africa, a study was conducted by Kizito *et al.*, (2007), that included a group of 155 SCD patients, 47 of whom had bacteraemia. A positive blood culture for *Staphylococcus aureus* accounted for 60% of these cases, while another common organism isolated was *Staphylococcus epidermis* (9%). These findings are similar to the current study. This similarity could be due to high prevelance of these organisms in the environment and on the skin. *Haemophilus influenzae* and *Streptococcus pneumoniae* accounted for 19% and 6% of bacteraemia cases, respectively. *Streptococcus viridans* and *Escherichia coli* were the least common organisms causing bacteraemia.

In contrast to this study, *Streptococcus pneumoniae* was the main causative organisms of bacteraemia in western countries (Isaacman *et al.*, 2010; Overturf, 2003).

In the USA, a study conducted in the period between 1993-2001, on 248 febrile children with SCD demonstrated that *Streptococcus pneumoniae* accounted for 42% of bacteraemias, followed by *Salmonella species* (17%), *Staphylococcus aureus* (7%) and *Escherichia coli* (5%) (Rogovik *et al.*, 2010).

The study findings have been inconsistent with the study conducted by Williams *et al.* (2009) in Kenya. The most commonly detected organisms in 2157 bacteraemia cases from 38441 SCA patients (6%) were *Streptococcus pneumoniae* (41%), non-typhi Salmonella species (18%), *Haemophilus influenzae* type b (12%), *Acinetobacter species* (7%) and *Escherichia coli* (7%) (Williams *et al.*, 2009). This discrepancy may be attributed to the age of patients, number, duration of the study and possible vaccination against *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitides*.

Homozygous sickle patients have an increased risk of developing severe pyrogenic infections, related to diminish or absent splenic function, by repeated splenic infarction, which may lead to a chronic inflammatory state due to insufficient clearance of bacteria and subsequent leukocytes activation (Al-Jam'a *et al.*, 2000).

Staphylococcus aureus was the most commun isolated organism in this study and this is in agreement with Valour *et.al.*, who *reported that Staphylococcus aureus* accounts for 2-5% of community aquired pneumonia, and 20-30% of cases of hospital aquired pneumonia (Valour *et al.*, 2013).

Similar study showed that *Staphylococcus aureus* was the most frequent hospital aquired infection from frequent hospitalisation (Schaumburg *et al.*, 2013).

This study has demonstrated a high incidence of urinary tract infection 32.7% in a mixed population of SCA patients in acute VOC. The data are in agreement with other studies that have reported also a high incidence of urinary tract infections in SCD (Hawasawi *et al.*, 1998; Kizito *et al.*, 2007). In the present study the most common isolated organisms from urine samples were *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis*, while Kizito *et al.*, (2007) found *Escherichia coli* as the common causative organism.

In another study conducted in Saudi Arabia on 53 SCD patients, the incidence of urinary tract infection was 69 %, but the most common organism was *Escherichia coli* (Hawasawi *et al.*, 1998).

In contrast to the present finding, Hawasawi *et al.*, (1998) demonstrated that *Streptococcus pneumoniae* was the most common organism in SCD patients with bacteraemia. Similarly, Rogers *et al.*, (1990) reported that invasive infection by encapsulated organisms, such as *Streptococcus pneumoniae* and *Haemophilus influenzae* was common in young children with SCA, who showed an increased risk of *pneumococcal* bacteraemia and *Haemophilus influenzae* bacteraemia compared to age-matched children without SCA.

In Saudi Arabian patients with SCA, included in this study, *pneumococcal* infection has low risk of morbidity and this could be contributed to the introduction of vaccines (Hawasawi *et al.*, 1998).

Mortality has significantly decreased in university of Texas southwestern medical center, since the introduction of universal vaccination for *Streptococcus pneumoniae* and *Haemophilus influenzae* in SCA patients (Rogers *et al.*, 1990).

Conclusively, this study reflects the magnitude of the problem in Saudi Arabia, namely a high incidence of *Staphylococcus aureus*, (non MRSA) and *Pseudomonas aeruginosa* infection among SCA patients with acute VOC. Generally, since infection is conttributing risk factor for VOC in SCA patients, early treatment of infection is important for manegment. The introduction of antibiotic prophylaxis, universal vaccination and early detection of infection are highly recommended for managing patients with SCA.

CHAPTER 4

CHAPTER 4: ANALYSIS OF LEUKOCYTES IN SICKLE CELL ANAEMIA

4.1 Introduction

Sickle cell anaemia patients undergo various pathophysiological crises that includes systemic inflammatory processes. Therefore, it is important to evaluate the role of the leukocytes in the different clinical presentations. Under normal conditions, leukocytes migrate through all body tissues; the cells exist in the blood are those in transit between different tissues. Each cell population has a particular pattern of migration, which is dependent on the state of cell differentiation and activities (Roitt *et al.*, 2002). During infection phagocytic cells, including neutrophils and monocytes leave the bone marrow and migrate to sites of infection (Takeshi & Shin, 2003). The phagocytic process can be separated into several major stages: chemotaxis, migration of phagocytes, attachment of particles to the cell surface of phagocytic cell, ingestion and intracellular killing by oxygen dependent and oxygen independent mechanisms (Roitt *et al.*, 2002).

Sickle cell anaemia is considered to be a chronic inflammation state. Numerous inflammatory markers are elevated in the steady state and further increased during VOC, including C reactive protein (CRP), tumor necrosis factor-alpha (TNF-α), leukocytes, platelets, α-2 macroglobulin, transferrin, fibrinogen, and interleukins 1, 4, 6 and 8 (IL-1 IL-4, IL-6, and IL-8) (Belcher *et al.*, 2000; Hebbel *et al.*, 2004). Inflammation appears to play an important role in vaso-occlusion, (Lard *et al.*, 1999). Leukocytes have been involved in the production and secretion of injurious

substances leading to tissues injury. The neutrophil is described as a ‘messy eater’ due to phagocytosis and the killing of microbial organisms, which is followed by the release of different cytotoxic proteins.

These proteins include collagenase, chymotrypsin-like cationic proteins, elastase, hydrolase and proteases (Okpala, 2004b). Cytotoxic proteins also attract and recruit other leukocytes to inflammatory site. During this process leukocytes releases highly reactive oxygen radicals that lead to oxidative damage and cytotoxicity. All these processes lead to the expression of ligands for adhesion molecules on leukocytes and at the site of inflamed endothelium with further lumen occlusion, ischaemic organ damage and eventually VOC. Elevated leukocytes counts in patients with SCA, was suggested to be a risk factor for morbidity and mortality (Okpala, 2006).

Increasing evidence suggests that white blood cells (WBC), especially neutrophils, may be involved in the initiation and propagation of VOC in SCA (Kasschau *et al.*, 1996). Elevated total WBC counts are common in SCA patients and are associated with an increased risk of early death (Kasschau *et al.*, 1996). Adhesion of activated neutrophils to endothelium in patients with SCA would impede passage of RBC and WBC, lead to endothelial damage which would eventually increase risk for VOC (Kasschau *et al.*, 1996; Ramakrishnan *et al.*, 2010). Acquired functional asplenia in SCA is associated with inefficient phagocytosis of opsonized bacteria. Wong *et al.*, (1995) studied the cellular immunity and leukocyte subsets count among patients with SCA. They found that there was a broad-based leukocytosis in SCA patients (Wong *et al.*, 1995). All T-and B- cell subsets participate in the lymphocytosis in SCA patients. Lard *et al.*, (1999) studied the activation state of leukocytes in SCA, and study has demonstrated significant increase in L-selectin and neutrophil markers in SCA with acute VOC as compared to control subjects.

Immunophenotyping through flow cytometry is a major tool in the study of the characteristics of leukocytes as it allows the identification and description of cell subsets that cannot be recognized by classical morphologic examination (Givan, 1992; Marie-Christine & Martini, 1997). With this technique, monoclonal antibodies specific to individual epitopes are conjugated with fluorescent labels and are used to identify cell surface antigens. The labeled cells can then be detected and enumerated by flow cytometry (Schmitz, 2003, 2004).

The aims of flow cytometry in this study were:

- To determine leukocyte phagocytic function in neutrophils and monocytes, in Saudi and non-Saudi SCA patients in steady state and normal control subjects, and compared to Saudi and non-Saudi normal control subjects.
- To assess gender difference in leukocyte phagocytic function, in both Saudi and non-Saudi SCA patients, in steady state and normal control subjects.
- To compare leukocyte immunophenotyping of Saudi and non-Saudi SCA patients in steady state and in normal control subjects.
- To compare differences in leukocyte immunophenotyping between Saudi and non-Saudi SCA patients in acute VOC and normal control subjects.
- To assess for gender differences in leukocyte immunophenotyping in SCA patients; in both steady state and in acute VOC.
- To compare differences in the expression of L-selectin in leukocytes in Saudi and non-Saudi SCA patients in acute VOC, with that of normal control subjects.
- To assess for gender difference in the expression of L-selectin in leukocytes in SCA patients in acute VOC.

4.2 Methods

Activated neutrophil and monocyte phagocytic function was performed as previously described in Chapter 2, section 2.3.

Immunophenotyping assay procedure was performed as previously described in Chapter 2, sections 2.4 and 2.5.

4.3 Results

Leukocyte Phagocytic Function in Sickle Cell Anaemia

Serum samples from patients with SCA in a steady state and normal control subjects were analysed in order to determine whether there were any differences between the two groups. Activated neutrophil and monocyte phagocytic activity was performed as illustrated in Figure 4.1 and Figure 4.2.

Statistical analysis using an independent Student *t*-test was performed in order to compare changes in phagocytic activity markers between steady state and normal control subjects. Measurements were expressed as mean ± standard deviation (SD), *t*-test and *p*-value, with $P<0.05$ as an indicator of statistical significance.

The current study included 14 male SCA patients (25%), 17 female patients (30%), 12 (21.4%) male and 13 female (23.2%) normal control subjects

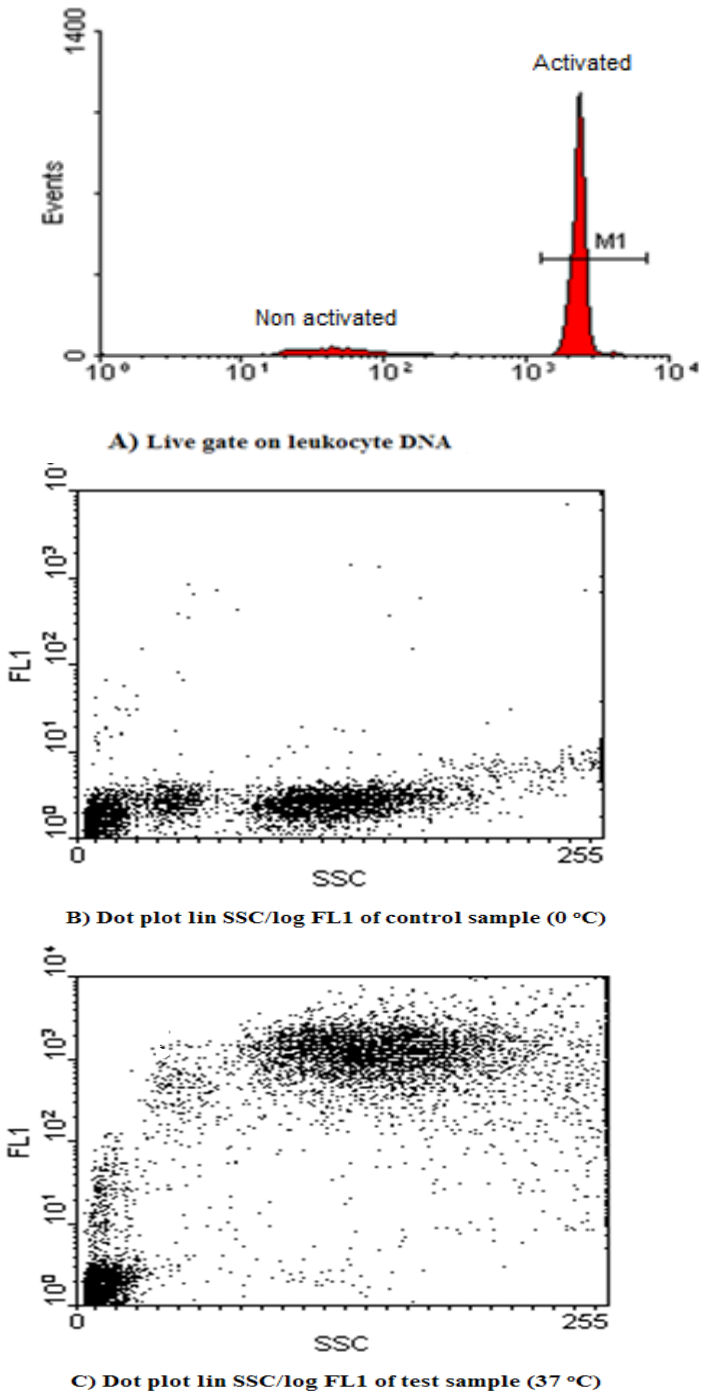

Figure 4.1 Activated (C) and non activated (B) leukocytes using flow cytometry. (*FL1: Fluorescence-1 intensity). This figure shows the increased fluorescence of neutrophil and monocyte , upon activation (4.1A)

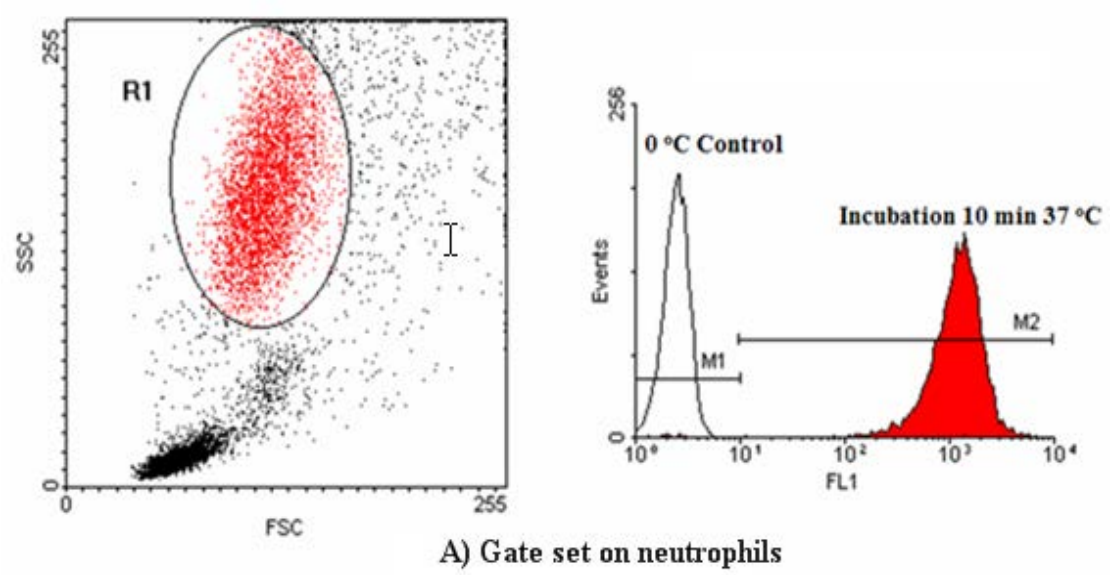

Figure 4.2 Dot plots FSC/SSC and FL1 histograms of the phagocytosis test. This figure shows the activated neutrophil (A) and monocytes populations (B) following gating.

Phagocytic Activity

Figure 4.1. and 4.2 are examples of the phagotest results. The mean ± SD of activated leukocytes phagotest values, for the two groups, are presented in Table 4.1. The mean ± SD of neutrophils percentage phagocytosing bacteria (% activated neutrophils) was 93.53 ± 6.61 % for SCA patients during steady state and 93.54 ± 5.59% for control subjects. Notably, there was no significant difference in the activated neutrophil percentage in SCA patients, in steady state, compared with normal control subjects. The mean ± SD of bacteria per cell (activated neutrophils) was 851.3 ± 36.74 for SCA patients during steady state and 840.43 ± 34.61 for normal control subjects. Accordingly, no significant differences between the two groups were found. The mean ± SD of monocytes phagocytosing bacteria percentage (activated monocytes %) were 81.36 ± 10.49% for SCA patients during steady state and 86.87 ± 6.1% for control subjects. Noteworthy, there was no significant difference in the percentage of activated monocyte phagocytic activity in SCA patients in steady state compared with normal control subjects.

The mean ± SD of bacteria per cell (activated monocytes %) was 754.33 ± 45.83 for SCA patients during steady state and 777.63 ± 49.76 for control subjects. This difference is statistically insignificant.

Phagocytic Activity in Male and Female Patients

The means (± SD) of leukocyte phagocytic activity (neutrophils and monocytes) in males and females SCA patients in steady state and in normal control subjects are presented in Table 4.2. The mean ± SD for the percentage of activated neutrophils was 92.13 ± 7.68% in male SCA patients with steady state, whilst in female with SCA patients in steady state was 94.69 ± 5.56%. There was no significant difference found

in neutrophils percentage of phagocytic activity between the two groups. Moreover, the mean ± SD of activated neutrophils percentage in male control subjects was 95.96 ± 2.92%, and in female control subjects was 91.3 ± 6.59%.

Table 4.1 Comparison of percentage of activated leukocytes (phagotest) between sickle cell anaemia patients in steady state and normal control subjects

Phagotest results	Sickle cell patients during steady state		Normal control subjects		*t*-test	*p*-value
	Mean	±SD	Mean	±SD		
Neutrophils (%) of phagocytes which have ingested bacteria (activated neutrophils %)	93.53	6.61	93.54	5.59	0.002	0.99
In neutrophils number of bacteria per cell	851.3	36.74	840.43	34.61	1.12	0.261
Monocytes (%) of phagocytes which have ingested bacteria (activated monocytes %)	81.36	10.49	86.87	6.1	2.45	0.182
In monocytes number of bacteria per cell	754.33	45.83	777.63	49.76	1.82	0.741

There was no significant difference, in terms of percentage, regarding the neutrophil phagocytic activity, between the two groups. Moreover, the mean ± SD for activated monocytes was 83.07 ± 7.95% in male SCA patients in steady state, whilst in female SCA patients with steady state was 79.95 ± 12.27%. Furthermore, no significant difference in the percentage of phagocytic activity for monocytes was established between the two groups. The mean ± SD for activated monocytes percentage in male control subject was 89.86 ± 2.58% and female control subjects was 84.11 ± 7.16%. In addition, there was no significant difference, in terms of percentage of monocyte phagocytic activity, between the two groups.

Mean Fluorescence Intensity (MFI) in Male and Female Patients

Table 4.2 shows the mean ± SD for MFI of neutrophils and monocytes in both male and female SCA patients in steady state, and in normal control subjects. The mean ± SD for MFI of neutrophils was 848.74 ± 41.92 and 853.41 ± 33.04 in male and female SCA patients in steady state respectively. There was no significant difference in terms of the MFI of neutrophils phagocytic activity between the two groups. The mean ± SD for MFI of neutrophils was 826.75 ± 25.44 in male normal control subjects and for female normal control subjects was 853.06 ± 37.98. There was no significant difference noted in the MFI of neutrophils phagocytic activity between the two groups. The mean ± SD for MFI of monocytes was 752.76 ± 50.37 in male SCA patients in steady state, and in female SCA patients in steady state was 755.62 ± 43.38. Furthermore, there was no significant difference in the MFI of monocytes phagocytic activity between the two groups. In male normal control subjects, the mean ± SD MFI of monocytes was 760.06 ± 31.47, and for female normal control subjects, the mean ± SD was 793.85 ± 58.74. Accordingly, no significant difference in the MFI of monocytes phagocytic activity was noted between the two groups.

Table 4.2 Comparison of phagocytic activity in male and female sickle cell anaemia patients during steady state and normal control subjects.

	Cells	Gender	N	% activated cells		*t-test*	*P*-value	MFI		*t*-test	*P*-value
				Mean	±SD			Mean	±SD		
Sickle cell patients in steady state	Neutrophils	M	14	92.13	7.68	1.08	0.29	848.74	41.92	0.35	0.731
		F	17	94.69	5.56			853.41	33.04		
	Monocytes	M	14	83.07	7.95	0.82	0.42	752.76	50.27	0.17	0.872
		F	17	79.95	12.27			755.62	43.38		
Normal control Subjects	Neutrophils	M	12	95.96	2.92	2.25	0.06	826.75	25.44	2.02	0.065
		F	13	91.3	6.59			853.06	37.98		
	Monocytes	M	12	89.86	2.58	2.63	0.07	760.06	31.47	1.77	0.092
		F	13	84.11	7.16			793.854	58.74		

*MFI: Mean fluorescence intensity

Phagocytic Activity in Saudi and Non- Saudi Patients

A comparative data for mean ±SD of leukocyte phagocytic activity (neutrophils and monocytes) in Saudi versus non- Saudi SCA patients in steady state and in normal control subjects are presented in Table 4.3. The mean ± SD of activated neutrophils (%) was 94.43 ± 6.67% in Saudi SCA patients in steady state and in non-Saudi was 92.45 ± 6.61%. Notably, there has been no significant difference found in terms of percentage of neutrophil phagocytic activity between the two groups. The mean ± SD for the percentage of activated neutrophils in Saudi control subjects was 92.4 ± 5.99 and in non-Saudi was 96.85 ± 1.87. There was no significant difference, regarding the percentage of neutrophil phagocytic activity between the two groups. The mean ± SD of percentage of activated monocytes was 84.5 ± 6.73 in Saudi SCA patients in steady state and 77.51 ± 13.02 in non- Saudi. There was no significant difference in the percentage of monocyte phagocytic activity between the two groups. The mean ± SD of percentage of activated monocytes in Saudi control subjects was 85.67 ± 6.27 and in non-Saudi was 90.68 ± 3.81. Moreover, there was no significant difference in terms of the percentage of monocyte phagocytic activity between the two groups.

Mean Fluorescence Intensity (MFI) in Saudi and Non-Saudi Patients:

Table 4.3 shows the mean ± SD for MFI of neutrophils and monocytes in Saudi and non-Saudi SCA patients in steady state and in normal control subjects. The mean ± SD for MFI of neutrophil was 854.35 ± 32.29 in Saudi SCA patients in steady state and the mean ± SD in non-Saudi was 847.60 ± 42.47. Moreover, there was no significant difference in MFI of neutrophils phagocytic activity between the two groups. The mean ± SD for MFI of neutrophils was 836.69 ± 35.68 in Saudi normal control subjects, whilst the mean ± SD for non-Saudi was 852.29 ± 30.75. Importantly, there was no significant difference in the MFI of neutrophil phagocytic

activity between the two groups. The mean ± SD for MFI of monocytes in SCA patients in steady state in the case of Saudi and non-Saudi samples was found as 760.88 ± 39.42 and 746.38 ± 53.03 respectively. There was no significant difference in the MFI of monocytes phagocytic activity between the two groups. In Saudi normal control subjects the mean ± SD for MFI of monocytes was 776.33 ± 49.93 and for non-Saudi was 781.76 ± 53.7. There was no significant difference established in terms of the MFI of monocytes phagocytic activity between the two groups.

Table 4.3 Comparison of phagocytic activity between Saudi and non-Saudi sickle cell anaemia patients in steady state and in normal control subjects.

	Cells	Ethnic group	N	% activated cells		*t*-test	*P*-value	*MFI		*t*-test	*P*-value
				Mean	±SD			Mean	±SD		
Sickle cell patients in steady state	Neutrophils	Saudi	17	94.43	6.67	0.83	0.42	854.35	32.29	0.5	0.612
		non-Saudi	14	92.45	6.61			847.6	42.47		
	Monocytes	Saudi	17	84.5	6.73	1.94	0.06	760.88	39.42	0.87	0.391
		non-Saudi	14	77.51	13.02			746.38	53.03		
Normal control subjects	Neutrophils	Saudi	19	92.4	5.99	1.74	0.09	836.69	35.68	0.96	0.353
		non-Saudi	6	96.85	1.87			852.29	30.75		
	Monocytes	Saudi	19	85.67	6.27	1.84	0.08	776.33	49.93	0.23	0.821
		non-Saudi	6	90.68				781.76	53.7		

*MFI: Mean fluorescence intensity

Leukocyte Immunophenotyping in Steady State

Major leukocyte population

The percentage of total T lymphocytes, T helper cells, T suppressor cells, B lymphocytes, NK cells, monocytes and neutrophils in peripheral blood were estimated based on the expression of ($CD3^+$+$CD7^+$), $CD4^+$, $CD8^+$, $CD19^+$, (CD16+ 56^+), $CD14^+$ and $CD64^+$ respectively. Statistical analysis observed for the proportions of these cells are outlined in Table 4.4.

The mean ± SD of the percentage of total T lymphocytes ($CD3^+$+ $CD7^+$) was 72.0 ± 9.4 for SCA patients during steady state and 58.5 ± 5.8 for normal control subjects. Accordingly, there was a significant higher percentage of ($CD3^+$+ $CD7^+$) total T lymphocytes in SCA patients in steady state compared with normal control subjects (P<0.001). The mean ± SD of the percentage of T helper lymphocyte cells ($CD4^+$) was 39.2 ± 8.7 for SCA patients during steady state and 33.5 ± 5.6 for normal control subjects. This reflects a significant higher percentage of $CD4^+$ T helper lymphocyte cells was found in SCA patients in steady state compared with normal control subject (*P*<0.002). The mean ± SD of the percentage of T suppressor lymphocyte cells $CD8^+$ was 29.0 ± 5.8 for SCA patients during steady state and 21.4 ± 5.0 for normal control subjects. This represents a significantly higher percentage of $CD8^+$ T suppressor lymphocyte cells in SCA patients in steady state, compared with normal control subjects (*P*<0.001). Furthermore, the results for the $CD19^+$ B lymphocytes showed that the mean ± SD was 18.6 ± 6.5% for SCA patients during steady state and 12.7 ± 5.7% for normal control subjects. There was also a higher significant percentage of $CD19^+$ B lymphocytes in SCA patients in steady state when compared with normal control subjects (*P*<0.001). The mean ± SD of (CD16+ 56^+) NK cells percentage was 22.4 ± 10.7 for SCA patients during steady state and 18.4 ± 7.5 for normal control

subjects. Furthermore, there was a significant difference of (CD16+ 56^{+}) NK cells in SCA patients in steady state when compared with normal control subjects (P=0.05).

Table 4.4 Statistical analysis of percentage of leukocytes in sickle cell anaemia patients in steady state compared to the normal control subjects, using Student's *t*-test

Leukocyte	Case/control	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	Steady state	36	72	9.4	7.21	0.001
	Control	34	58.5	5.8		
T helper cells ($CD4^+$)	Steady state	36	39.2	8.7	3.32	0.002
	Control	34	33.5	5.6		
T-suppressor cells ($CD8^+$)	Steady state	36	29	5.8	5.82	0.001
	Control	34	21.4	5		
B lymphocytes ($CD19^+$)	Steady state	36	18.6	6.5	4.03	0.001
	Control	34	12.7	5.7		
NK cells ($CD16^+$+56^+)	Steady state	36	22.4	10.7	1.83	0.05
	Control	34	18.4	7.5		
Monocytes ($CD14^+$)	Steady state	20	2.8	1.7	0.12	0.898
	Control	20	1.8	1.3		
Neutrophils ($CD64^+$)	Steady state	20	94.1	4.7	0.93	0.371
	Control	20	95.3	3.7		

The mean ± SD of $CD14^+$ monocytes percentage was 2.8 ± 1.7 for SCA patients during steady state and 2.8 ± 1.3 for normal control subjects. Notably, there was no significant difference of $CD14^+$ monocytes in SCA patients in steady state compared with normal control subjects. Results for $CD64^+$ neutrophils percentage showed that the mean ± SD was 94.1 ± 4.7 for SCA patients during steady state and 95.3± 3.7 for normal control subjects. Furthermore, there was no significant difference of $CD64^+$ neutrophils in SCA patients in steady state compared with normal control subjects.

Major Leukocyte Population in Male and Female Patients

Table 4.5 shows the statistical analysis of leukocyte percentage in male and female sickle cell anaemia patients in steady state. The mean ± SD of the percentage of total T-lymphocyte cells ($CD3^+$+ $CD7^+$) was 60.1 ± 9.6 for male SCA patients during steady state and 57.5 ± 9.3 for females. No significant difference was found between genders in relation to ($CD3^+$+ $CD7^+$) total T lymphocyte. The mean ± SD of the percentage of $CD4^+$ T helper lymphocyte cells was 34.1 ± 7.4 for male SCA patients during steady state and 33.1 ± 9.6 for females. Markedly, there was no significant difference established in regard to gender-related $CD4^+$ T helper lymphocyte cells. The mean ± SD of the percentage of $CD8^+$ T suppressor lymphocyte cells was 24.2 ± 5.8 for male SCA patients during steady state and 19.7 ± 5.2 for females. No significant difference was apparent in gender-related $CD8^+$ T suppressor lymphocyte cells. The mean ± SD of the percentage of $CD19^+$ B lymphocytes was 19.0 ± 5.5 for male SCA patients during steady state and 18.3 ± 7.2 for females. Accordingly, there was no significant difference found in gender- related $CD19^+$ B lymphocytes.

Table 4.5 Statistical analysis of percentage of leukocytes in male and female sickle cell anaemia patients in steady state, using Student's *t*-test

Leukocyte	Sex	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	Male	14	60.1	9.6	0.81	0.422
	Female	22	57.5	9.3		
T helper cells ($CD4^+$)	Male	14	34.1	7.4	0.33	0.742
	Female	22	33.1	9.6		
T suppressor cells ($CD8^+$)	Male	14	24.2	5.8	2.42	0.062
	Female	22	19.7	5.2		
B lymphocytes ($CD19^+$)	Male	14	19	5.5	0.34	0.757
	female	22	18.3	7.2		
NK cell (CD16+56^+)	Male	14	22.2	9.9	0.12	0.943
	female	22	22.5	11.3		
Monocytes ($CD14^+$)	Male	7	2.8	2.3	0.13	0.971
	Female	13	2.8	1.5		
Neutrophil ($CD64^+$)	Male	7	94.2	3.9	0.04	0.972
	Female	13	94.1	5.2		

The mean ± SD of the percentage of (CD16+ 56^{+}) NK cells was 22.4 ± 9.9 for male SCA patients during steady state and 22.5 ± 11.3 for females. There was no significant difference found in gender-related (CD16+ 56^{+}) NK cells. The mean ± SD of the percentage of $CD14^{+}$ monocytes was 2.8 ± 2.3 for male SCA patients during steady state and 2.8 ± 1.5 for females. In addition, there was no significant difference apparent in gender-related $CD14^{+}$ monocytes. The mean ± SD of the percentage of $CD64^{+}$ neutrophils was 94.2 ± 3.9 for males SCA patients during steady state and 94.1 ± 5.2 for females. Furthermore, there was no significant difference apparent in terms of gender- related $CD64^{+}$ neutrophils.

Major Leukocyte Population in Saudi and Non- Saudi Patients

Table 4.6 shows the percentage of leukocytes in Saudi and non-Saudi sickle cell anaemia patients in steady state. The mean ± SD of the percentage of total T lymphocyte ($CD3^{+}$+ $CD7^{+}$) was found as 57.6 ± 8.4 and 59.5 ± 10.5 for Saudi and non-Saudi SCA patients respectively during the steady state. Moreover, there was no significant difference in ($CD3^{+}$+ $CD7^{+}$) total T lymphocyte in Saudi SCA patients in steady state compared with non-Saudi. The mean ± SD of the percentage of $CD4^{+}$ T helper lymphocyte was 32.5 ± 8.4 for Saudi SCA patients during steady state and 34.6 ± 9.2 for non-Saudi. Moreover, there was no significant difference in $CD4^{+}$ T helper lymphocyte cells in Saudi SCA patients in steady state compared with non-Saudi. The mean ± SD of the percentage of $CD8^{+}$ T suppressor lymphocyte was 22.0 ± 6.1 for Saudi SCA patients during steady state and 20.8 ± 5.6 for non-Saudi. Accordingly, there was no significant difference in $CD8^{+}$ T suppressor lymphocyte in Saudi and non-Saudi SCA patients in the steady state.

Table 4.6 Statistical analysis of percentage of leukocytes in Saudi and non-Saudi sickle cell anaemia patients in steady state, using Student's *t*-test

Leukocyte	Nationality	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	Saudi	19	57.6	8.4	0.616	0.541
	Non-Saudi	17	59.5	10.5		
T helper cells ($CD4^+$)	Saudi	19	32.5	8.4	0.712	0.482
	Non-Saudi	17	34.6	9.2		
T suppressor cells ($CD8^+$)	Saudi	19	22	6.1	0.623	0.542
	Non Saudi	17	20.8	5.6		
B lymphocytes ($CD19^+$)	Saudi	19	18.7	6.7	0.088	0.93
	Non-Saudi	17	18.5	6.6		
NK lymphocytes ($CD16+56^+$)	Saudi	19	22.5	12	0.101	0.92
	Non-Saudi	17	22.2	9.2		
Monocytes ($CD14^+$)	Saudi	10	2.9	1.8	0.054	0.951
	Non-Saudi	10	2.8	1.8		
Neutrophil ($CD64^+$)	Saudi	10	92.8	6.1	1.288	0.212
	Non-Saudi	10	95.4	2.1		

The mean ± SD of the percentage of $CD19^+$ B lymphocytes was 18.7 ± 6.7 for Saudi SCA patients during steady state and 18.5 ± 6.6 for non-Saudi. In addition, there was no significant difference in the percentage of $CD19^+$ B lymphocytes in Saudi SCA patients in steady state compared with non-Saudi. The mean ± SD of the percentage of ($CD16+56^+$) NK cells was 22.5 ± 12.0 for Saudi SCA patients during steady state and 22.2 ± 9.2 for non-Saudi. Notably, there was no significant difference in ($CD16+56^+$) NK cells between Saudi and non-Saudi SCA patients in steady state. The mean ± SD of the percentage of $CD14^+$ monocytes was 2.9 ± 1.8 for Saudi SCA patients during steady state and 2.8 ± 1.8 for non-Saudi. Furthermore, there was no significant difference in $CD14^+$ monocytes in Saudi SCA patients in steady state compared with non-Saudi. The means ± SD of the percentage of neutrophils $CD64^+$ was 92.8 ± 6.1 for Saudi SCA patients during steady state and 95.4 ± 2.1 for non-Saudi. Moreover, no significant difference in the percentage $CD64^+$ neutrophils in Saudi SCA patients in steady state was established when compared with non-Saudi.

The estimation of absolute numbers of T helper lymphocyte and T suppressor lymphocyte in peripheral blood was based on expression of $CD4^+$ and $CD8^+$ respectively. The absolute numbers for these cells in the study population are outlined in Table 4.7. The mean ± SD of the absolute number of $CD4^+$ T helper lymphocyte cells was 2.6 $x10^9$/L ± 1.7 for SCA patients during steady state and 2.0 $x10^9$/L ±1.1 for normal control subjects. Essentially, there was no significant difference established in absolute $CD4^+$ T helper lymphocyte in SCA patients in steady state compared with normal control subjects. The mean ± SD of the absolute number of $CD8^+$ T suppressor lymphocyte was 1.7 $x10^9$/L ± 1.0 for SCA patients during steady state and 1.5 $x10^9$/L ±1.0 for normal control subjects. There was no significant difference in the $CD8^+$ absolute T suppressor lymphocyte in SCA patients in steady state compared with normal control subjects.

The ratio of $CD4^+$ T helper lymphocytes to $CD8^+$ T suppressor cells was 1.5 $x10^9$/L ± 0.6 for SCA patients during steady state and 1.3 $x10^9$/L ± 0.4 for normal control subjects (Table 4.8). Remarkably, there was a significant difference in $CD4^+$:$CD8^+$ ratio, in SCA patients when compared with normal control subjects (P<0.05).

Table 4.7 Statistical analysis of T lymphocytes in sickle cell anaemia patients in steady state compared to the normal control subjects, using Student's *t*-test

Leukocyte	Sample type	N	Mean ($x10^9$/L)	±SD	*t* -test	*P*-value
T helper cells $CD4^+$ (absolute)	Steady state	36	2.6	1.7	1.956	0.552
	Control	34	2	1.1		
T suppressor $CD8^+$ (absolute)	Steady state	36	1.7	1	0.636	0.527
	Control	34	1.5	1		

Table 4.8 Ratio of T lymphocyte cells $CD4^+$ helper to $CD8^+$ T suppressor in sickle cell anaemia patients in steady state compared to the normal control subjects, using Student's *t*-test

	Sample type	N	Ratio	±SD	*t* -test	*P*-value
$CD4^+/CD8^+$ cell ratio	Steady state	36	1.5	0.6	2.205	0.03
	Control	34	1.3	0.4		

Leukocyte Immunophenotyping in Acute Vaso-Occlusive Crisis

Major Leukocyte Population

The percentage of the total T lymphocytes, T helper, T suppressor, B lymphocytes, NK cells, monocytes and neutrophils in peripheral blood were estimated, based on expression of $CD3^+$+ $CD7^+$, $CD4^+$ and $CD8^+$, $CD19^+$, ($CD16+56^+$), $CD14^+$, $CD64^+$ and $CD62^+$ L (lymphocytes, neutrophils, monocytes) respectively, Lymphocytes ($CD62^+L/CD19^+$) , neutrophils ($CD62^+L/CD64^+$) and monocytes ($CD62^+L/CD14^+$). The percentage values for these phenotypic cells in study population are outlined in Table 4.9.

The mean ± SD of the percentage of T lymphocytes ($CD3^+$+ $CD7^+$) was 69.84 ± 8.73 for SCA patients in acute VOC and 69.48 ± 10.11 for normal control subjects. There was no significant difference in $CD3^+$+ $CD7^+$ T lymphocytes of SCA patients in acute VOC, when compared with normal control subjects. The mean ± SD of the percentage of $CD4^+$ T helper lymphocytes was 44.04 ± 8.77 for SCA patients in acute VOC and 44.50 ± 10.05 for normal control subjects. There was no significant difference of $CD4^+$ T lymphocyte helper cells of SCA patients in acute VOC, as compared to normal control subjects. The mean ± SD of the percentage of $CD8^+$ T suppressor lymphocyte cells was 23.69 ± 7.59 for SCA patients in acute VOC and 24.10 ± 7.24 for normal control subjects. Moreover, there was no significant difference of $CD8^+$ T suppressor lymphocytes of SCA patients in acute VOC when compared with normal control subjects. The mean ± SD of the percentage of B lymphocytes $CD19^+$ was 16.01 ± 7.45 for SCA patients in acute VOC and 11.71 ± 5.14 for normal control subjects. There was significant difference in the percentage of $CD19^+$ B lymphocyte cells in SCA patients in acute VOC when compared with normal control subjects (P=0.002). The mean ± SD of the percentage of $CD16+56^+$ NK cells was 14.62 ± 7.19

for SCA patients in acute VOC and 11.68 ± 6.34 for normal control subjects. There was a significant difference in NK cells ($CD16+56^{+}$) in SCA patients in acute VOC when compared with normal control subjects ($P<0.05$). The mean ± SD of the percentage of $CD14^{+}$ monocytes was 91.03 ± 5.78 for SCA patients in acute VOC and 89.02 ± 6.65 for normal control subjects. Furthermore, there was no significant difference of $CD14^{+}$ monocytes in SCA patients in acute VOC when compared with normal control subjects. The mean ± SD of the percentage of neutrophils $CD64^{+}$ was 94.27 ± 6.84 for SCA patients in acute VOC and 93.68 ± 4.69 for normal control subjects. There was no significant difference in $CD64^{+}$ neutrophils in SCA patients in acute VOC when compared with normal control subjects. The mean ± SD of the percentage of monocytes ($CD62^{+}L/CD14^{+}$) was 66.73 ± 13.54 for SCA patients in acute VOC and 51.81 ± 17.91 for normal control subjects. Remarkably, there was a significant difference in the percentage of monocytes ($CD62^{+}L/CD14^{+}$) in SCA patients in acute VOC when compared with normal control subjects ($P=0.01$). The mean ± SD of the percentage of neutrophils ($CD62^{+}L/CD64^{+}$) was 93.54 ± 7.50 for SCA patients in acute VOC and 89.96 ± 5.31 for normal control subjects. Importantly, there was a significant difference in the percentage of neutrophils ($CD62^{+}L/CD64^{+}$) for SCA patients in acute VOC when compared with normal control subjects ($P<0.05$). The mean ± SD of the percentage of lymphocytes ($CD62^{+}L/CD19^{+}$) was 55.49 ± 11.23 for SCA patients in acute VOC and 45.14 ± 12.59 for normal control subjects. There was a significant difference in the percentage of lymphocytes ($CD62^{+}L/CD19^{+}$) of SCA patients in acute VOC, when compared with normal control subjects ($P=0.01$).

Table 4.9 Statistical analysis of percentage of leukocytes in sickle cell anaemia patients in acute vaso-occlusive crisis compared to the normal control subjects, using Student's *t*-test

Leukocyte	Samples	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	VOC	52	69.84	8.73	0.182	0.856
	Control	43	69.48	10.11		
T helper cells ($CD4^+$)	VOC	52	44.04	8.77	0.233	0.814
	Control	43	44.5	10.05		
T suppressor cells ($CD8^+$)	VOC	52	23.69	7.59	0.273	0.786
	Control	43	24.1	7.24		
B lymphocytes ($CD19^+$)	VOC	52	16.01	7.45	3.207	0.002
	Control	43	11.71	5.14		
NK cells (CD16+56^+)	VOC	52	14.62	7.19	2.091	0.039
	Control	43	11.68	6.34		
Neutrophils ($CD64^+$)	VOC	52	94.27	6.84	0.483	0.63
	Control	43	93.68	4.69		
Monocytes ($CD14^+$)	VOC	52	91.03	5.78	1.556	0.118
	Control	43	89.02	6.65		
CD62 L monocytes ($CD62^+L/CD14^+$)	VOC	47	66.73	13.54	4.208	0.011
	Control	37	51.81	17.91		
CD62 L Neutrophils ($CD62^+L/CD64^+$)	VOC	47	93.54	7.5	3.397	0.034
	Control	37	89.96	5.31		
CD62 L lymphocytes ($CD62^+L/CD19^+$)	VOC	47	55.49	11.23	3.917	0.011
	Control	37	45.14	12.59		

Major Leukocyte Populations in Male and Female Patients

Table 4.10 shows the leukocytes percentage in male and female sickle cell anaemia patients in acute VOC compared to the normal control subjects. The mean ± SD of the percentage of the T lymphocytes ($CD3^+$+ $CD7^+$) was 67.99 ± 9.76 for male SCA patients in acute VOC, and 71.08 ± 7.86 for females. There was no significant difference established in gender-related ($CD3^+$+ $CD7^+$) T lymphocytes. The mean ± SD of the percentage of $CD4^+$ T helper lymphocyte cells was 41.46 ± 8.21 for male SCA patients in acute VOC and 45.78 ± 8.82 for females. Moreover, there was no significant difference determined in gender-related $CD4^+$ T helper lymphocytes. The mean ± SD of the percentage of $CD8^+$ T suppressor lymphocyte cells was 24.2 ± 8.54 for male SCA patients in acute VOC and 23.28 ± 6.99 for females. Furthermore, there was no apparent significant difference in gender- related $CD8^+$ T suppressor lymphocytes. The mean ± SD of the percentage of $CD19^+$ B lymphocytes was 17.15 ± 9.58 for male SCA patients in acute VOC and 15.22 ± 5.60 for females. There was no notable disparity established in gender-related $CD19^+$ B lymphocytes. The mean ± SD of the percentage of (CD16+56+) NK cells was 15.01 ± 6.85 for male SCA patients in acute VOC and 14.36 ± 7.50 for females. There was no significant difference found in gender-related ($CD16+56^+$) NK cells. The mean ± SD of the percentage of $CD14^+$ monocytes was 92.95 ± 4.42 for males SCA patients in acute VOC and 89.72 ± 6.27 for females. Moreover, there was no apparent significant difference in gender-related $CD14^+$ monocytes. The mean ± SD for the percentage of neutrophils $CD64^+$ was 95.40 ± 3.68 for male SCA patients in acute VOC and 93.50 ± 8.30 for females. There was no apparent significant difference in gender-related $CD64^+$ neutrophils. The mean ± SD of the percentage of monocytes ($CD62^+L/CD14^+$) was 62.26 ± 12.52 for male SCA patients in acute VOC and 69.49 ± 13.60 for females.

Table 4.10 Statistical analysis of percentage of leukocytes in male and female sickle cell anaemia patients in acute vaso -occlusive crisis, using Student's *t*-test

Leukocyte	Sex	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	Male	21	67.99	9.76	1.252	0.21
	Female	31	71.08	7.86		
T helper cells ($CD4^+$)	Male	21	41.46	8.21	1.783	0.081
	Female	31	45.78	8.82		
T-suppressor cells ($CD8^+$)	Male	21	24.28	8.54	0.462	0.65
	Female	31	23.28	6.99		
B lymphocytes ($CD19^+$)	Male	21	17.15	9.58	0.91	0.363
	Female	31	15.22	5.6		
NK cells (CD16+56^+)	Male	21	15.01	6.85	0.313	0.752
	Female	31	14.36	7.5		
Monocytes ($CD14^+$)	Male	21	92.95	4.42	1.035	0.064
	Female	31	89.72	6.27		
Neutrophils ($CD64^+$)	Male	21	95.4	3.68	1.123	0.261
	Female	31	93.5	8.3		
CD62L monocytes ($CD62^+$L/$CD14^+$)	Male	18	62.26	12.52	1.822	0.072
	Female	29	69.49	13.6		
CD62L Neutrophils ($CD62^+$L/$CD64^+$)	Male	18	93.53	5.31	1.107	0.063
	Female	29	89.36	8.27		
CD62L lymphocytes ($CD62^+$L/$CD19^+$)	Male	18	53.02	11.77	1.183	0.245
	Female	29	57.01	10.8		

No significant difference was found in gender-related ($CD62^+L/CD14^+$) monocytes. The results of neutrophils in terms of percentage ($CD62^+L/CD64^+$) mean ± SD was 93.53 ± 5.31 for male SCA patients in acute VOC and 89.36 ± 8.27 for females. There was no significant difference found in gender-related ($CD62^+L/CD64^+$) neutrophils. The mean ± SD of the percentage of lymphocytes ($CD62^+L/CD19^+$) was 53.02 ± 11.77 for male SCA patients in acute VOC and 57.01 ± 10.80 for females. There was no significant difference found in gender-related ($CD62^+L/CD19^+$) lymphocytes.

Major Leukocyte Population in Saudi and Non- Saudi Patients

Table 4.11 shows the leukocytes percentage in Saudi and non-Saudi sickle cell anaemia patients with VOC. The mean ± SD of the percentage of T lymphocytes ($CD3^+$+ $CD7^+$) was 69.81 ± 8.75 for Saudi SCA patients in acute VOC and 69.88 ± 8.91 for non-Saudi patients. There was no significant difference in ($CD3^+$+ $CD7^+$) T lymphocyte cells in Saudi SCA patients in acute VOC when compared with non-Saudi patients. The mean ± SD of the percentage of $CD4^+$ T helper lymphocyte cells was 45.08 ± 9.65 for Saudi SCA patients in acute VOC and 42.37 ± 7.03 for non-Saudi patients. There was no significant difference in $CD4^+$ T helper lymphocyte cells in Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of $CD8^+$ T suppressor lymphocyte cells was 23.88 ± 7.34 for Saudi SCA patients in acute VOC and 23.37 ± 8.15 for non-Saudi. There was no significant difference in $CD8^+$ T suppressor lymphocyte cells in Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of B lymphocytes $CD19^+$ was 15.20 ± 6.96 for Saudi SCA patients acute VOC and 17.28 ± 8.18 for non-Saudi. Moreover, there was no significant difference in $CD19^+$ B lymphocyte cells in Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of ($CD16^+$+ 56^+) NK cells was 14.86 ± 6.63 for Saudi SCA patients acute VOC and 14.24 ± 8.16 for non-Saudi. There was no significant difference in (CD16 + 56^+) NK cells in Saudi SCA patients in acute VOC

when compared with non-Saudi. The mean ± SD of the percentage of $CD14^+$ monocytes was 91.44 ± 5.92 for Saudi SCA patients acute VOC and 90.37 ± 5.62 for non-Saudi. There was no significant difference in $CD14^+$ monocytes in Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of neutrophils $CD64^+$ was 93.95 ± 7.89 for Saudi SCA patients acute VOC and 94.78 ± 4.82 for non-Saudi. There was no significant difference in $CD64^+$ neutrophils in Saudi SCA patients in acute VOC as compared to non-Saudi. The mean ± SD of the percentage of monocytes ($CD62^+L/CD14^+$) was 68.49 ± 12.87 for Saudi SCA patients in acute VOC and 63.31 ± 14.54 for non-Saudi. There was no significant difference in the percentage of monocytes ($CD62^+L/CD14^+$) of Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of neutrophils ($CD62^+L/CD64^+$) was 91.29 ± 7.97% for Saudi SCA patients in acute VOC and 90.30 ± 6.68 for non-Saudi. Furthermore, there was no significant difference in the percentage of neutrophils ($CD62^+L/CD64^+$) of Saudi SCA patients in acute VOC when compared with non-Saudi. The mean ± SD of the percentage of lymphocytes ($CD62^+L/CD19^+$) was 57.06 ± 11.83 for Saudi SCA patients in acute VOC and 52.42 ± 9.55 for non-Saudi. There was no significant difference in the percentage of lymphocytes ($CD62^+L/CD19^+$) of Saudi SCA patients in acute VOC when compared to non-Saudi.

Table 4.11 Statistical analysis of percentage of leukocytes in Saudi and non-Saudi sickle cell anaemia patients in acute vaso-occlusive crisis, using Student's *t*-test

Leukocyte	Nationality	N	Mean	±SD	*t* -test	*P*-value
T lymphocytes ($CD3^+$+$CD7^+$)	Saudi	32	69.81	8.75	0.03	0.97
	Non-Saudi	20	69.88	8.91		
T helper cells ($CD4^+$)	Saudi	32	45.08	9.65	1.08	0.28
	Non-Saudi	20	42.37	7.03		
T suppressor cells ($CD8^+$)	Saudi	32	23.88	7.34	0.232	0.81
	Non Saudi	20	23.37	8.15		
B lymphocytes ($CD19^+$)	Saudi	32	15.2	6.96	0.971	0.33
	Non-Saudi	20	17.28	8.18		
NK lymphocytes (CD $16+56^+$)	Saudi	32	14.86	6.63	0.293	0.76
	Non-Saudi	20	14.24	8.16		
Monocytes ($CD14^+$)	Saudi	32	91.44	5.92	0.642	0.61
	Non-Saudi	20	90.37	5.62		
Neutrophils ($CD64^+$)	Saudi	32	93.95	7.89	0.423	0.67
	Non-Saudi	20	94.78	4.82		
CD62L monocytes ($CD62^+L/CD14^+$)	Saudi	31	68.49	12.87	1.251	0.21
	Non-Saudi	16	63.31	14.54		
CD62L Neutrophils ($CD62^+L/CD64^+$)	Saudi	31	91.29	7.97	0.426	0.67
	Non-Saudi	16	90.3	6.68		
CD62L lymphocytes ($CD62^+L/CD19^+$)	Saudi	31	57.06	11.83	1.354	0.18
	Non-Saudi	16	52.42	9.55		

4.4 Discussion

Leukocyte Phagocytic Function

The aim of this study was to compare the phagocytic function of neutrophils and monocytes, in Saudi and non- Saudi SCA patients.

Okpala, (2004b) studied neutrophil function in 74 steady state adult patients with SCA compared with 50 normal haemoglobin (HbAA) healthy control subjects. The results showed significantly reduced neutrophil ability to phagocytose *Candida albicans*. However the study was carried out in moderate and severe cases of SCA. Okpala further noted that phagocytic competence correlates positively ($P < 0.001$) to the clinical severity of the disease. The findings of his study were based on *Candida albicans* infection, which was not tested in the current study and may explain the discrepancy between the findings.

The results in Table 4.2 showed no significant statistical differences in the percentage of activated neutrophils and monocytes between male and female SCA patients in steady state. Similarly, no significant statistical difference was found in normal control subject.

The current study demonstrated that there was no significant difference in MFI of neutrophils and monocytes in male and female SCA patients in steady state compared to normal control subjects.

The measurements of the phagocytic function of neutrophils and monocytes were analysed further in order to clarify whether population differences can be seen

(Table 4.3). The study has demonstrated no significant difference in the percentage of activated neutrophils, monocytes and MFI of phagocytic function between Saudi and non–Saudi SCA patients in steady state. In normal control subjects, there was no significant difference between Saudi and non-Saudi subjects, no similar studies have been reported previously from Saudi Arabia.

Lard *et al.*, (1999) analysed the activation state of neutrophils by measuring neutrophil $CD64^+$. The study included a group of 42 steady state SCD patients, 15 SCD patients in VOC and 30 healthy volunteers. In this study, a significant difference was reported in the activation state of $CD64^+$ neutrophil and $CD14^+$ monocytes, in steady state non- symptomatic SCA patients compared to normal control subjects. Furthermore, neutrophil activation was more pronounced during VOC. One possible explanation for these results may be that they used a flow cytometric analysis to measure the activation state of neutrophils by targeting or determining the level of expression of neutrophils' antigens such as $CD64^+$ and CD 14^+, rather than measuring phagocytic function directly, as in the study presented here.

In vivo studies suggest that adherent leukocytes bind RBC and contribute to the micro vascular pathology that characterises SCA. Increasing evidences indicate that activated neutrophils could play an important role in the initiation and propagation of vaso-occlusive processes in SCA (Lard *et al.*, 1999).

Leukocyte Immunophenotyping in Steady State

The percentage of total ($CD3^+$+ $CD7^+$) T lymphocytes, $CD4^+$ T helper lymphocytes, $CD8^+$ T suppressor lymphocytes, and $CD19^+$ B lymphocytes and ($CD16+56^+$) NK cells were significantly increased in steady state SCA patients as shown in Table 4.4

The broad based increase in total ($CD3^+$+ $CD7^+$) T lymphocyte, $CD4^+$ T helper lymphocyte cells and $CD8^+$ T suppressor lymphocyte cells reflects lymphocyte activation caused either by infections and tissue damage or increased bone marrow production.

The finding of a significant increase in the percentage of $CD19^+$ B lymphocyte cells reflects immune stimulation secondary to tissue damage. The results reported in this study confirmed previous findings by Wong *et al.*, (1995); this study was conducted on 173 SCA patients and 131 normal control subjects. Wong *et al.*, (1995) found that the percentage of T and B lymphocytes was significantly higher in SCA patients. Monocytes $CD14^+$ and CD (16^+ + 56^+) NK cells were increased as percentages in SCA patients as compared with control subjects. Wong *et al*. reported that the increase in NK (CD16+ 56^+) cells could be due to antibody-dependent cellular cytotoxicity mechanisms that provide important and effective responses to viral infections.

The results in Table 4.4 display the percentage of $CD14^+$ monocytes and $CD64^+$ neutrophil in SCA patients in steady state and normal control. There was no significant difference in $CD14^+$ monocytes and $CD64^+$ neutrophil in SCA patients in steady state as compared to normal control subjects. On the contrary, Wong *et al.*, (1995) reported an increase in $CD14^+$ monocytes percentage of SCA patients. However, they suggested that this could be due to haemolysis or tissue damage.

The present study compared absolute $CD4^+$ T helper lymphocyte cells in SCA patients in steady state and normal control subjects (Table 4.7), showed no significant difference in the $CD4^+$ T helper lymphocyte cells in SCA patients in steady state as compared to normal control subjects. Also there was no significant difference in the $CD8^+$ absolute T suppressor lymphocyte cells in SCA patients in steady state as

compared to normal control subjects. The ($CD4^+$:$CD8^+$) ratio showed significant difference. Wong *et al*., (1995) reported that during sickle cell crisis the ($CD4^+$:$CD8^+$) ratio was variably affected.

Previous study by Donadi & Falcão in 1987 found that the numbers of ($CD3^+$+$CD7^+$) total lymphocytes, $CD4^+$ T helper lymphocyte cells, $CD8^+$ T suppressor lymphocyte cells and $CD20^+$ B lymphocytes were elevated in SCA patients (Donadi & Falcão, 1987).

Groom *et al.*, (1991) reported an increased number of lymphocytes ($CD2^+$ T lymphocytes, $CD4^+$ T helper lymphocytes, $CD8^+$ T suppressor lymphocytes, $CD20^+$ B lymphocytes and CD16+ 56^+NK) in acute VOC patients compared to normal control subjects (Groom *et al.*, 1991). The $CD8^+$ T suppressors increased by 3-4 folds, $CD4^+$ T helpers increased by 2 folds, NK CD16+56^+ increased 4-5 folds as well as $CD20^+$ B lymphocytes. However $CD8^+$ T suppressors only doubled at the time of acute VOC. Therefore, $CD4^+$ T helper and $CD8^+$ T suppressor ratio ($CD4^+$:$CD8^+$) was normal in SCA patients in steady state, compared to normal control subjects. The number of $CD20^+$ B lymphocytes exceeds that seen in patients with medical and trauma emergencies (Groom *et al.*, 1991).

In the present study there was no significant difference in the percentage of ($CD3^+$ + $CD7^+$) total T lymphocyte, $CD4^+$ T helper lymphocytes, $CD8^+$ T suppressor lymphocytes and $CD19^+$ B lymphocytes, (CD16+56^+) NK cells $CD14^+$ monocytes and $CD64^+$ neutrophils in peripheral blood between male and female SCA patients in steady state.

With regards to nationality the percentage of total T lymphocyte ($CD3^+$+ $CD7^+$), T helper lymphocyte cells $CD^{4+,}$ T suppressor lymphocytes $CD8^+$, and $CD19^+$ B

lymphocytes, ($CD16+56^+$) NK cells, $CD14^+$ monocytes and $CD64^+$ neutrophils in peripheral blood, there was no significant difference between Saudi and non–Saudi steady state SCA patients and normal control subjects.

These results confirm the work of Bisset *et al.*, (2004) who established reference ranges for lymphocyte phenotypes in healthy Swiss adults. Neutrophil counts vary among different ethnic groups (Martineau *et al.*, 2007).

Al-Qouzi *et al.*, (2002) and Koffi *et al.*, (2003) demonstrated that the ($CD4^+$:$CD8^+$) ratio in SCA patients was 1.5:1.0, compared to 1.3:1.0 in normal control subjects. Both Saudi and non Saudi individuals were recruited to the study and no significant difference was found between the studied groups (Koffi *et al.*, 2003). This confirms earlier findings in the immunophenotyping of lymphocytes in Saudi men (Al-Qouzi *et al.*, 2002).

Leukocyte Immunophenotyping in Acute Vaso-Occlusive Crisis

The results obtained in this study showed that the percentage of $CD19^+$B lymphocytes, $CD16^+$+56^+ NK cells were significantly increased in SCA patients with acute VOC. The percentage of other cells such as total ($CD3^+$+$CD7^+$) T lymphocytes, $CD4^+$ T helper lymphocytes, $CD8^+$ T suppressor lymphocytes, $CD14^+$ monocytes and $CD64^+$ neutrophils were not significantly increased in SCA patients with acute VOC.

The finding of a significant increase in the percentage of $CD19^+$ B lymphocyte cells reflects immune stimulation secondary to tissue damage.

L-selectin expression of ($CD62^+$L/$CD14^+$) monocytes, ($CD62^+$L/$CD64^+$) neutrophils and ($CD62^+$L/$CD19^+$) lymphocytes were significantly elevated in SCA patients in

acute VOC as compared to normal control subjects. This confirmed a positive role for L-selectin expression in the pathogenesis of VOC.

These results are in agreement with Okpala *et al.*, 2002, who reported that high expression of L-selectin on leukocytes in steady-state, which predisposes patients to severe manifestations, and contributed to the pathogenesis of crisis. In addition Inwald *et al.*, (2000) reported that vaso-occlusive events in childhood SCA are related to inflammatory cell activation, and to interactions between sickle erythrocytes and vascular endothelium (Inwald *et al.*, 2000).

Prior to this study, different observation was reported by Lard *et al.*, (1999) who analysed ($CD62^{+}L/CD64^{+}$) neutrophil L- selectin in SCD patients in VOC. The study showed significant reduction in ($CD62^{+}L/CD64^{+}$) neutrophil L-selectin expression in SCD patients in VOC compared to normal control subject. The explanation was thought to be due to the shedding of L-selectin during neutrophil activation. Therefore, the surface L- selectin expression was low.

In order to explore the L-selectin expression on the other leukocytes, $CD62^{+}L$ in conjunctions with other markers was analysed, e.g ($CD62^{+}L/CD19^{+}$) for B lymphocytes and ($CD62^{+}L/CD14^{+}$) for monocytes. Another study by Okpala, (2006) revealed similar findings, when he showed that L-selectin expression on monocytes, neutrophils and lymphocytes was elevated in SCD patient during VOC.

In addition, Okpala, (2006) found that L-selectin expression was more elevated in complicated SCD patients. These findings suggest that high levels of L-selectin and other adhesion molecule markers predispose SCD patients to severe manifestations and complications. These leukocyte selectin adhesive interactions contribute to blood

vessel occlusion in SCD, which is the major mechanism of organ damage (Chiang & Frenette, 2005; Okpala, 2006).

The current study demonstrated that there was no significant statistical difference for the percentage of various leukocyte subtypes and L-selectin expression between male and female SCA patients in acute VOC. The results for leukocyte markers were analysed further, to identify any population difference, between Saudi and non Saudi SCA patients during VOC. No significant difference was found in the expression of different leukocyte markers or L-selectin in the studied groups. This indicates that neither the ethnicity nor the gender had any effect on peripheral blood leukocyte phenotype in patients with SCA.

In conclusion, the phagocytic activity was intact in SCA patients, with no nationality or gender differences, when compared with normal control subjects. To the best of the current knowledge, there are no previous publications regarding nationality related differences therefore the findings of this study are novel.

Flow cytometric analysis of peripheral blood leukocyte subpopulations from patients with SCA in steady state, denotes that there was an elevation in the percentage of T helper lymphocytes, T suppressor lymphocytes, B lymphocytes and NK cells

During VOC, B lymphocytes and NK cells are increased, L-selectin expression on neutrophils, monocytes and lymphocytes correlates with their activation. This is due to immune stimulation, infections and tissue damage. Therefore, correlation with serum L-selectin can be utilised in the future as a marker of activation during acute VOC. This finding in the Saudi population has not been reported in prior studies.

CHAPTER 5

CHAPTER 5: Soluble E and P selectin in sickle Cell Anaemia in Steady State and Vaso-Occlusive Crisis

5.1 Introduction

The endothelial cell participates in numerous functions of vascular physiology. Cell adhesion molecules play a major role in the recruitment and binding of inflammatory cells to the vascular endothelium. Endothelial cell adhesion molecules including E-selectin and P-selectin induce specific inflammatory cells to roll across the endothelial surface. Strong adhesive interactions develop on endothelial VCAM-1 and ICAM-1 to specific ligands on the surface of inflammatory cells (Alon & Feigelson, 2002). The expression of these CAM is under the influence of inflammatory cytokines, such as TNF-α and IL-1β (Duits *et al.*, 1996).

In animal models, adhesion molecules were found to mediate vaso-occlusion in mouse models of SCA (Belcher *et al.*, 2000; Matsui *et al.*, 2001). A number of studies indicate that interactions between sickle reticulocytes, leukocytes, and endothelial cells via these adhesion molecules occur in patients with SCA, and may contribute to disease pathology (Hebbel *et al.*, 2004; Turhan *et al.*, 2002).

Adhesion molecule-dependent reticulocyte, monocyte and endothelial interactions in the postcapillary venules were suggested to contribute to the pathophysiology of VOC (Frenette, 2004). Cytokines by up-regulating CAM, modulate the role of the endothelium in coagulation, inflammation and vaso-regulation (Brown *et al.*, 2001). High levels of inflammatory cytokines were found in the plasma of patients with SCA

(Duits *et al.*, 2003; Makis *et al.*, 2000). High levels of adhesion molecules such as soluble endothelium-derived E and P selectin have been found in the sera of patients with SCA (Matsui *et al.*, 2002). Such findings might have significant impact on targeted therapy aimed at prevention of VOC. Furthermore, sE-selectin and sP-selectin levels in patients with SCA were associated with pulmonary hypertension, organ dysfunction, and mortality (Kato *et al.*, 2005).

Aims:

- To investigate the levels of the adhesion molecules sE-selectin and sP-selectin in the serum of SCA patients in both steady state and acute VOC, and compared with those in the normal control subjects.
- To determine the difference in the level of the adhesion molecules, sE-selectin and sP-selectin between Saudi and non-Saudi SCA patients in both steady state and in acute VOC; and compared to normal control subjects.

5.2 Methods

Soluble E-selectin and sP-selectin were measured through quantitative sandwich enzyme immunoassay (Bisset *et al.*, 2004), as described in chapter 2, section 2.6

5.3 Results

The sickle cell anaemia patients with acute VOC aged 18-45 years old took part in this study. The age of SCA in steady state ranged between 15 and 45 years. The age of normal control subjects ranged between 18 and 45 years.

Serum samples from SCA patients with acute VOC, steady state and control subjects were analysed in order to determine whether there were any disparities in regard to the levels of sE-selectin and sP-selectin in these groups.

Table 5.1 shows the mean ± SD for serum level of sE-selectin for the three groups. The mean ± SD of serum level of sE-selectin was 106.90 ± 23.51 ng/ml for SCA patients in acute VOC, 66.63 ± 14.57 ng/ml for SCA patient in steady state and 57.25 ± 21.67 ng/mL for normal control subjects.

ANOVA analysis of variance showed significant ($P = 0.001$) differences in means of sE-selectin between SCA patients with acute VOC, steady state and control subjects. Post hoc demonstrated that SCA patients with acute VOC showed significantly ($P = 0.001$) higher sE-selectin levels than steady state and control subjects. Moreover, steady state patients showed significantly ($P < 0.01$) higher sE-selectin levels compared to control subjects.

Table 5.2 shows the mean ± SD for serum level of sP-selectin for the three groups. The mean ± SD of serum level of sP-selectin was 212.0 ± 73.32 ng/mL for SCA patients with VOC, 153.81 ± 28.60 ng/mL for SCA patient in steady state and 127.73 ± 22.75 ng/mL for control subjects.

ANOVA analysis of variance showed significant ($P = 0.001$) differences in means of sP-selectin, between SCA patients in acute VOC, steady state and control subjects. Post hoc demonstrated that SCA patients with acute VOC showed significantly higher ($P < 0.001$) sP-selectin levels than steady state and control subjects. Moreover, steady state patients showed significantly higher ($P < 0.01$) sP-selectin levels compared to control subjects.

Table 5.1 Statistical analysis (ANOVA) of soluble E-selectin in normal control subjects compared to sickle cell anaemia patients in steady state and acute vaso–occlusive crisis

	Number	Mean (ng/ml)	±SD	*P*- value
Control	84	57.25	21.67	
Steady state	43	66.63	14.67	0.01
Vaso-occlusive crisis	41	106.9	23.51	0.001

Table 5.2 Statistical analysis (ANOVA) of soluble P-selectin in normal control subjects compared to sickle cell anaemia patients in steady state and acute vaso–occlusive crisis

	Number	**Mean (ng/ml)**	**±SD**	***P*- value**
Control	84	127.73	22.75	
Steady state	43	153.81	28.6	0.01
Vaso-occlusive crisis	41	212	73.32	0.001

Soluble Adhesion Molecule Markers in Saudi Non-Saudi Subjects

Table 5.3 shows the mean ± SD of the serum levels of sE-selectin for Saudi and non-Saudi control subjects. The mean ± SD of serum levels of sE-selectin was 59.57 ± 22.45 ng/ml for Saudi control subjects and 54.7 ± 20.77 ng/ml for non-Saudi control subjects. No significant difference between the two groups was found, using the independent *t*-test.

Table 5.4 shows the mean ± SD of sP-selectin for Saudi and non- Saudi control subjects. The mean ± SD of serum levels of sP-selectin was 123.36 ± 20.64 ng/ml for Saudi control subjects and 132.55 ± 24.23 ng/ml for non-Saudi control subjects. Importantly, no significant difference was established in the serum levels of sP-selectin between Saudi control subjects and non-Saudi control subjects using the independent *t*-test.

Soluble E-selectin serum levels mean ± SD for Saudi and non-Saudi SCA patients in steady state are presented in Table 5.5. The mean ± SD of sE-selectin serum levels was 65.34 ± 14.42 ng/ml for Saudi and 67.86 ± 15.14 ng/ml for non-Saudi subjects. There was no significant difference in serum levels of sE-selectin between the two study groups.

Table 5.6 shows the mean ± SD of sP-selectin serum levels in steady state for the two groups. The mean ± SD of serum levels of sP-selectin was 112.95 ± 27.74 ng/ml for Saudi SCA patients and 114.64 ± 30.02 ng/ml for non-Saudi. There was no significant difference in the serum levels of sP-selectin in SCA patients in steady state when compared with non-Saudi patients.

Table 5.3 Statistical analysis of soluble E-selectin in Saudi and non-Saudi normal control subjects

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi control	44	59.57	22.45	1.028	0.307
Non- Saudi control	40	54.7	20.77		

Table 5.4 Statistical analysis of soluble P-selectin in Saudi and non-Saudi normal control

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi control	44	123.36	20.64	1.88	0.064
Non- Saudi control	40	132.55	24.23		

Table 5.5 Statistical analysis of soluble E-selectin in Saudi and non-Saudi patients with sickle cell anaemia in steady state

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi steady state	21	65.34	14.42	0.56	0.58
Non-Saudi steady state	22	67.86	15.14		

Table 5.6 Statistical analysis of soluble P-selectin in Saudi and non-Saudi patients with sickle cell anaemia in steady state

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi steady state	21	112.95	27.74	0.91	0.853
Non-Saudi steady state	22	114.64	30.02		

Soluble E-selectin level mean ± SD in acute VOC is presented in Table 5.7. The mean ± SD of sE-selectin serum level was 106.2 ± 25.13 ng/ml for Saudi SCA patients in acute VOC and 108 ± 21.51 ng/ml for non-Saudi SCA patients in acute VOC. No significant difference was found in the serum levels of sE-selectin in SCA patients in acute VOC when compared with non-Saudi SCA patients in acute VOC.

Table 5.8 shows the mean ± SD sP-selectin serum level in SCA patients with acute VOC for the two groups. The mean ± SD of serum level of sP-selectin was 211.2 ± 67.03 ng/ml for Saudi and 213.25 ± 84.53 ng/ml for non-Saudi. There was no significant difference in the serum levels of sP-selectin in Saudi SCA patients in acute VOC when compared with non-Saudi SCA patients in acute VOC.

Table 5.7 Statistical analysis of soluble E-selectin in patients with sickle cell anaemia in acute vaso-occlusive crisis in Saudi and non-Saudi patients

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi Patients with Vaso-occlusive crisis	25	106.2	25.13	0.24	0.81
Non-Saudi Patients with Vaso-occlusive crisis	16	108	21.51		

Table 5.8 Statistical analysis of soluble P-selectin in sickle cell anaemia patients in acute vaso-occlusive crisis in Saudi and non-Saudi patients

Nationality	Number	Mean (ng/ml)	±SD	*t*-test	*P*-value
Saudi Patients Vaso-occlusive crisis	25	211.2	67.03	0.09	0.93
Non-Saudi Patients with Vaso-occlusive crisis	16	213.25	84.53		

5.4 Discussion

This study compared differences in the serum levels of sE-selectin and sP-selectin in three subject groups namely SCA patients in acute VOC, patients in steady state and normal control subjects (Table 5.1 and Table 5.2). The three groups were age matched. Patients with SCA in acute VOC and steady state showed significantly higher serum levels of sE-selectin and sP-selectin than normal control subjects ($P < 0.001$). Sickle cell anaemia patients in acute VOC had significantly higher ($P < 0.001$) serum level of sE-selectin and sP-selectin compared to SCA patients in steady state. These finding were in agreement with previous studies (Blum *et al.*, 2005; Kato *et al.*, 2005).

Similar observations were reported by Blum *et al.*, (2005) in regards to endothelial adhesion and dysfunction in acute VOC among SCA patients compared to healthy volunteers. The results showed a significant difference in the level of sE-selectin among patients with SCA in both crisis and steady state as compared with control subjects ($P < 0.001$). The level of sE-selectin was approximately doubled among patients with sickle cell crisis as compared to healthy volunteers control subjects. In addition, there was a trend towards increased levels of sE-selectin among SCA patients in acute crisis versus those in steady state. This finding confirms that the heightened levels of sE-selectin confer increased risk of endothelial dysfunction, inflammation and endothelial cell activation. These abnormalities characterize not only the sickle cell crisis but also the steady state pathophysiology of sickle cell anaemia.

Prior to this study, an extensive work was performed in 2002 by Turhan *et al.*, on E-selectin and P-selectin as major adhesion molecules that regulate binding to sites of inflammation, and their role in SCA patients in acute crisis. Some studies, using sickle

transgenic mouse model, have revealed increased adhesive interaction between circulating erythrocytes and leukocytes to endothelium, via E-selectin and P-selectin and promote VOC. P-selectin is reported to be expressed on endothelial cell surface within minutes of endothelial stimulation. On the other hand E-selectin is synthesized de novo and requires several hours to be expressed following endothelial stimulation. A role for P-selectin in mediating leukocytes and erythrocyte interactions and adhesion to endothelium in mice with SCA has been demonstrated by Turhan *et al.*, (2002) using both blocking monoclonal antibodies and P-selectin knockout mice. Sickle mice deficient in E-selectin and P- selectin were found to be protected from vaso-occlusion. This confirms that P-selectin could be considered as a candidate molecule for new therapeutic approaches, for the vaso- occlusive manifestations of SCA (Matsui *et al.*, 2001), by blocking of the P-selectin molecule.

A previous study by Mathews *et al.*, (2002), reported that P-selectin was measured in SCD patients and compared to normal controls subjects. P-selectin immunoreactivity was associated with intraretinal vessels, adjacent to the preretinal neovascular formation, in subjects with proliferative retinopathy (Mathews *et al.*, 2002), these data suggest that the highest P-selectin immunoreactivity was associated with the vaso-occlusive phase of SCD, in patients with proliferative retinopathy and might play an important part in the vaso-occlusive phase of sickle cell retinopathy.

Saleh *et al.*, (1998), studied the effect of hydroxyurea on vascular endothelium, adhesion molecule expression and cytokine production of sE-selectin and P-selectin in, the steady state SCA patients (Saleh *et al.*, 1998). Remarkably, hydroxyurea therapy was associated with significant reduction in levels of sE-selectin and sP-selectin compared to normal control subjects.

The result of this study have shown that serum levels of sE-selectin and sP-selectin were significantly increased in patients with SCA in acute VOC and steady state. However, they were most significant in SCA patients in acute VOC. The data from other studies, showed a similar results to this study, in relation to level of sE-selectin and sP-selectin (Blum *et al.*, 2005; Kutlar & Embury, 2014).

Kato *et al.*, (2005) included a group of 160 adult SCA patients in steady state. Serum level of sE-selectin and sP-selectin were significantly higher in patients with SCD in steady state compared to normal control subjects ($P < 0.001$ and $P < 0.001$) respectively. Their study demonstrated that elevated levels of serum sE-selectin and sP-selectin were a marker of inflammatory stress and disease severity.

A study conducted by Mohan *et al.*, (2005) included a group of 64 patients with SCD. The study revealed that serum level of sE-selectin was significantly higher ($P < 0.001$) in SCD patients compared to HbAA normal control subject ($P < 0.001$) (Mohan *et al.*, 2005). This study indicated that raised plasma sE-selectin in SCD was an inflammatory marker and implied endothelial activation.

Brown et al., (2001) found that the exposure of endothelial cells to sickle cells demonstrated increased expression of E-selectin. This finding reflects the significant role of expressed E-selectin in the pathophysiology of sickle- related complication. The reduction in E-selectin expression may be of value in the treatment of SCA. The results of this study have confirmed that sE-selectin and sP-selectin are markers of endothelial surface activation and indicator of disease severity.

Furthermore, there was no significant difference in serum levels of sE-selectin and sP-selectin among the Saudi and non Saudi SCA patients, either in steady state or acute

VOC, or when comparing the results of SCA patients with normal control subjects (Blum *et al.*, 2005).

In conclusion, sE-selectin and sP-selectin are valuable markers, in the management of SCA. They are indicators for endothelial surface activation and correlate with disease severity. This study is novel to Saudi patients and, to the best of the current knowledge, no similar work has previously conducted in the Middle East.

CHAPTER 6

CHAPTER 6: General Discussion, Future Work and Conclusion

6.1 General Discussion

Sickle cell anaemia has a prevalence of 0.26% in Saudi Arabia, while up to 4.2 % may have sickle cell trait gene (Al-Hamdan *et al.*, 2007). Sickle cell anaemia is a systemic disorder caused by a mutation that replaces adenine with thymidine, in the sixth codon of the beta-globin gene. This mutation leads to the production of sickle haemoglobin (HbS) which is predisposed to polymerisation, upon deoxygenation of haemoglobin. Sickle haemoglobin polymerisation and denaturation causes oxidant damage to the red blood cell membrane, and subsequently, the unique morphological abnormality of sickled red blood cells. Associated with such damage is abnormal erythrocyte cation homeostasis, which in turn results in dehydrated, dense, and finally irreversibly sickled cells. Red cell abnormalities can result in both haemolysis and vaso-occlusion. Vaso-occlusion is a complex event mediated by interaction between red cell, leukocyte and endothelial cells, and activation of inflammation and coagulation pathways. Haemolysis and the release of cell-free hemoglobin contribute to dysregulation of nitric oxide pathway.

Vaso-occlusion of blood vessels results in tissue ischaemia, infarction in various tissues and susceptibility to infections, leading to progressive end organ damage. The clinical manifestations of the sickle cell anaemia are the product of various genes and enviromental factors, acting in concert with the protein lesion underlying the red cell abnormality. Leukocytes adherence to the vascular endothelium, stimulates the latter to increase its expression of ligands for adhesion molecules; causing an inflammatory

response and tissue damage which in turn leads to vaso-occlusion (Johnson & Telen, 2008; Okpala, 2004b). The clinical implication of the role of leukocytes in the pathophysiology of VOC was highlighted by the fact that high leukocyte count is associated with more severe clinical course. (Okpala, 2004b)

This study is the first of its kind, in the Western region of the Kingdom of Saudi Arabia, that addresses health related issues for SCA patients. More specifically, it aimed to uncover some of the physiological/immunological patterns in the immune system and the relationship between infection and SCA.

Infection is an important risk factor in the pathogenesis of VOC in patients with SCA, therefore early and intensive treatment of infection is required to treat acute crisis (Booth *et al.*, 2010). Patients with SCA have an increased risk of developing severe bacterial infections, partly due to impaired cellular and humoral immunity (Magnus *et al.*, 1999; Steinberg, 1999), and the characteristic functional asplenia. Recurrent splenic sequestration, known as autoinfarction, is caused by splenic sequestration of red blood cells. The nonfunctional spleen is unable to filter bacteria from the blood stream. This leads to a chronic inflammatory state, due to insufficient clearance of bacteria and subsequent leukocytes activation (Al-Jam'a *et al.*, 2000; Lard *et al.*, 1999; Okpala, 2004b; Rogers *et al.*, 1990). Functional asplenia increases the risk of invasive infection by encapsulated organism, such as *Streptococcus pneumoniae* and *Haemophilus influenzae*. Other factors contributing to increased incidence of infections, include nutritional deficiency particularly zinc, as well as genetic and environmental interactions (Hasanato, 2006; Prasad, 2002; Prasad *et al.*, 1999; Tamouza *et al.*, 2002).

Prior to universal vaccination children with HbSS had an increased risk of both *pneumococcal* and *Haemophilus influenzae* sepsis, compared to age-matched children without SCA (Rogers *et al.*, 1990).

As one of the objectives of this study, was to determine the incidence of infection in SCA, and to identify the most common causative organisms in these patients. The most common organism isolated from blood was *Staphylococcus aureus*, which is not in concordance with other studies (Ramakrishnan *et al.*, 2010). The other common infections found in the current study are shown in (Table 3.3). Both *pneumococcal* and *Haemophilus influenzae* sepsis was not high due to previous vaccination. Another important objective met in this study, was to assess the leukocyte function in SCA patients in steady state and compared to normal control subjects. Leukocyte function was measured by the Phagotest, a well established test which uses flow cytometry to assess the uptake of fluorescent bacteria. Phagotest is originally used in the diagnosis of combined immune deficiency states and chronic granulomatous disease (Hirt *et al.*, 1994).

Overall, the phagocytic function was well preserved with no differences between patients, in steady state and normal control subjects. This is in agrrement with findings of Lard *et al*., (1999).

Flow cytometry analyses in the present study, demonstrated significantly higher levels of total T lymphocytes, both helper and suppressor, B lymphocytes and NK cells in patients with SCA during steady state, as compared to normal control subjects, and this elevation represent the reactivity of the immune system in response to chronic inflammatory state in SCA patients. No significant difference was found between Saudi and non-Saudi, or between male and female patients. These finding are

consistent with those of Wong *et al*., (1995). Thus, current findings suggest that there is no impairment in the defense mechanism for these patients.

Results show clearly that there was no significant difference in the percentages of neutrophils and monocytes that display phagocytic activity in SCA patients in steady state as compared to normal control subjects. It is important to consider the possibility that intact phagocytic function may contribute to steady state. Supporting these results is the data for the immunophenotyping that shows no significant difference of monocytes or neurtophils between steady state and normal control subjects.

Previous studies have argued for a deficiency in the immune response for SCA patients as indicated by the reduction of T lymphocytes (Adedeji, 1985; Kaaba & al-Harbi, 1993). In contrast, this study shows indications of not only intact immuophenotyping of lymphocytes and NK- cells but rather a significant increase in cytokine receptors for these cells in steady state SCA patients compared to normal control subjects.

Given that the patients in this study are SCA steady state survivors, it is possible that T lymphocytes and NK cells overactivation is providing a protective mechanism. This protective mechanism could be complemented by the increase of liberation of the adhesion molecules of sE and sP selectin.

One of most important and novel findings of the present study, was the presence of high levels of L-selectin on neutrophils, monocytes and lymphocytes during acute VOC. Accordingly, as markers for leukocytes activation, L selectin can be used as a predictor for VOC, which allows for early diagnosis and intervention (Bunting *et al.*, 2002; Okpala, 2006).

The use of ELISA technology revealed significant differences in sE-selctin and sP-selectin expression, between patients with VOC and those in steady state of the disease. This is in agreement with the findings from Blum *et al.*, (2005). Thus, sP-selectin and sE-selectin can be used as markers of disease severity. Furthermore, therapeutic modalities targeting these inflammatory mediators maybe therapeutic options for sickle cell disease. Thus, further studies are needed to examine the potential new therapeutic approaches for clinical complications of SCA including VOC. These therapeutic approaches could be directed to reverse the activation of endothelial cell and/or to develop and use antagonists for selectin ligand.

The phagocytic activity in patients with SCA is not impaired, compared to matched controls and cannot be implicated in the high incidence of infection. Susceptibility to infection is compounded by failure to implement a national vaccination protocol for SCA patients. Environmental and, genetic factors may contribute to this problem.

Overall, the results indicate a high level of infection in VOC patients compared to other studies, supporting the consensus that VOC predisposes individuals to infective agents (Hawasawi *et al.*, 1998; Kizito *et al.*, 2007; Schlitt & Keitel, 1960). However it is worth noting that almost two thirds of VOC patients were infection free, suggesting that infections are more likely to be associated with VOC but not the cause or an ultimate outcome. This could be supported by the finding that the most frequent organism (*S. aureus*) is mostly present in the blood and not necessarily in ischeamic parts. Therefore, treatment for VOC is important to be given alongside infection-targeted treatment, and may even contribute to the preventions of further infections.

Analysis of SCA patients with VOC indicates a hyper responsive immune system, as reflected by the significant rise in the cytokine receptors on T lymphocytes and NK cells. In addition, VOC are associated by an elevation in sE and sP selectin adhesion

molecules. Also in comparison with steady state patients, the expression for L-selectin increases significantly during VOC. In addition, the data show an increased expression in L-selectin on neutrophils, monocytes and lymphocytes in VOC patients.

When testing associated impact of socio-demographic characteristics of SCA patients, including gender and ethnicity, on the state of the immune system, no significant difference was seen in our data. This result may be due to non bias of genetic factors. Further personalised analysis may be required to investigate detailed differences, especially for VOC patients that are subjected to continuous infections. The coinheritance of α-thalassaemia and sickle cell genes, in the Eastern province, interferes with the sickling process and therefore preserves the splenic function, which may explain the lower rates of infection.

Due to the various ethnic origins among Saudis, a number of SCD haplotypes are seen. The severe Benin haplotype is the most common, while the Arab – Indian type is less common. This variation provided a unique opportunity, in this study, to compare the differences between these haplotypes.

The clinical implications of these results could be utilized to improve treatment outcomes. Implementation of national management protocol for SCA is highly recommended. Moreover, vaccination program should be enforced and patient identification card issued. All these strategies would help further reductions in the incidence of infection, being a major trigger for VOC.

6.2 Limitations

The study has some limitations including the difficulty to recruit matched healthy controls. Thus we recruited random, healthy, volunteer blood donors as normal control subjects. Volunteer blood donors were readily available and willing to participate. Moreover, the demographics of the donor population were similar to the study patients.

Only fresh blood was used for flowcytometry testing and all samples were analysed within 24 hours of collection. Thus, samples could not be tested in batches alongside the ELISA testing. The more accurate flowcymetry beads methodology was not available at the time of the study. Alternatively, the manual technique was used.

Although the flow of patients coming to the haematology clinic or emergency room varied, which affected the number of samples used in the study, the statistical measure of power has established post hoc using G power program and was found to be in the range of 80-85 %. A larger number of patients would lend more power to the statistical analysis.

Furthermore, follow up of sickle cell patients in clinical studies is difficult, as their visits to the out-patient clinics are usually irregular due to repeated hospitalization and lack of socioeconomic support. Finally, the scarcity of literature on the association of clinical complications of sickle cell disease and leukocyte adhesion markers among Saudi patients, made it difficult to compare our results to others.

6.3 Recommendations

In the way to improve the clinical outcome, and the quality of life, for patients with sickle cell anaemia, this work highlights the importance of using adhesion molecules, on cell membranes, in the diagnosis and treatment. Early disease detection through neonatal screening program should be implemented. Premarital screening program for the detection of sickle cell anaemia and other haemoglobinopathies should be in effect. Attention should be focused on educating patients about the potential benefit of antibiotic prophylaxis, along with the role of pneumococcal and Haemophilus influenzae vaccination in minimizing the risk of infections. In addition more research is needed to explore cell adhesion molecules, a targeted approach to counteract the disease manifestations.

6.4 Scope for Further Studies

A “pan anti-adhesive” agent is a novel potential therapeutic target, to inhibit selectin-mediated cell interactions between sickled red blood cells, leukocytes and endothelial cells, which is implicated in the pathophysiology of vaso-occlusion. Selectin inhibition reduces leukocyte rolling and adhesion, which in turn may increase and restore microvascular blood flow. Therefore, it holds promise as a novel therapeutic approach for the treatment of vaso-occlusion. Moreover, it has the potential to reduce disease related morbidity and end-organ damage. Further clinical research of this therapeutic approach may help improve quality of life for sickle cell disease patients and reduce the burden of disease management on the healthcare system throughout Saudi Arabia.

6.5 Conclusion

The overall findings of the current study support data from other reports on patients with SCA in different parts of the world. The present results confirm that cell adhesion molecules play a major role in the pathophysiology of SCA, as reflected by the novel finding of increased levels of L-selectin on the surface of leukocytes, as well as the increased levels of sE-selectin and sP-selectin in serum. High levels of these markers suggest that these could be used to monitor VOC and could be targeted for novel therapeutic approach. However, a multi-center study is recommended to further elucidate the nature of interaction between haplotype and cell adhesion molecules in the pathogenesis of VOC. The findings of this study may also be significant to the quality of life of SCA patients.

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Appendix 1

The Bioethical and Research Committee

Faculty of Medicine

Memo

To: Dr Salwa Al Najar

From: Professor H Nasrat

CC: File

Date: 16th February 2003

Re: Immuno Phynotyping of Sickle cell population in Saudi Arabia

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This is to certify that the research titled:

Immuno Phynotyping of Sickle cell population in Saudi Arabia

Submitted by:

Dr Salwa Al Najar (hematology)

Has been reviewed by the committee with respect to protecting the rights and welfare of human subjects involved in the research project and/or experimental animals utilized. The methods employed are adequate for obtaining the information required and satisfy the required ethical principles and does not involve undue risk in the light of the potential medical benefits to be derived there from.

Decision:

The committee approves the above mentioned proposal as fulfilling the ethical requirements.

Professor Hassan A Nasrat

Chairman of the Bioethical and Research Committee

Appendix 2

Patient's Information:

Name: **Hospital No. :**

Sex: **Date of Birth:**

Tel. No. : **Nationality:**

Ethnic origin: **History of infection:**

Clinical and laboratory Status:

Presence of vaso -occlusive Crisis and infection

Signs of infection	**Yes**	**No**	**Laboratory result**		
Fever			HBsAg		
Chest infection			Anti HCV		
GIT symptoms			Anti HIV		
urinary tract infection			**Complete blood culture**		
Skin infection			WBC:		
Clinical picture			Hb:		
Vaso-occlusive crisis			Platelets:		
			Hb Electrophoresis:		
			1. HbS		
			2. HbF		
			3. HbA2		
			Culture and sensitivity		
			Organism		

Appendix 3

KING ABDULAZIZ UNIVERSITY HOSPITAL
BLOOD TRANSFUSION SERVICES (DONOR SERVICES)
DONOR QUESTIONNAIRE (ENGLISH)

Donors demographical data :
Name :_____ Age:_____ Nationality:_____
Hafeeza/iqama number:_____ Address_____ Occupation :_____
Tel-home:_____ tel-work:_____ mobile:_____
P.O.Box (if available):_____ e-mail (if available):_____
Martial Status: Single ☐ Married ☐ Divorced ☐ **Gender**: male ☐ female ☐
Type of Donation: Volunteer ☐ Autologous ☐ Replacement for patient ☐ Patient file number:_____
First time donation ☐ previous donation ☐ how many times ____ last donation date:_____
Donation Type: regular ☐ apheresis ☐
To Protect your health & the patient receiving blood, please read these questions carefully and answer them accurately:

No	Question	Put √ on the appropriate answer	Yes	No
1	Are you feeling well and healthy today?			
2	Would you be willing to be a regular donor and accepting blood bank calls whenever there is a need?			
3	Have you ever rejected as a blood donor before?			
4	Have you had yellow jaundice, liver disease, hepatitis since age of 11 year old or found to be positive for hepatitis?			
5	Do you have an infection now or are you taking antibiotics now?			
6	Have you suffered from a blood disease or had a tendency to bleed?			
7	Have you had any unexplained fever, swollen glands or weight loss?			
8	Have you ever suffered from convulsions, seizures or fainting spells?			
9	Have you or any one of your relatives ever had a disease called CREUTZFELDT-JAKOB DISEASE?(CJD)			
10	Have you ever taken any growth hormone injection?			
11	Have you ever had Brain surgery or brain converting graft?			
12	From 1980 through 1996, did you spend time more than3 months in UK or have you got any blood transfusion there? received blood products from the same country during the same date?			
13	Have you been under a Doctor's care or taking any medication now?			
14	Are you a citizen from or have visited a country with endemic of malaria?			
15	Have you ever had malaria in the past?			
16	Are you a blood relative of the intended recipient?			
17	Have you been injected with Bovine (beef) insulin since1980?			
IN THE LAST YEAR				
18	Have you traveled abroad?			
19	Have you ever had any surgery in the past? Or Have you received any blood or blood products?			
20	Have you had a tattoo or an accidental needle prick?			
21	Have you come in contact with any one with Hepatitis or HIV?			
22	Have you had any vaccination recently?			
23	Have you had any disease or taking any medication now?			
24	Have you imprisoned or incarceration in a correctional institution for more than 72 consecutive hours?			
FOR LADIES				
25	Are you pregnant or have been pregnant in the past 6 weeks?			

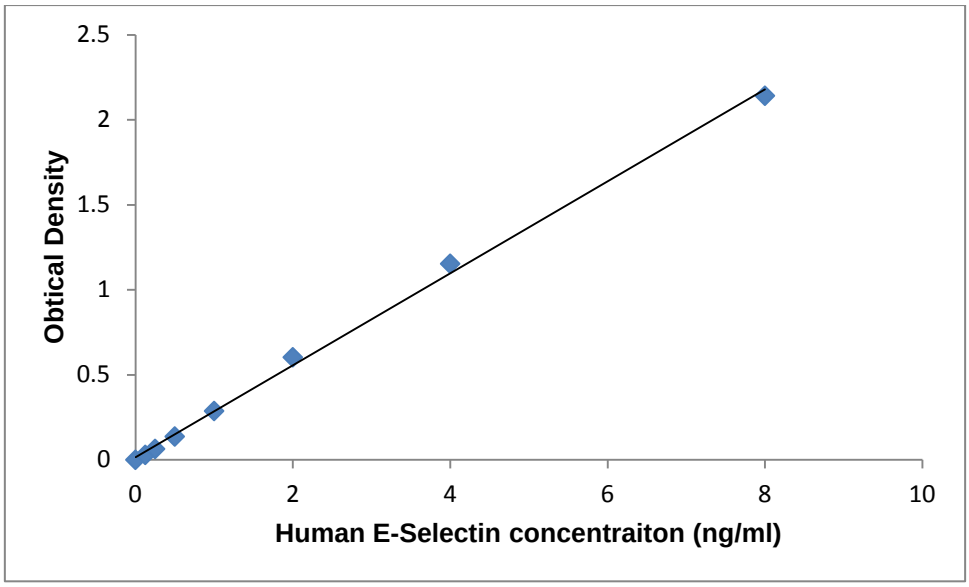
FOR ALL DONORS: you should never give blood if
- You are a carrier of Hepatitis "B" or " C" Virus or AIDS
- You have ever had sex with another man/woman even safe sex
- You have ever had sex with anyone with Hepatitis or HIV even once
- You are taking any Narcotic Drugs

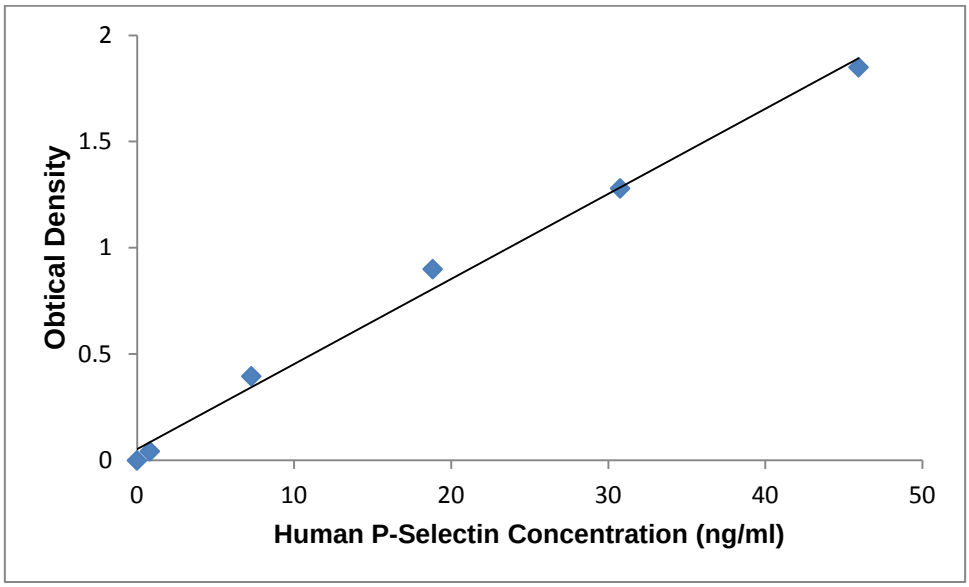
After reading the above information, do you still feel that Your blood is suitable for blood transfusion? Yes ☐ No ☐

Donor acknowledgment
I (name) _____ acknowledge that
- All educational materials was given to me and it has been read and understood,
- I was given all opportunity to ask all concerned questions
- I have provided accurate information to the best of my knowledge and ability.
- I accepted that blood transfusion services has the full authority to use my blood in helping patient's ,research purpose or in any other purpose that they found it useful
- I acknowledge that I have no rights to ask for the blood that I donated later.
- I am authorizing blood transfusion services staff to collect 450 ml of blood from me.
- I accepted that my blood will be tested for the following (hepatitis B HBsAg, Hepatitis C HCV, AIDS HIV1.2,syphilis and HTLV1.2) and HIV1 RNA HCV RNA
- I have been informed that there are circumstances in which infectious diseases are not performed
- I have been informed that I may be called blood bank within 24 hours time if I feel that my blood should not be used for transfusion.
- If any of these tests or the information recorded on this form indicate that I should no longer donate blood or blood components because of a risk of transmitting AIDS or other diseases, my blood will not be used. I will be notified, and my name will be entered on a list of deferred donors. (confidentially)

Signature:_____ Date :_____

Appendix 4





Standared curve for the ELISA.