Characteristics of cardiovascular dysfunction and pulminory hypertension in patients with sickle cell disease

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Characteristics of cardiovascular dysfunction and pulmonary hypertension in patients with sickle cell disease

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DECLARATION

I declare that I am the sole author of this thesis and that the work contained within is my own unless otherwise indicated.

Sitali Mushemi-Blake

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List of Abbreviations

2D	Two dimensional
3D	Three dimensional
A wave	Transmitral A wave
AA	Normal genotype
CI	Cardiac Index
CRP	C-reactive protein
CT	Chest tomogram
E wave	Transmitral E wave
E/A wave	Transmitral E/ transmitral A wave ratio
Echo	Echocardiography
ECG	Elecrocardiogram
EF	Ejection fraction
FEV_1	Forced expiratory volume in one second
FMD	Flow mediated dilatation
FRC	Functional residual capacity
FVC	Forced vital capacity
Hb	Hemoglobin
HbAS	Heterozygous sickle cell genotype
HbF	Fetal hemoglobin molecule
HbSC	Heterozygous sickle cell hemoglobic C disease
HbSthal	Heterozygous sickle cell thalaseamia disease
HbSS	Heterozygous sickle cell
H_2O_2	hydrogen peroxide
HU	Hydroxyurea
ICA	Extracrainial transcranial Doppler
K_{CO}	Gas transfer per lung volume
LDH	Lactate dehydrogenase
LV	Left ventricle
LVEDV	Left ventricular end-diastolic volume
LVESV	Left ventricular en-systolic volume
LVH	Left ventricular hypertrophy
LVRI	Left ventricular remodeling index
LV SDI	Left ventricular systolic dyssynchrony index
MI	Myocardial infarction
MRI	Magnetic resonance
N-BNP	N-type brain natriuretic peptide
NO	Nitric oxide
NOS	Nitric oxide synthase
OH•	Hydroxyl radical
O_2	Oxygen
$O_2^{-\bullet}$	Superoxide anion radical
PAP	Pulmonary arterial pressure
PCW	Pulmonary capillary wedge
PEF	peak expiratory flow
PHT	Pulmonary hypertension
PVH	Pulmonary venous hypertension
PVR	Pulmonary vascular resistance
RV	Right ventricle

RV_{pleth}	Residual volume
RVOTI	Right ventricular outflow index
SCD	Sickle cell disease
SV	Stroke volume
STE	Speckle tracking echocardiography
TAPSE	Tricuspid annular displacement
TLC_{pleth}	Total lung capacity
TDI	Tissue dopper imaging
TRV	Tricuspid regurgitation velocity
VA	Alveolar volume
VC_{pleth}	Vital capacity
WBC	White blood cell

ABSTRACT

Background

Sickle cell disease (SCD) is a hereditary hemoglobinopathy that causes chronic complications due to repetitive vaso-occlusive events and hemolysis, and can lead to multiorgan failure and shortened life expectancy. Among a spectrum of cardiovascular manifestations in these patients, pulmonary hypertension (PHT) has been stated to pose the highest concern. The exact prevalence of PHT in SCD is controversial; clinical studies using echocardiography have suggested PHT to be highly prevalent and a major determinant of outcome. The contribution of cardiac and endothelial dysfunction to SCD also remains poorly understood.

Aims

(1) To characterise cardiac function in SCD patients using new 3-dimensional echocardiographic imaging techniques.

(2) To determine the prevalence of PHT in SCD patients by using advanced echocardiographic methods and establish mechanisms involved in the development of PHT.

(3) To assess whether SCD patients have endothelial dysfunction independent of other vascular risk factors.

Methods

Detailed 2D, 3D and Doppler echocardiography studies were performed in 122 consecutive unselected SCD patients and 30 healthy age- gender- and ethnicity-matched controls to assess cardiac function and look for PHT. A sub-

group of patients underwent invasive assessment of pulmonary vascular resistance (PVR) by right heart catheterisation. CT lung scan and lung function tests were also performed in patients suspected to have PHT. Vascular endothelial function was assessed by flow-mediated forearm vasodilatation in twenty stable adolescent SCD patients without other risk factors and fifteen healthy age-matched controls.

Results

Results indicate that patients with SCD have significantly enlarged cardiac dimensions associated with elevated cardiac index (CI) that correlated with the degree of anaemia. Cardiomegaly in SCD patients was not associated with significant contractile dysfunction as assessed by regional myocardial deformation. A high proportion (>30%) of SCD patients had a tricuspid regurgitation jet velocity ≥ 2.5m/s but non-invasive estimation of PVR revealed that only a minor proportion (<5%) had elevated values. These findings were confirmed by right heart catheterisation. The raised tricuspid regurgitant velocities in SCD patients may be driven more by elevated CI than by elevated PVR. Finally, it was found that young patients with SCD had evidence of subclinical vascular endothelial dysfunction.

Taken together, these results provide new information about the prevalence and underlying mechanisms of cardiovascular dysfunction in patients with SCD.

Chapter 1

Introduction

1. Sickle cell disease

Sickle cell disease (SCD) is an autosomal recessive disorder that occurs throughout the world. The distribution of SCD often coincides with that of falciparum malaria parasites, therefore making it common among people from sub-Saharan Africa, Mediterranean countries, Southern India and their descendants (African Americans) (Weatherall et al., 2005). It is estimated that around 200 000 to 300 000 people are born every year with SCD worldwide (Siddiqui and Ahmed, 2003). According to a global epidemiology report implemented by the World Health Organization (WHO), the increase in occurrence of SCD in western society is primarily owed to migration (Modell and Darlison, 2008). As there was variation in the range of estimates of SCD derived from different ethnic groups in the United Kingdom (Hickman et al., 1999), work was initiated for the WHO to essentially plan for screening programs to be set up in and across England which was implemented by October 2006 (Modell and Darlison, 2008). Initial statistics gave an estimated incidence of 1 in 2000 in SCD at birth, making SCD the most common serious inherited disorder in England (Telfer et al., 2007).

1.1 Genetics of sickle cell disease

The genetic defect for SCD occurs due to a single amino acid substitution (glutamine to valine) at the sixth residue in each of the two β subunits of the haemoglobin (Hb) molecule leading to formation of haemoglobin S (HbS) (Lane, 1996). By far the most common and severe form of SCD occurs in patients homozygous for sickle mutation (HbSS) which accounts for 0.15% of all African American children (Naoman et al., 2009). In contrast, patients heterozygous for

sickle mutation (HbAS)– prevalence of 8-10% in African Americans (Anzalone et al.) and as high as 25-30% in western Africa (Fleming, 1989)- are often asymptomatic or present with minimal symptoms (Lane, 1996; Schutt and Meier, 2005). Conventional tests performed on uncomplicated cases of HbAS usually show no haemolysis at rest. However, a number of studies have reported sickling of haemoglobin molecules in HbAS individuals during intense exercise and that the rate of sickling is enhanced at higher altitudes. Furthermore, autopsies performed on U.S army recruits who died suddenly during strenuous exercise between 1968 and 1969 found a link between extreme exercise and sickling of red blood cells (Mitchell, 2007).

The genetic defect for SCD can coexist with other mutations of the haemoglobin molecule i.e. β-thalassemia, haemoglobin C (HbSC), $D_{Los\ Angeles}$, E Lepore, O_{arab}, C_{Harlem} or Quebec-Chori (Chang and Kan, 1981; Kan and Dozy, 1978; Konotey-Ahulu et al., 1968; Witkowska et al., 1991). Although the clinical manifestations resulting from coinheritance vary significantly, depending on the mutant β-globin gene, more severe complications (table 1) tend to be observed among sickle β- thalassemia and homozygous patients (HbSThal), with only a few HbSC patients hospitalized per year due to frequent episodes of pain and acute chest syndrome (Lane, 1996; Wood et al., 2008).

Table 1.1. Sickle cell disease variants and disease severity

Sickle cell disease gene variation	Genes affected	Clinical complications
HbSS	Homozygous mutation in both copies of β-globin alleles	Severe
HbS/β°thal	Heterozygous mutation leading to inheritance of two mutated beta Sickle and Thalassemia alleles causing complete absence of β-globin molecules	Severe
HbS^{+}thal	Same as above but inheritance of this genotype results in decreased production of β-globin molecules	Mild
HbAS	Mutation in only one beta allele resulting in Sickle trait. Patients with trait produce normal and abnormal β-globin molecules	Mild
HbSC	Heterozygous mutation of HbS and HbC allele. Patients have higher Hb levels, lower rates of haemolysis and lower white blood cells	Mild/Moderate

1.1.2 Pathophysiology of SCD

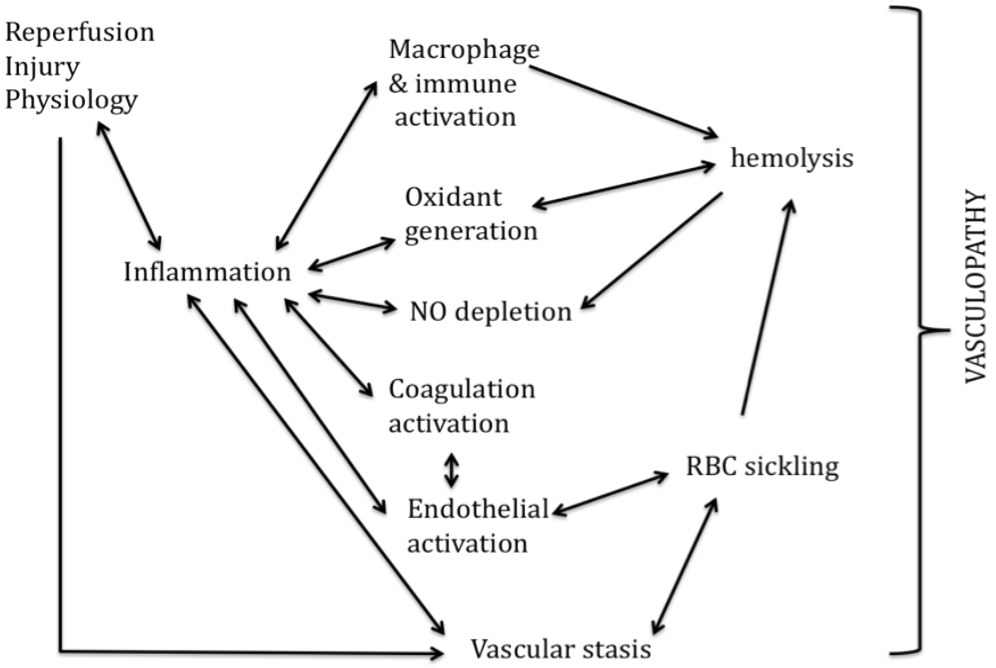
Abnormal HbS is less soluble than normal haemoglobin (HbA) and tends to readily form elongated crystals inside red blood cells, particularly when exposed to low oxygen tension (Frenette and Atweh, 2007; Siddiqui and Ahmed, 2003). These elongated crystals alter the shape of red blood cells (to a sickle shape), thereby preventing their passage through the microvasculature. Aggregation of malformed red blood cells in turn occludes pre-arterioles and arterioles, thus leading to regular cycles of tissue ischaemia and reperfusion injuries (vascular occlusion crisis). Long-term cycles of tissue ischaemia account for the painful, vascular-occlusive crises suffered by patients with SCD and can potentially affect any organ in the body. Furthermore, haemolytic anemia observed in SCD

patients is a consequence of HbS crystals rupturing red blood cells and leaking into the circulation (Lane, 1996).

1.1.2.1 Reperfusion injury

Reperfusion injury constitutes a major problem in the pathophysiology of sickle cell vasculopathy. Although the vaso-occlusive process is simplified above, the actual mechanisms involved are complex and multi-factorial and involve interaction of erythrocytes with leucocytes, plasma proteins, endogenous vaso-active substances and the vascular endothelium (Bunn, 1997; Frenette, 2002). Many biological processes (such as systemic inflammatory state, abnormal expression of adhesion molecules, deficiency and resistance of NO production) interlink to form positive feedback loops. The extensive list of processes involved is highlighted in Figure 1 (Morris, 2008). In addition, the pathophysiology of microvascular occlusion differs from that of large conduit vessels (Frenette, 2002).

Figure 1.1. Multiple biological processes interconnect and contribute to sickle cell vasculopathy. Adapted from Kato et al, 2009 (Kato et al., 2009).



1.1.3 Clinical manifestations of sickle cell disease

As highlighted, SCD is a complex disorder with multi-organ complications. Most SCD infants are healthy at the time of birth, but become symptomatic later on in infancy as levels of fetal haemoglobin (HbF) – the primary haemoglobin produced by the fetus to carry oxygen in a low oxygen environment - decline (Henderson, 1950; Lane, 1996; SOX, 2002). Generally, HbF production after birth (or when babies reach the age of one or two years) reduces when the γ-gene is switched down and the β-gene is switched on to facilitate production of HbA in adulthood. Researchers have shown that individuals with SCD who have increased levels of HbF tend to experience milder symptoms and longer survival because HbF inhibits sickling of red blood cells (Charache et al., 1992; Higgs and Wood, 2008; Koshy et al., 2000; Steinberg et al., 1997).

The outlook for patients with SCD has improved dramatically in developed countries since 1960, when Sir John Dacie described sickle cell as 'essentially a disease of childhood' (Platt et al., 1994). Autopsy studies performed in 1973 (Diggs, 1973) reported a median survival age of 14.3 years, with 20% of deaths occurring in the first couple of years. However, more recent cooperative studies of sickle cell have shown marked improvement in patient survival (life expectancy of 50 years) (Claster and Vichinsky, 2003). Advances in medical management, neonatal screening, and patient education have all contributed to improved life expectancy in SCD patients. Unfortunately, as the patient population ages, new complications arise accounting for serious patient morbidity and mortality **(table 1-2).**

Table 1.2. Important Clinical Manifestations of SCD during childhood and Adolescence

Chronic manifestations	Acute Manifestations
Anaemia	Acute chest syndrome *
Avascular necrosis	Aplastic crisis *
Cardiomegaly and functional murmurs	Bacterial sepsis or meningitis *
Cholelithiasis	Haematuria, including papillary necrosis
Delayed growth and sexual maturation	Priapism
Functional asplenia	Recurrent vaso-occlusive pain
Jaundice	Splenic sequestration *
Leg ulcers	Stroke *
Proteinuria	
Pulmonary hypertension *	
Proliferative retinopathy	
Restrictive lung disease *	
Splenomegaly	
Transfusional haemosiderosis	

**** Potential cause of mortality***

1.1.4 Role of the endothelium

The endothelium, an organ once viewed as an inert 'cellophane-like' membrane lining the circulatory system whose primary function was to maintain vascular permeability (Cines et al., 1998) is in fact a very active heterogeneous monolayer cell system whose multiple roles range from controlling vasomotor tone, haemostatic balance, permeability of nutrient substances, monitoring of cell to cell interaction and proliferation to adaptive immunity (Aird, 2007). In an adult human, the endothelium weighs approximately 1 Kg and covers a surface area of 1 to 7 m^2 (Cines et al., 1998; Kharbanda and Deanfield, 2001). The endothelium is involved in many disease states either as a primary cause or consequence of altered pathophysiological conditions (Aird, 2008) which makes it a prime target for future therapeutic strategies. One potential problem that has faced translation from bench to bedside research is the perception that all endothelial cells are the same. Which off course isn't the case, for instance endothelial cells that line postcapillary venules are responsible for mediating leukocyte trafficking, while arterial endothelial cells regulate vasomotor tone and tissue perfusion (Aird, 2008). Regardless of individual function, a critical balance between potent mediators such as nitric oxide, prostacyclin, angeotensin II, endothelin and endothelium-derived hyperpolarizing factor is required in order to maintain a balanced homeostatic state (Cannon, 1998; Kharbanda and Deanfield, 2001; Lim et al., 2008; Murdoch et al., 2011; Verma and Anderson, 2002). When this balance is disturbed it predisposes the vasculature to vasoconstriction, platelet activation, leukocyte adherence, thrombosis, inflammation, oxidative stress and atherosclerosis (Kharbanda and Deanfield, 2001).

In 1980, Furchgott and Zawadzki first demonstrated the involvement of endothelial cells during relaxing processes of isolated rabbit aorta while experimenting with acetylcholine (Furchgott and Zawadzki, 1980). In their observations, administration of acetylcholine to aorta arterial vessels lacking endothelial cells had vasoconstrictive effects as a result of acetylcholine acting directly on muscarinic receptors of vascular smooth muscle cells (Beny and Brunet, 1988; Furchgott and Zawadzki, 1980). Further experiments led to Furchgott and Ignarro suggesting that the endothelial derived relaxing factor may be nitric oxide (Ignarro et al., 1987). Later that year Palmer et al examined the suggestion that nitric oxide (NO) was the EDRF (Palmer et al., 1987). In their experiments, of endothelial cell cultures biological activity, stability and susceptibility to inhibition by haemoglobin and superoxide dismutate bioassays found EDRF to have identical characteristics to NO.

Indeed NO, a free radical produced in the endothelial cells plays a pivotal role in the maintenance of vascular tone and reactivity (Verma and Anderson, 2002). The half-life of NO in biological systems tends to be short i.e < 4 seconds (Beckman and Koppenol, 1996), therefore NO cannot be stored in cells but is instead synthesized upon demand in the endothelial cells from L-arginine circulating the body as a product of the urea cycle (Moncada and Higgs, 1995). The endothelium acts as a signal transducer, induced stimuli in the form of physical stress or chemically by substance P or acetylcholine instantly cause a rise in intracellular calcium concentration. Raised calcium concentrations lead to increased blood flow/shear stress and activation of the enzyme endothelial nitric

oxide synthase (eNOS) which can be located on caveolin complex found in the plasma membrane (Kharbanda and Deanfield, 2001). Activation of eNOS subsequently leads to complex intracellular interactions between eNOS and caveolin structure and conversion of L-arginine to NO with L-citrulline being formed as a byproduct (Feron et al., 1998; Harrison, 1997; Vallance and Chan, 2001).

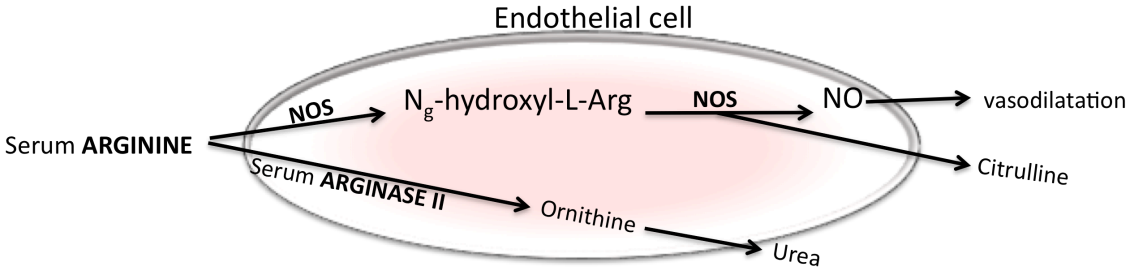
Once synthesized nitric oxide rapidly diffuses across the cell membrane where it binds the cytoplasmic enzyme guanylate cycle, thereby increasing intra-cellular concentration of a second messenger cyclic guanosine-3',5-monophosphate (cGMP) (Shah, 1992). The rise in cGMP inhibits phosphatidylinositol hydrolysis causing a cascade of reactions that result in a net reduction in intracellular calcium concentration thereby inducing smooth muscle cell relaxation. The bioactivity of NO is reduced by oxyhaemoglobin molecules in blood and during reactions with free radicals such as superoxide ($O^{-\bullet}$) to produce a slow reactive but damaging oxidant called peroxynitrite ($ONOO^-$) (Li and Shah, 2004; Noronha et al., 2005)

1.1.5 Role of vascular endothelial dysfunction in manifestation of SCD

Regular cycles of ischaemia and reperfusion injury during vascular-occlusive crisis lead to the release of reactive oxygen species (such as the superoxide anion radical ($O_2^{-\bullet}$)), which promote endothelial dysfunction through pro-oxidant and pro-inflammatory stress (Gladwin and Kato, 2005; Kato et al., 2009; Morris, 2008; Morris et al., 2003). Intra-vascular haemolysis also releases HbS and serum arginase into plasma, which also interferes with the production of NO.

Serum arginase hydrolyses L-arginine, the main substrate for the production of NO into ornithine and urea. In turn, ornithine competitively inhibits the transport of L-arginine into endothelial cells. Furthermore, the interaction of HbS with pro-oxidants propagates a chain of oxidative reactions resulting in the formation of potent pro-oxidants (such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\bullet}$)). In conjunction with endothelial dysfunction, micro-infarction secondary to microvascular occlusion is another important source of tissue damage and inflammation. With time, regular cycles of injury lead to a proliferative vasculopathy, which, as outlined above, can affect multiple organs throughout the body.

Figure 1.2. Mechanisms contributing to impaired biosynthesis of nitric oxide in SCD during haemolysis. Under normal physiological conditions, nitric oxide synthase (NOS) hydrolyses the substrate L-Arginine to nitric oxide (NO) and citruline. However, in cases of pulmonary hypertension/ sickle cell crisis, it is speculated that NO production is decreased because the enzyme arginase II competes with NOS for available L-Arginine. Arginase hydrolyses arginine to orthinine and urea. Orthinine can inhibit NO production by inhibiting L-arginine transport into the endothelial cell, hence reducing the amount of substrate available for NO production (Morris et al., 2003).



1.2 Cardiopulmonary complications in SCD

1.2.1 Cardiac abnormalities

Cardiovascular abnormalities are nearly always apparent and a well recognised complication in SCD with cardiac enlargement and systolic murmurs being the most common findings (Covitz et al., 1995; Lester et al., 1990; Lindsay et al., 1974; Mueller et al., 2006). Although many studies have attempted to understand the heart in SCD, there are apparent inconsistencies in the literature regarding cardiac functional changes and autopsy findings. Moreover, although several mechanisms have been proposed to explain cardiac involvement - such as progressive dyspnea, thrombotic occlusion of coronary vessels, chronic anaemia etc (Caldas et al., 2008) -none of these studies have attempted to describe the nature of the cardiac remodelling involved in this disease.

1.2.2 Cardiovascular pathphysiology

James Herrick reported the first diagnosis of cardiac abnormalities in SCD in 1910. He reported evidence of cardiac enlargement and a praecordial murmur in an African American patient who was later misdiagnosed as having rheumatic heart disease, due to musculoskeletal symptoms (Lane, 1996; Tsironi and Aessopos, 2005). Since then, cardiovascular pathology in SCD has been documented in both adults and children, with most clinical studies emphasizing the importance of cardiopulmonary disease and cor pulmonale. Its however surprising that despite long standing interests, characterisation of the underlying pathophysiological mechanisms remains poor.

Long-term stresses due to low oxygen content of arterial blood due to anaemia and inability of the lungs' to load red blood cells are believed to play a role in the 'compensatory' increased cardiac output (Covitz et al., 1995; Lindsay et al., 1974). Indeed elevated cardiac output has long been reported in SCD patients with haemoglobin levels less that 7g/dL (Varat et al., 1972). A study by Duke and Abelmann in 1969 comparing cardiac output before and after treatment with 100% oxygen in patients with chronic anaemia found that anaemia hyperdynamic state correlated with age, with more compensatory mechanisms being present in most young patients. Furthermore, in that study administration of oxygen lowered cardiac output significantly. Since the heart rate in SCD patients is within normal range (Jaja et al., 2000; Martins Wde et al.), it is postulated that increase in cardiac output is achieved by means of increased stroke volume.

Although it has been established that the coronary arteries of SCD patients are generally free of arteriosclerotic disease, there still remains uncertainty regarding myocardial fibrosis. One report on 52 SCD patients conducted by Gerry et al 1978, describes evidence of cor pulmonale associated with right ventricular hypertrophy and dilation, but not of fibrosis (Gerry et al., 1978; Haywood, 2009). However, other autopsy studies such as Uzsoy *et al* and Baroldi describe elements of myocardial degradation and fibrosis (Falk and Hood, 1982; Metivier et al., 2000). Furthermore, a review of postmortem examinations of seventy SCD adults and children conducted between 1950 and 1982 found evidence of myocardial infarction and fibrosis in 9.7% of patients (Martin et al., 1996). The pathophysiological mechanism responsible for

myocardial infarction (MI) remain unclear; however, anaemia, myocardial hypoxia and occlusion of intramural coronary arteries are believed to independently play an active role.

1.2.3 Diagnosis of cardiac dysfunction

Sickle cell crisis causes diffuse body pain and acute chest pain, which often makes it difficult to suspect MI. More so, these patients tend to be young with either non-specific or no changes on the electrocardiogram. In any case, when MI is suspected, the criteria set out by the World Health Organization (WHO) for diagnosis of MI are followed. Diagnosis of MI is based on 2 or 3 findings: patient symptoms consistent with MI, changes in the electrocardiogram and cardiac enzyme markers of myocardial necrosis (Pannu et al., 2008).

Echocardiography is a simple non-invasive way of assessing cardiac structure and function. Two-dimensional echocardiography has contributed significantly to the understanding of cardiopulmonary manifestations in SCD. However, it still remains unclear whether SCD causes cardiac systolic dysfunction. On one hand, studies such as Klinefelter (1942) who evaluated 27 patients with the homozygous phenotype using M-mode and two dimensional echocardiography studies and found ejection fraction– a measure of systolic function- to be significantly reduced compared to controls with 11% of the cohort having systolic dysfunction (Gacon and Donatien, 2001; Klinefelter, 1942). Lamers and colleagues in 2006 used load-dependent echocardiographic indexes such as M-Mode, fractional shortening and corrected ejection time to assess systolic function in SCD patients aged 3 months to 18 years, again lower systolic

function was present among SCD patients compared to controls (Lamers et al., 2006).

In contrast to the studies above, other reports suggest normal left ventricular function (Covarrubias et al., 1980; Lester et al., 1990). Moyssakis *et al* evaluated left ventricular systolic function in forty patients with sickle beta thalassaemia using echocardiography methods such as ejection fraction, circumferential fibre shortening velocity and end systolic stress/volume ratio and found systolic function to be normal (Moyssakis et al., 2005). Similarly, one study by Arslankoylu and colleagues (Arslankoylu et al., 2009) used echocardiography and Doppler to examine thirty-two patients with SCD. In their study left ventricular function by ejection fraction method was normal, and measures of myocardial performance index showed a hyperdynamic picture.

Systemic hypotension is a common feature among SCD patients with a lower prevalence of hypertension (3.2% v 60%) than the general black population (Foucan et al., 1999; Gordeuk et al., 2008; Hatch et al., 1989; Johnson and Giorgio, 1981; Kramer et al., 2004; Rodgers et al., 1993). The cause of lower blood pressure in patients is not apparent though postulated that lower arterial stiffness due to endothelial dysfunction, blunted plasma volume expansion due to abnormalities in renal vascular system responsible for sodium and water excretion and body weight may all play a role (Homi et al., 1993; Pegelow et al., 1997). In a recently published study, serial blood pressure evaluations made over a period of 24 months in 163 homozygous SCD patients found an association between elevated systemic blood pressure, traditional estimates of pulmonary hypertension and increased mortality rates (Gordeuk et al., 2008).

Some investigators have even reported an association between cerebrovascular accidents and elevated blood pressure measurements in male patients (Rodgers et al., 1993). Although labeled ‘elevated’, the systemic blood pressure in these patients was still within normal limits.

Sudden death reports have been documented among homozygous and heterozygous SCD patients (Romero Mestre et al., 1997). This raises concern because significant arrhythmias have been observed in SCD patients including morphological cardiac abnormalities in the conductive system such as sinus node degeneration and prolonged corrected QT interval on electrocardiogram (James, 2000). Postmortem studies have shown degenerative changes of the myocardium and conductive system which may contribute to prolonged repolarisation patterns seen on the electrocardiogram (James et al., 1994). Twenty-four hour continuous monitoring of thirty SCD patients in acute crisis detected a high incidence of arrhythmias in 80% of the cohort (Maisel et al., 1983). A study of corrected QT, a risk factor for ventricular arrhythmias, in the electrocardiogram of 76 SCD children found long QT syndrome in 38% of patients enrolled (Liem et al., 2009). Prolonged corrected QT interval observed in the Leim’s study was not associated with left ventricular hypertrophy or presence of arrhythmia. The exact mechanism responsible for prolonged QT and occurrence of arrhythmias remains unclear.

1.2.4 Clinical features

Physical examination of patients with SCD often demonstrates signs of a hyperdynamic circulatory state. These findings include a wide pulse pressure,

active praecordial impulses, with a laterally displaced and prominent apex beat. In the absence of heart failure, the jugular venous pressure is usually normal, but heart sounds are usually loud with a split second sound. Systolic murmurs are common with the third heart sound reflecting hyperdynamic state rather than cardiac failure. Although clinical heart failure and death is a relatively rare entity, a high proportion of sickle patients experience dyspnoea on moderate exertion, which makes it difficult to determine presence or absence of superimposed cardiac disease (Falk and Hood, 1982).

Undoubtedly, with continued medical support for patients with SCD, life span is increasing. Therefore, cardiovascular abnormalities may have time to develop. The deviations in cardiac patterns described so far provide clues regarding cardiac structure and function. However, a clearer definition of sickle cell heart disease would be of great value.

1.3 Pulmonary hypertension in SCD

The term pulmonary hypertension (PHT) refers to raised pressure in the pulmonary circulation resulting from an increase in vascular resistance, which leads to an increased workload for the right heart and may also impair systolic ventricular function (Farber and Loscalzo, 2004; Huisman et al., 2005). PHT can arise from acute or chronic constriction of the pulmonary vasculature, pulmonary thromboembolic disease, parenchymal lung disease or secondary to raised left heart pressures. PHT has been suggested to be a frequent complication of SCD, and may be a manifestation of a number of pathological processes acting on the lung, heart and vasculature. It has been suggested that

PHT in SCD in the absence of significant coronary artery disease acts as an independent predictor of death- with an associated 40% mortality rate at two years (Jison and Gladwin, 2003; Sachdev et al., 2007).

The exact prevalence of pulmonary hypertension in SCD remains unclear. Echocardiography studies performed to date (which estimate pulmonary artery pressures but not resistance) suggest that around 20-60% of all SCD patients have resting pulmonary hypertension (Castro et al., 2003; Derchi et al., 2005; Gladwin et al., 2004; Lin et al., 2005; Vichinsky, 2004). Data collected from assessment of PHT using the gold standard technique of right heart catheterization, are indeed, few and far between (Anthi et al., 2007; Castro et al., 2003; Parent et al.). One such study is that by Castro *et al* 2003 whose group retrieved haemodynamic data on 34 adult SCD patients catheterised between 1991 and 2001 while hospitalized for painful sickle episodes. In this study PHT was found in 20 patients. A high proportion of patients with SCD and PHT tend to have raised pulmonary capillary wedge pressure, suggestive of left ventricle contribution. Although left ventricular systolic function (on echocardiography) is usually normal at the time of catheterisation, techniques employed to assess systolic function are usually influenced by loading condition such as preload, which is usually high in anaemia. Furthermore, invasive studies performed so far do not explain the pathogenesis of PHT in SCD.

Histological examinations on lung tissue in SCD patients at the time of death suggest that PHT is more prevalent than previously recognised i.e. up to 75%. In addition, these pathological findings also report vascular changes such as

medial hypertrophy, dilatation of pulmonary vasculature, intimal fibrosis, pulmonary occlusion and plexiform lesion within the pulmonary vasculature (Haque et al., 2002; Machado, 2007).

1.3.1 Pathophysiology of pulmonary hypertension

The precise cause and pathophysiology of pulmonary hypertension in patients with haemolytic anemia have not been well defined. Chronic intravascular haemolysis may disturb nitric oxide bioavailability, promoting endothelial dysfunction, smooth muscle dystonia, enhancement of coagulopathy, and increased oxidant and inflammatory stress, all of which may lead to the development of proliferative changes seen in patients with pulmonary hypertension (Butrous et al., 2008).

1.3.2 Diagnosis of pulmonary hypertension

The diagnosis of PHT follows the algorithm established for other causes of PHT (Barst et al., 2004), which involves two stages: detection and cardiopulmonary characterization. According to the National Health Lung and Blood Institute/National Institutes of Health (NHLBI/NIH) guidelines, PHT is defined as a sustained elevation in pulmonary artery pressure (PAP) at rest, with a systolic PAP > 30mmHg, mean PAP > 25mmHg and pulmonary vascular resistance >3 wood units (Pauwels et al., 2001).

Right heart catheterisation is the gold standard method for diagnosis, and is used clinically to establish the severity of pulmonary hypertension and also provide information on sites of vascular resistance as well as the contribution of

the left ventricle to PHT. The disadvantages of cardiac catheterization are that it is an invasive investigation and relatively expensive and not suitable as a screening test. Due to the suspected high prevalence of PHT in SCD patients and its association with high mortality rates, there is increasing demand for non-invasive screening of all adults.

Doppler echocardiography is favoured clinically because it is simple, inexpensive and well tolerated by all patients. Doppler echocardiography provides information such as: heart structure, function and an estimation of pulmonary artery pressure (PAP) from the spectral display of tricuspid regurgitation. A tricuspid regurgitation velocity $\geq$ 2.5 m/s by Doppler is conventionally taken to suggest PHT due to a raised pulmonary vascular resistance. However, this traditional method of estimating PAP can be problematic especially in patients with low hemoglobin concentration and therefore a high cardiac output. In this case, it may be the elevated cardiac output rather than a raised pulmonary vascular resistance that drives increased pressures (mean pulmonary arterial pressure – mean pulmonary artery wedge pressure = PVR x Cardiac output). In other words, the estimated pulmonary pressure may provide an exaggerated estimate of the pulmonary vascular resistance. Another fall back is that, tricuspid regurgitation by echocardiography does not provide information regarding primary sites of pulmonary vascular resistance (i.e. arterial or venous).

1.3.3 Symptoms of pulmonary hypertension

The typical clinical presentation of PHT in SCD patients can be challenging,

because symptoms such as dyspnoea resemble those of chronic anaemia. Therefore, a high index of suspicion is necessary. More specific symptoms can range from syncope, lower limb odema and those resembling angina pain. Physical findings are suggestive of right ventricular dysfunction where the jugular vein is distended with a loud right ventricular S3 gallop and accentuated pulmonary S2 heart sounds. In severe cases, these patients can also present with ascites and peripheral odema.

Indeed, important questions need to be addressed regarding pathogenesis of pulmonary hypertension in SCD. Right heart studies have so far reported raised capillary wedge pressure, suggestive of left ventricular disease (Gladwin et al., 2004; Machado, 2007; Parent et al.). More so another issue surrounding overestimation of PHT as a consequence of high-output state remains to be addressed and clarified. Finally, an important consequence of PHT is the development of right ventricular dilatation and failure (Derchi et al., 2005; Haque et al., 2002) - but the detection of this problem remains challenging and sub-optimal.

1.4 Established and novel echocardiographic methods for analysis of cardiopulmonary function

Echocardiography is a noninvasive clinical tool capable of displaying cross-sectional ‘slices’ of the beating heart. It’s use is owed to Edler and Hertz who first described ‘a signal that moved with cardiac action’, of which Edler subsequently identified as the echoes produced by the motion of the anterior mitral valve leaflet (Siggers et al., 1971). Nowadays, this technology has

evolved so much that current scanning machines have the ability to accurately reconstruct heart structures in 3-dimensions. Clinically, echocardiography (echo) plays a useful part in the diagnosis and understanding of the pathophysiological processes that occur in cardiovascular diseases. In SCD alone, echo has contributed in the understanding cardiopulmonary manifestations. It is for this reason, that echo has become a compulsory screening procedure in SCD patients suspected of having pulmonary hypertension.

1.4.1 Two-dimensional echocardiography, M-mode imaging and Doppler

Two-dimensional (2D) echocardiography provides multiple tomographic real time images of the heart. It requires the operator to rotate the scanning transducer at various degrees and placing the transducer in four views to appreciate left ventricular function and hemodynamic information. A 2D echocardiogram is usually performed along with other imaging modalities such as M-Mode and Doppler. M-mode allows for assessment of chamber size, volume and wall function, while Doppler techniques measure blood flow direction, blood flow velocities and myocardial tissue velocity.

The principles of Doppler were first described in 1842 by a mathematician/scientist from Austria called Johann Christian Doppler who based his theory on the motion stars (Kisslo and David, 1987; Reinold, 2004). Nearly twenty years after Doppler's descriptions, Japanese scientist suggested the use of backscattered ultrasound frequency in determining the motion of blood in vessels (Bollinger and Partsch, 2003). This idea was taken further by

Donald Eugene Stradness who invented a Doppler ultrasound flow meter that has now evolved into more sophisticated instrumentations.

Measurement of LV volume and ejection fraction

M-mode imaging modality assumes the shape of the left ventricle (LV) is elliptical; therefore single minor axis dimensions are cubed to derive the final volume (Greaves, 2000; Walther et al., 1986). This method of calculating final LV volumes has led to large errors being generated (Barbier et al., 2011) such that volume assessments made by M-mode are no longer recommended during clinical follow up of patients. Two-dimensional echo uses a biplane method of discs (Simpson's rule) that makes fewer geometrical assumptions (Chukwu et al., 2008). However, 2D echo still assumes that the shape of the LV is elliptical. The problem arises when the shape of LV is altered in cardiomyopathies or aneurysms post-myocardial infarction. Furthermore, poor inter- and intra-observer variability have been closely associated with these two (M-mode and 2D) traditional methods of assessing cardiac structure and function (Rademakers, 2006).

1.4.2 Real-time 3-D echocardiography

Real time three-dimensional (3D) echocardiography (echo) is becoming increasingly important, because it allows more accurate image analysis, quantification and measurements to be made, therefore providing better clinical diagnosis (Bhan et al., 2010; Monaghan, 2006). In addition, 3D echo allows detailed morphological and dimensional datasets to be obtained in just four consecutive cardiac cycles. This has been achieved by development of full

matrix array transducers (e.g. X34, Philips medical system), which use 3-4000 elements to facilitate 3D imaging at a frame rate of 20-25 MHz (Badano et al., 2007; Bhan et al., 2008; Monaghan, 2006). Indeed, the techniques' ability to carry out repeated non-invasive assessments of left ventricular shape, size and function aids clinical cardiologists in following up patients affected by heart disease.

Multiple studies comparing established and novel echocardiographic techniques have shown a significant relationship between 3D echo volumes, cardiac magnetic resonance (clinical gold standard) and post-mortem studies (Brown et al., 2010). For instance, in multicenter study conducted at four different institutions accuracy of real time 3D left ventricular volumes compared to cardiac magnetic resonance in 92 patients found a high correlation between left ventricular volumes and cardiac magnetic resonance (end-diastolic volume, r =0.91; end-systolic volume, r =0.93) (Mor-Avi et al., 2008). In another study, real time 3D, 2D and M-mode echocardiography comparisons with postmortem examinations in 28 sheep and 27 patients found the highest correlations with 3D mass as compared to 2D and M-mode techniques (r =0.92; r =0.69; r =0.77) (Qin et al., 2005). Volumes derived from 3D datasets can provide further information regarding the sphericity index. Most cases involving cardiac remodelling result in the heart becoming less elliptical and more spherical thereby reducing its ability to contract efficiently. 3D derived sphericity indexes have been shown to be an accurate predictor of remodelling following myocardial infarction (Monaghan, 2006).

1.4.3 Assessment of right ventricular function

In relation to the left ventricle, the right ventricle is located anteriorly in the thoracic cavity (Horton et al., 2009). Anatomically, this chamber can be divided into three parts, the inflow, the infudibulum (outflow) and the apex (Ho and Nihoyannopoulos, 2006; Horton et al., 2009). Therefore it is not surprising that right ventricular haemodynamics differ from that of the left heart (Biondi et al., 1988). Right heart function is dependent on ventricular impendence from the pulmonary circulation and extracardiac pressures. Qualitative assessments of the right ventricle can be made by two/three dimensional echocardiography, magnetic resonance imaging and invasive pressure-volume loops (Bleeker et al., 2006). Although there have been significant improvements in cardiac imaging, evaluation of the right haemodynamics and function can be challenging simply because the right ventricle has a complex shape, is heavily trabeculated and also sits directly behind the sternum (Bleeker et al., 2006; Haddad et al., 2008).

Cardiac magnetic resonance imaging (MRI) is regarded as a gold standard tool for assessing right ventricular volumes, flow velocities, presence of scarring tissue and function (Haddad et al., 2008). However, despite enhanced image quality and excellent reproducibility, MRI has a few limitations, such as the time taken to acquire and analyse data and excludes patients with pacemaker insitu.

Cardiac catheterisation provides direct measurement of haemodynamic data and accurate indexes such as pulmonary vascular resistance can be obtained.

Right heart cathetherisation also proves attractive because of its ability to quantify determinants of right ventricular function through pressure-volume loops (Bleeker et al., 2006). Furthermore, important information regarding right ventricular compliance, volumes, stroke work and function can be retrieved from these loops.

Traditional 2D echocardiography is the most widely used as an imaging modality. Images of the right ventricle are acquired in the three standard views namely, parasternal, apical and subcostal windows (Teske et al., 2008). However, quantitative assessments of RV volume and function using this modality can be cumbersome and limiting as the RV free wall is not fully visualized (Kaul et al., 1984). Rushmer *et al* first demonstrated that RV shortening occurs along its longitudinal axis, from base to apex with more pronounced shortening occurring in the apical region, unlike the LV which shortens relatively symmetrically in the longitudinal and transverse axes (Rushmer et al., 1953).

Tricuspid annular plane systolic excursion (TAPSE) is a simple echo-derived technique that measures right ventricular function by visually displaying M-mode motion of the triscuspid valve from base to apex throughout the entire cardiac cycle. The normal values of TAPSE obtained from M-mode echocardiography are between 2.4 and 2.6 cm and there are no significant differences between different age or gender groups (Andre, 2012). Subsequent comparative studies of TAPSE measurements with gold standard radionuclide angiography have demonstrated a good correlation (r =-0.76) (Kaul et al.,

1984). In addition studies have demonstrated that a depressed TAPSE portends a poor prognosis in patients with pulmonary hypertension (Forfia et al., 2006; Galie et al., 2009). Other studies have also shown good correlation of TAPSE values with brain natriuretic peptide, an endogenous peptide used as a marker for prognosis and risk stratification in chronic heart failure patients (Troisi et al., 2008). A value of TAPSE < 1.4 cm has been shown to be an independent predictor of mortality in patients with left ventricular failure (Engstrom et al., 2010).

1.4.4 Load independent assessment of ventricular function

Assessment of ventricular contractile function plays an important part in clinical practice and many physiological investigations (Sodums et al., 1984). Studies conducted by Suga alongside many others such as Starling using excised frog and canine hearts have demonstrated just how complex the contractile state is and how contractility is influenced by external and internal variables such as diastolic volume, arterial blood pressure, heart rate and inotropic stimulation (Markwalder and Starling, 1914; Roy, 1879; Suga, 1969; Suga and Sagawa, 1974).

The Frank-Starling law of the heart states, “the energy of contraction is a function of the length of the muscle fibre” (Frank, 1959; Katz, 2002; Sarnoff and Berglund, 1954; Starling, 1918). This law is based on the length tension relationship of myocardial sarcomeres which was first investigated by a German physiologist Otto Frank who studied the effects of stretch on the contractile function of isolated frog hearts (Chapman, 1960; Frank, 1895). With the aorta

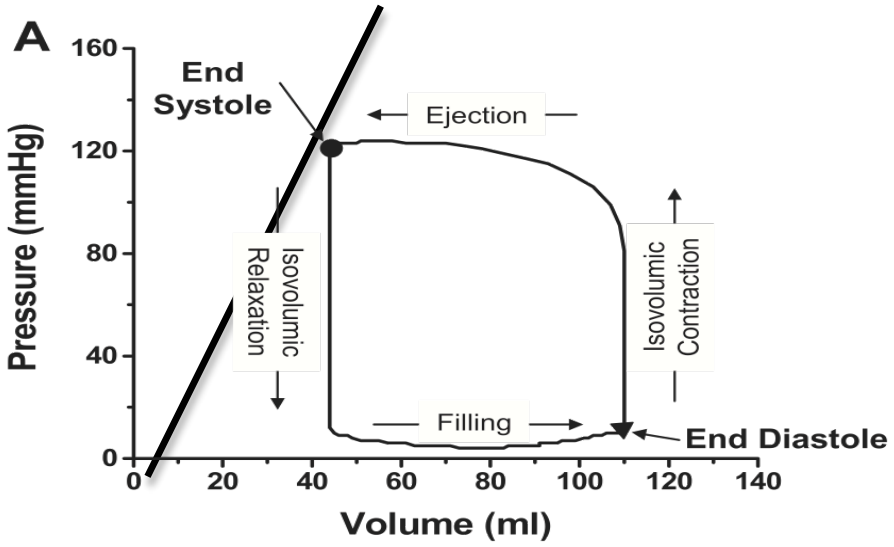
ligated, he injected fluid and observed that an increase in end-diastolic volume resulted in greater pressure being generated during systole. Indicating that myocardial tissue fibres energy for contraction depended on diastolic stretch. Further investigations by Ernest Starling in isolated canine hearts and lungs perfused with oxygen from a venous reservoir illustrated that diastolic stretch influenced stroke volume and that an increase in preload enhanced ventricular performance- a phenomenon called the Frank-Starling mechanism (Markwalder and Starling, 1914). In addition to preload, ventricular performance is also affected by afterload. The higher the afterload, the lower the stroke volume and ejection fraction.

Quantification of cardiac function independent of loading conditions has indeed been the goal of physiologists for nearly a century, but this remains challenging. Bearing in mind that there are large changes in loading in SCD due to increased circulating volume, it becomes clear why effects of SCD on the heart remains under debate. It is therefore important to examine contractile function using load independent techniques that are less sensitive to changes in preload or afterload. Clinical assessments of cardiac haemodynamics in vivo can be carried out with tools such as left ventricular pressure-volume tracings during heart catheterization or imaging tools such as echocardiography or MRI.

Invasive assessments of pressure-volume loops have been used for nearly 40 years to characterize in vivo cardiac performance in various pathophysiological disease states, drug therapies and many murine studies (Baan and Van der Velde, 1988; Burkhoff et al., 2005; Cingolani and Kass, 2011; Grieve et al.,

2004). This technique is certainly regarded as a more specific and reliable method. Since Frank Otto and colleagues' ventricular pressure relationships reports, a group by Suga and Sagawa in the 1970s and 80s demonstrated cardiac time pressure elastic behavior of the ventricle as a mechanical measure of contractile efficiency (Kass et al., 1986; Suga, 1990; Suga et al., 1982). The elastic model consisted of a schematic rectilinear end-systolic and end-diastolic pressure-volume relationship diagram reflecting four cardiac phases (ejection, isovolumic contraction, diastole and isovolumic relaxation) that are repeated during each contractile cycle. The relationship between left ventricular end-diastolic pressure and left ventricular end-diastolic volume corresponds to the passive stiffness of the left ventricle. The steeper the slope the stiffer the ventricle. Contractility or systolic function is represented by the slope of the end-systolic pressure volume relationship that connects end-systolic pressure with the x-axis volume intercept. The steeper the slope, the higher the contractility. Stroke work, the area under the pressure-volume curve increases with increased contractility. Although pressure-volume analysis may be regarded as a gold standard method for load-independent assessment of contractile function, it is limited by its invasive nature and the technical difficulties in measuring pressure and volume simultaneously. Therefore, non-invasive methods that are relatively load-independent are also sought.

Figure 1.3. Adapted from (Burkhoff et al., 2005) highlights the elastic properties of the ventricle. The elastance of the slope is a relationship between pressure and volume at any given time during systole normalized to the volume of chamber. The slope of end-systolic relationship intercepting volume on x-axis is a load independent measure of ventricular contractility.



1.4.5. Myocardial tagging and speckle tracking

An alternative approach to load-independent assessment of ventricular function is to assess regional myocardial deformation. This may be done by MRI or echocardiography. Magnetic resonance imaging, initially named nuclear magnetic resonance was first described by Nobel Prize winner Isidor Rabi in 1938 as a method for measuring molecular beams (Kolezhuk, 1996). Magnetic resonance imaging (MRI) has since expanded to be a powerful technique used universally to measure dynamics of molecules in solution and solid state in three dimensions. Clinical non-invasive MRI has proved to be informative in quantifying ventricular function and identifying the presence of regional wall motion abnormalities (de Roos et al., 1991; Moore et al., 1992). Cardiac MRI tagging is a technical approach applied to quantify cardiac myocardial radial

shortening motion in multiple directions by applying analysis tags superimposed on acquired MRI images as reference points (Moore et al., 2000). Conventional analyses of tagged images allow for load independent measurements of ventricular torsion to be made (Hansen et al., 1991).

MRI is an excellent method but maybe limited by its availability. Echocardiography is a simple and inexpensive non-invasive method for quantifying function (Miyatake et al., 1995; Schiller et al., 1989). Tissue Doppler imaging (TDI) and strain rate echocardiography are relatively new techniques that are constantly changing and improving to detect ventricular wall motion (Pellerin et al., 2003; Price et al., 2000). These predominantly research techniques offer objective means of quantifying regional and global ventricular function and may be relatively load-independent.

Tissue Doppler unlike conventional Doppler uses relatively low amplitude signals (40dB) to depict frequency shift in myocardial wall motion than signal amplitude reflected from red blood cells (Price et al., 2000). Tissue Doppler imaging (TDI) was first proposed by Isaaz and colleagues in 1989, however the technique at the time was limited by visualization of only the posterior wall (Isaaz et al., 1989). These days TDI uses three modalities; spectral pulse wave Doppler, B-mode imaging and M-mode colour Doppler and quantifies myocardial motion in multiple segments of the heart (Pellerin et al., 2003; Price et al., 2000; Sutherland et al., 1994). As a technique, TDI relies heavily on correct angle placement of the imaging cursor to myocardial wall segments. Misalignments of sample volumes to myocardial tissue motion direction can

lead to underestimation/overestimation of wall velocities leading to poor reproducibility of data. Although technological advances now allow angle adjustments to be made, TDI angle dependency remains a challenge, as contraction of cross sectional myocardial motion cannot be measured accurately. This drawback in TDI has led to the more recent development of strain rate/speckle tracking imaging.

Speckle tracking echocardiography (STE) is a more recent, non-invasive method for measuring relatively load-independent LV deformation both globally and regionally (Kim et al., 2009; Notomi et al., 2005a; Nottin et al., 2009; Sengupta et al., 2008; Wang et al., 2007). STE is conceptually similar to myocardial tagging in MRI. STE has opened new avenues as it quantifies characteristic speckle patterns created by deflected ultrasound beams from the myocardium. Speckle tracking is done on grayscale two-dimensional images and essentially tracks the displacement of segment points by calculating the change in length between two endpoints. STE has added benefits, it is angle and load independent, enabling better assessment of longitudinal, circumferential and radial cardiac muscle function. More so, STE's accuracy has been validated with gold standard techniques such as tissue tagging cardiac magnetic resonance imaging and invasive sonomicrometry (Notomi et al., 2005b; Wang et al., 2007). There are no reported studies to date documenting LV strain in SCD hearts.

1.5 Clinical assessment of endothelial function

Endothelial dysfunction, including reduced nitric oxide release is a hallmark of

many diseases states such as athesclerosis, pulmonary hypertension, cerebral ischaemia, hypercholesterolemia and diabetes (Benjamin et al., 2004; Katz et al., 2005; Lefer et al., 2001; Patti et al., 2005; Verma et al., 2003; Wolff et al., 2007). Endothelial-dependent dilatation in coronary arteries of healthy individuals regulates vasomotor tone, however as pathophysiological damage progresses vasoconstriction becomes more prominent (Davignon and Ganz, 2004). It is now possible to assess multiple functional properties of the endothelium, namely; vasomotor tone, vascular wall inflammation and coagulation (Davignon and Ganz, 2004; Drexler, 1997). There are several techniques available to investigate endothelial function e.g. magnetic resonance (MRI), coronary angiography, forearm plethysmography and flow mediated dilatation (FMD) (Pries et al., 2008).

Ludmer and colleagues (1986) were the first to describe an 'endothelial function test' in coronary arteries of patients (Ludmer et al., 1986). Their study of dose dependent responses to acetylcholine and nitroglycerin in eight patients with advanced coronary artery disease was able to demonstrate vasodilatation to acetylcholine in coronary arteries of normal subjects and selective vasoconstriction in patients with atherosclerosis. In 1995, Quyyumi *et al* further investigated effects of L-NG monomethyl arginine (L-LMMA) after administration of acetylcholine in 32 patients with angiographically normal coronary arteries. With this experiment, the group was able to confirm that the impaired response to acetylcholine was due to reduced bioavailability of endothelial-derived NO in the coronary circulation. Since these findings, coronary endothelial dysfunction has been demonstrated as a progressive vasoactive process in

hypercholesterolemia patients (Drexler and Zeiher, 1991). Currently, this technique is used to evaluate vasomotor tone by measuring changes in coronary artery diameter and can quantitatively assess the functional status of the coronary microvasculature using intracoronary Doppler ultrasound to measure blood flow while employing vasodilatator agents such as acetylcholine, bradykinin, substance P or serotonin (Celermajer, 1997; Drexler, 1999; Ludmer et al., 1986; Melikian et al., 2007). Although this technique is very useful, it is inappropriate as a screening tool for conducting basic clinical research especially in asymptomatic subjects as it is invasive and therefore carries a small risk of adverse events.

1.5.1 Flow mediated dilatation

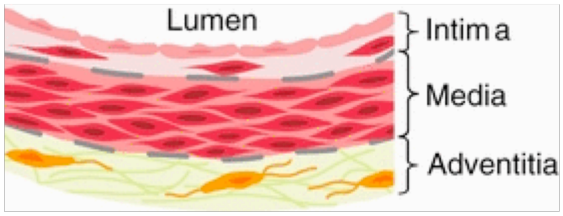
Celermajer and colleagues in 1992, were the first group to devise a non-invasive method for testing endothelial function using high-resolution ultrasound imaging in the femoral and brachial arteries of human subjects (Celermajer et al., 1992). Their group was able to demonstrate that hyperaemia caused after release of a temporary mechanical occlusion of large conduit arteries with a pneumatic tourniquet led to increased shear stress and flow-mediated dilatation (FMD) of the artery. In their report, impaired FMD was observed among smokers, patients with established coronary artery disease and children with familial chorlesterolaemia. Subsequent studies by Joannides et al in 1995 demonstrated that flow-mediated dilatation in conduit arteries was mediated by NO (Joannides et al., 1995). In their experiment, a catheter infusing the NOS inhibitor N^{G}-L-arginine placed in the brachial arteries of sixteen healthy volunteers resulted in blunted FMD confirming involvement of NO pathway.

Precise mechanisms underlying flow mediated dilatation remain unknown, although its magnitude is believed to vary depending on vessel size, vascular bed, and species (Miura et al., 2001). One mechanism surrounding initial detection of shear stress involves calcium-activated potassium channels in the endothelial cell membrane (Cooke et al., 1991; Olesen et al., 1988). Indeed, non-invasive FMD as a technique has revolutionized the perceived clinical role that vascular endothelium plays in many disease states.

1.5.2 Carotid anatomy and assessment

The arterial wall consists of interconnected cellular and extracellular components that conform three distinct layers; namely an intima layer made of a basal layer of endothelial cells, a media layer with an elastic smooth muscle lamina and an adventitia layer composed of collagen bundles, nerve fibre and fibroblasts (Martinez-Lemus; Wikstrand, 2007). Investigations into the progression of atherosclerosis suggest complex arterial media changes that are likely to precede appearance of atherosclerotic lesions (Salonen and Salonen, 1993).

Figure 1.4. adapted from (Wikstrand, 2007) illustrates the three different anatomical layers of an arterial wall.



Pignoli and colleagues were first to describe the use of carotid ultrasound as a non-invasive tool for identifying vascular disease and evaluating cardiovascular risk factors (Pignoli et al., 1986). In their assessments of carotid intima and medial walls, characteristic posterior wall ultrasound measurements showed good correlation with microscopic pathologic segments. Since then carotid artery intima-media ultrasound imaging has exploded as a clinical research technique in assessing early cardiovascular disease and risk of stroke (Simon et al., 2010). Carotid B-mode ultrasound provides useful information regarding vascular adaptation such as lumen diameter, intima-media thickness and presence and extent of plaques (Blankenhorn et al., 1993; Bots et al., 1997; Crouse et al., 1995; Cuspidi et al., 2009; Wang et al., 2006). In an intervention lipid-lowering trial, carotid intima media thickness in subjects treated with drugs showed significant progressive reduction correlating to angiographic stenosis (Blankenhorn et al., 1993). In another trial, an angiotensin II receptor blocker was found to have blunting effects on progression of intima-media thickness in the carotid artery (Cuspidi et al., 2009). Whether carotid intima-media thickness is altered in SCD patients remains unknown.

1.6 Summary

Although it is generally agreed that SCD causes compensatory demands on the heart and that cardiac enlargement is a common feature in most patients, there still remains a great deal of controversy surrounding mechanisms involved and degree of ventricular impairment. Indeed, reported studies have used traditional imaging modalities such as M-Mode, 2D and Doppler echocardiography that are limited by loading conditions.

The exact prevalence and mechanisms of pulmonary hypertension in SCD remain unclear. Traditional non-invasive method for diagnosing pulmonary hypertension using tricuspid regurgitation jet velocity method suggests a prevalence of up to 30%. However, this method assumes that TR is a good non-invasive estimate of pulmonary hypertension in patients with SCD.

Endothelial dysfunction may be a general abnormality that contributes to many if not most of the complications seen in SCD. Endothelial dysfunction in this patient group may be present from a very early stage and could possibly vary from patient to patient independent of the underlying haemoglobinopathy. The presence of endothelial dysfunction in SCD patients independent of conventional risk factors for cardiopulmonary diseases has not been studied well.

1.7 AIMS OF THESIS

The aims of this thesis are threefold:

i) To characterise in detail, the global and regional functional changes occurring within the heart of stable homozygous SCD patients.

ii) To evaluate the prevalence of PH in SCD patients and assess underlying mechanisms using echocardiography and right heart catheterisation.

iii) To determine whether abnormal endothelial dysfunction exists in stable SCD adolescents independent of risk factors such as age, blood pressure, smoking or hypercholesterolemia.

Chapter 2

General methodology

2.1 Patient recruitment and study design

All work described in this thesis was supported by the National Institute of Health Research (NIHR) Centre as a comprehensive Biomedical Research Centre award to Guys and St Thomas Trust NHS Foundation in partnership with Kings College London and Kings College NHS Foundation Trust. The study protocols were approved by the ethics committee (REC references 08/H0808/196 and 09/H0808/20) at King's College Hospital, London and written consent obtained from all participants prior to recruitment.

Patients with an electrophoresis diagnosis of Sickle Cell disease were recruited from the Haematology Outpatient Department during their routine clinic visits that are held once every week. Sickle cell disease variances in the study population were as follows: HbSS, HbS_{Thal}, HbSC and $HbSC_{Thal}$. Depending on their schedule, patients either had their echocardiography or FMD scans performed on the day of their clinic visit or were invited to have investigations at a time most convenient to them.

Patient inclusion criteria: An unselected group of male or female patients with a diagnosis of SCD. Subjects with sickle cell haemoglobinopathy and at steady state were eligible to take part in the observational studies.

Patient exclusion criteria: Patients with a cardiac disorder known to be caused by an alternative diagnosis.

Healthy volunteers: A group of 30 healthy control subjects who had no known

medical conditions and were not on regular medication were recruited from the general public. Healthy volunteers were recruited through general advertisement, friends and relatives of SCD patients.

People of similar geographic ancestry share similar mutations in their genes (Patel and Ye, 2011; Winkelmann and Hager, 2000). There is increasing evidence to suggest that race/ethnic genetic variances may play an important role in determining cardiovascular risk factors such as ventricular hypertrophy, systemic blood pressure, endothelial function as well as coronary artery disease (Cooper et al., 2000; Mensah et al., 2005; Winkelmann and Hager, 2000). In addition, previous investigations report variations in "normal" left ventricular parameters such as mass and ventricular volumes in different ethnic groups (Natori et al., 2006). One study by Zabalgoitia reported differences in left ventricular concentric hypertrophy in 3 different races, namely Caucasians, African Americans and Hispanics (Zabalgoitia et al., 1998). In another study, echocardiography measurements of left ventricular mass and wall thickness found left ventricular hypertrophy to be predominant amongst African Americans than Caucasian groups with known systemic hypertension (Koren et al., 1993). For this reason, it was fundamental that controls from the same ethnic/race background were used so that data obtained reflected 'normal' parameters for the specific study population group.

Exclusion criteria: Participants with SCD trait or cardiac abnormalities such as significant valvular disease on echocardiogram were excluded from the study.

Furthermore, participants were excluded from the study if it was not possible to obtain adequate echocardiography images.

2.2 Anthropomorphic and baseline characteristics

Each subject had their anthropomorphic and baseline characteristics recorded immediately prior to a study. Height (m) and weight (kg) measurements in each participant was measured in accordance to previously published guidelines (Power et al., 1997). Standing height was measured using a wall-mounted Harpenden standiometer (Holtain Ltd, Dyfed, UK), with a resolution of 0.1 cm. The patient/subject was asked to remove any footwear and stand with heels, back and head against the wall. The chin was lifted so the line of sight was perpendicular to the thorax. Once correctly positioned, the carriage was lowered to the top of the subject's head. The weight was measured using a set of electronic scales (Seca, Hamburg, GmbH). The subject was asked to sit on the scale with feet resting on the footrest. Once stable, weight was recorded to the nearest 0.05 Kg. Body surface area- BSA (m^2) was derived from ([Height (cm) x Weight (kg)]/3600)$^{1/2}$ equation.

All participants had their systolic and diastolic pressure measured according to previously published guidelines (O'Brien et al., 2003). Blood pressure readings were taken in a quiet, calm environment in a supine position after resting for 20 minutes. The blood pressure cuff was wrapped so that the cuff covered 80% of the upper arm, at midpoint between the shoulder and elbow.

Oxygen saturation was measured using a pulse oximeter (Welch Allyn medical,

NY, USA) in accordance with standard guidelines (Severinghaus and Koh, 1990). A 30 second self-testing period was left for full detection of waveforms before displayed values were recorded. The accuracy of detection of heart rates by the pulse oximeter was compared with heart rate obtained by ECG tracing.

2.3 Assessment of laboratory markers

Blood results from the patients' most recent outpatient clinic visit were obtained from the hospital database nearest to the day of scan. Control subjects' blood samples were collected after recording anthropomorphic and baseline characteristics. Samples were analysed immediately in the hospital biochemistry laboratory at King's College Hospital. All blood samples were processed by hospital technicians blinded to the identity of subjects.

2.3.1 Haemoglobin variant of SCD

All variations of SCD (SS, SC, Sβ thalassemia) were determined by haemoglobin electrophoresis in the biochemistry laboratory and results retrieved from the hospital biochemistry database.

2.3.2 Markers of haemolysis

Lactate dehydrogenase (LDH - a widespread cytosolic enzyme), reticulocyte count, aspartate transaminase and bilirubin were obtained from the most recent electronic biochemistry database.

2.3.3 Markers of systemic inflammation

C-reactive protein (CRP) is a protein found in blood during periods of

inflammation. The assay is a highly sensitive test, commonly used clinically to predict cardiovascular events (Sattar et al., 2007). CRP was measured on blood samples using high-sensitivity turbidmetric immunoassay (WAKO chemicals, Neuss, Germany) on the Cobas Mira Analyser (Roche Diagnostics, Lees, UK) in the hospital biochemistry laboratory.

2.3.4 Markers of cardiac dysfunction

Markers such as brain natriuretic peptide (BNP), troponin I and T are sensitive indicators of cardiac tissue injury. As a biologically active peptide consisting of 32 amino acids, BNP has both vasodilator and natriuretic properties (Hobbs et al., 2002). BNP is often released from ventricular myocytes in response to stretching of the chamber due to volume expansion and pressure overload. BNP can give useful information for differentiating causes of breathlessness due to heart failure from other causes such as age, renal failure or comorbid illnesses (Felker et al., 2006). Blood samples for BNP were collected from consenting participants and stored in our biochemistry laboratory for future analysis.

There are three different troponins, namely: troponin C, troponin T and troponin I. Cardiac troponins T and I are protein components of striated muscle with different sensitivity levels for cardiac injury. These tend to be release following cardiac damage such as myocardial infarction or angina. Although sensitive, absence in troponin does not exclude diagnosis of heart disease. Both troponin T and I maybe elevated in patients with chronic renal failure however, this increase in troponin due to renal complications levels remain high during serial

measurements (Bodi et al., 2002; Ebell et al., 2000).

2.4 Cardiac assessments

2.4.1 Electrocardiogram (ECG)

A standard 12 lead ECG examination was performed during quiet respiration in a supine position using a Marquette Hellige (Milwaukee, Wisconsin, USA) ECG recorder. The electrodes were carefully placed on precordial and limb locations and ECGs recorded at paper speed of 25 mm/s. Measurement of ECG PR interval, QRS duration, QT interval, QRS axis, Q,R,S, and T wave voltages including ST segments were made in lead V1, V2, V5 and V6. P wave voltage was measured in lead II alone.

2.4.2 Echocardiography studies

Echocardiographic digital images were obtained using a Philips iE33 ultrasound system (Philips Medical Systems, Best, the Netherlands). Patients were scanned in the left decubitus position. Once comfortable, ultrasound gel (Medgraphics, UK) was applied to the ultrasound probe prior to placing the probe on patient's chest.

2.4.3 Two-dimensional echo

Detailed 2-dimensional transthoracic echocardiography (2D) studies were performed in accordance with the guidelines set by the European Association of Echocardiography (Evangelista et al., 2008). Conventional 2D and Doppler studies were captured using an S5-1 transducer. All 2D images were initially obtained from the parasternal long axis and M-mode recordings of the LV taken from real time 2D images in the parasternal long axis view at the level of the mitral valve leaflets. Further images of the LV and right ventricle (RV) were

obtained in the parasternal short axis view, apical four-chamber view, apical two-chamber view and apical long axis view. Images were optimized by adjusting frame rate and temporal resolution and also by zooming in on regions of interest. Digital 2D, colour Doppler and spectral Doppler images acquired with a transthoracic 2D transducer were stored onto a dedicated hospital database and data analysis performed off-line using Xlera analysis software (Philips).

2.4.4 Doppler techniques

Aortic, mitral, tricuspid and pulmonary valve flows were assessed by colour flow Doppler. Flow velocity was measured using pulse wave and continuous wave Doppler. Mitral flows were obtained in the apical four chamber view at the tips of the mitral valve. Mitral flow velocity scale was reduced to allow for a clear velocity time blood flow display. The mitral flows were all biphasic with the first peak ‘E’ wave representing early filling and ‘A’ wave presenting late diastolic filling. Diastolic function of the LV was assessed by measuring pulsed spectral Doppler transmitral E and A wave velocities, enabling calculations of transmitral E/A ratio and E wave deceleration time. Tricuspid flows were clearly seen in the parasternal short axis view at the tips of the tricuspid valve. Like the mitral Doppler flow pattern, biphasic filling was present in all subjects.

Continuous Doppler was used to capture aortic forward flows in the five chamber view. Left ventricular outflow Doppler profiles were acquired in the five chamber view 5mm below the tips of the aortic valve. To avoid sampling aortic forward flows, pulse wave Doppler was used. Continuous wave Doppler was

used for pulmonary valve flows, which were clearer in the parasternal short axis view. Guided pulse wave Doppler flows of the right ventricular outflow tract were taken in the parasternal short axis view just below the pulmonary valve.

Optimized tricuspid regurgitation (TR) spectral Doppler signals were assessed in the parasternal long axis, parasternal short axis, apical and subcostal views. Pulmonary hypertension was suspected when peak end systolic tricuspid regurgitant jet velocity (TRV) was $\geq$ 2.5 m/s.

Tissue Doppler echocardiography is a modality that detects shifts in ultrasound frequency from low-velocity motion signals of myocardial tissue (Ho and Solomon, 2006). Therefore, less load dependent diastolic function was assessed using tissue Doppler imaging (TDI) (Sohn et al., 1997). Pulse wave TDI was performed by activating TDI function on the machine. Sample volumes were obtained from the right ventricular free wall, left ventricular septal and lateral walls at the level of the tricuspid and mitral valve annulus. Left ventricular diastolic function assessments by tissue Doppler were assessed as a ratio of (E/E') early diastolic transmitral waveforms (E) and TDI of the lateral wall (E'). Right ventricular tissue Doppler assessments were taken as the ratio of tricuspid valve E wave and RV free wall E' Doppler profile.

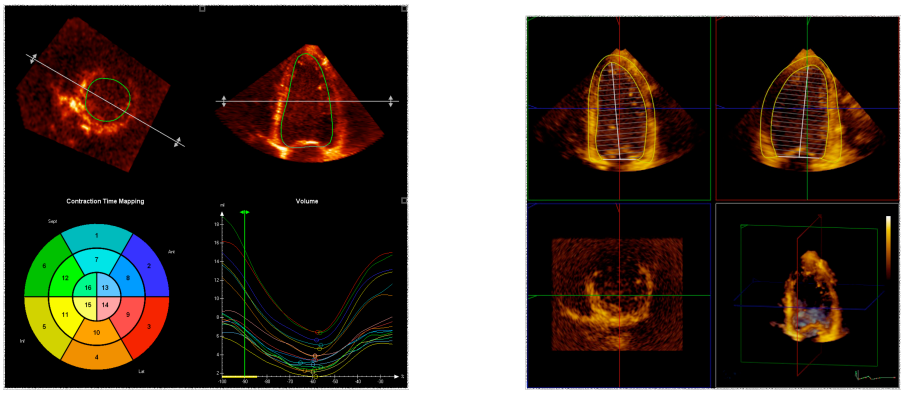
2.4.5 Three-dimensional echo

Real time 3-dimensional echocardiography (RT3DE) was used for all calculations of left ventricular (LV) volumes, ejection fraction and mass. A fully sampled matrix array transducer (x4) was used to acquire digital datasets

during a single breathhold. All LV dataset analyses were performed off-line using LV analysis software (TomTec Imaging Systems, Unterschleisheim, Germany and Research Arena imaging systems). LV endocardial contours were traced semi-automatically, excluding any trabeculation within the LV cavity and papillary muscle. Comparative qualitative and quantitative differences were performed by blinded independent investigators (one technician and one clinician) based in the Echocardiology department.

Figure 2.1. (a) TomTech research arena 3-D volume datasets of LV volumes showing SAX view, four chamber view, sixteen segment model and time volume curve (**b**) Philips QLAB 3-D full volume datasets for mass quantification. Apical four chamber view and two chamber view, SAX and left ventricle four chamber cast.

(a) **(b)**

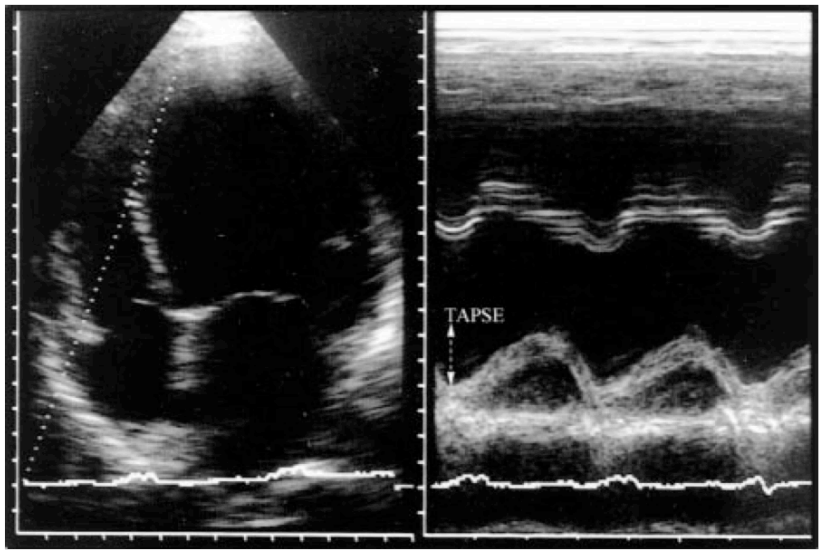


2.4.6 Tricuspid annular plane systolic excursion (TAPSE)

The detailed principles of TAPSE are covered in the introduction. Right ventricular systolic function by TAPSE was determined in the four chamber view, with the M-mode cursor carefully placed at the junction of the tricuspid valve plane in the RV free wall. Echoes generated were received and registered

as longitudinal motion of the RV base. The maximal TAPSE was determined by total excursion of the tricuspid annulus obtained from M-mode tracings. All tracings were analysed as averages of three beats.

Figure 2.2. Left panel: apical four chamber view with the m-mode cursor positioned at the lateral portion of the tricuspid annulus. Right panel: M-mode recording of the TAPSE from tricuspid annulus.



2.4.7 Speckle tracking

Speckle tracking is primarily based on the interference/ scatter of ultrasound resulting in irregular 'fingerprint' speckled patterns acquired from specific regions of interest (Marwick et al., 2009; Pavlopoulos and Nihoyannopoulos, 2008). Speckled ultrasound patterns from interest regions are automatically displayed on a visual time/velocity graph highlighting the motion of the myocardium throughout the cardiac cycle.

For global two-dimensional strain analysis, digital loops consisting of five cardiac cycles were acquired from the parasternal short-axis view at the level of

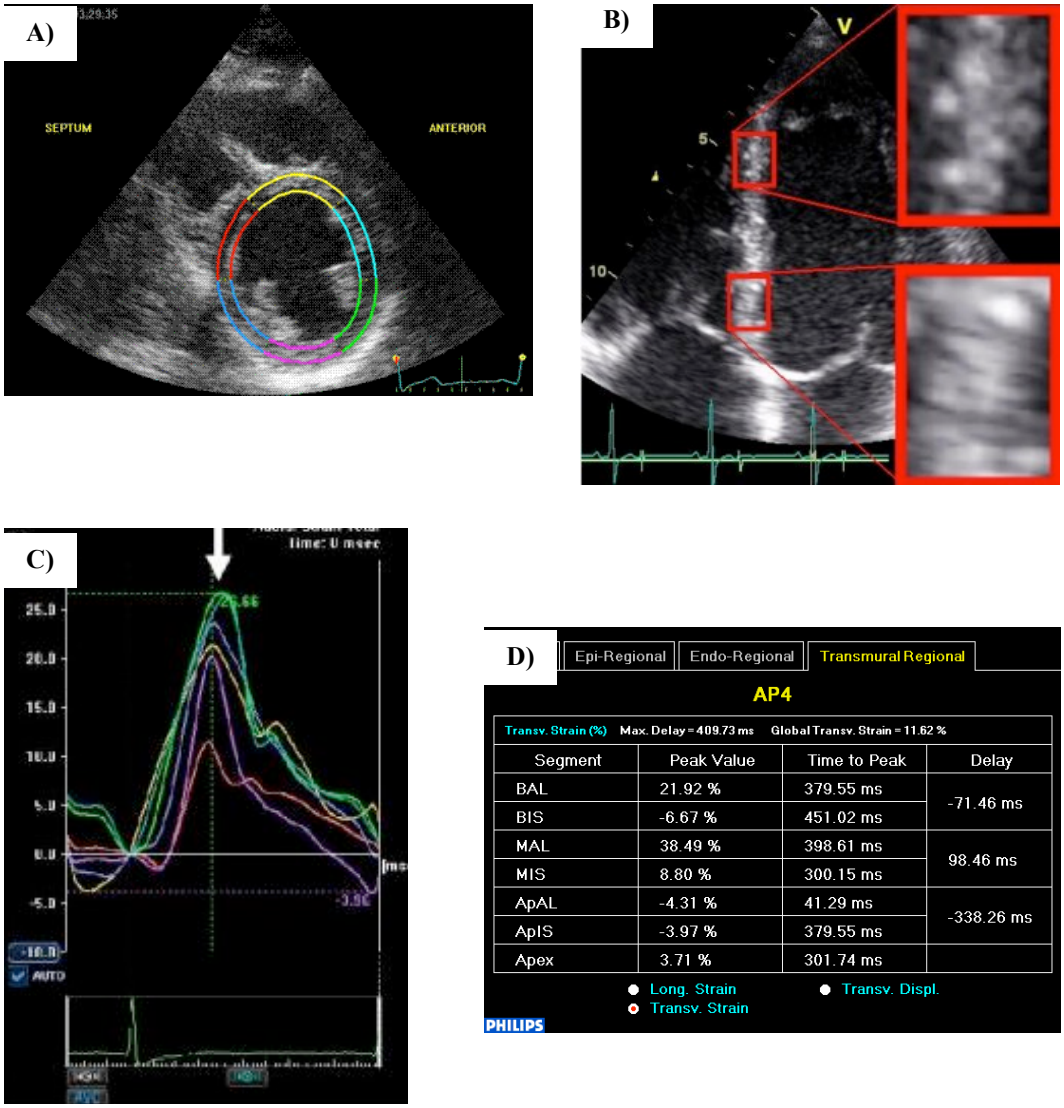
the mid-papillary muscle as well as in the apical four-chamber view. A high frame rate i.e. >200 fps was used to acquire these digital images. Data analysis was performed offline using software that allowed for semi-automated analysis of ventricular dynamics (Philips Q-lab). Endomyocardial radial strain and strain rate were calculated in the anterior, posterior, inferior and lateral segments of the LV in both short and long axis views. The region of interest (ROI) was placed in the myocardial muscle, avoiding any tissue velocity aliasing and blood pool data. The ROI position was adjusted manually when the box swerved away from the myocardium. Peak systolic strain was defined as the negative value on the strain curve during the entire cardiac cycle. Strain rate was averaged in parasternal short axis, apical four-chamber and apical two chamber views. Averaged data consisted of an average of three cardiac cycles.

The equation adapted for strain-rate is shown below:

$$\text{Strain rate (\%)} = \frac{V_1 - V_2}{d} \times 100$$

Where V_1 represents the segmental end-diastolic myocardial tissue velocity deformation and V_2 the end-systolic myocardial tissue velocity deformation divided by the distance travelled between the two points (V_1 and V_2) in the chosen ROI (Sutherland et al., 2004).

Figure 2-3. Modified A) Cross sectional view of the left ventricle in parasternal short axis view B) four chamber views of the left ventricle highlighting regions of interest on 2D gray scale images. The bottom panels show time velocity myocardial motion displays and regional strain data in a SCD patient.



2.5 Pulmonary function tests

2.5.1 Lung volume measurements

All pulmonary function tests were performed in accordance with the American Thoracic Society guidelines (Laszlo, 2006) in the hospital lung function laboratory. Total lung capacity (TLC_{pleth}), functional residual capacity (FRC_{pleth}), vital capacity (VC_{pleth}) and residual volume (RV_{pleth}) were assessed using a constant volume whole body plethysmograph (Morgan TLC, Morgan Medical, Rainham, UK). Each participant was instructed to sit in the plethysmograph box and an equilibration period (2 minutes) was allowed prior to test for changes in box temperature and pressure. The subject was initially instructed to inspire normally through a mouthpiece attached to the equipment. Panting manoeuvres were initially performed and measurements of mouth/box pressure recorded accordingly. The shutter valve was subsequently closed and five panting breaths were performed before releasing of shutter. The subject was instructed to expire to residual volume then inspire to TLC. Lung volume measurements were repeated twice and the mean of two results within 5% of each other were recorded. All lung volumes were corrected for body temperature, pressure and water vapour saturated conditions.

2.5.2 Gas transfer

Total lung gas transfer of inhaled carbon monoxide (DL_{CO}), gas transfer per unit lung volume (K_{CO}), alveolar volume (VA) and vital capacity (VC_{SB}) were recorded during a single breath technique (Masterscreen PFT, Viasys Healthcare, UK). The subject, while wearing a nose-clip and seated upright was asked to breath through the test equipment via a mouthpiece. The subject was

then asked to inspire maximally, expire maximally, followed by a maximal breath in. The test gas (5% helium, 0.28% carbon monoxide, 19% oxygen and balanced nitrogen) was automatically introduced to the system at the beginning of the second maximal inspiration manouver. The subject was asked to hold their breath for ten seconds while the pneumotchograph shutter connected to mouthpiece automatically maintained a closed seal. After ten seconds, the shutter re-opened and the subject was instructed to expire to residual volume. The first 750 ml of expired air were discarded and regarded as anatomical dead space. The next 750 ml expired air were then collected for analysis. These measurements were performed twice and a mean of two values within 5% of each other recorded accordingly. Steady state haemoglobin levels (obtained at last outpatient clinic visit) were used to correct DL_{CO} and K_{CO} values.

2.5.3 Spirometry

Forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and peak expiratory flow (PEF) measurements were performed using a heated pneumotachograph (Jaeger Masterscreen PFT, Viasys Healthcare, UK). The FEV_1/FVC ratio was expressed as a percentage.

The technique was explained to all participants prior to procedure. While in the seated position and wearing a nose-clip, subjects were asked to breathe normally via a mouthpiece attached to a pneumotachograph. Subjects were allowed time to adjust to equipment set-up prior to conducting the test. Once relaxed, subjects were then instructed to inspire slowly to total lung capacity (TLC), followed by maximal forced expiration to residual volume, as hard and as

fast as possible to measure FEV_1, FVC and PEF. Real-time flow-volume and volume-time traces were obtained automatically and volume measurements calculated on JLAB (version 4.0) software. A minimum of three flow-volume loops results within 5% of each other were recorded and the highest FEV_1 flow-volume analysed. Any traces showing evidence of premature termination of expiration, leaking, slow rise to peak expiration or cough were rejected.

Restrictive lung pathology was diagnosed according to the reference ranges set by the American Thoracic Society (1991); restrictive pathology was indicated when TLC_{pleth} was below eight percent of the predicted normal values according to height. Obstructive lung pathology was indicated when FEV_1/FVC was below 81.4 percent (males) or 82.3 (females) and mixed obstruction/restriction indicated when both TLC and FEV_1/FVC were reduced.

2.5.4 Bronchodilator test

The airway bronchodilator test consisted of 200mcg of salbutamol administered via a spacer. A total period of twenty minutes activation time was allowed prior to re-testing FEV_1. Positive bronchodilator response was considered when a twelve percent increment in the forced expiratory volume rate was measured.

2.5.5 Airway reactivity

Bronchial hyper-reactivity was performed using a Turboaire Challenger system (Equilibrated Bio systems Inc., Melville, New York, USA). Inlet pressure at the Turboaire Challenger was maintained at 80PSI by means of a two stage variable regulator. All subjects were allowed to breath a cooled, -20°C gas

mixture (air with 5% CO2) via a mouthpiece connected to a two-way non re-breathing valve (Hans Rudolph Inc., Kansas City, Missouri, USA). Inspired air temperature was regulated on a digital thermometer (TAC49200 Equilibrated Bio Systems Inc., New York, USA) and real-time ventilation monitored by target ventilation meter displaced on digital monitor. Measurements of FEV1 as described in the spirometry section were recorded in 5 minutes intervals for a total of twenty minutes. The test was terminated and regarded positive when FEV1 measurements fell below 12% baseline readings.

Lung function results were corrected for patient height and ethnicity in accordance with the European Respiratory Society(Quanjer et al., 1993).

2.6 Computer tomography (CT) X-ray imaging

All high-resolution CT imaging was conducted on a dual detector helical CT scanner (HiSpeed NX/I, GE Medical systems, Milwaukee, WI, USA). Subjects were placed in both supine and prone positions. Digital images were captured during full inspiration. A high spatial-frequency (bone) algorithm was used to reconstruct all captured images and study images stored on CD for further analysis.

Extent/severity of lung pathology, i.e. presence of pulmonary hypertension, established lung disease or parenchyma were assessed blindly by two thoracic radiologists, unaware of echocardiographic and lung function test results.

2.7 Measurement of flow mediated dilatation

Peripheral endothelial function can be assessed by a technique known as flow-mediated dilatation (FMD). FMD is performed non-invasively using ultrasound-guided measurement of brachial artery diameter. It is designed to reflect endothelial-dependent relaxation of a conduit artery when exposed to an increase in blood flow (shear stress). This technique has been widely used as a research tool to quantify endothelial function, with particular focus on endothelial-derived relaxing factor or nitric oxide (Moens et al., 2005). In principle, FMD involves initial occlusion of venous outflow by inflating a pneumatic sleeve around the forearm to 250 mmHg for 5 min, then rapidly deflating it. The technique of FMD has played an important part in understanding vascular physiology, pathophysiology and pharmacology processes that occur in situ under the influence of hormonal, neuronal and locally controlled factors, such as room temperature. In healthy subjects FMD values range from 5 to 15% (De Roos et al., 2003), however, a reduction in flow-mediated dilatation is indicative of impaired endothelial function. FMD is a predictive measure of adverse cardiovascular risk factors (Brevetti et al., 2003).

2.7.1 Protocol for Forearm-mediated dilatation studies (FMD)

Studies took place in a quiet, temperature regulated room (22-26°C) with the participants quietly lying supine for 10 minutes prior to test. Resting blood pressure (in the left arm) and heart rate were recorded as described above. For support cushions were placed under the arm and wrist/hand of subjects. Three standard ECG electrodes were attached to the participants' chest and checks were made to ensure that ECG tracings were tracking clearly with no electrical

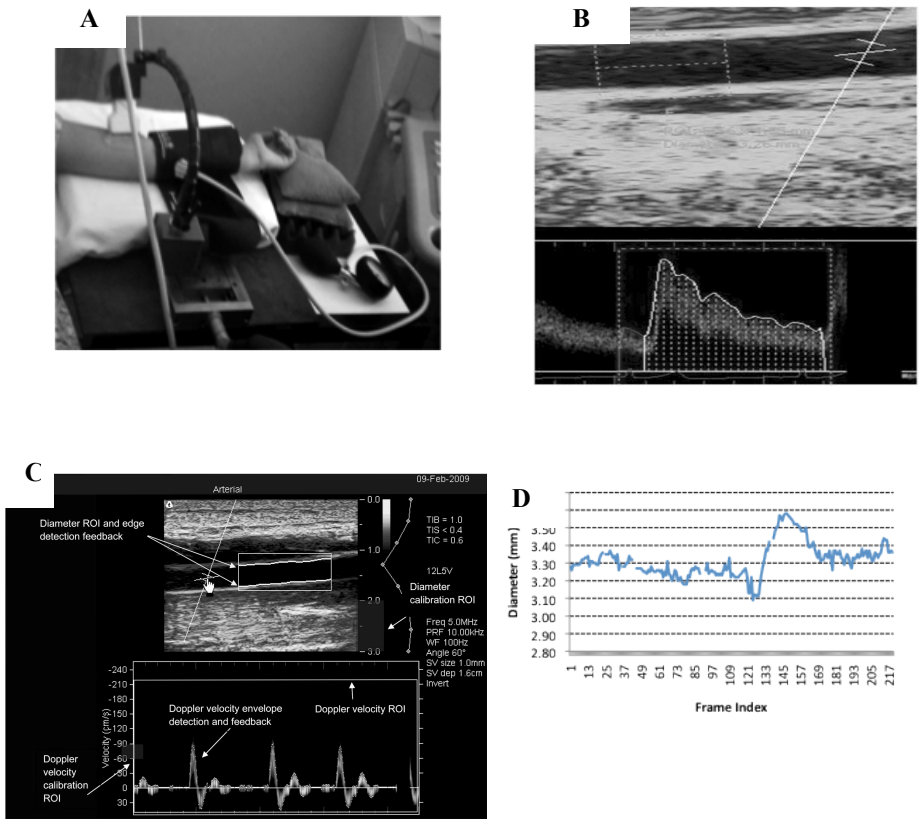
interference or muscle tremor. A paediatric pneumatic cuff (Welch Allyn DS 54, single tube sphygmomanometer) was then placed on the right arm, just below the elbow distal to the medial epicondyle as per established guidelines for assessing FMD (Corretti et al., 2002). The right arm was extended laterally to approximately 70-80° with the hand rotated such that the palm was facing up.

A 1cm wide strip of imaging gel was applied to the right arm 5cm above the elbow crease on the crown of the biscep muscle. A 6-11 MHz multi-frequency linear-array probe attached to a high resolution ultrasound machine (Aspen, Acuson, Mountain View, CA) anchored to a sterotactic clamp was carefully placed on top of the imaging gel and longitudinal two-dimensional images of the brachial artery optimized by micrometer adjustments. Brachial artery B-mode images were further optimized by adjusting dynamic range, time correction gains, frequency, focus number resolution and gain settings. The brachial artery image was enhanced by zooming into the region of interest.

A Pulse wave Doppler cursor was placed parallel to the vessel walls and Doppler angle set at 70°. The ECG trigger was switched on to ensure B-mode images were updated at the R-wave of the ECG (end diastole). The subject was informed that the test was about to begin and also encouraged to keep still throughout the imaging process. Baseline FMD recordings were captured one minute prior to cuff inflation. A count down was made and FMD cuff inflated to 250mmHg for a period of 5 minutes and images recorded accordingly. Post cuff inflation/hyperemia Doppler and B-mode images were recorded for another 5 minute before terminating test. Checks were made to ensure subjects were

feeling normal before leaving the examination room.

Figure 2.4. A) FMD study showing an ultrasound probe that was held in place by a sterotactic clamp and adjustments made using a micrometer **B)** B-mode image of brachial artery computer software generated Doppler tracing obtained from region of interest showing brachial flows during hyperemia **C)** ROI definition of near and far borders adapted from (Hopkins et al., 2009) **D)** Time velocity presentation of FMD study in compatible Excel spreadsheet.



2.7.2 Brachial diameter analysis

To avoid lengthy waiting times, analyses were performed off line after participants had left the examination room. All assessments were performed on Brachial Imager software (Medical Imaging Applications, Iowa City, IA). The

border detection software was initially calibrated to differentiate blood vessel properties such as lumen diameter and intima-media wall thickness. Initial calibration also enabled software to identify potential artifacts. The ROI was left in the same position throughout test to facilitate reproducibility. Although the initial selection of ROI was determined by the operator, all proceeding analysis of entire FMD study were carried out without investigator bias. All image sequence analysis information i.e. baseline/inflation/deflation were stored on a study file and later transferred onto a compatible excel spreadsheet. Measured frame-to-frame near (anterior) and far (posterior) walls were reflected in a graph.

The percent FMD was computed using the formula shown below.

$$\frac{\text{maximal diameter} - \text{baseline diameter}}{\text{baseline diameter}} \times 100$$

2.7.3 Reproducibility of FMD

The reproducibility of FMD was assessed by re-scanning 10 randomly selected subjects seven days after the initial scan. Correlations for repeated measurements of baseline diameter, maximum diameter and % FMD in subjects were between 0.78 and 0.9. Intra-observer and inter-observer correlation coefficients for % FMD was 0.72 and 0.89, further calculations by Bland-Altman analysis may be useful in determining accuracy of these measurements.

2.7.4 Brachial blood flow analysis

Using specific Doppler blood flow analysis menu, a ROI setting was chosen which allowed for definition flow pattern from the onset of R wave. The software was trained to detect the entire width and height (envelope) of the waveform in the ROI corresponding to R wave on ECG. Calibrations of velocity/time were set and automated analyzing initiated once software was able to accurately distinguish Doppler wave onset from ECG tracing. Subsequent flows were checked and transported onto Excel spreadsheet as described in FMD protocol.

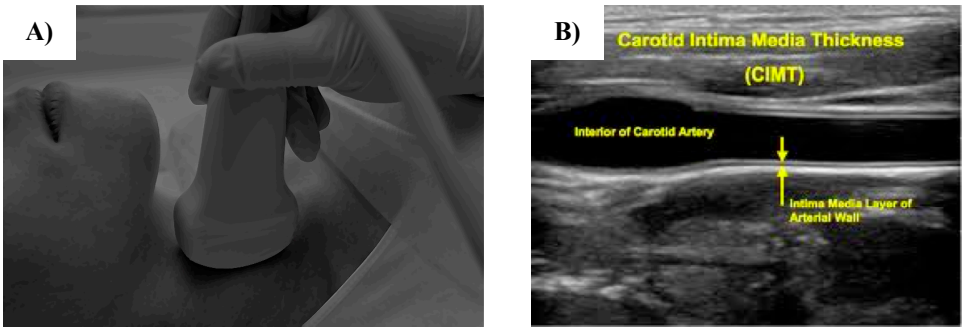
2.8. Common carotid artery imaging and analysis

Common carotid artery intima-media wall imaging using high resolution B-mode ultrasonography has emerged as a surrogate marker of cardiovascular disease events (Aminbakhsh and Mancini, 1999). Different non-invasive ultrasound modalities such as M-mode, B-mode (Roman et al., 2006) and echo-tracking derived distension waves (Van Bortel et al., 2001) have been used to measure carotid artery intima media thickness and diameter. In the current study, B-mode ultrasound was used to measure both the left and right common carotid intima-media.

Participants were examined immediately after FMD studies in the supine position. Initially, longitudinal and cross sectional images of common carotid artery were taken between the chin and shoulders to identify presence of atherosclerosis lesions and beginning (1cm)/ dilatation of the carotid bulb used as a reference point. The probe was then manipulated so that far wall intima-

media wall was parallel to the transducer footprint and that both the anterior/posterior walls were in focus. Five cardiac cycles triggered on the R wave of the QRS complex were acquired and analysis made off line using semi-automated computerized tracking software (Medical Imaging Applications, Iowa City, IA). Systolic and diastolic thickening of arterial wall measurements were calculated as the distance between the first bright line (lumen-intima interface) and leading edge of the second bright line (media-adventitia interface).

Figure 2.5 A) Imaging of common carotid artery **B)** B-mode digital image of carotid artery showing bulb and near/far walls.



2.9 Statistical analysis Comparisons of continuous variables in SCD patients and healthy controls were made using student *t-test* and data presented as mean ± SD. Non continuous variables were analysed using χ^2 or Mann-Whitney U test. Associations between the continuous variables were done by linear regression using Pearson Correlation coefficient. All p values <0.05 were considered statistically significant with Bonferroni correction for multiple testing. To identify factors independently associated with mechanisms responsible for occurrence of PHT in our patient cohort, a linear multivariable regression model was constructed based on a selection of demographic and clinical variables that

showed a good correlation on Pearson correlation analysis. Odds ratios (B) and 95% confidence intervals (CI) were calculated for covariates in models. Data analysis were performed using SPSS statistical software (version 17, SPPSS Inc).

All statistical analyses performed in this manuscript were carried out by myself. Initial help was sort and given by my supervisors, colleagues and statistical staff at King's College London.

Chapter 3

Characterisation of cardiac dysfunction in sickle cell disease

3.0 Introduction

Echocardiographic measures of left ventricular (LV) volume and systolic function provide meaningful predictors of prognosis in many cardiovascular disease states (Dujardin et al., 1999; Grayburn et al., 2005; Macron et al., 2010), making quantification of LV parameters imperative during clinical decision-making. Assessments of LV size and ejection fraction have been documented to predict outcomes in major clinical trials such as the vasodilators in heart failure trial (V-HeFT) (Cintron et al., 1993), the Studies Of Left Ventricular Dysfunction (SOLVD) (Quinones et al., 2000) and the Valsartan in Heart Failure Trial (Val-HeFT) (Wong et al., 2002).

Recent advances in echocardiography technology have led to better visualization of ventricular chambers than traditional two-dimensional (2D) methods that rely on geometrical assumptions. Owing to improved spatial and temporal resolution, including software tracking algorithms, three-dimensional (3D) echocardiography now provides accurate detection of endocardial borders allowing accuracy in estimation of left ventricular volumes and mass (Soliman et al., 2008; Sugeng et al., 2006). Furthermore, 3D echocardiography also allows for differentiation of pathophysiological disease states, thereby, identifying patients at risk of developing heart failure (De Castro et al., 2007).

If life expectancy is indeed increasing with improved medical management of SCD, it is of great importance to clearly understand and describe pathophysiological changes that occur in the heart.

The aims of this chapter were to characterise in detail, global and regional functional changes occurring within the heart of stable homozygous SCD patients. To achieve this, electrocardiography, standard transthoracic echo as well as novel 3-D and speckle tracking echocardiography imaging techniques were employed.

3.1 Methods

3.1.1 Recruitment criteria

Patient inclusion criteria:

For the main study, only a selected group of male and female patients with the homozygous diagnosis of SCD (HbSS) were enrolled. Subjects with sickle cell haemoglobinopathy with co-existing controlled systemic hypertension, diabetes and at steady state (clinically stable, not in crisis for the past 6 weeks) were eligible to take part in the observational studies.

Patient exclusion criteria: Patients with other SCD genotypes or HbSS patients with a cardiac disorder known to be caused by an alternative diagnosis.

Healthy volunteers: A group of 30 healthy control subjects who had no known medical conditions and were not on regular medication were recruited from the general public. Healthy volunteers were recruited through general advertisement, friends and relatives of SCD patients.

3.1.1 Echocardiography examination

Detailed 2D and 3D echocardiography examinations were performed as

described in general methods (chapter 2).

3.2 Results

3.2.1 Patients

The study protocol was approved by the ethics committee at King's College Hospital, London and written consent obtained from all participants prior to recruitment. Subjects with the homozygous sickle cell haemoglobinopathy and at steady state (not in crisis within last 4 weeks) were eligible to take part in the observational study; 81 patients were enrolled from April 2008 to October 2011. For comparison, thirty healthy volunteers matched for patients' age, gender and ethnic origin were enrolled from the general public.

3.2.2. Clinical characteristics

Table 3-1 depicts demographic and laboratory data of HbSS patients compared to thirty controls. The patient's mean age was 34 $\pm$12 years, mean BSA 1.7 g/m^2 of whom 54 (67%) were female. Patients and controls were similar at baseline in terms of age, gender and ethnic origin (table 3.1). However, HbSS had significantly lower body surface area ($P<0.001$) than control subjects. In addition, HbSS subjects had lower systolic and diastolic blood pressure ($P<0.05$) and oxygen saturation. No statistical differences were found in the heart rates.

Past medical history assessments showed significant differences, in that SCD patients had more A&E admissions than controls groups. SCD patients presented with history of acute chest syndrome, respiratory disease and

pulmonary hypertension. More patients had controlled systemic hypertension than controls. Significantly higher records of vasculopathy complications such as stroke, priapism and leg ulcers were also seen among patients. Furthermore SCD patients had more renal complications that accounted for high numbers of patients receiving transfusion therapy. No vasculopathy complications or previous medical history of acute chest syndrome, respiratory disease, systemic hypertension, liver disease, pulmonary embolism, stroke, renal disease, leg ulceration or priapism were documented in any of the control subjects. None of the controls were receiving hydroxyurea therapy or blood transfusion.

Table 3.1. Demographic and clinical characteristics of HbSS patients compared with control subjects matched for age, gender and ethnicity.

Patient characteristics	**Controls (n=30)**	**Patients (n=81)**	**P value**
SCD genotype			
Haemoglobin SS	-	81 (100)	
Sβthalassemia	-	-	
Haemoglobin SC	-	-	
Haemoglobin SC thalassemia	-	-	
Black Ethnic origin, no.(%)	30 (100)	81 (100)	
Age — yrs	38 ± 9	34 ±12	0.64
Female — no.(%)	18 (60)	54 (67)	0.7
BSA — g/m^2	2.0 ±0.2	1.7 ±1.2	<0.001
Blood pressure — mmHg			
Systolic	123 ±14	113 ±14	<0.02
Diastolic	76 ±22	66 ±18	<0.001
Mean arterial Pressure	92 ±11	82 ±10	<0.001
Oxygen saturation — (%)	98 ±2	95 ±5	<0.0001
Heart rate — bpm	72 ±12	77 ±11	0.86
>2 A&E visits in past 5 yrs — no.(%)	-	7 (7)	<0.0001
History of acute chest syndrome — no.(%)	-	8 (10)	<0.0001
History of respiratory disease — no. (%)	-	16 (22)	<0.0001
History of pulmonary embolism — no.(%)	-	7 (7)	<0.0001
Controlled systemic hypertension — no. (%)	-	8 (10)	<0.0001
History of liver disease — no.(%)	-	8 (10)	<0.0001
History of stroke — no.(%)	-	8 (10)	<0.0001
History of renal dysfunction — no.(%)	-	10 (12)	<0.0001
History of leg ulcers — no.(%)	-	9 (11)	<0.0001
History of priapism — no.(%)	-	7 (7)	<0.0001
Hydroxylurea therapy — no.(%)	-	15 (18)	<0.0001
Regular transfusion programme — no.(%)	-	14 (17)	<0.0001

Abbreviations: yrs, years; BSA, body surface area; BP, blood pressure.

Laboratory parameters are shown in Table 3.2. Comparisons of haemoglobin found significantly lower concentrations of haemoglobin in SCD patients versus controls (P<0.0001). The white blood cell count was significantly raised (P =0.04) with concomitantly high platelet counts observed amongst patients than controls (P<0.0001). SCD patients had significantly higher levels of HbF.

Table 3.2. Laboratory results of HbSS patients compared with control subjects matched for age, gender and ethnicity.

Patient characteristics	Normal ranges	Controls (n=30)	Patients (n=81)	P value
Haemoglobin — g/dL	11.5 - 15.5	13 ±1.1	8.5 ±1.6	<0.0001
White cell count —mm^2	4.00 - 11.00	7.4 ±3.1	10.4 ±2.0	0.04
Platelet count —mm^2	150 - 450	252 ±66	395 ±157	<0.0001
Haemoglobin F —%	< 1	-	9.6 ±9.3	-
Bilirubin —µmol/L	3 – 20	-	67 ±9.6	-
LDH —U/L	< 240	-	416 ±128	-
Reticulocyte —U/L	50 – 150	-	334 ±151	-
Asparate Transaminase —U/L	10 – 50	-	48 ±23	-
CRP —mg/L	< 5.0	-	8.3 ±6.9	-
eGFR —ml/min/1.73	-	-	91.1 ±31	-

Abbreviations: LDH, lactate dehydrogenase; CRP, C-reactive protein; eGFR, estimated glomerular filtration rate.

3.2.3 Electrocardiogram features

Electrocardiogram characteristics of HbSS patients and controls were compared as reported in Table 3.3. Electrocardiogram features of cardiac rhythm, PR interval, QRS axis and previous evidence of myocardial infarction did not reveal any differences between the two groups.

The Sokolow-Lyon index criterion (i.e. S wave in V_1 plus R wave in V_5 or V_6 should be $\geq$ 35mm) (Sokolow and Lyon, 1949) was used to diagnose left ventricular hypertrophy (LVH) in all participants. The criterion for LVH was reached in 42% of HbSS patients versus 13% of controls ($P < 0.01$). Ponderal corrected LVH voltage demonstrated significantly higher voltages in HbSS patients than controls ($P <0.001$). ECG criteria for left atrial enlargement was seen in 5% of SCD patients, versus 0% in controls, which was not statistically significant ($P=0.4$). The corrected QT interval was significantly higher in SCD patients than controls ($P<0.02$). All other parameters such as dominant rhythm, PR interval, QRS axis, depolarization abnormalities (i.e. ST segments, bundle branch block) were not statistically different between the two groups. None of the patients and controls had any ECG evidence of previous myocardial infarction. Therefore no pathological Q waves were seen in the ECGs.

Table 3.3. ECG characteristics in HbSS patients versus control subjects. Left atrial and LV voltage criteria are expressed as a percentage change in a given population.

Characteristics	**Controls (n=30)**	**Patients (n=78)**	**P value**
Rhythm	Sinus	Sinus	
PR interval, sec	165 ±29	167 ±21	0.4
Corrected QT interval, msec	389 ±31	410 ±25	<0.02
QRS axis, °	61 ±31	58 ±21	0.52
Increased LVH voltage, no.%	4 (13)	33 (42)	<0.01
Ponderal corrected LVH, mVKg/m^3	1.6	2.5	<0.001
Left atrial enlargement, no.%	0	4 (5)	0.4
ST segment depression, no.(%)	0	2 (2)	0.4
Left bundle-branch block	0	0	
Previous MI, no. %	0	0	
Q wave amplitude, mV	0	0	

Abbreviations: LVH, left ventricular hypertrophy; MI, myocardial infarction

3.2.4 Structural changes by 3D echocardiography

Left ventricular chamber morphology and function

An initial assessment of left ventricular diastolic volume, uncorrected for patient body surface area was made. Left ventricular end-diastolic volume was significantly higher in HbSS patients compared to controls (P< 0.001) – Figure 3.1. These echocardiographic findings were then corrected for patients' body surface area to eliminate gender and growth relationships. Interestingly results in Figure 3.2, still showed a statistically significant difference between HbSS patients and control group. Similar findings were also observed for left

ventricular end-systolic volumes (Figure 3.3 and Figure 3.4).

Figure 3.1. Unadjusted left ventricular end-diastolic volume in HbSS patients and control subjects. Patients had significantly increased end-diastolic volumes.

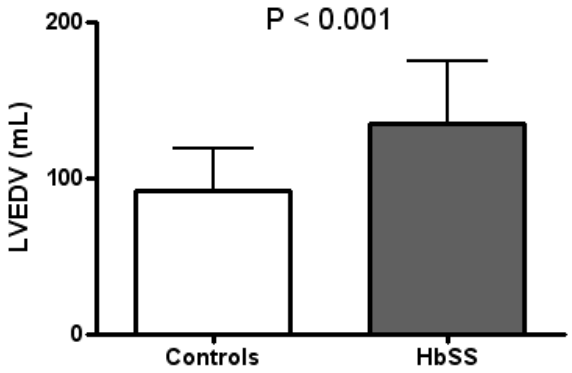


Figure 3.2. Adjusted left ventricular end-diastolic volume in HbSS patients and controls. Left ventricular volumes were corrected for subject's body surface area. Increased end-diastolic volume was evident after correction for body surface area.

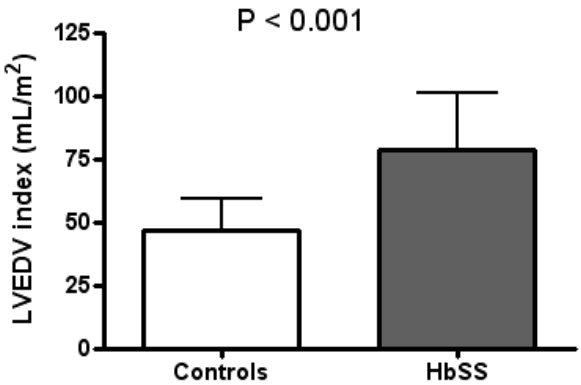


Figure 3.3. Unadjusted left ventricular end-systolic volume in homozygous HbSS patients compared to control subjects. HbSS patients had enlarged end-systolic volumes than controls.

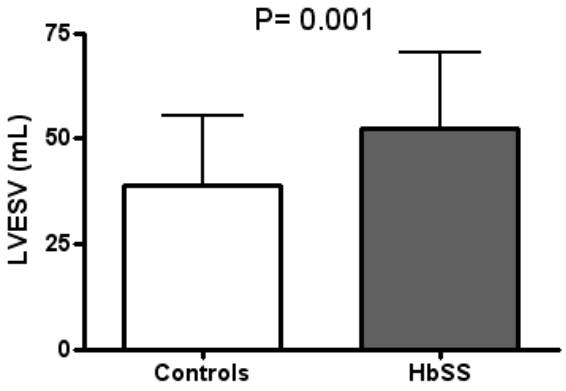
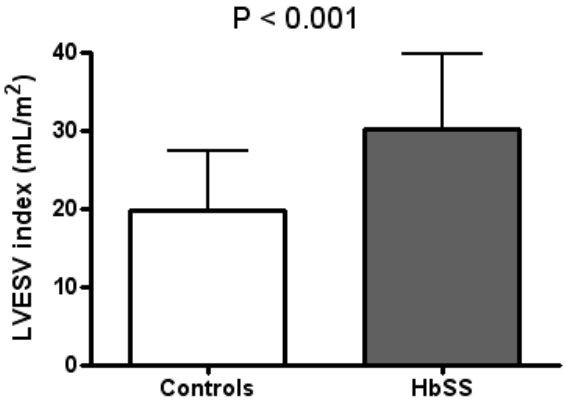
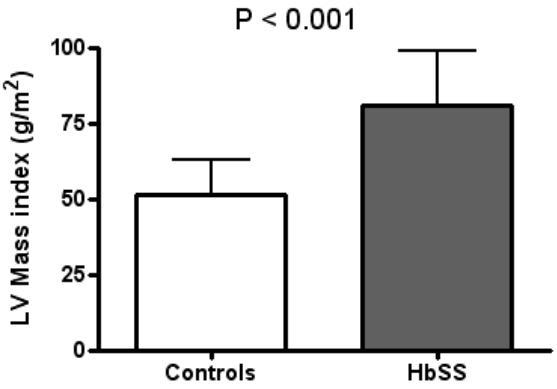


Figure 3.4. Adjusted left ventricular end-systolic volume in homozygous HbSS patients and controls. Actual volumes were corrected for body surface area. Increased end-systolic volumes were demonstrated after indexing for body surface area.



To evaluate the impact of increased left ventricular volumes, comparisons of left ventricular mass indices assessed by 3D echocardiography in HbSS patients and controls were made. Left ventricular mass index was higher in HbSS patients (P< 0.001) - Figure 3.5. This finding remained significant when eight of the patients with controlled systemic hypertension were excluded (81 ±18 g/m^2 vs 51 ±11 g/m^2, P<0.001).

Figure 3.5. Left ventricular mass index between HbSS patients and control subjects. Mass measurements were normalised for each patient's body surface area. There was significantly increased LV mass in HbSS patients.



Assessment of Left ventricular systolic function was conducted by measuring ejection fraction. No differences were seen in EF between HbSS and control groups (Figure 3.6). Comparisons of volume ejected out of the left ventricle per heartbeat (stroke volume - SV) and cardiac index (CI) showed significantly higher values of both SV and CI amongst HbSS patients than controls (Figure 3.7 and 3.8).

We also quantified LV stroke work index, which controls for differences in cardiac loading and therefore provides a better assessment of global contractile function (Glower et al., 1985). The data revealed that LV stroke work was significantly increased in HbSS patients compared to control (P<0.001)(Figure 3.9).

Figure 3.6. Left ventricular ejection fraction, expressed as % in HbSS patients compared to control subjects. No significant differences were demonstrated in the ejection fraction.

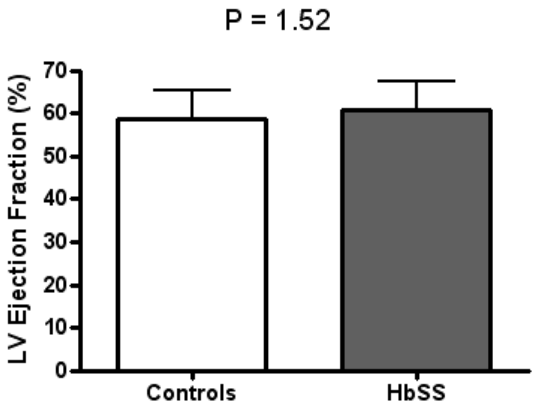


Figure 3.7. Left ventricular stroke volume index in HbSS patients compared to control subjects. Significantly increased stroke volume was observed in HbSS patients compared to controls.

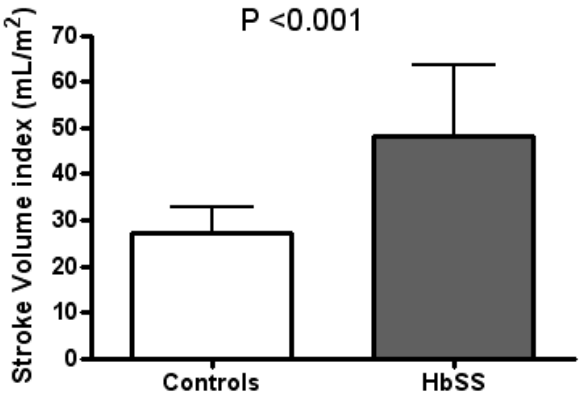


Figure 3.8. Left ventricular cardiac index in HbSS and control subjects. There was significantly increased cardiac index in HbSS patients versus control subjects.

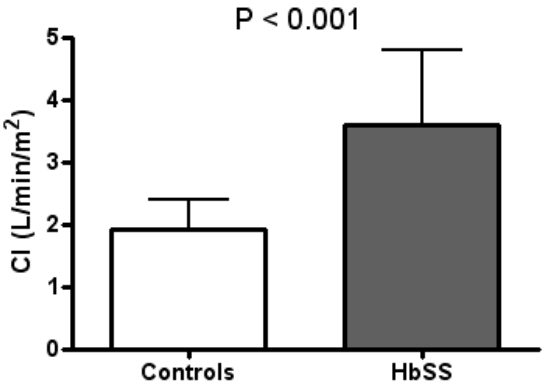
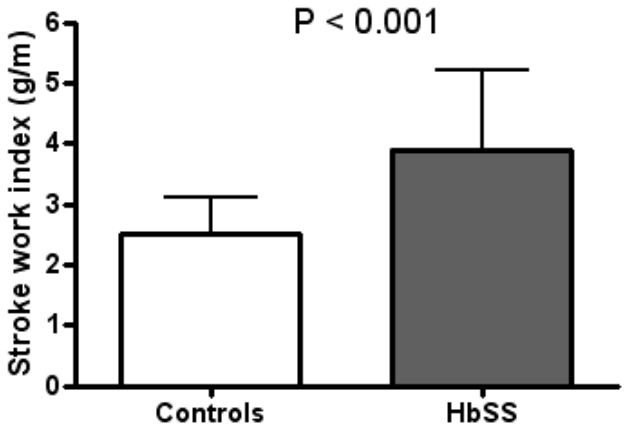


Figure 3.9. Stroke work index in HbSS patients compared to control subjects. The stroke work index was significantly higher in HbSS patients.



3.2.5 Gender differences

We evaluated whether gender differences were apparent among HbSS patients. Interestingly indexed LV systolic and diastolic volumes remained significantly larger when gender comparisons between HbSS and controls were made (Figure 3.10). Differences were also present in other parameters such as cardiac index (CI) and LV mass index (Figure 3.12 to Figure 3.14).

Figure 3.10. Adjusted left ventricular end-diastolic volume index in male and female HbSS patients. There was significantly increased left ventricular end-diastolic volume index in male and female HbSS patients.

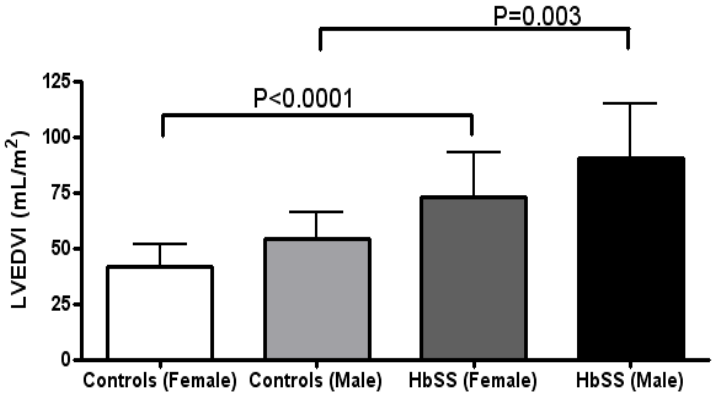


Figure 3.11. Adjusted left ventricular end-systolic volumes in male and female HbSS patients. Increased end-systolic volume index was demonstrated in male and female HbSS patients.

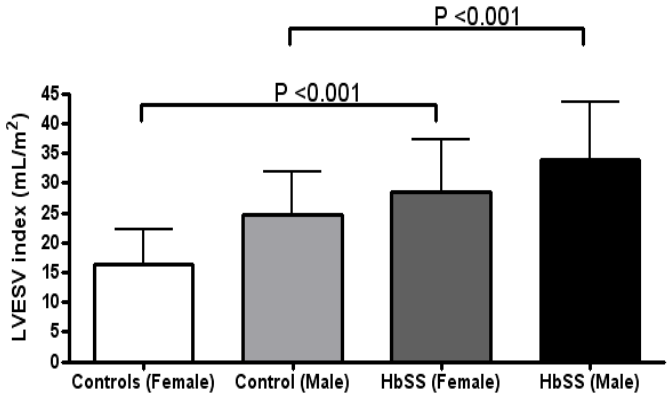


Figure 3.12. Adjusted cardiac output in male and female HbSS patients. Increased cardiac index was demonstrated in male and female HbSS patients.

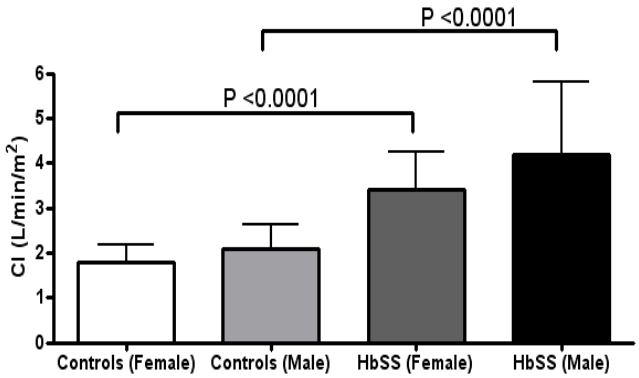
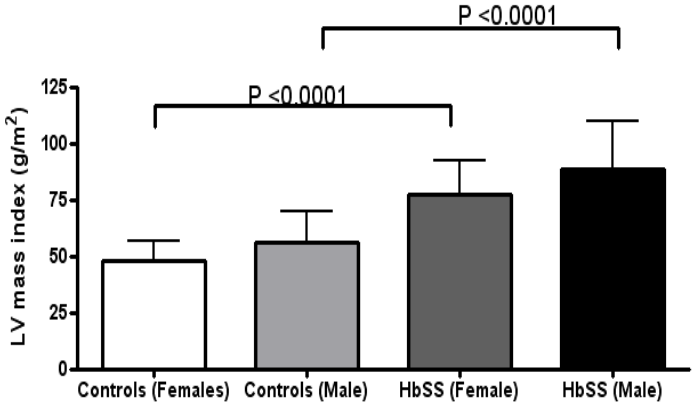


Figure 3.13. Adjusted LV mass in male and female HbSS patients. Increased left ventricular mass index was demonstrated in male and female HbSS patients.



3.2.6 Potential causes of left ventricular adaptation

The data above shows that patients with sickle cell disease have increased cardiac volumes and increased LV mass. To investigate the possible causes for this, correlation analyses were made between echocardiographic parameters of

LV chamber size and various clinical and laboratory markers of sickle cell disease. A significant negative association was found between LV end-diastolic index and Hb levels (Figure 3.14). Measurements of cardiac index strongly correlated with haemoglobin concentrations (Figure 3.15). Furthermore, our data demonstrated a negative association between cardiac index or LV mass and Hb levels (r =0.53, P =0.01) - Figure 3.15 and Figure 3.16. These findings would be consistent with the hypothesis that anaemia leads to a hyperdynamic circulation with increased cardiac output that in turn leads to increased LV mass. A significant inverse correlation was demonstrated between mass index and circulating haemoglobin concentration (r= -0.26, P=0.05) – Figure 3.17 or circulating HbF (Figure 3.18).

Figure 3.14 Correlation of left ventricular end-diastolic volume index with circulating haemoglobin concentration. Significant inverse correlation was shown between end-diastolic volume and haemoglobin concentration.

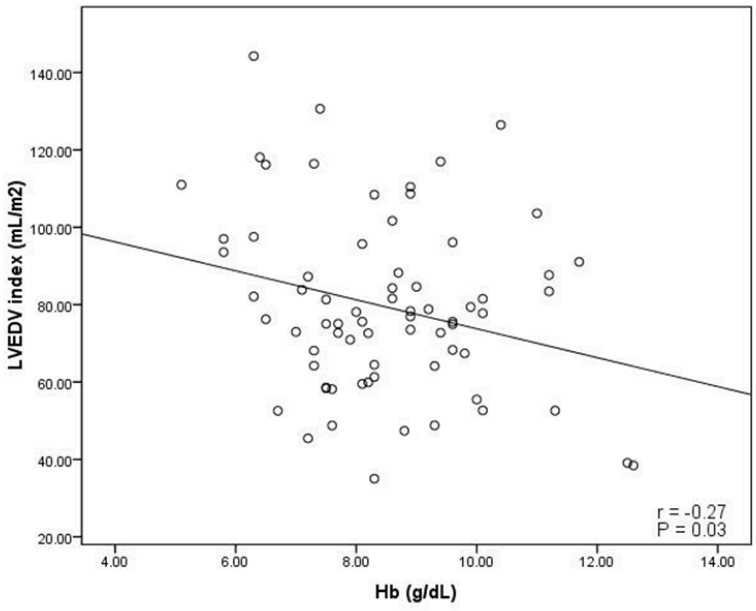


Figure 3.15. Correlation between cardiac index and circulating haemoglobin levels in HbSS patients. A significant inverse correlation was demonstrated between cardiac index and haemoglobin concentration (r = -0.3, P =0.02).

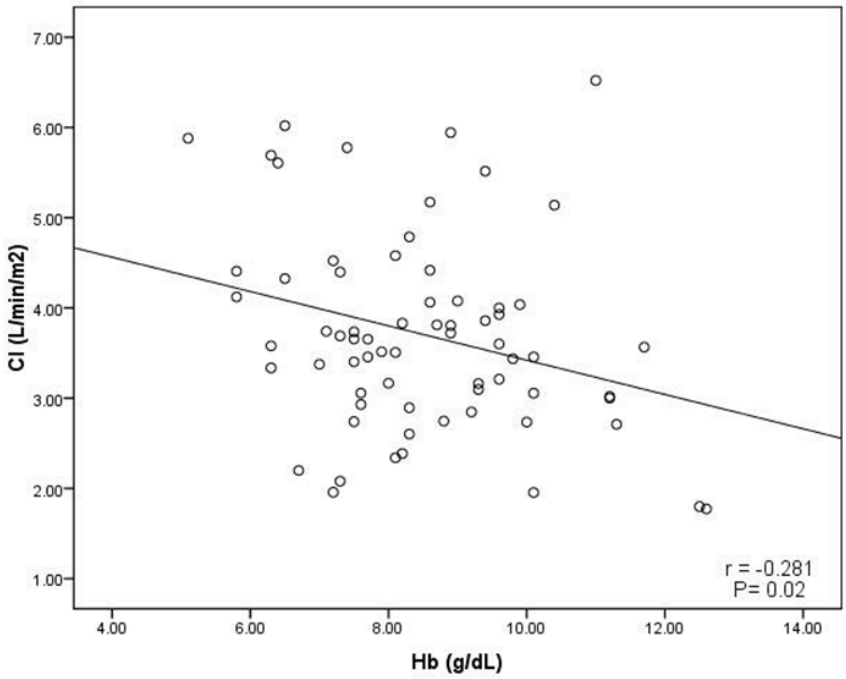


Figure 3.16. Correlation between left ventricular mass index and cardiac index in HbSS patients. A significant positive correlation was demonstrated in left ventricular mass index and cardiac index (r =0.5, P <0.001).

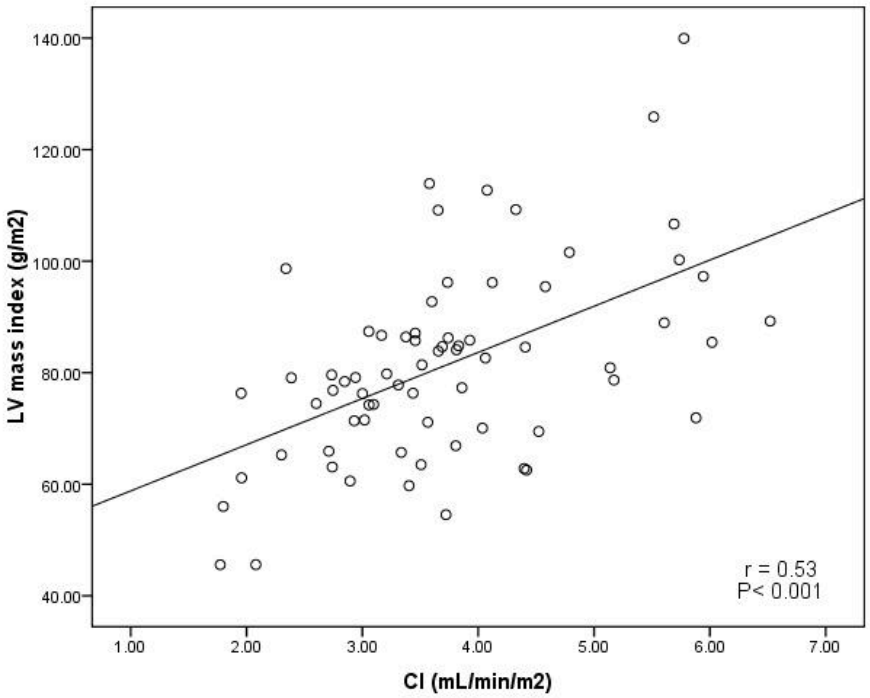


Figure 3.17. Correlation between left ventricular mass index and haemoglobin levels. An inverse correlation was demonstrated (r =-0.26, P =0.05).

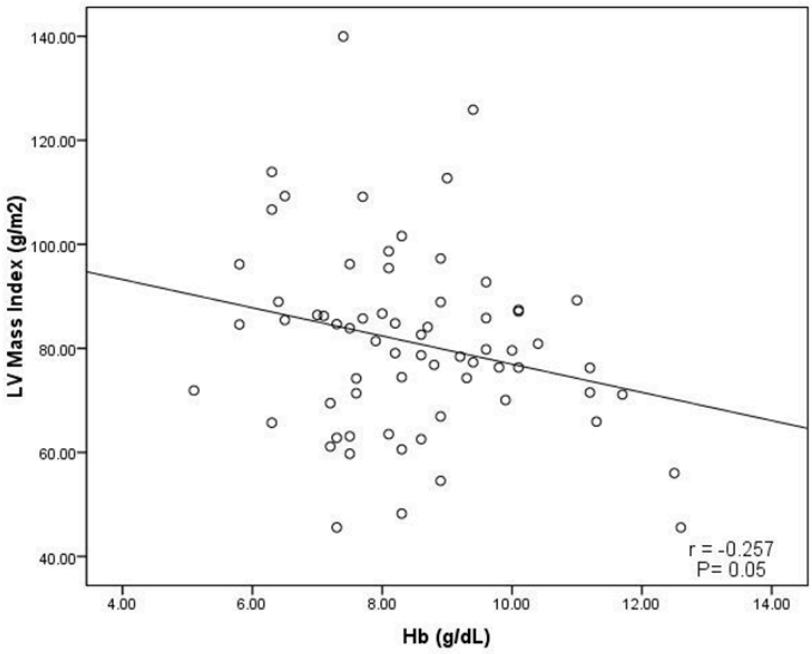
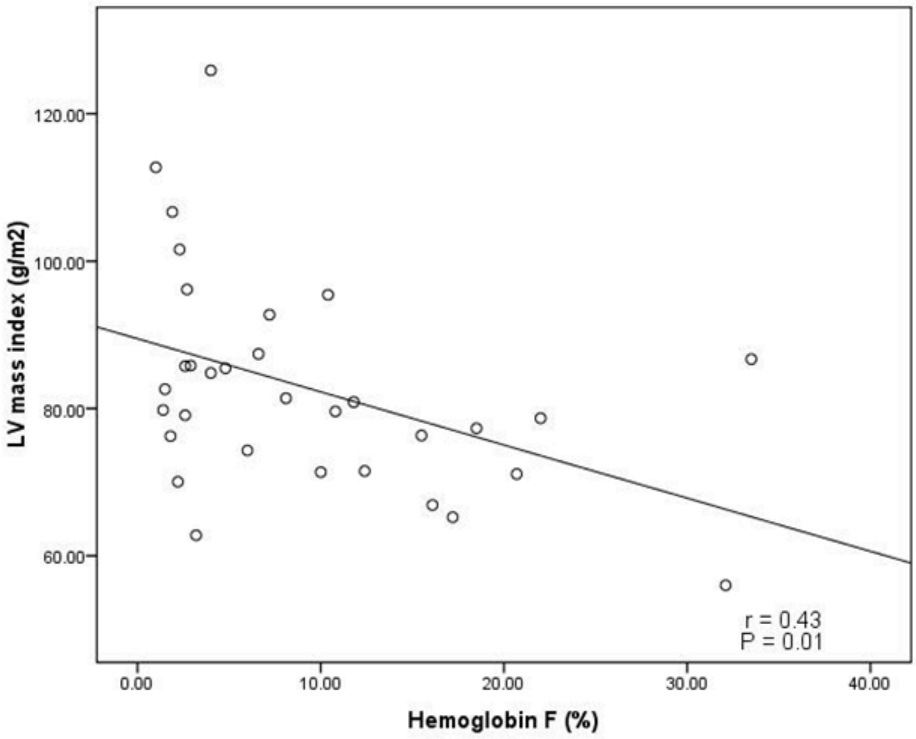


Fig 3.18. Correlation of left ventricular mass index with HbF (%) in HbSS patients. A positive correlation was shown (r =0.43, P =0.01).



We also found (Table 3-4) positive correlations when left ventricular mass index was associated to markers of haemolysis such as the reticulocyte count, and bilirubin and lactate dehydrogenase (LDH) levels. No correlation was found with other parameters such as white cell count, renal dysfunction (cystatin C, urea, creatinine, glomerular filtration rate) or hypercoagulability (platelet count). No correlation was shown when pearson correlation analyses were made between asparate transaminase and LV mass index.

Table 3.4. Pearson correlation coefficients (r) and 2-tailed P values showing correlation of left ventricular mass index and laboratory biomarkers of sickle cell disease.

	R Value	**P Value**
Haemolysis		
Bilirubin, μmol/L	0.28	0.03
LDH, UL	0.28	0.03
Reticulocyte, UL	0.31	0.02
Inflammation		
White blood cell count	0.002	0.98
Renal dysfunction		
Cystisin C	0.19	0.12
Creatinine	0.08	0.52
eGFR	0.16	0.23
Hypercoagulation		
Platelet	-0.06	0.64
Liver		
Asparate Transaminase	0.23	0.07

During patients' clinic visits, routine measurements of oxygen saturation by pulse oximetry were also taken. We therefore looked for any correlation between left ventricular echocardiography indices (such as end-diastolic volume and mass) and patients' oxygen saturations, obtained on the same day. As shown in Figures 3.19 and 3.20, no correlation between O_2 saturation and LVEDV or mass index was found.

Figure 3.19. Correlation of left ventricular end-diastolic volume index with oxygen saturation in HbSS patients. No association was found.

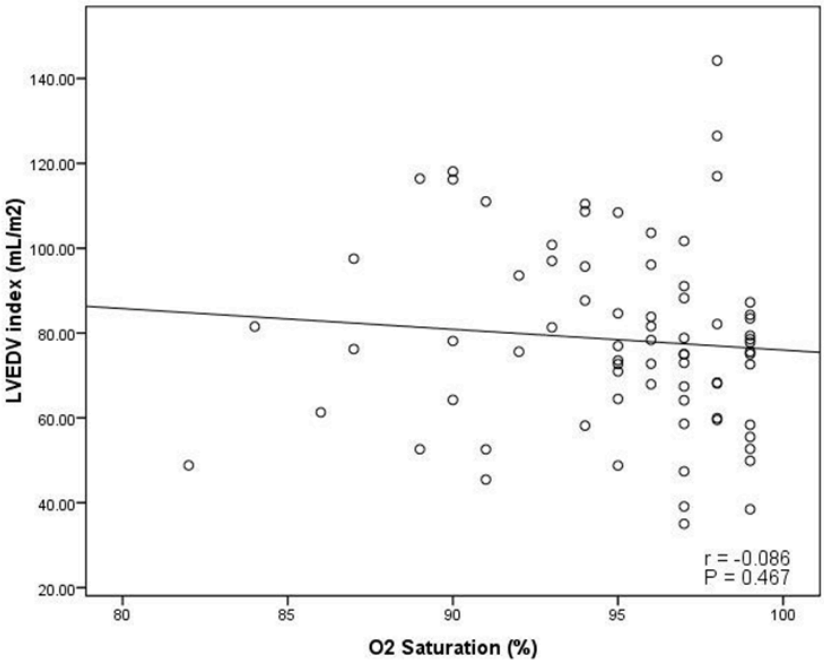
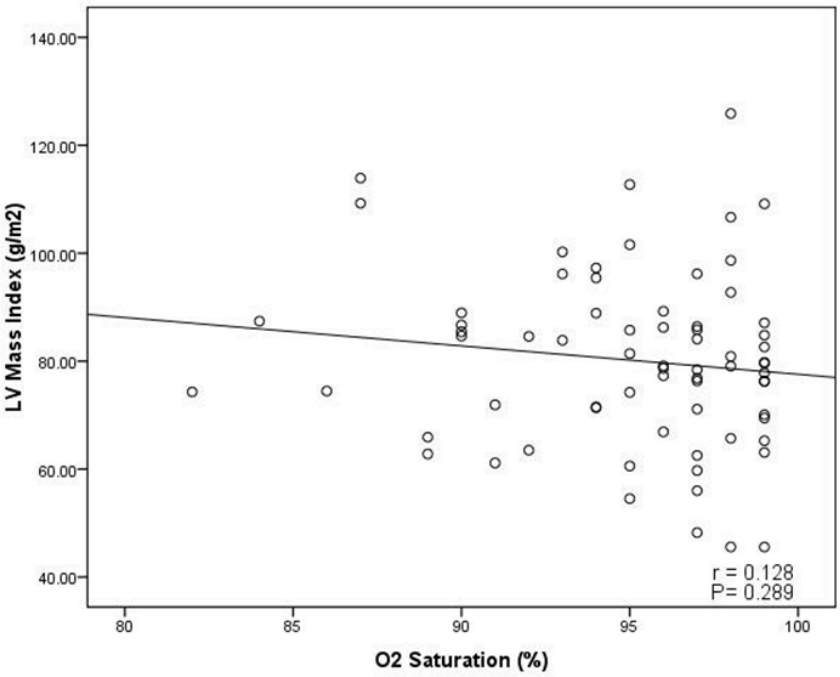


Figure 3.20. Correlation of left ventricular mass index with oxygen saturation in HbSS patients. No association was demonstrated (r =0.13, P =0.29).



Independent predictors of left ventricular mass

We undertook a multivariable linear analysis to study possible deteminants of LV mass. This showed that body surface area, Hb and asparate aminotransferase were determinants of LV hypertrophy (Table 3.5).

Table 3.5. Independent predictors of left ventricular mass index in stepwise multivariable linear regression analysis.

	β, Estimated increase in LV mass index (±SE)	*P*
Age, yrs	0.25 (.13)	0.68
Gender	-14 (8)	0.10
Blood pressure, mmHg		
Systolic	.13 (0.16)	0.7
Diastolic	0.14 (0.01)	0.86
Body surface area, m^2	74 (21)	0.002
Haemoglobin S, g/dL	-6.8 (2.3)	0.01
Aspartate Aminotransferase, U/L	0.5 (0.2)	0.01

Variables entered into the model were oxygen saturation, blood pressure, body surface area, age, haemoglobin S, aspartate aminotransferase, gender and genotype.

3.2.7 Left ventricular diastolic dysfunction

Diastolic dysfunction is a feature of pathological hypertrophy (Zile and Brutsaert, 2002). Assessment of characterized patterns of the transmitral blood flow Doppler velocities depicting of left ventricular early (E) and late (A) filling velocities can provide useful information regarding diastolic function (Nagueh et al., 2009; Ommen, 2001). The ratio E/A waves, transmitral deceleration time of the E wave from maximum point to baseline including tissue Doppler mitral annulus E/e' ratio were calculated from Doppler profiles as highlighted below. Data comparing diastolic function in SCD patients and controls did not show any significant statistical differences.

Tissue Doppler assessment of left ventricular diastolic function in our homozygous SCD patients showed the same prevalence rate as the control group (4%, $P>0.05$). Interestingly, no correlation was found between left ventricular diastolic function and left ventricular mass index (Figure 3.21). However, results in Figure 3.22 showed a significant correlation between left ventricular diastolic function and patient age ($r = 0.4$, $P < 0.001$).

Table 3.6. Comparison of left ventricular diastolic function using non-invasive echocardiography spectral and tissue Doppler modalities.

Patient characteristics	Controls (n=30)	Patients (n=76)	P value
Transmitral E/A ratio	1.2 ±0.36	1.6 ±0.57	0.06
Transmitral E/e' ratio	7.2 ±2.2	7.5 ±2.3	0.75
Mitral deceleration time (m/s)	0.25 ±0.2	0.23 ±0.2	0.7

Figure 3.21. Correlation of left ventricular diastolic function (expressed as mitral E/e' ratio) with left ventricular mass index. No association was shown.

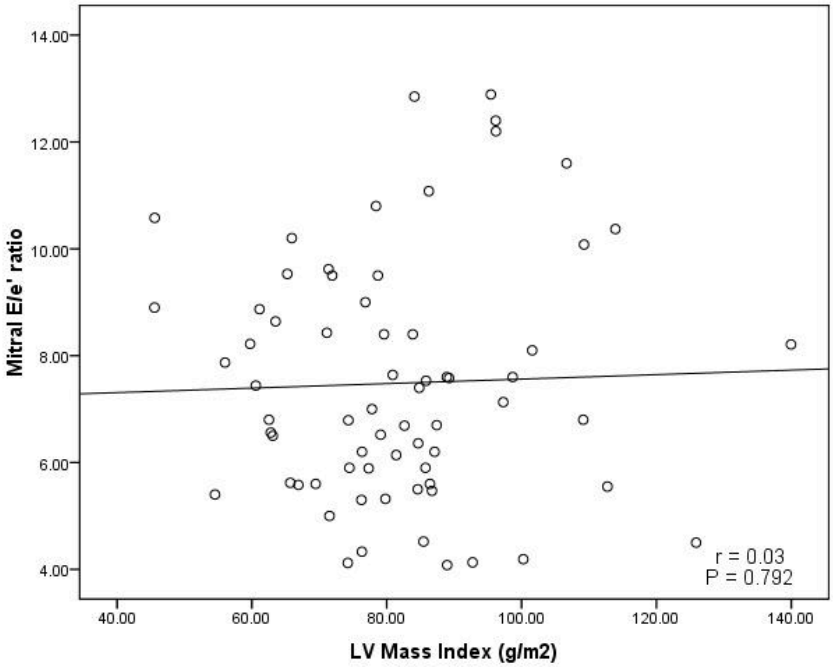
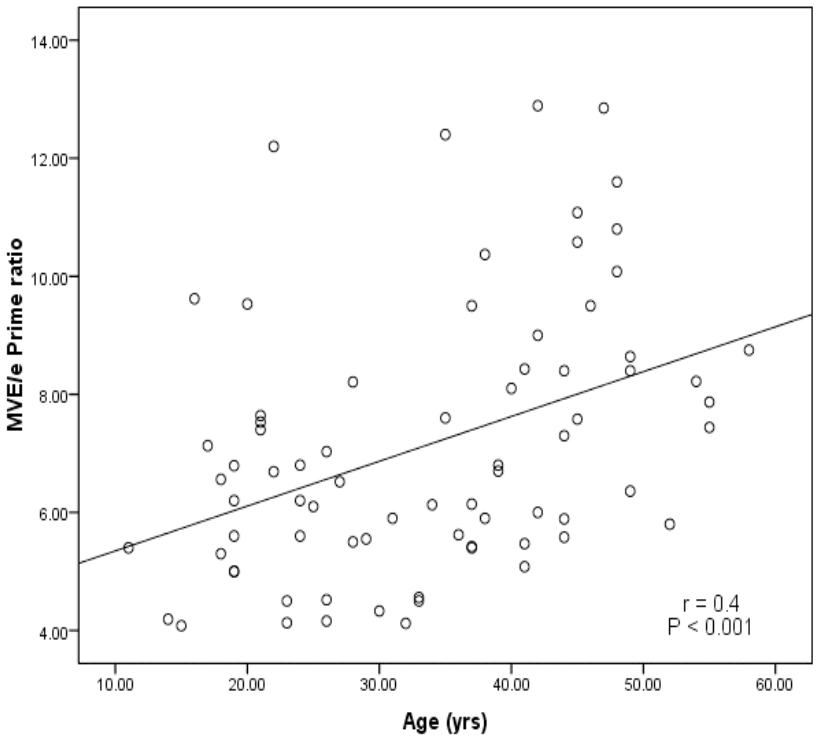


Figure 3.22. Correlation between left ventricular diastolic function expressed as mitral E/e prime ratio and age of HbSS patients. Left ventricular diastolic function was positively associated with patient's age (r =0.4, P <0.001).



3.2.8 Left ventricular shape

Previous studies in failing hearts have demonstrated a shift in LV shape (i.e. from elliptical to spherical), which is attributed to poor contractile performance and is a predictor of LV remodelling and outcome (Hung et al., 2004; Monaghan, 2006; van Dalen et al.; Wong et al., 2004). To assess changes in global LV shape, 3D derived sphericity index was calculated as end-diastolic volume divided by volume of the sphere (Monaghan, 2006). A significantly (P<0.001) larger sphericity index was observed in HbSS patients compared to controls. Differences in sphericity index were present in both diastolic and systolic phases (Figure 3.23 and Figure 3.24).

Left ventricular remodelling index (LVRI) serves as a useful differentiator of ventricular adaptation processes, i.e. physiological versus pathological remodelling. It is calculated from 3D datasets as the ratio of LV mass over LV end-diastolic volume (De Castro et al., 2007). The ratio increases in pathological LV remodelling but remains normal (1.03 g/ml) in physiological LV hypertrophy (De Castro et al., 2007). Despite the increase seen in LV end-diastolic volume and mass indexes, comparisons of LVRI in HbSS patients and controls showed no significant differences (Figure 3.26) i.e. remodeling index remained normal.

Systolic dyssynchony index (SDI) is a 3D echocardiographic parameter that quantifies variations in systolic contraction within the LV (Kapetanakis et al., 2005). In heart failure, the SDI maybe elevated as a result of regional segments reaching peak ejection volumes at different times (Kapetanakis et al., 2005; Marsan et al., 2008). In the current study comparisons of SDI in HbSS patients and controls showed no differences (Figure 3.26).

Figure 3.23. Left ventricular sphericity index in diastole of HbSS patients vs control subjects. The diastolic sphericity index in HbSS patients was significantly larger than controls.

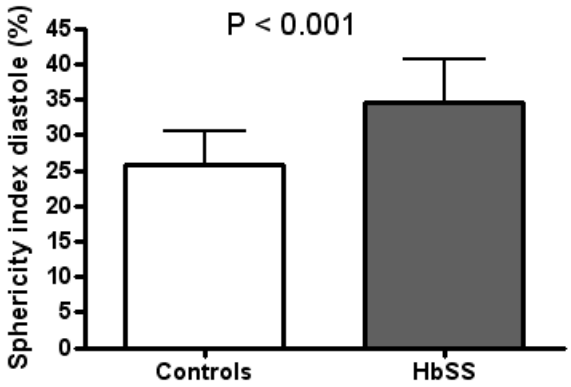


Figure 3.24. Systolic left ventricular sphericity index in HbSS patients compared to controls. Significantly larger systolic sphericity index was demonstrated in HbSS patients.

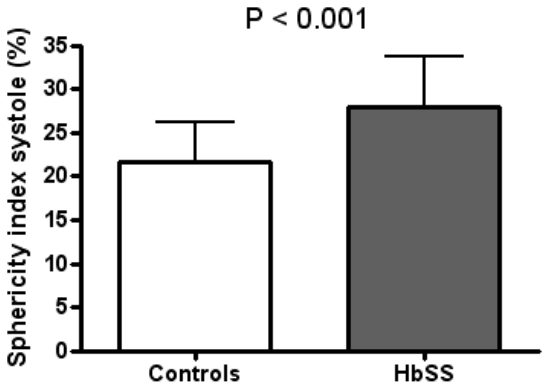


Figure 3.25. Left ventricular remodelling index in HbSS patients compared to control subjects. No differences were seen in remodelling index.

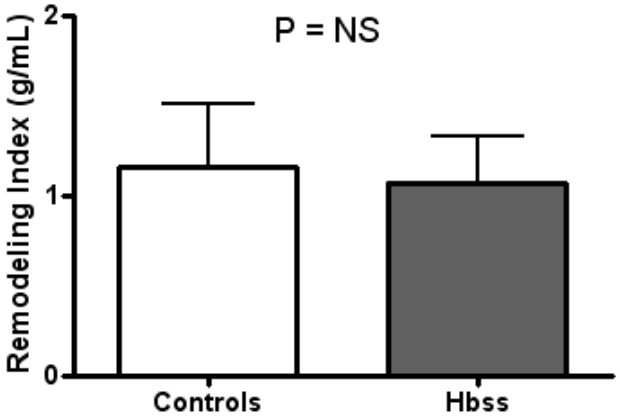
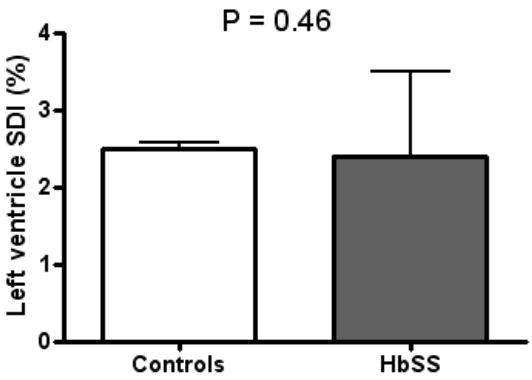


Figure 3.26. Left ventricular systolic dyssynchrony index, expressed as (%) in HbSS patients compared to control subjects. No differences were demonstrated in systolic dyssynchrony index between patient and controls subjects.



3.2.9 Analysis of left ventricular strain

Left ventricular strain can help distinguish between normal and abnormal muscle contraction and deformation. Detailed analyses of LV global, longitudinal, circumferential and radial strain were made in 3 standard LV regions namely, basal, mid and the apex. When looking specifically at

parameters of longitudinal, circumferential and radial strain in a subgroup of HbSS patients (i.e. those with the most enlarged hearts n =22) versus controls (n =13), no statistically significant differences were found between patients and controls (Table 3.7 to 3.9).

Since iron overload arising from multiple blood transfusion therapy could potentially lead to cardiac hypertrophy, we also compared strain patterns in transfused patients versus non-transfused. However, results highlighted in Table 4.0 showed no differences between the two groups.

Table 3.7. Peak systolic longitudinal strain rate of left ventricular segments in HbSS and control groups. No statistical difference was demonstrated in HbSS patients and control subjects.

Region of interest	Controls (n=13)	Patients (n=22)	P value
Basal anterior lateral, %	-26.0 $\pm$11.3	-22.0 $\pm$7.9	0.32
Basal interventricular septum, %	-14.4 $\pm$5.3	-15.5 $\pm$6.0	0.61
Mid-anterior lateral, %	-15.0 $\pm$6.2	-11.9 $\pm$5.7	0.17
Mid-interventricular septum, %	-15.6 $\pm$5.2	-14.7 $\pm$4.2	0.62
Apical anterior lateral, %	-10.7 $\pm$5.5	-17.6 $\pm$3.6	0.56
Apical interventricular septum, %	-15.5 $\pm$8.2	-20.4 $\pm$2.0	0.40

Table 3.8. Peak systolic circumferential strain of left ventricular segments in HbSS and control groups. No differences were shown in segmental strain rate between HbSS patients and control subjects.

Region of Interest	Controls (n=13)	Patients (n=22)	P value
Basal septal, %	-18.0 4.3	-19.6 ±5.5	0.44
Basal inferior lateral, %	-19.3 ±6.5	-16.9 ±3.15	0.36
Basal anterior, %	-14.0 ±5.9	-18.7 ±3.47	0.16
Basal inferior, %	-14.7 ±2.9	-16.6 ±3.1	0.18
Basal anterior lateral, %	-16.9 ±4.7	-17.9 ±4.8	0.76
Basal inferior septal, %	-19.3 ±4.0	-19.7 ±4.3	0.85

Table 3.9. Peak systolic radial strain rate of left ventricular segments in HbSS and controls. No differences were demonstrated between HbSS patients and control subjects.

Region of Interest	Controls (n=13)	Patients (n=22)	P value
Basal anterior septal, %	23.0 ±6	25.2 ±15	0.65
Basal inferior lateral, %	25.7 ±16.6	26.8 ±11.0	0.88
Basal anterior, %	22.7 ±10.3	18.9 ±16.4	0.75
Basal inferior, %	22.1 ±8.7	25.9 ±14.3	0.47
Basal anterior lateral, %	18.3 ±5.7	16.9 ±16.9	0.83
Basal inferior septal, %	15.5 ±8.2	20.4 ±2.0	0.59

Table 4.0. Peak longitudinal strain rate of left ventricular segments in transfused and non-transfused HbSS patients. No differences were observed between the two groups.

Region of interest	Transfused (n=9)	Non-transfused (n=13)	P value
Basal anterior Lateral, %	-22.8 ±9	-21.6 ±7.6	0.76
Basal interventricular septum, %	-12.7 ±7.7	-15.2 ±3.7	0.43
Mid-anterior Lateral, %	-9.0 ±3.1	-12.9 ±4.2	0.38
Mid-interventricular septum, %	-15.5 ±5.1	-14.3 ±3.9	0.60
Apical anterior lateral, %	-18.1 ±5.5	-17.5 ±3.8	0.88
Apical interventricular Septum, %	-19.5 ±5.0	-19.8 ±2.0	0.87

3.3 Discussion

Cardiopulmonary abnormalities are well-recognized acute complications in SCD (Fitzhugh et al.). Although many researchers have attempted to understand the clinical manifestation, there still remains a lot of controversy around exact prevalence and impact of both cardiac and pulmonary diseases in this patient group partly related to variations around the time of acute versus chronic presentations. More so, as medical support for patients increases life span (Platt et al., 1994), important questions arise regarding the type of cardiovascular abnormalities likely to appear with time and what long-term consequences they may have.

This study focus was on stable SCD patients expressing the homozygous genotype associated with high morbidity and mortality rates and is thus representative of the commonest type of SCD. Given the severe nature of the disease in HbSS patients, it has been suggested that cardiopulmonary abnormalities are a common feature (Gladwin and Vichinsky, 2008; Hoffman).

Overall clinical characteristics (Table 3-1) in this study were in agreement with previous reports (Serjeant et al., 1973; Serjeant et al., 1994; Zago et al., 1980). Significantly higher incidence of acute chest syndrome, respiratory and systemic hypertension were present in our patients as well as previous diagnosis of pulmonary emboli. Notably, HbSS patients as a group had lower BSA, systolic and diastolic pressure when compared to control subjects. Low blood pressure in homozygous SCD patients has been associated with lower ventricular afterload and decreased viscosity (leading to decreased arteriolar tone) and renal dysfunction (Adams-Campbell et al., 1993; Johnson, 2005)

Examinations of cardiac conduction system in SCD have shown evidence of degeneration of the sinus node, atrioventricular node and bundle of His, all of which are suspected to result in electrical instabilities (Akgul et al., 2007; James et al., 1994). In this study, electrocardiographic features in HbSS patients compared to controls were investigated. Only one patient in all eighty one HbSS patients enrolled had rhythm disturbances, which is not statistically different from the normal population. The rhythm abnormality observed in the patient was in the form of isolated supraventricular extrasystoles. No significant differences were found in terms of QRS axis. Significantly longer corrected QT interval was documented in our cohort of HbSS patients, this was in agreement with previous reports by other researchers documenting prolonged corrected QT intervals in their study population (Liem et al., 2009; Muller et al., 2006). Furthermore in this study, five percent of HbSS patients reached the criteria for left atrial enlargement on ECG, which was later confirmed on echocardiography. It is postulated that an increase in preload may be the likely cause of atrial

enlargement. However, this was not statistically different from the control group.

Electrocardiogram findings showed an increased LV voltage with evidence of left ventricular hypertrophy in 42% of HbSS patients tested compared to only 13% of controls (P = 0.01). Several electrocardiographic studies comparing healthy black population to other ethnic groups have reported marked differences in the appearance of ECG tracings attributed to cardiovascular risk factors such as systemic hypertension (Greene and Kelly, 1959; Rawlins et al.; Spencer et al., 2004; Wasserburger, 1955). High prevalence rates of LV voltage amplitude and repolarisation abnormalities (ST-segment and T wave changes) are frequent findings documented among black individuals. In this context, it is important to note that our control population was age, gender and ethnically-matched.

Left ventricular voltage magnitude on the ECG can be influenced by factors such as ventricular muscle mass, age, orientation of the heart in the chest, electrolyte imbalance and body habitus (Howard and Gertler, 1952; Kilty and Lepeschkin, 1965; Luskin and Whipple, 1961; Simonson and Keys, 1952; Surawicz, 1986). With these factors in mind, it is possible that the high LV voltage seen in HbSS patient population could likely be due to the patients' thinner body build. However, correction of ECG LVH by ponderal index (Kilty and Lepeschkin, 1965) and echocardiography studies, further confirmed evidence of LVH in our patient group. Although LVH strain (ST depression and T wave inversion) was evident in a few patients and control subjects, no statistical differences were found. No significant differences were found when

QRS axis deviation comparisons were made.

It is well established that an increase in cardiac output is the haemodynamic consequence of reduced oxygen-carrying capacity in anaemia (Brannon et al., 1945). It is therefore perhaps not surprising that there was a significant increase in left ventricular diastolic volume and cardiac index amongst HbSS patients. Furthermore, increased left ventricular volume index was significantly associated with severity of anaemia (haemoglobin concentration), consistent with the above hypotothesis. Other reported studies (Covitz et al., 1995; Johnson et al.; Rees et al., 1978) have also shown similar findings.

Comparisons of left ventricular end-diastolic volume with oxygen saturation showed no relationship. One study by Johnson *et al* reported that lower oxygen saturation predicted SCD patient's LV mass, while in the same study no association was found between LV mass and haemoglobin (Johnson et al.). Perhaps our study findings are not surprising because oxygen saturation readings obtained by pulse oximetry are known to be prone to error as they depend on many physiological components, and are not a reliable substitute for measuring arterial partial pressures of oxygen or ventilation. Furthermore, the Hb count probably provides a better measure of the stimulus for measured cardiac output than a single measure of oxygen saturation (Gibson, 1996).

Studies examining accuracy of pulse oximetry in SCD found poor specificity in detection hypoxia due to the right ward shift in the oxygen dissociation curve (Blaisdell et al., 2000; Fitzgerald and Johnson, 2001; Ortiz et al., 1999). A

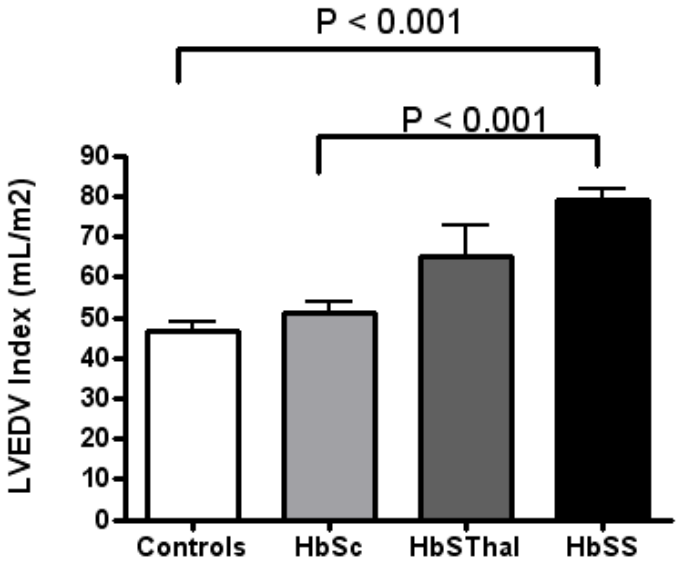
number of physiological variables including haemoglobin concentration, arterial blood flow to vascular beds, temperature of the digital area where the oximetry sensor is located, percentage of inspired oxygen, ventilation-perfusion mismatch and venous return to the probe location might affect measurements. Other possible sources of error can arise from factors such as hypotension, carbon monoxide poisoning (carboxyhaemoglobin absorbs more light than oxygen, thereby giving false positively high readings) and disturbance in light absorption from probe to surrounding skin tissue (Amar et al., 1989; Barker and Tremper, 1987; Keidan et al., 1997).

Furthermore, blood viscosity is a measurement of the resistance of blood to flow formed by shear stress or strain. Blood consists of plasma and red blood cells; therefore, viscosity of blood depends on both these factors which in turn can influence resistance, circulation and consequently tissue perfusion (Lenz et al., 2008). Blood viscosity cannot be accurately measured clinically owed to lack of stable measurement techniques and principles (Shibata et al., 2002). Novel use of viscosity-dependent fluorescence molecular rotors appears sensitive as they rely on two modes of relaxation, fluorescence and free volume of viscosity. Pulse oximetry used routinely to detect hypoxia may occasionally demonstrate some abnormalities in severe anemia where blood viscosity is reduced markedly (Haidekker et al., 2002), therefore, results should be viewed with caution.

Sickle cell disease is an example of balanced polymorphism. Generally individuals who are heterozygous (i.e. HbAS, HbSc/ HbSthal) at particular gene

locus have fewer episodes of crisis and are resistant to the malarial parasite than those bearing the homozygous (HbSS) copy of the gene (Elliott et al., 2007; Menzel et al.). Here, the question of polymorphism was addressed as there are no articles in the literature focusing on how different SCD genotypes influence cardiac structure; hence left ventricular volumes in a total of 120 SCD patients recruited were grouped according to SCD genotype, i.e. HbSS, HbSthal and HbSc (Figure 3.27). The results demonstrated that remodelling of the left ventricle depend on the patients' genotype. Patients with the severe form of sickle cell (HbSS) have the largest left ventricular volumes compared to those with a milder form of the disease (HbSc). If anything, the mildest form of SCD - HbSC, showed similar left ventricular chamber volumes as the normal group, when we evaluated LV diastolic volumes in a larger SCD with varying genotypes (Figure 3.27).

Figure 3.27. Left ventricular diastolic volume index in three SCD genotype compared to control group. Increased ventricular volumes in SCD depend on severity of disease/ genotype.



Laplace's law plays a fundamental role in understanding cardiac function in health and disease. The basic principles that govern this law can be used to estimate myocardial wall stress from intraventricular pressure, radius of curvature and wall thickness (Burkhoff, 2001). In brief, this law states that: a higher ventricular pressure or larger chamber radius generate more tension, whereas thicker wall muscle results in less tension being generated by the heart. The heart responds to increased demands by undergoing a process known as remodelling. The term 'cardiac remodelling' refers to the changes that occur in cardiac structure and function as a result of a series of adaptations to normalize wall stress, thereby reducing myocardial oxygen consumption (Knaapen et al., 2007; Strauer, 1979). Remodelling can occur due to either volume/pressure overload or cardiac injury (e.g. myocardial infarction). Depending on the type of remodelling that occurs (i.e. physiological or pathological), the clinical likelihood of developing heart failure can be greatly increased (Cohn et al., 2000; Konstam et al., 2003; Swynghedauw, 1999). It is therefore of interest to evaluate this in our study population.

Left ventricular hypertrophy occurring in response to disease stresses is associated with significant systolic and diastolic dysfunction (Cuocolo et al., 1990; Elliott and McKenna, 2004; Federmann and Hess, 1994; Gaasch and Zile, 2004; Norton et al., 2002). Studies in hypertensive patients have demonstrated that systolic function decreases with increasing hypertrophy (Devereux et al., 1983; Elliott and McKenna, 2004; Ganau et al., 1990). Pathological remodelling is a complex mixture of pathways, caused by factors such as myocardial infarction, hormone, genetic and pressure overload (due to

aortic stenosis or hypertensive heart disease), leading to parallel replication of sarcomeres (cardiac hypertrophy) (Carabello, 2002a; McMullen and Jennings, 2007; Wakatsuki et al., 2004). There is generally an increase in cardiac fibroblasts, leading to excessive interstitial fibrillar collagen production (i.e. fibrosis) and proliferation. These collective structural alterations arise in order to counter balance an increase in systolic wall stress, and often contribute to mechanical stiffness and reduced filling of the heart in diastole. In addition, apoptosis of hypertrophic cardiac muscle cells reduces muscle contractile force and leads to development of ventricular dysfunction. A combination of apoptosis and diastolic dysfunction together increases risk of developing malignant cardiac arrhythmias and heart failure.

This type of remodelling can be seen where there is a requirement to generate a higher cardiac output than normal, such as elite athletes and pregnancy (Basavarajaiah et al., 2008; Mone et al., 1996; Rawlins et al.; Weeks and McMullen; Wisloff et al., 2001). In these settings, modest chamber dilation occurs as new sarcomeres are added in-series to existing sarcomeres with further remodeling occurring in the extracellular matrix components (Hutchinson et al., 2009; Swynghedauw, 1999). This process serves to normalize left ventricular end-diastolic dimension to wall thickness ratio, thus allowing greater generation of stroke volume, to compensate for the volume overload present. Fibrosis caused by collagen overproduction does not occur in this type of remodeling and there is no increase in the incidence of heart failure. Pathological increases in volume load (eg mitral regurgitation or arterio-venous shunts) induce eccentric cardiac remodelling that has some features similar to

physiological remodeling (Gorman et al., 2005). However, excessive and persistent volume overload usually leads to pathological remodelling in the long-term.

Since the current study findings demonstrated increased left ventricular volumes in HbSS patients, it was important to assess how the ventricle adapts to this increase in wall tension. Previous cardiac studies in SCD patients employed either traditional 2D or M-mode echocardiography techniques that have been proven to yield poor reproducibility in both inter- and intra observor data. Therefore we also investigated changes in left ventricular mass using 3D echo, which has been proven by postmortem and cardiac MRI studies to provide accurate mass measurements (Brown et al., 2010; Monaghan, 2006). In this study HbSS patients were found to have a significantly higher left ventricular mass index than control subjects. This increase in ventricular mass was significantly associated with circulating haemoglobin S levels ($r = 0.26$, $P = 0.05$). Although correlations cannot precisely identify drivers for the increased cardiac volumes, we also looked at the possible impact of haemolysis, as it is a hallmark of SCD. In the current cohort of HbSS patients a significant association was found between steady state clinical markers of haemolysis (LDH, biliruibin and reticulocyte count) and left ventricular mass index, further supporting contribution of anaemia to cardiac remodelling.

Foetal haemoglobin (also known as hemoglobin F- HbF) is produced in large quantities during gestation because HbF has the ability to carry oxygen at low oxygen tension (Bertles, 1974; de Araujo and Jamra, 1967; Huisman, 1980).

Subsequently after birth, levels of HbF drop as the γ- gene is switched down and β gene is switched on to facilitate the production of normal haemoglobin A (Higgs and Wood, 2008). Since HbF cells survive longer and inhibit polymerization of HbS red blood cells, researchers have reported high levels of HbF to correlate with low morbidity and mortality rates in SCD, (Charache et al., 1992; Higgs and Wood, 2008; Koshy et al., 2000; Steinberg et al., 1997).

To-date, no studies have assessed the association between HbF and cardiac structure and function. Correlative analysis of left ventricular mass index and levels of HbF in our study indicate that patients with low levels of plasma HbF are likely to have higher left ventricle mass. Although the mechanism by which HbF helps to maintain cardiac structure was not fully explored, it is possible that an increase in HbF percentage reduced ventricular workload by increasing overall oxygen carrying capacity. A study by Fabry et al found that increasing HbF concentration ameliorated SCD pathology particularly renal dysfunction (Fabry et al., 2001). Perhaps another possible mechanism could be that SCD patients with high HbF concentrations have smaller ventricles because their ventricular preload is reduced through maintained renal function.

Gender is well known to effect left ventricular hypertrophy (Hammond et al., 1986). Women have been reported to have a higher prevalance of left ventricular mass index than men (33% vs 23.7%) in the young and older population (Liebson et al., 1993; Savage et al., 1987). We further investigated whether differences observed in LV mass were driven by gender. Therefore, measures were made to group data in patients and controls according to their

sex. Interestingly, even after corrections for gender was made, the significant increase in LV mass index remained evident.

A multivariable regression model (table 3.5) was generated using variables known to increase left ventricular mass such as patient gender, blood pressure and age (Krumholz et al., 1995; Levy et al., 1990) as well as laboratory parameters related to SCD. In this model, body surface area, haemoglobin levels and aspartate aminotransferase were the only variables independently associated with changes in left ventricular mass index.

Studies in hypertensive patients and pathological LVH in general have demonstrated that diastolic dysfunction often precedes systolic dysfunction (Federmann and Hess, 1994). Therefore it was of interest to assess whether SCD patients had diastolic dysfunction, which might suggest pathological LVH. The gold standard assessment of left ventricular diastolic function requires invasive measurement of LV pressure and volume (Little and Downes, 1990; Mirsky, 1984). However, Doppler echocardiography can provide quite good non-invasive assessment of diastolic function. Conventional echocardiography Doppler measurements of transmitral flow together with tissue Doppler of the mitral annulus (E/e' ratio) have been shown to provide useful clinical information concerning left ventricular filling pressures and outcome (Geske et al., 2007; Ommen et al., 2000).

Tissue Doppler E/e' in current HbSS study found prevalence of LV dysfunction to be the same as controls i.e. only 4%. Previous studies have suggested left

ventricular diastolic dysfunction to be related to poor prognosis in patients with SCD (Caldas et al., 2008; Lewis et al., 1991; Sachdev et al., 2007). Published data by other researchers have shown varying levels of diastolic dysfunction. For example Arslankoylu et al's study reported no diastolic abnormalities (Arslankoylu et al., 2009) whereas Sachdev and Machado's study, reported diastolic dysfunction as present in 18% of the patient population tested (Sachdev et al., 2007). However, it is important to highlight that the traditional echocardiography diastolic methods used in these studies depend on multiple interrelated factors, such as heart rate and mitral valve incompetence, including atrial and ventricular compliance. In the current study, the only parameter that correlated with left ventricular diastolic function (Figure 3.22) was patient age (r 0.4, P< 0.001), which is well known to relate to diastolic dysfunction in the normal population (Miller et al., 1986; Villari et al., 1997).

The presence of LV systolic dysfunction in SCD would again suggest pathological LVH. There is controversy regarding left ventricular systolic function in SCD. A study by Rees *et al* (Rees et al., 1978) reported LV systolic dysfunction (as assessed by 2D echo derived EF and velocity of shortening of myocardial fibres during ejection) in a significant proportion of young SCD patients who had dyspnea and fatigue. However, these incidences are highly load-dependent. Other contradictory studies, including those performed by Sachdev *et al* (Sachdev et al., 2007), show no impairment in LV systolic function as assessed by 2D echo derived EF. Left ventricular systolic function in the current study was assessed by multiple methods. We found no differences in EF between sickle cell patients and the control group as assessed by 3D

echocardiography. However, although EF is a widely used clinical measure of cardiac systolic function, care has to be taken when interpreting findings because EF is highly influenced by loading conditions (Carabello, 2002b). Stroke work index controls for differences in cardiac loading, and thereby provides a better assessment of contractile function. Here, non-invasive comparisons of stroke work index were calculated using mean arterial pressure and 3D derived stroke volume index. Results showed a significantly higher stroke work index in HbSS patients than controls ($P< 0.001$), arguing against LV systolic dysfunction and suggesting if anything better LV function.

Speckle tracking is a new echocardiographic imaging technique that can provide useful information on the presence, location and extent of segmental scarring, fibrosis and regional contractile function independent of loading conditions (Kang et al., 2008; Popovic et al., 2007). Speckle tracking echocardiography has been widely used in detecting subclinical changes of LV myocardial walls in hypertension patients (Geyer et al., 2010). Strain rate studies performed in 20 hypertensive patients with LVH reported regional LV systolic function impairment despite having good global ejection fraction (Chen et al., 2007). In another study, speckle tracking assessments in 56 patients with untreated hypertension reported regional LV impairment correlating to measurements in collagen (Kang et al., 2008). Again, similar to the previous study, the global systolic function (ejection fraction) in untreated hypertensive patients was normal.

Systolic function analyses by speckle tracking technology have not been done

in SCD patients. Regional strain was evaluated in twenty two HbSS patients compared to thirteen controls (Tables 3-4 to Table 3-6). Strain rate measurements of longitudinal, circumferential and radial strain were made in the basal, mid and apical regions of the LV from 2D images acquired in the apical four chamber and parasternal short axis views. The mean peak systolic strain in HbSS patients was similar to those seen in the control group suggesting normal regional contractile function. Torsion assessments of hypertensive patients document abnormalities in untwist mechanics in hypertensive patients (Han et al., 2008; Takeuchi et al., 2007), in our study speckle tracking twist and untwist mechanisms were not assessed because this specified programme is not available on the QLab Advanced Quantification Software version 6.0 (Philips).

Red-cell transfusion therapy serves to correct for low haemoglobin levels, thereby reducing the number of circulating sickle cells and thus improving oxygen carrying capacity of blood. It has been documented that transfusion reduces incidence of cardiac stroke and improves morbidity and mortality (Josephson et al., 2007; Switzer et al., 2006). However long-term therapy has potential adverse effects, such as iron overload (Switzer et al., 2006), because humans are not capable of effectively excreting excessively high non physiological levels of iron (Brittenham; Inati et al.). Iron complications in sickle cell anaemia often develop later as a result of iron entering multiple specific cells such as cardiomyocytes, pituitary, hepatic and pancreatic cells. Iron accumulation in multiple organs leads to cellular dysfunction, apoptosis and necrosis (Brittenham; Darbari et al., 2006; Finch et al., 1982). Little is known

about the impact of myocardial iron-overload in long-term transfused patients. It is theoretically possible that the increased LV mass in SCD patients is due to myocardial iron overload. However, results in our study did not show statistical differences between patients receiving repeated transfusion therapy (n=7) and those not on transfusion (n=15). To definitively look for myocardial iron overload, it would be necessary to undertake cardiac MRI (Anderson et al., 2001; Mavrogeni et al., 1998).

Studies have demonstrated that patients with dilated LV often develop globular/spherically shaped ventricles that exhibit both reduced contractility and filling (D'Cruz et al., 1989; Tumkosit et al., 2007). Until recently, spherical indices measured by 2D echocardiography failed to characterise changes in regional LV geometry. Three dimensional echocardiography has been shown to visualise LV endocardial walls better and also validated to accurately quantify LV parameters (Mor-Avi et al., 2009).

As there is no data published on the shape of the LV in SCD and how ventricular adaptation affects contractility, we evaluated LV shape by assessing spherical remodelling using 3D echocardiography. The results of this study demonstrated significantly larger LV sphericity index, both in diastole and systole among HbSS patients, suggesting probable pre-adaption of the LV to a globular shape.

Three dimensional echocardiography derived left ventricular remodelling index (LVRI) expresses the relationship between LV mass and LV end-diastolic

volume. A simple ratio of LV mass divided by end-diastolic volume can serve as a useful differentiator of physiological and pathological adaptation. De Castro and colleagues were the first to compare left ventricular remodelling index using 3D echocardiography in a large cohort of 220 subjects consisting of elite athletes, patients with hypertensive heart disease and patients with dilated cardiomyopathy (De Castro et al., 2007). Their study reported lower values of LVRI in patients with dilated cardiomyopathy (0.55g/mL) and higher LVRI values in patients with hypertensive cardiomyopathy (2.4 g/mL), when compared to healthy subjects. However, no differences were observed between athletes and controls subjects' LVRI. In their study, they concluded that the balanced proportions of mass and volume index observed in elite athletes were suggestive of normal physiological adaptation. In the current study, the LVRI in HbSS patients compared to control subjects did not show any difference, which could suggest that the LV in SCD is remodelling eccentrically, i.e. physiologically.

Different segmental activation times within the LV are a common and problematic phenomenon in heart failure patients (Kapetanakis et al., 2005). Left ventricular dyssynchrony leads to reduced efficiency because blood does not synchronously move around the heart (Cleland et al., 2006). Left ventricular dyssynchrony index (SDI) (derived from 3D data on time to peak segmental contraction) is a widely used clinically index for assessing ventricular contraction in heart failure (Hawkins et al., 2006). SDI in the current study failed to show differences between HbSS patients and control subjects indicating that during the systolic phase of contraction all segments are activated at the same

time.

3.4 Summary

In summary, the current study used novel non-invasive echocardiography imaging techniques to characterise cardiac adaptation in SCD. The study findings indicate that SCD patients have increased LV volumes and LV mass which are probably related to anaemia and chronically higher cardiac output. However, the LV remodelling appears to be physiological rather than pathological since we did not find evidence of systolic or diastolic dysfunction. Longer-term follow up of such patients would be required to show whether LV remodelling is detrimental.

Chapter 4

Prevalence and mechanism of pulmonary hypertension in sickle cell disease

4.0 Introduction

Sickle cell anaemia is a hereditary hemoglobinopathy that limits the quality of life due to chronic complications resulting from repetitive vaso-occlusive events and haemolysis (Lane 1996; Siddiqui and Ahmed 2003). Ischaemia reperfusion injuries arising from vaso-occlusive events can subsequently lead to multiorgan failure and shortened life expectancy (Farmakis and Aessopos 2011). Although clinical severity and presentation can vary depending on the type of gene inherited, individuals with the homozygous sickle cell genotype pose a significant medical and social-economic burden, due to lifelong medical management and frequency of hospitalisation (Lane 1996; Wood, Hsu et al. 2008).

Recent advances in the management and treatment of sickle cell disease (SCD) patients have seen a growing number of case reports surrounding cardiopulmonary complications emerging with time (Lane 1996; Gladwin and Kato 2005; Parent, Bachir et al. 2011). In particular, great emphases has been placed on pulmonary hypertension (PHT) as it seems to be a major determinant of outcome (Haque, Gokhale et al. 2002; Machado, Anthi et al. 2006). Clinical studies using echocardiography and right heart catheterisation have reported that mortality rates maybe up to 50% over a period of two years in adult patients found to have PHT (Castro, Hoque et al. 2003; Anthi, Machado et al. 2007; Sachdev, Machado et al. 2007). Hence, calling for more rigorous non-invasive echocardiography screening measures to be made in SCD patients suspected of having PHT. However, the accuracy of non-invasive echocardiography screening for pulmonary hypertension in SCD patients and selected threshold

criteria remain under debate. It is also unclear whether the relationship between suspected PHT and increased mortality is a caused one or whether PHT is a matter of severe SCD per se.

Pulmonary arterial hypertension in general is a crippling disease that is characterised by progressive increment of pulmonary vascular resistance and pulmonary artery pressures (Loscalzo 1992; Humbert, Sitbon et al. 2004; Hyduk, Croft et al. 2005). If left untreated, PHT can lead to right ventricular failure and death (Gladwin, Sachdev et al. 2004; Hyduk, Croft et al. 2005). Pulmonary arterial hypertension can be classified in two groups, primary PHT and secondary PHT associated with various conditions that share identical obstructive pathological changes of the pulmonary microcirculation. It should be noted, however, that pulmonary hypertension can also occur due to venous hypertension e.g. abnormalities of left heart function that lead to elevated pulmonary venous pressures. The importance of this is that treatments targeted at pulmonary arterial hypertension (such as phosphodiesterase inhibitors or endothelin antagonists) will not be of value in this setting. While it seems clear that increased pulmonary pressures are increased in many patients with SCD the exact mechanisms underlying this, the type of hypertension and the impact on the right heart remain unclear.

Transthoracic echocardiography is a non-invasive clinical imaging technique widely used for monitoring cardiovascular pathophysiological disease processes (Scherrer-Crosbie, Steudel et al. 1998). The main method for detecting PHT in SCD has so far been the quantification of tricuspid

regurgitation jet velocity by Doppler echocardiography which correlates with pulmonary systolic pressure (Kato, McGowan et al. 2006; Sachdev, Machado et al. 2007; Gladwin and Vichinsky 2008). However, this does not provide information on whether PHT is arterial or venous nor on its mechanism.

The past two to three decades have seen considerable improvements and integration of new echocardiography imaging techniques, such as three-dimensional echocardiography (Monaghan 2006; Bhan, Kapetanakis et al. 2008). The right ventricle's complicated eccentric ecliptical shape makes it challenging to visualize when using two-dimensional echocardiography (Shiota, Jones et al. 1998). Three-dimensional echocardiography is now becoming the preferred method for clinically assessing the right ventricle (RV) as it enables better visualisation and quantification of complex geometrical changes. Furthermore, real-time three-dimensional echocardiography has been validated with gold standard magnetic resonance imaging with strong correlations found in volume and functional studies (Grewal, Majdalany et al. ; Leibundgut, Rohner et al. ; Niemann, Pinho et al. 2007).

4.1 Study aims

In consideration of conflicting reports from clinicians and investigators surrounding the prevalence and underlying mechanisms of pulmonary hypertension in SCD, a number of questions remain unanswered. The aim of the present study was to evaluate the prevalence, underlying mechanisms and hemodynamic impact of PHT using a combination of methods including validated real-time three-dimensional imaging techniques in adult SCD patients.

4.2 Methods

4.2.1 Study design

The study was conducted at King's College Hospital, London. Two main prospective studies were carried out which aimed at establishing the prevalence of pulmonary hypertension, its mechanisms if possible, and prospectively defining reliable non-invasive clinical echocardiographic methods for diagnosing pulmonary hypertension in our sickle cell patients.

The study began in April 2008 and was completed by October 2011. Patients in a stable condition from the local outpatients haematology department were included in the study. A total of 125 consecutive unselected SCD patients with the following genotypes: HbSS, HbS_{thal}, HbSC and $HbSC_{thal,}$ aged 18 years and above were evaluated. Criterion for exclusion were active manifestation of painful crisis/recent acute chest syndrome within 6 weeks, pregnancy, patients receiving medication for pulmonary hypertension, right ventricular outflow obstruction, left ventricular outflow (LVOT) obstruction, aortic stenosis or poor echocardiography imaging windows. One patient was excluded due to pulmonary stenosis and another due to poor echocardiogram imaging windows; thereby bringing the total number of SCD patients recruited to 123. No patients were on medication for PHT. All patients evaluated were screened by means of medical history, physical examination, laboratory studies including two non-invasive echocardiography methods (see below). For comparison, a group of thirty normotensive subjects without SCD and matched for age, gender and ethnicity were recruited from the community at large.

A sub-group of patients from the first study suspected clinically and by TR jet velocity of having PHT, underwent further invasive investigations using the gold standard method of right heart catheterisation. A total of 18 SCD patients were referred to have right heart catheterisation and repeat echocardiography studies performed on the same day as catheterisation. These patients were also referred for lung function tests and CT chest examination.

4.2.2 Echocardiography studies

Complete two-dimensional (2D) echocardiography examinations were performed in accordance with the European Association of Echocardiography guidelines (Evangelista, Flachskampf et al. 2008). Patients were imaged in the left lateral decubitus position using a commercially available Philips IE33 ultrasound-imaging machine equipped with 2.5MHz, stand-alone and matrix array transducers. Valvular regurgitations were visualized in standard parasternal long axis and short axis views; apical two, four, and five-chamber views and subcostal views and stored digitally while scanning in real time. An average of 3 cardiac cycles were taken in all patients with sinus rhythm and an average of 5 cardiac cycles if patients had arrhythmia. Tricuspid E/A ratios were calculated with distinctly identifiable E and A waves. Conventional colour-coded tissue Doppler from the tricuspid valve (E') annulus in the four chamber view were performed and E/E' ratio calculated to determine right ventricular diastolic function. All 2-dimensional image analyses were made off line on Xlera Imaging software.

TAPSE

Right ventricular function assessments were performed by TAPSE method, which is described in detail in the general methods chapter.

Speckle tracking strain analysis

Speckle tracking imaging and analysis are fully described in the general methods chapter.

Three-dimensional echocardiography

Description of detailed three dimension LV and RV acquisition and analysis are described in the general methods section.

Assessment of pulmonary hypertension by echocardiography

Transthoracic echocardiography was used as a screening test for the presence of pulmonary hypertension in all participants. For comparison, two technically different approaches for estimating pulmonary hypertension by echocardiography were applied:

- Tricuspid regurgitation jet velocity
- Pulmonary vascular resistance

Tricuspid regurgitation jet velocity

Tricuspid regurgitation (TR) jet measurements, are based on the intensity of colour Doppler which is identified as retrograde systolic flow originating from the tricuspid valve directed down towards the right atrium (RA). Echocardiographically, the retrograde flow arises due to a pressure difference

between the pulmonary artery and right atria during the systolic phase of contraction measured in velocity time units using spectral Doppler modality.

In the current study, subjects with identifiable TR (i.e. height, width and intensity of the jet) had their TR jet velocities measured by carefully placing continuous wave Doppler cursor parallel to the regurgitant jet, ensuring an accurate beam-to-flow alignment. Most Doppler TR signals were adequately acquired from a standard 2.5MHz transducer. However, in a few subjects, the maximum TR jet could only be obtained with a stand-alone probe. Only the highest TR velocities in the four standard views were recorded. It must be highlighted that in a majority of published SCD studies, pulmonary artery systolic pressure of at least 25mmHg corresponding to a TR value of 2.5m/s has been applied as cut off margin for diagnosis of PHT, which denotes mild PHT without the inclusion of right atrial pressure (Simonneau, Robbins et al. 2009; Farmakis and Aessopos 2011). With this in mind mild PHT was defined as TR $\geq$2.5 to 2.9 m/s and moderate PHT as TR $\geq$ 3m/s.

Routinely, clinical estimates of pulmonary artery systolic pressure are derived from a simplified Bernoulli equation [Pressure = 4(TR velocity)^2 + right atrial pressure]. Essentially, the equation converts velocity measurements to pressure estimates in mmHg (Yock and Popp 1984; Galie, Hoeper et al. 2009; Simonneau, Robbins et al. 2009). The Bernoulli equation, determined by pressure differences between two locations, is based on the principle of energy conservation in a circulating system by balancing pressure and velocity flows.

There are three main components involved in determination of pressure gradient:

1) A change in the cross sectional area of flow
2) Flow acceleration due to the pressure drop
3) Blood viscosity

Right atrial pressure estimates are made from the diameter and respiratory variation of B-mode images of the inferior vena cava, which is best visualised in the subcostal view. However, there are a lot of variations in inferior vena cava size due to changes in the central blood volume, which have led to several researchers omitting RA pressure in the calculations and estimates of PASP (Kircher, Himelman et al. 1990; Fisher, Forfia et al. 2009).

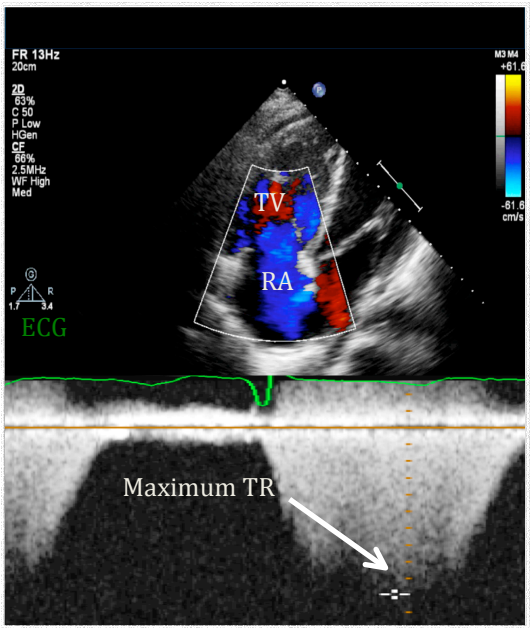


Figure 4.1 Illustrates a 2D echocardiography image in a patient with tricuspid regurgitation (TR) seen here in blue arising from the tricuspid valve (TV) to the right atrium (RA) - top panel. The bottom panel represents a continuous-wave Doppler recording of TR with the long arrow highlighting maximum TR velocity in m/s.

Pulmonary Vascular Resistance

Assessment of pulmonary vascular resistance (PVR) is crucial for the diagnosis and management of pulmonary arterial hypertension (Roule, Labombarda et al. 2010). Indeed PVR is an essential component for heart and liver transplant patients (Braunwald and Colucci 1984). Pulmonary vascular resistance is directly related to transpulmonary pressure gradient and transpulmonary flow. The simplified Bernoulli equation, neglects pressure recovery turbulent and viscosity effects which could lead to potential overestimation in patients with subvavular stenosis or severe anaemia (Cape, Jones et al. 1996).

The non-invasive echo estimate of pulmonary vascular resistance (PVR_{echo}) has been validated with invasive right heart cathetherisation studies, however it's accuracy in SCD patients has not been tested ((Abbas, Fortuin et al. 2003). Variable for PVR_{echo} are derived from velocity measures of tricuspid regurgitation and pulmonary flow (cardiac output). The measurement for right ventricular outflow tract integral (RVOTI) was obtained from optimised views of the pulmonary trunk were obtained in the parasternal short axis view and a sample volume cursor placed proximal to the right ventricular outflow tract, 1-2 mm below the pulmonary valve using a sweep speed of 100 to 200 mm/s. Timing of pulmonary valve opening, peak velocity and valve closure were determined as time from the onset of QRS complex on the electrocardiogram (ECG) tracing.

The highest peak TR velocities obtained from multiple views was used to determine subjects' pulmonary vascular resistance. A simplified validated

equation for non-invasive calculation of PVR_{echo} (in Wood units) was applied as previously reported (Abbas, Fortuin et al. 2003).

$$PVR_{echo}(\text{Wood units}) = \frac{10 \times TR\ (\text{meters/second})}{RVOTI\ (\text{centimetres})}$$

Where PVR represents pulmonary vascular resistance in Wood units (WU); TR is the maximum measure tricuspid regurgitation velocity in meters per second and RVOTI representing time velocity integral for right ventricular outflow tract flows.

4.2.3 Oxygen therapy

Oxygen therapy was administered to patients with TR jet velocity $\geq$2.5m/s. Oxygen treatment was administered via nasal prongs at 5L/min. Echocardiography measurements of TR, PVR and cardiac output were performed before and after twenty minutes oxygen therapy. Full echocardiography images of the LV, RV and spectral Doppler profiles were obtained before hand, however, only images required for measurements of TR, PVR, TAPSE and cardiac output were taken after oxygen treatment as patients became restless and refused to spend more than 2 hours having images of their hearts taken. Repeat recordings of blood pressure and oxygen saturation were also taken immediately after oxygen therapy.

4.2.4 Haemodynamic measurements

Pulmonary artery catheterisation is an invasive procedure that uses balloon tipped catheters originally designed by Swan and Ganz in 1970 (Swan, Ganz et al. 1970) and now widely used to monitor haemodynamic parameters of the right heart in a variety of cardiopulmonary disease states and also allows for

blood samples to be obtained from the right atrium, right ventricle and pulmonary artery (Adams 2004). Right (± left) heart catheterisation procedures in the current study were performed in 18 consenting patients. All patients were admitted as day cases and asked to refrain from eating eight hours before the procedure. Only stable patients, free from sickle crisis at the time of catheterisation were included in the study.

All patients received inspired oxygen to maintain oxygen level > 97% for at least 15 minutes. The patient was carefully positioned and local anaesthesia consisting of 1% lidocaine administered to the right groin prior to guidewire insertion. A balloon-tipped, flow directed, pulmonary artery thermodilution catheter (7.5F, Ecossa Medical Ltd, Cumberland, Strathclyde) was advanced through the guidewire via the right femoral artery to enable continuous monitoring of the systemic blood pressure. The catheter is a multiple lumen device that comprises of a pulmonary artery lumen, right atrial lumen, thermistor and a flotation balloon. The catheter is 110cm long with a maximum diameter of 12 mm. The catheter is coated with heparin to inhibit thrombus formation. A period of 30 minutes was allowed after insertion of catheter to allow readings to settle.

The catheter was zeroed and referenced to atmospheric pressure prior to recording stable readings of parameters under fluoroscopic guidance. The catheter was advanced into the mid portion of the right atrium via subclavian artery. Once correctly positioned waveforms of the right atrial pressure corresponding to ECG tracings at end-expiration were recorded accordingly.

The catheter was advanced further across the tricuspid valve into the right ventricle and downstream to the pulmonary artery where mid-expiration recordings of right ventricular pressure, pulmonary artery systolic, diastolic and mean pressures were recorded. The balloon was inflated with approximately 1.0 to 1.5 mL air, thereby occluding the pulmonary artery. Pulmonary capillary wedge pressure waveforms reflecting transmitted pressure of the left atrium were taken. Pulmonary artery wedge pressure provides an indirect measure/ assessment of left atrial pressure but does not reflect true left ventricular preload (i.e., left ventricular end-diastolic volume) if there is obstruction between the left atrium and left ventricle and when left ventricular compliance is abnormal (Weed 1991).

In addition to pressure measurements, pulmonary artery catheter measurements of cardiac output were determined by thermodilution technology. With thermodilution method, repeated bolus injections of cool saline of known temperature were injected into the proximal port of the catheter into the right ventricle where the temperature of the bolus dropped as blood moved around the ventricle. The drop in temperature of the 'mixed' blood was re-measured downstream past the distal port of the thermistor. Signal changes in input and output saline temperatures were constructed into a modified Steward-Hamilton thermodilution curve to calculate cardiac output. An average of 4 cardiac output values were recorded. Estimated calculations of pulmonary vascular resistance (PVR) were made from thermodilution measurements of cardiac output as demonstrated below.

$$PVR = \frac{\text{mean pulmonary arterial pressure – pulmonary wedge pressure}}{\text{Cardiac output}}$$

Normal right atrium pressure can vary from 0 to 7 mmHg, elevation in right atrium pressure are seen in conditions such as pulmonary hypertension, when left to right shunts are present (Rich, Dantzker et al. 1987; Sharkey 1987). Normal right ventricular systolic pressure can vary from 15 to 25 mmHg, elevated values are seen in cases such as pulmonary hypertension, pulmonary stenosis or in acute pulmonary emboli. Normal pulmonary artery systolic pressure varies from 15 to 25 mmHg and elevated PA pressure are observed in conditions which cause a rise in pulmonary vascular resistance such as hypoxic vaso-occlusion, pulmonary artery hypertension or pulmonary vascular diseases.

For comparison, TR and PVR_{echo} Doppler echocardiography studies were repeated within 24 hours of right heart catherisation studies.

4.2.5 High-resolution computed tomography

A dual detector helical CT scanner (HiSpeed NX/I, GE Medical systems, Milwaukee, WI, USA), was used for assessment of the lung parenchyma, extent/severity of lobular lung abnormalities such as ground glass opacification/emphysema, including prominence of segmental and subsegmental airways. Two thoracic radiologists blinded to the purpose of the study reviewed prone images.

4.2.6 Pulmonary function tests

Pulmonary function tests measuring forced expiratory volume (FEV_1), forced vital capacity (FVC), residual volume (RV), carbon monoxide transfer coefficient (Kco) and total lung capacity (TLC) were performed in the hospital lung function laboratory using a heated pneumotchograph (Jaeger Masterscreen PFT, Viasys Healthcare, UK). Values were expressed as percentages of predicted values for the patient's age, gender and height.

4.2.7 Biomedical measures

Measures of plasma haemoglobin concentration, bilirubin, reticulocyte count and lactate dehydrogenase (LDH) were recorded as detailed in the methods chapter.

4.2.8 Statistics

Detailed statistical analysis of results are described in the general methods chapter.

4.3. RESULTS

4.3.1 Study patients

Between April 2008 and October 2011, a total of 125 consecutive SCD patients (Figure 4.2) were randomly recruited to take part in the study. Two patients were ineligible because of pulmonary stenosis and poor echo imaging windows, leaving 123 eligible patients with mean ($\pm$SD) age 37+14, 80 (65%) of whom were women. All underwent non-invasive echocardiogram to assess prevalence of PHT in SCD using echo measures of TR and PVR_{echo}. For comparison, thirty

age, gender and ethnically matched normotensive subjects with no SCD served as controls. Eighteen SCD patients were referred and consented to undergo catheterisation. The baseline characteristics of the participants are shown in Table 4.1, and a breakdown of screening protocol highlighted in Figure 4.2.

Figure 4.2 Characteristics of 125 SCD enrolled patients screened and randomized for study. Among these 18 patients who underwent right heart catheterisation. SCD denotes sickle cell disease, TR tricuspid regurgitation.

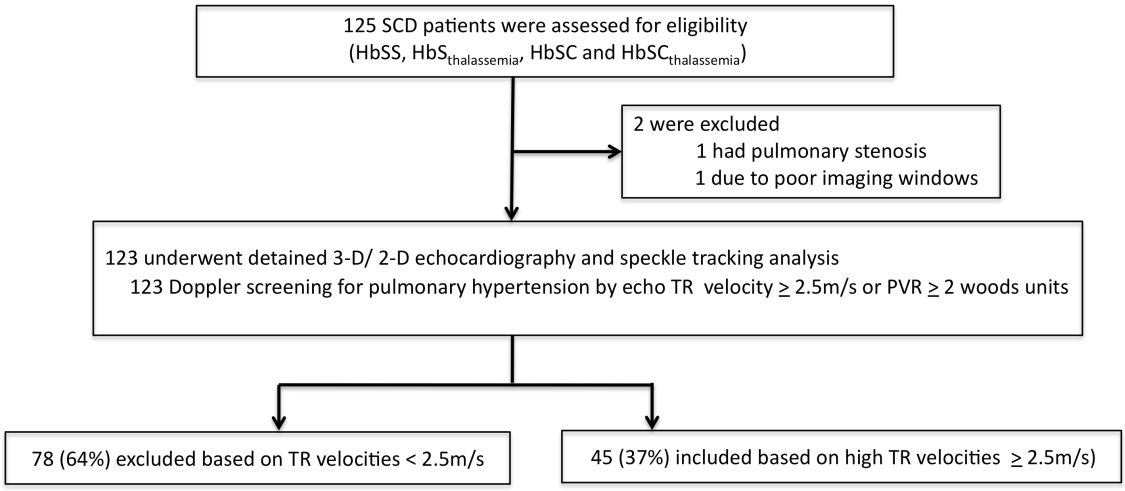


Table 4.1. Clinical characteristics of study participants.

Patient characteristics	Controls (n=30)	Patients (n=123)	P value
Number	30	123	
SCD phenotype			
Haemoglobin SS	-	80 (66)	
Sβthalassemia	-	14 (12)	
Haemoglobin SC	-	22 (18)	
Haemoglobin SC thalassemia	-	5 (4)	
Age — yrs	38 ±9	37 ±14	ns
Female	18 (60)	80 (65)	ns
BSA — g/m^2	2.0 ±0.2	1.7 ±1.9	<0.001
Tricuspid regurgitation Velocity > 2.5m/s	3.0(10)	45(37)	<0.001
Pulmonary vascular resistance >2 woods units on echo	0(0)	22(18)	<0.05
Blood pressure — mmHg			
Systolic	123 ±14	115 ±14	<0.05
Diastolic	76 ±9.8	68 ±10	<0.001
Oxygen saturation	98 ±2	95 ±4	<0.0001
>2 A&E visits in past 5 yrs	-	40 (35)	<0.0001
History of acute chest syndrome	-	9 (7)	<0.0001
History of respiratory disease	-	20 (16)	<0.0001
Controlled systemic hypertension	-	16 (13)	<0.0001
History of liver disease	-	10 (8)	<0.0001
History of pulmonary embolism	-	10 (8)	<0.0001
History of stroke	-	9 (7)	<0.0001
History of renal dysfunction	-	9 (7)	<0.0001
History of leg ulcers	-	9 (7)	<0.0001
History of priapism	-	7 (6)	<0.0001
Hydroxyurea therapy	-	18 (15)	<0.0001
Regular transfusion programme	-	14 (11)	<0.0001
Haemoglobin — g/dL	13 $\pm$1.1	9.2 $\pm$2.0	0.001
White cell count —$no./mm^2$	7.4 $\pm$3.1	9.5 ±3.9	0.03
Platelet count —$no./mm^2$	252 $\pm$66	355 $\pm$162	<0.0001
Blood urea nitrogen—Normal range (3.3-6.7mmol/L)	-	12 ±7.6	
Creatinine—Normal range (45-120 μmol/L)	-	82 ±68	
Lactate dehydrogenase — Normal range (<240 IU/L)	-	371 ±143	
Bilirubin — Normal range (3-20 μmol/L	-	56 ±71	
Asparate aminotransferase — Normal range (10-50 IU/L)	-	44 ±22	
Alkaline phosphate — Normal range (30-130 IU/L)	-	92 ±46	
eGFR — mL/min	-	88 ±28	
Cystatin C — Normal range (0.63 -1.61mg/L)	-	0.98 ±0.6	

Abbreviations: yrs, years; BSA, body surface area; BP blood pressure.

4.3.2 Prevalence and severity of pulmonary hypertension

Two technically similar echocardiography methods, TR and PVR_{echo} were used to screen for pulmonary hypertension. Patients with sickle cell disease as a group were found to have significantly higher mean TR and PVR_{echo} values than the control group ($P < 0.001$ and $P < 0.05$). The prevalence of pulmonary hypertension in SCD population varied significantly, depending on method used. Of 123 patients, 45 (37%) were found to have TR jet velocities $\geq$ 2.5m/s; 22 (18%) had PVR of $\geq$ 2 woods units. Distribution chart demonstrated that 27% of SCD patients had TR velocity ranging between 2.5-3 m/s, which is mild in nature (Figure 4.3). Less than 10% of the study cohort had TR jet velocities between 3-3.5 m/s, which is moderate in severity and only 3% had severe TR velocities > 3.5m/s. Distribution chart of PVR by echo demonstrated that a large proportion (65%) of patients had PVR_{echo} values between 1 to 2 woods units (Figure 4.4). Fifteen percent of the study cohort had PVR values of 2 to 3 woods units and only two percent had PVR echo greater than 3 woods units.

Figure 4.3. Demonstrates the distribution of TR in SCD patients. Most patients had TR jet velocity <2.5m/s. Twenty seven percent had TR jet velocity between 2.5 and 3m/s. Very few patients < 5% had severe TR jet velocities greater than 3.5m/s.

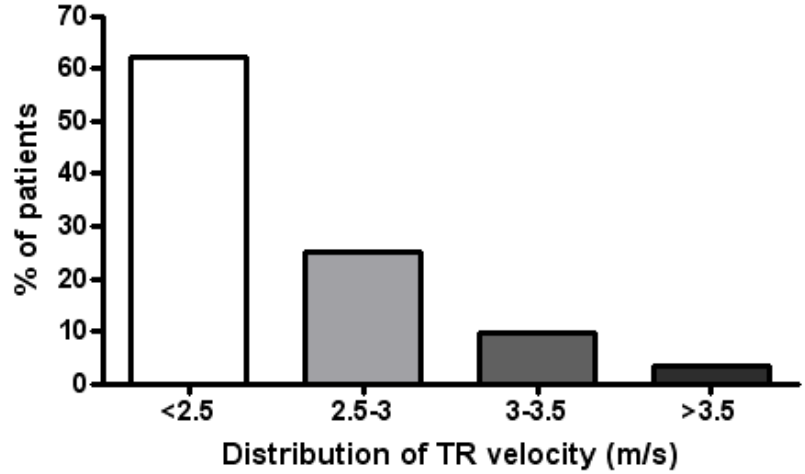
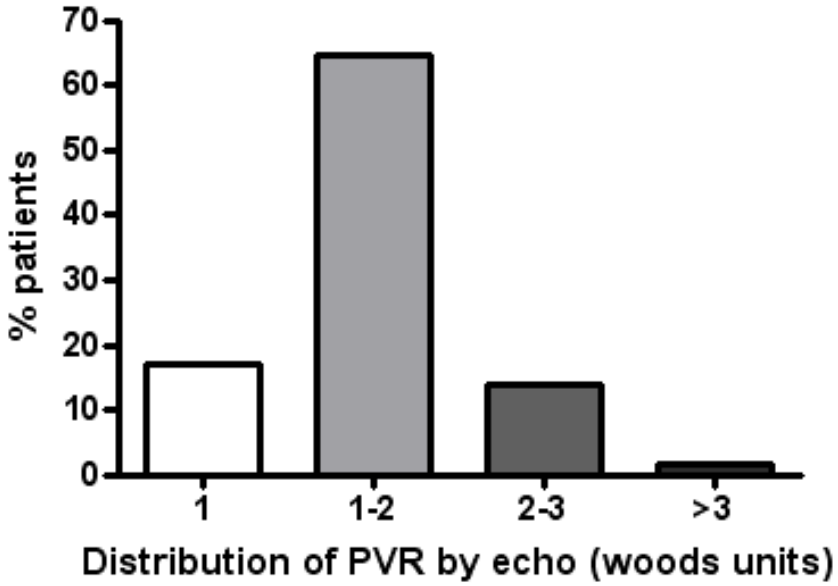


Figure 4.4. Distribution of PVR_{echo} in SCD cohort. A large proportion of patients had PVR_{echo} ranging between 1-2 woods units. Only a small proportion (2%) had PVR_{echo} > 3 woods units.



4.3.3 Right ventricular structure and function

Assessment of right ventricular structure and function by 3-dimension echocardiography found higher right ventricular systolic and diastolic volumes among sickle cell patients than controls either as absolute values or normalised for BSA (Figure 4.5 to Figure 4.8). Right ventricular volumes were highly influenced by the patients' genotype, with patients carrying the homozygous sickle gene having significantly higher mean volumes.

Figure 4.5 Right ventricular diastolic volumes in SCD patients with different phenotypes compared to control subjects'. Volumes are uncorrected for body surface area.

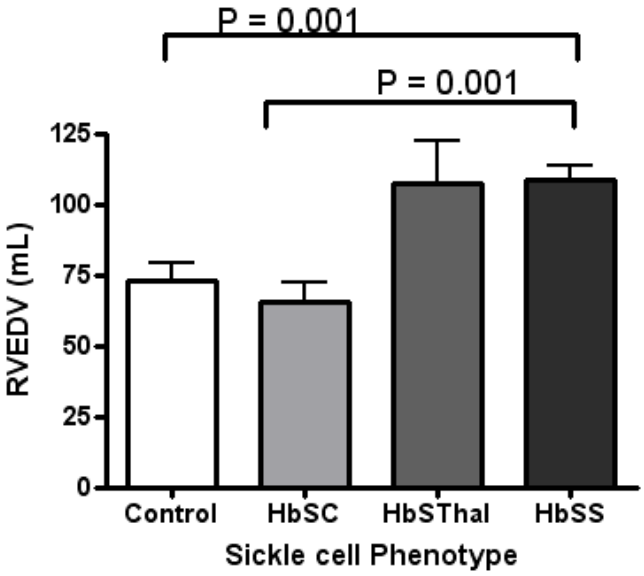


Figure 4.6 Indexed right ventricular diastolic volumes in SCD patients with different phenotypes compared to control subjects'.

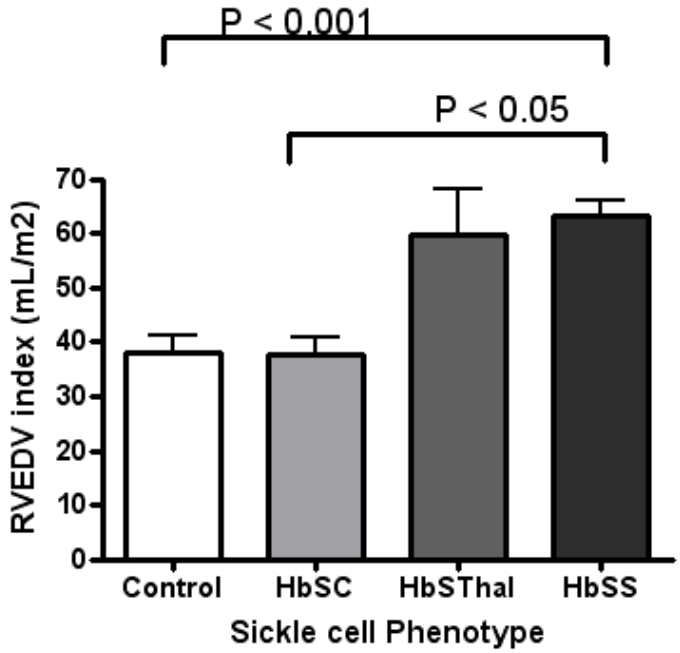


Figure 4.7 Right ventricular systolic volumes in SCD patients with different phenotypes compared to control subjects'. Volumes are uncorrected for body surface area.

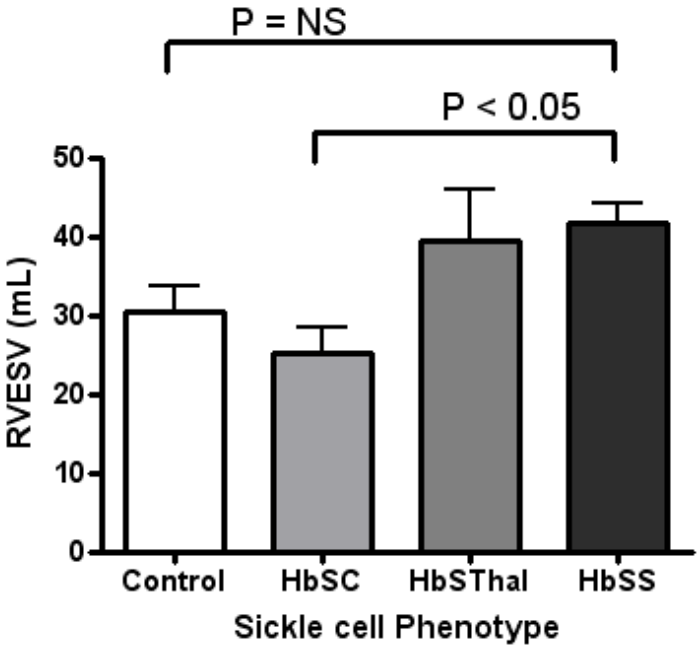
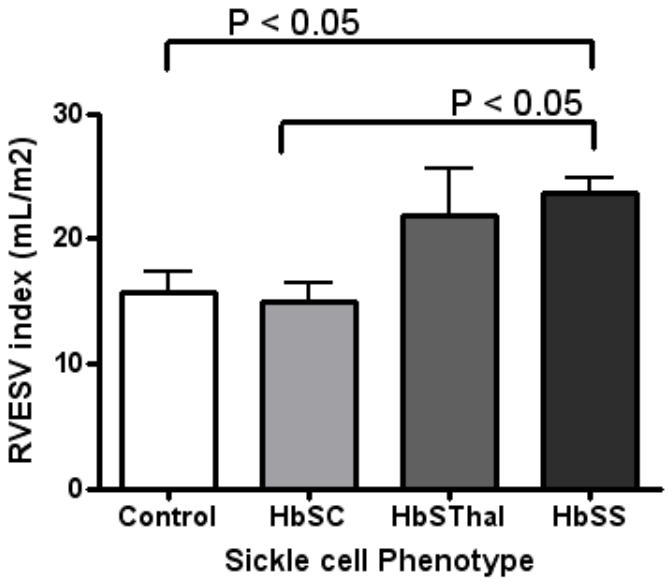


Figure 4.8 Indexed right ventricular systolic volumes in SCD patients with different phenotypes compared to control subjects'.



Patients with SCD did not have a global hyperdynamic systolic function (by ejection fraction). However, the longitudinal systolic function as assessed by tricuspid annular plane systolic excursion (TAPSE) method was significantly higher (Figure 4.10). Right ventricular diastolic function as measured by tissue Doppler E/E' ratio did not show any difference between sickle patients and control subjects. Multivariable analysis of large right ventricular volumes found the main independent determinants to be haemoglobin concentration and female gender (Table 4.2).

Figure 4.9 Comparison of right ventricular systolic function by ejection fraction in sickle cell disease patients and controls group.

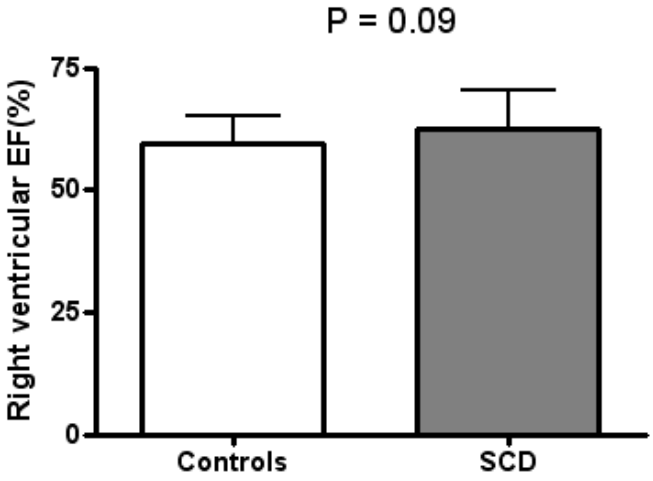


Figure 4.10 Right ventricular systolic function measured by tricuspid annular plane systolic excursion (TAPSE) in sickle cell patients compared to control group.

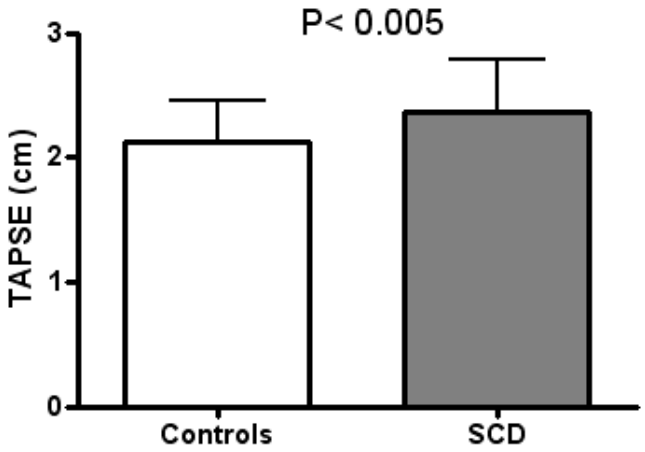


Figure 4.11 Comparison of right ventricular diastolic function by tissue Doppler E/e' ratio in sickle cell disease patients and controls.

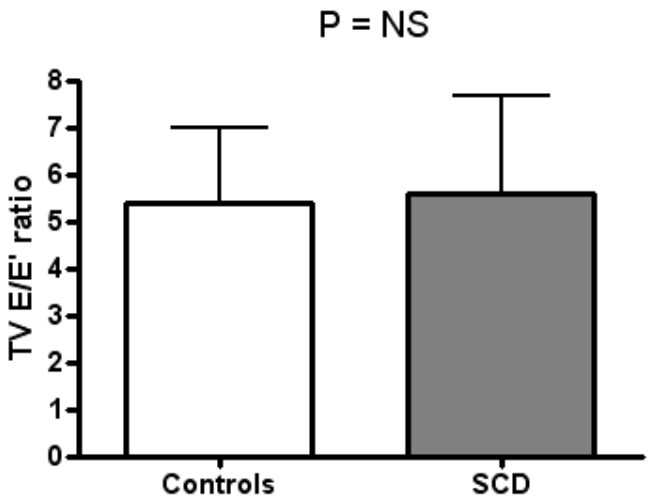


Table 4.2. Multivariable analysis model of independent determinants of large RV diastolic volumes in SCD.

	Beta	P value
Age — yrs	-0.21	0.06
Body surface area —m^2	0.09	0.43
Blood pressure —mmHg		
Systolic	-0.10	0.58
Diastolic	-0.01	0.97
Oxygen saturation — %	0.03	0.76
Haemoglobin saturation — g/dL	-0.44	0.002
LDH concentration — g/dL	-0.01	0.90
Patient gender — female	-0.25	0.03

†LDH,lactate deydrogenase

4.3.4 Determinants of raised TR velocity

Patients with TR $\geq$ 2.5m/s and those with TR <2.5 m/s were compared for clinical, laboratory and echocardiography indices (Table 4.3). Patients with tricuspid jet velocity $\geq$ 2.5m/s tended to be older and had a higher proportionate history of regular transfusion. They also had increased cardiac chamber volumes, hyper dynamic function, increased cardiac output and worse anaemia and evidence of hemolysis. There was an association between tricuspid jet velocity and tissue Doppler measurements of diastolic function in the left heart. There was also no evidence that those with raised TR velocity had RV dysfunction, in fact, TAPSE suggested RV hyperfunction.

Table 4.3 - Clinical, laboratory and echocardiographic parameters for patients divided according to TRV ≤2.5 and ≥2.5 m/s.

	TRV ≤2.5 m/s (n = 70)	**TRV ≥2.5 m/s (n = 53)**	**p value**
Clinical parameters			
Age (years)	35±13	39±14	0.08
Female gender (%)	46 (66)	33 (64)	0.80
BSA (kg/m^2)	1.7±0.2	1.8±0.2	0.16
Mean blood pressure (mmHg)	85±11	83±11	0.50
Oxygen saturation (%)	97±3	94±5	0.01
Medical History			
History of smoking (%)	3 (4)	2 (4)	0.90
History of hypertension (%)	6 (7)	10 (19)	0.09
Proteinuria	12 (17)	22 (42)	<0.01
History of acute chest syndrome (%)	6 (7)	3 (6)	0.56
History of pulmonary embolism (%)	2 (3)	8 (15)	0.01
History of liver disease (%)	6 (9)	4 (8)	0.86
History of priapism (%)	3 (4)	4 (8)	0.42
History of embolic stroke (%)	4 (6)	5 (10)	0.42
History of leg ulceration (%)	3 (4)	6 (12)	0.13
Regular blood transfusion (%)	4 (6)	10 (19)	0.02
Hydroxyurea therapy (%)	8 (11)	10 (19)	0.23
Laboratory markers			
Haemoglobin (mg/dl)	9.9±2.0	8.3±1.6	<0.01
Reticulocyte count (%)	298±157	314±120	0.54
White-cell count (No/mm^3)	9.2±3.7	9.9±4.2	0.35
Platelet count (No/mm^3)	339±155	376±171	0.24
eGFR (ml/min)	87±29	90±28	0.54
Bilirubin (µmol/l)	49±38	53±33	0.64
Aspartate aminotransferase (U/l)	43±27	45±16	0.73
Alkaline phosphatase (U/l)	96±51	86±37	0.25
Lactate dehydrogenase (U/l)	337±122	427±149	<0.01
Echocardiographic parameters			
LV ejection fraction (%)	61±6	61±8	0.84
TAPSE (cm)	2.3±0.4	2.5±0.4	0.02
LV lateral wall E/e' ratio	6.9±2.2	7.9±2.5	0.01
RV free wall E/e' ratio	5.4±1.8	5.8±2.4	0.44
TRV (m/s)	1.9±0.4	2.9±0.5	<0.01

†Plus –minus values are means ±SD. The body-surface area is the weight in kilograms divided by the height in meters.

¤ yrs, years; BSA, body surface area; BP blood pressure;TAPSE, tricuspid annular annular plane systolic excursion.

Model building for multivariable analysis required clinical characteristics from previously published articles and a shortlist of variables from carried forward from the univariate model (Pearson correlation for continuous, Mann-Whitney for non-continuous variables as highlighted in table 4.3) with cut off p-values values set at <0.005. Multivariable analysis model consisted of patient age, body surface area, systolic blood pressure, diastolic blood pressure, oxygen saturation, haemoglobin S concentration, lactate dehydrogenase and patient gender. Multivariable predictors of high tricuspid regurgitation jet velocity found low haemoglobin to be the only independent determinant of high TR velocity (Table 4.4).

Table 4.4. Multivariable analysis model highlighting determinants of raised TR in SCD.

	Beta	P value
Age — yrs	0.17	0.10
Body surface area —m^2	0.17	0.09
Blood pressure —mmHg		
Systolic	0.14	0.41
Diastolic	-0.25	0.15
Oxygen saturation — %	0.08	0.44
Haemoglobin saturation — g/dL	-0.37	0.006
LDH concentration — g/dL	0.05	0.67
Patient gender — female	0.004	0.97

†LDH,lactate deydrogenase

4.3.5 Determinants of high PVR measurements on echo

Data of patients with PVR $\geq$ 2 woods units and those with < 2 woods units were compared, similar to previous subsection. Patients with pulmonary vascular resistance values on echocardiography $\geq$ 2 woods units were associated with increasing patient's age and female gender (Table 4.5). As suspected, patients with PVR_{echo} $\geq$ 2 woods units did not have a have evidence of haemolysis or worse anaemia. Cardiac ventricular volumes were not significantly increased. Patients with PVR $\geq$ 2woods units did not demonstrate a hyper dynamic function.

Table 4.5. Clinical, laboratory and echocardiography indices in patients with high/low PVR_{echo}.

	PVR_{Echo} <2 WU (n = 101)	PVR_{Echo} >2 WU (n = 22)	p value
Clinical parameters			
Age (years)	35±13	47±15	0.01
Female gender (%)	73 (68)	6 (40)	0.03
BSA (kg/m^2)	1.7±0.2	1.8±0.2	0.04
Mean blood pressure (mmHg)	83±11	89±12	0.15
Oxygen saturation (%)	96±6	96±3	0.88
Medical History			
History of smoking (%)	5 (5)	0 (0)	0.39
Hypertension (%)	10 (9)	6 (40)	<0.01
Proteinuria (%)	31 (29)	3 (20)	0.47
History of acute chest syndrome (%)	9 (8)	0 (0)	0.24
History of pulmonary embolism (%)	8 ( )	3 (20)	0.08
History of liver disease (%)	9 (8)	1 (7)	0.82
History of priapism (%)	7 (7)	0 (0)	0.31
History of embolic stroke (%)	7 (7)	2 (13)	0.35
History of leg ulceration (%)	6 (6)	3 (20)	0.05
Regular blood transfusion (%)	9 (8)	5 (33)	<0.01
Hydroxyurea therapy (%)	16 (15)	2 (13)	0.87
Laboratory markers			
Haemoglobin (mg/dl)	9.2±2.0	9.4±2.0	0.71
Reticulocyte count (%)	309±142	271±136	0.40
White-cell count (No/mm^3)	9.2±4.0	8.7±2.6	0.29
Platelet count (No/mm^3)	356±163	352±128	0.94
eGFR (ml/min)	87±27	101±35	0.20
Bilirubin (µmol/l)	58±37	30±12	<0.01
Aspartate aminotransferase (U/l)	44±23	42±17	0.75
Alkaline phosphatase (U/l)	92±47	93±41	0.93
Lactate dehydrogenase (U/l)	374±140	357±169	0.77
Echocardiographic parameters			
LV ejection fraction (%)	61±6	59±10	0.47
TAPSE (cm)	2.4±0.4	2.4±0.4	0.99
LV lateral wall E/e' ratio	7.3±2.5	7.0±1.5	0.53
RV free wall E/e' ratio	5.6±2.1	5.2±2.1	0.52
PVR_{Echo} (WU)	1.3±0.4	2.4±0.4	<0.01

† Plus –minus values are means ±SD. The body-surface area is the weight in kilograms divided by the height in meters.

¤ yrs, years; BSA, body surface area; BP blood pressure;TAPSE, tricuspid annular annular plane systolic excursion.

4.3.6 Determinants of raised pulmonary vascular resistance

Multivariable model of raised pulmonary vascular resistance was constructed using known determinants of increased pulmonary hypertension and Pearson correlation analysis performed in table 4.5, with cut off values of P<0.005. Variables consisted of patient's age, body surface area, blood pressure, oxygen saturation, lactate dehydrogenase and patient gender (Table 4.6). From this model patient's age was demonstrated as the main independent determinant of raised pulmonary vascular resistance on echocardiography.

Table 4.6. Multivariable analysis model highlighting determinants of raised PVR_{echo} in SCD.

	Beta	P value
Age — yrs	0.29	0.01
Body surface area —m^2	0.11	0.30
Blood pressure —mmHg		
Systolic	-0.07	0.72
Diastolic	-0.07	0.73
Oxygen saturation — %	0.04	0.70
Haemoglobin saturation — g/dL	-0.16	0.26
LDH concentration — g/dL	-0.08	0.53
Patient gender — female	-0.15	0.18

†LDH,lactate deydrogenase

4.3.6 Evaluation of hypoxic pulmonary hypertension

Twenty patients with TR jet velocities $\geq$ 2.5m/s were treated with 5 L/min of oxygen through nasal prongs for a duration of 20 minutes and then echocardiography repeated. Most patients became restless after 2 hours of

scanning therefore repeat echocardiography parameters were limited to TR, PVR, TAPSE and CO. Treatment with oxygen resulted in a significant increment in oxygen saturation. However, there were no significant differences demonstrated in cardiac output, pulmonary vascular resistance, TR or right ventricular systolic function (assessed by TAPSE) after oxygen therapy.

Table 4.7. Effect of acute oxygen therapy on SCD with raised TR velocity

	Off Oxygen	On Oxygen	P Value
Oxygen Saturation, %	95 (± 5)	99 (± 0.5)	<0.005
Heart rate, bmp	73 (± 12)	69 (± 11)	0.39
Blood pressure, mmHg			
Systolic	116 (± 15)	119 (± 22)	0.60
Diastolic	69 (± 11)	68 (± 10)	0.76

Figure 4.12. Comparison of Doppler echocardiography TR jet velocities in SCD patients before and after oxygen therapy.

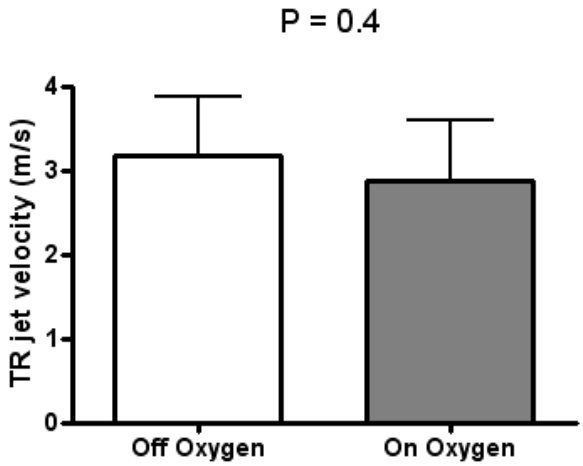


Figure 4.13. Comparison of PVR_{echo} in SCD patients before and after oxygen therapy.

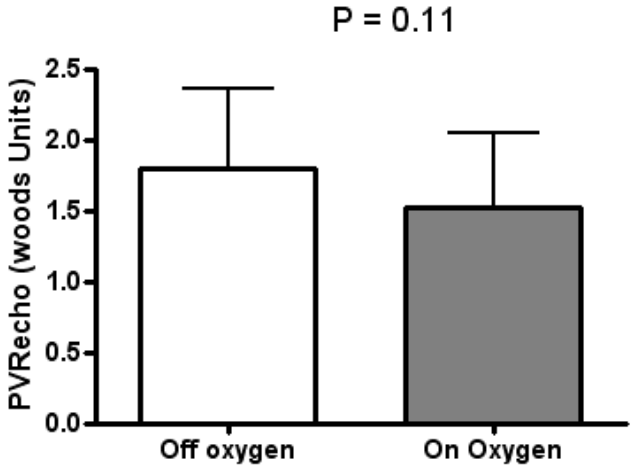


Figure 4.14 Comparison of right ventricular systolic function by TAPSE in SCD patients before and after oxygen therapy.

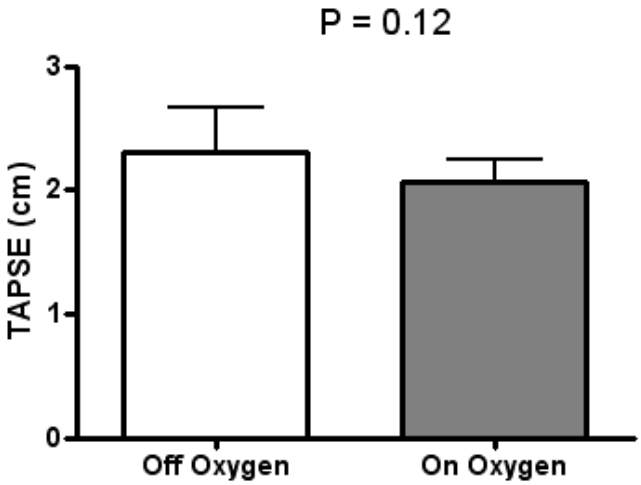
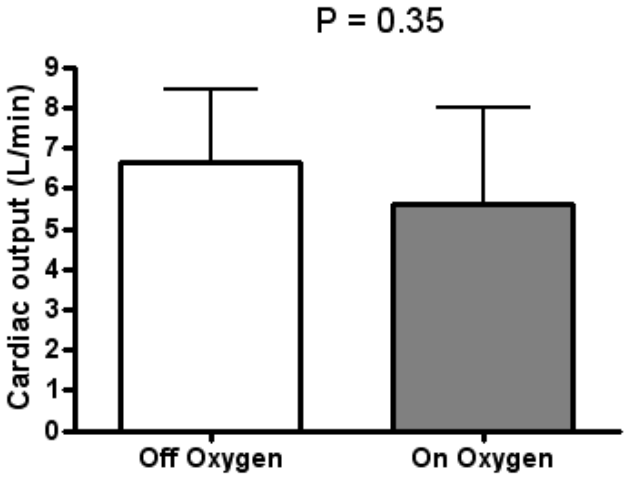


Figure 4.15 Comparison of cardiac out-put in SCD before and after oxygen therapy.



4.3.7 Right heart catheterisation

Among the 18 patients who underwent right heart catheterisation, 2 (11%) had confirmed pulmonary hypertension. Pearson correlation of TR with invasive measurements of PVR showed a moderate strength (r =0.65, P =0.004). Comparative analyses of TR and gold standard right heart cathetherisation data (Figure 4.17) demonstrate that the TR method yielded a very high false positive rate. Similarly, correlative analyses of PVR_{echo} with invasive measured PVR also showed moderate strength (r =0.68, P =0.002), Figure 4.18. However, unlike TR, no false positive estimates of pulmonary artery hypertension were demonstrated when PVR_{echo} was compared to invasively measured PVR, Figure 4.19. Receiver operating characteristic curve (ROC) was attempted to determine TR and PVR specificity and sensitivity, however, sample size and events outcomes were too small for the statistical programme to generate meaningful data.

Figure 4.16 Correlation of echocardiography Doppler derived TR velocity with invasive measurements of pulmonary vascular resistance in SCD patients.

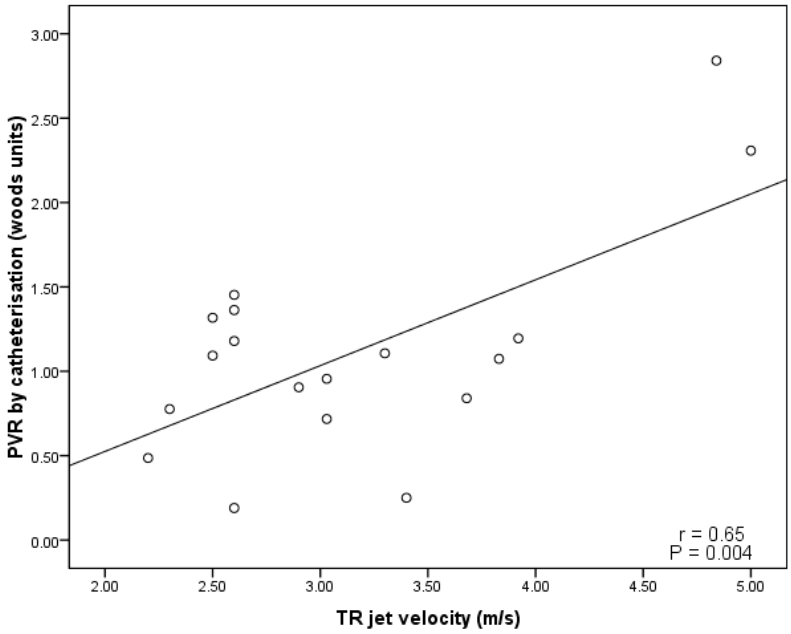


Figure 4.17 Validation of echocardiography Doppler measurements of TR velocity with invasive measures of pulmonary vascular resistance in SCD patients.

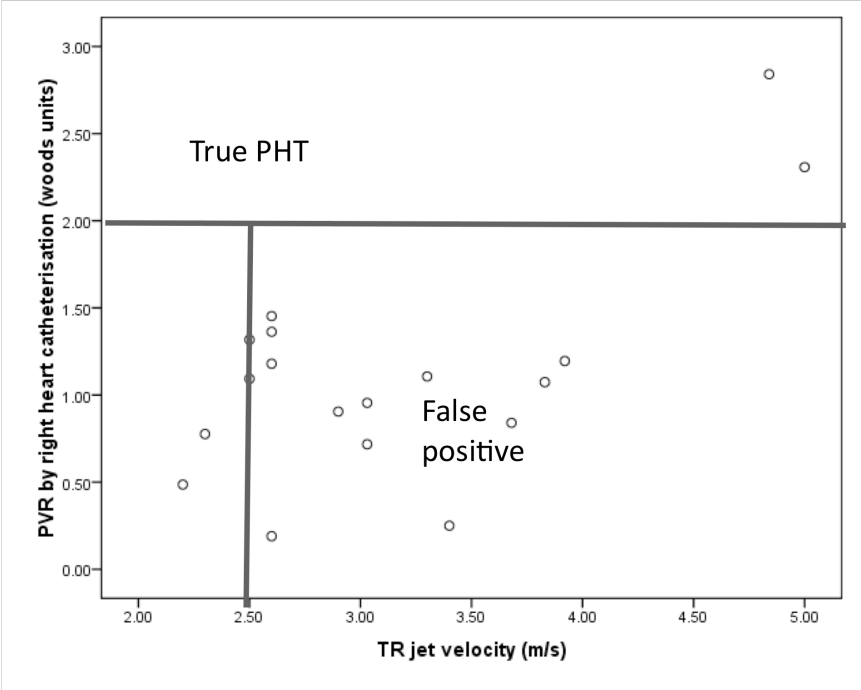


Figure 4.18. Correlation of echocardiography Doppler derived PVR_{echo} with invasive measurements of pulmonary vascular resistance in SCD patients.

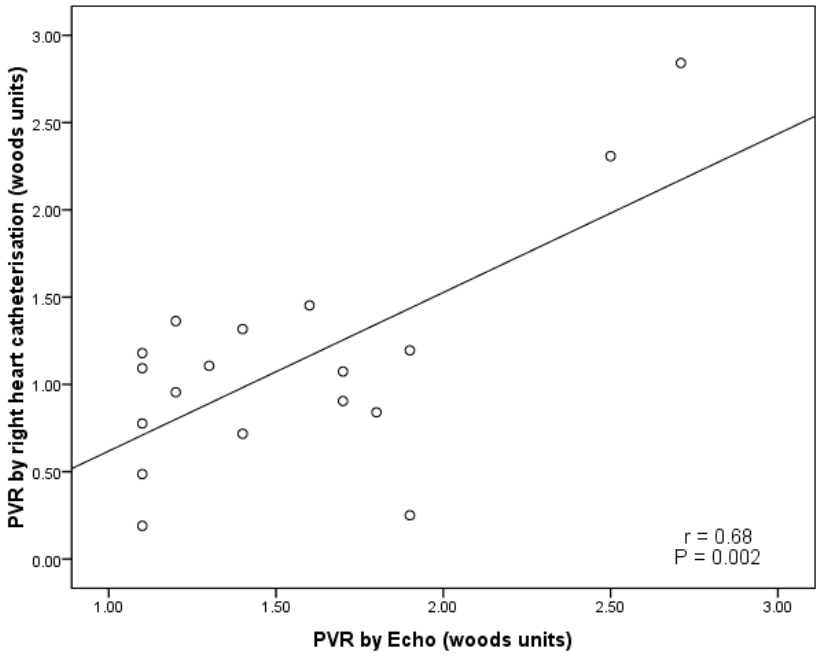
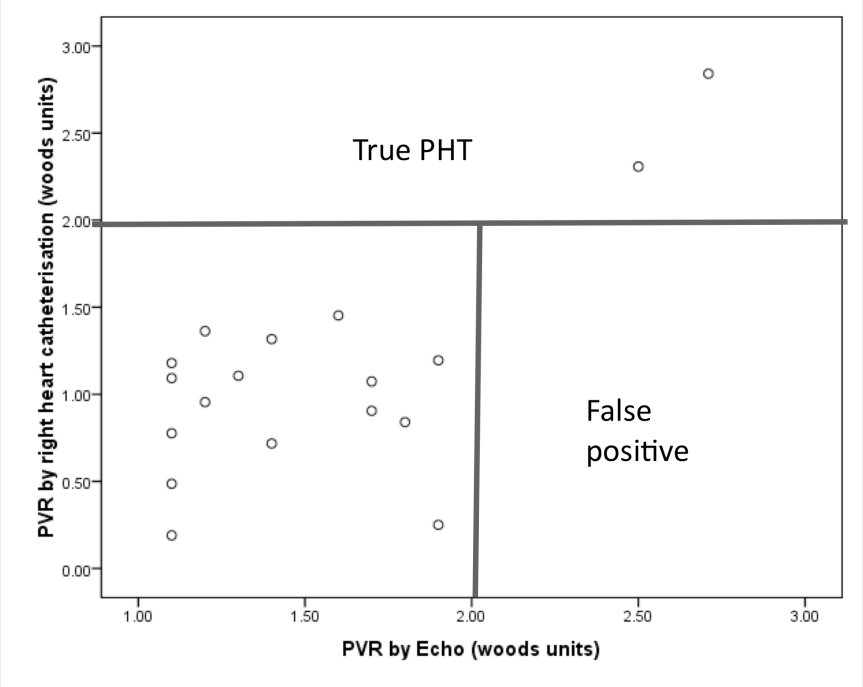


Figure 4.19. Validation of Doppler PVR_{echo} with invasive measures of pulmonary vascular resistance in SCD patients.



Our data demonstrates that there were a high proportion (44%) of patients with raised pulmonary venous hypertension (pulmonary capillary wedge pressure of >15mmHg) compared to true pulmonary arterial hypertension caused by raised pulmonary vascular resistance. Patients with PAH had raised right atrial pressure. Similarly, the pulmonary artery systolic, mean and diastolic pressure measurements were raised. PAH patients were severely anaemic with raised cardiac output. Assessments of left ventricular diastolic function by spectral and tissue Doppler showed normal function (Table 4.8).

Figure 4.20. Frequency of invasively diagnosed pulmonary arterial hypertension and pulmonary venous hypertension in SCD patients.

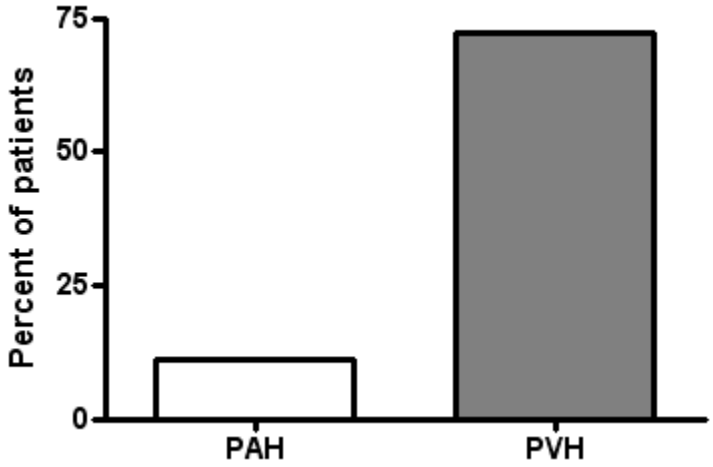


Table 4.8. Hemodynamic parameters of SCD patients with invasively diagnosed pulmonary arterial hypertension and raised capillary wedge pressure.

	PAH n=2	Raised PCWP n=8
RAP (mmHg)	8 ±0.0	10.1 ±3.4
PAS (mmHg)	53.5 ±0.7	35.4 ±9.0
PAD (mmHg)	24 ±0.0	18.4 ±5.4
mPAP (mmHg)	36 ±1.4	24.9 ±6.0
CO (L/min)	8.9 ±0.2	7.5 ±1.8
PCW (mmHg)	13 ±4.2	16.7 ±2.1
PVR (woods units)	2.6 ±0.38	1.1 ±0.6
TPR (dyn.sec.cm^{-5})	292 ±35	195 ±47
Hb concentration (g/dL)	7.8 ±0.3	7.5 ±1.1
Mitral E/a ratio	1.8	1.8 ±1.0
Mitral Ee' ratio	5.5	9.3 ±2.7

Abbreviations; RAP, right atrial pressure; PAS, pulmonary artery systolic pressure; PAD, pulmonary atery diastolic pressure; mPAP, mean pulmonary artery pressure; CO, cardiac output; PCW, pulmonary capillary wedge pressure; PVR, pulmonary vascular resistance, TPR, temperature pulse respiration; Hb, haemoglogin

Pearson correlative analyses were performed to determine whether measurements of PVR or pulmonary capillary wedge pressure (PCWP) were influenced by indexes such as haemoglobin concentration or cardiac output. Data in Figure 4.21 demonstrates no association between invasive measures of PVR and CO. No association was shown when invasive measures of PCWP pressure were correlated with thermodilution cardiac output (r =0.23, P =0.93). Furthermore, no association was demonstrated between PCWP and haemoglobin S concentration (r =-0.37, P =0.15). As hypothesised invasive values of PVR were not driven by severity of anaemia (r =0.2, P =0.4).

Figure 4.21. Correlation of invasive pulmonary vascular resistance to cardiac output in catheterised SCD patients. No association was demonstrated.

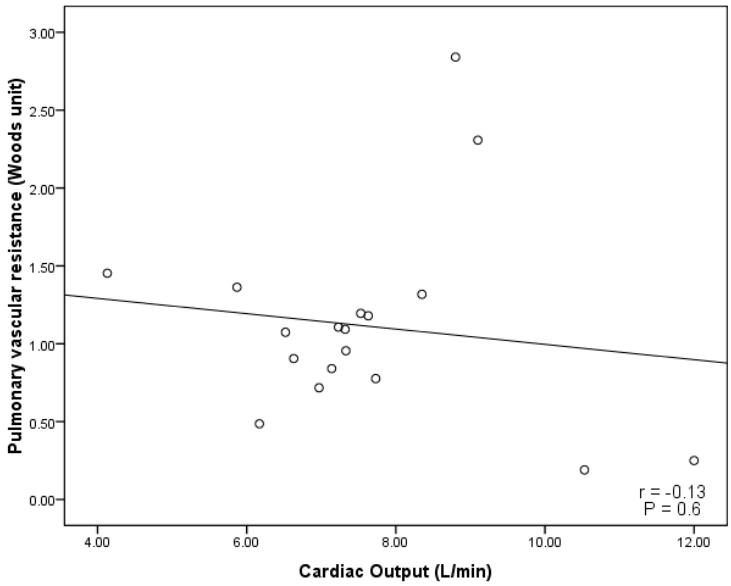


Figure 4.22. Correlation of invasive pulmonary capillary wedge pressure to cardiac output in catheterised SCD patients.

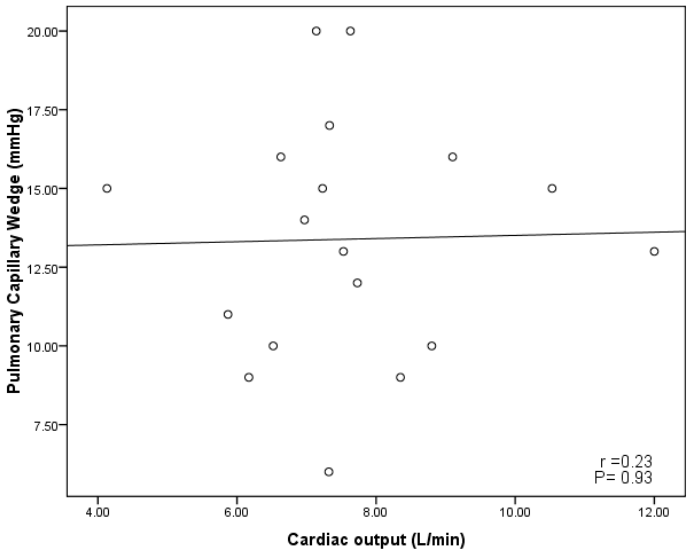


Figure 4.23. Correlation of invasive pulmonary wedge pressure to circulating haemoglobin concentration in catheterised SCD patients.

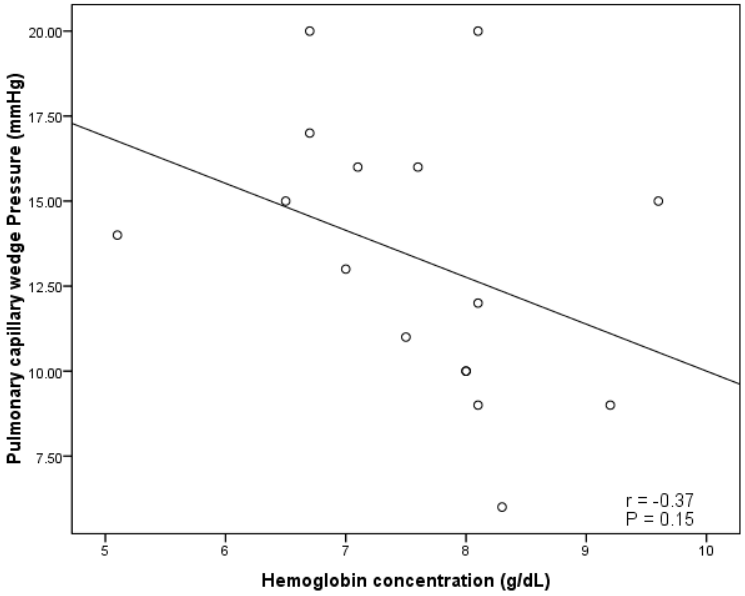
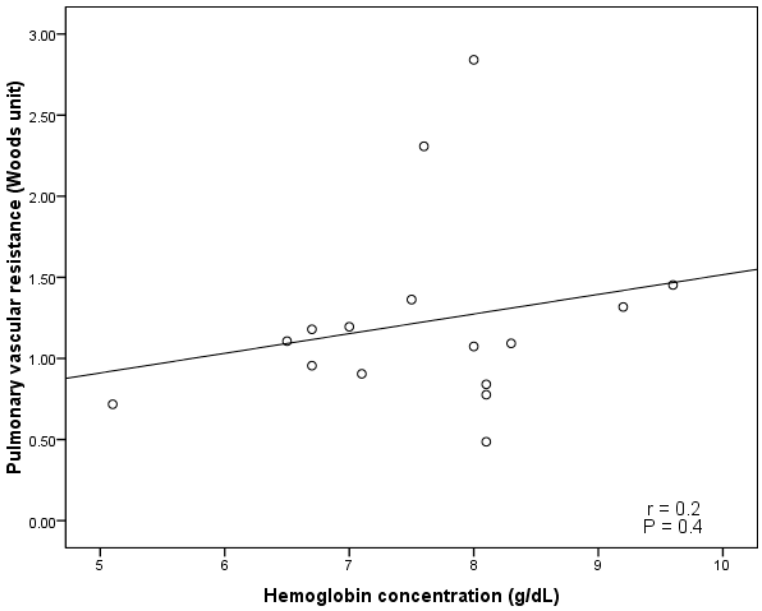


Figure 4.24. Correlation of invasive pulmonary vascular resistance to haemoglobin concentration in catheterised SCD patients.



Sixteen of the patients who underwent invasive right heart catheterisation had lung function tests and CT studies performed to determine whether there was underlying lung pathology. Lung function test data demonstrated mean reductions in forced expiratory volume (FEV_1), vital capacity (VC) and total lung capacity (TLC), Table 4.9. Normal CT findings were seen in a small proportion (6%) of patients. Fibrosis of lung tissue was demonstrated in up to 25% of patients with parachymal appearance on CT seen in 12% of the study cohort (Table 4.10). A large proportion of patients had reticular patterns (31%) and general loss of lower lobular volume.

Table 4.9. Pulmonary function findings in SCD at time of right heart catheterisation.

	Actual Values	% Predicted
Lung function		
FEV_1	2.0 (± 0.64)	63 ±17
VC	2.54 (± 0.74)	67 ±4
FEV_1/FVC ratio	77.6 (± 10.6)	-
TLC	4.56 (± 0.53)	5.7 ± 0.8
TLCO	5.95 (± 1.94)	48 ±32
KCO	1.52 (± 0.46)	-

Forced expiratory volume in a second (FEV_1); vital capacity (VC); total lung capacity (TLC); carbon monoxide diffusion coefficient (Kco).

Table 4.10. High resolution computer tomography findings in SCD patients (n=16).

		% of Patients
Computer Tomography		
	Normal	6
	Fibrosis	25
	Parachymal	12
	Pronounced pulmonary vessel	13
	lower lobe volume loss	25
	Reticular pattern	31

4.4 Discussion

In this large unselected cohort of SCD patients, two non-invasive Doppler methods for diagnosing PHT namely, PVR_{echo} and TR were used to assess the prevalence of pulmonary hypertension in SCD patients. The accuracy of the two imaging methods were compared to gold standard invasive measurements of PVR.

Novel real-time three-dimensional imaging techniques were initially used to address the hemodynamic effects of PH on the right ventricle and its adverse impact on cardiac outcomes. The study results show that patients with SCD have significantly higher right ventricular volumes and systolic function compared to control subjects. Observations of increased right ventricular volumes remained unchanged when volumes were indexed for body surface

area. Increased right ventricular volumes were demonstrated to be a consequence of anaemia and increased CO, a finding that has not been reported previously. In the current study, multivariable analysis of clinical features and laboratory findings demonstrate that female gender and chronic anaemia are independently associated with increased right ventricular volumes in SCD patients.

Multivariable models development in the current study identified variables from univariate analyses of clinical risk factors using Pearson regression analyses with cut off values set at $P<0.005$ (Czopowicz, Kaba et al. 2012). In addition, a literature search from published manuscripts indentified known clinical characteristics used on multivariable models for PHT in SCD populations (Sutton, Castro et al. 1994; Gladwin, Sachdev et al. 2004; Sachdev, Kato et al. 2011). Therefore, multivariable models consisted of patient age, body surface area, systolic blood pressure, diastolic blood pressure, oxygen saturation, haemoglobin S concentration, lactate dehydrogenase and patient gender.

In agreement with previously published retrospective studies, > 30% of SCD patients in the current study had TR velocity $\geq$ 2.5m/s (Gladwin, Sachdev et al. 2004; Gladwin and Vichinsky 2008; van Beers, van Eck-Smit et al. 2008; Minniti, Sable et al. 2009; Gladwin 2011). Clinical features associated with high TR velocities were: previous history of respiratory disease, regular transfusion, increased blood urea nitrogen, high levels of lactate dehydrogenase and severe anaemia. These data suggest that there is a link in SCD patients between TR velocities and high cardiac output state resulting from anaemia. However, in

contrast to previous studies, laboratory and clinical history investigations to determine contribution of multi-organ dysfunction found no association between increased TR velocities and acute chest syndrome, leg ulcers, renal, liver dysfunction, bilirubin, creatinine and alkaline phosphate.

Three-dimensional echocardiography studies found strong correlations between cardiac output, left ventricular volume, left ventricular mass, left atrial size and raised TR velocities. These observations may suggest that elevated TR readings are driven by raised cardiac output associated with chronic haemolysis. TR velocities may also be increased due to post-capillary reasons such as elevated left ventricular preload. In addition, our data found an association between TR and load independent measurements of left ventricular diastolic function – as assessed by tissue Doppler. Further analysis of three-dimensional right ventricle showed that right heart volumes and function were significantly higher in patients with increased TR velocities. As suspected, multivariable analysis of clinical and laboratory findings found severity of anaemia to be the only independent measure associated with increased TR velocity.

Previous studies have found an overestimation of the prevalence of pulmonary hypertension in up to two-thirds of SCD patients (Simmons, Santhanam et al. 1988; Aleem, Jehangir et al. 2007) because the enrolment protocols included patients who were critically ill with low hemoglobin levels (Ahmed, Siddiqui et al. 2004; Suell, Bezold et al. 2005). Other investigators have associated comorbidity i.e. end organ dysfunction to be a secondary cause of pulmonary

hypertension in SCD and thalassemia patients (Gladwin, Sachdev et al. 2004). In the exception of a few studies where pulmonary hypertension was confirmed by right heart catheterisation (Anthi, Machado et al. 2007; Parent, Bachir et al. 2011; Fonseca, Souza et al. 2012), the vast majority of studies in SCD have used a single echocardiography method as a screening tool.

Put together, the current study findings support the argument that elevated echocardiography derived TR values in SCD patients result due to an overestimation of pulmonary artery pressures caused by chronic anaemia and high cardiac output state. Mean pulmonary artery pressure (PAP) is a product of cardiac output and pulmonary vascular resistance (mean PAP – mean PCW pressure = CO x PVR). Therefore an increase in either cardiac output or higher pulmonary vascular resistance can lead to an increase in pulmonary artery pressure.

Thalassemia is an inherited haemolytic disease associated with chronic anaemia due to reduced synthesis of globin chains that produce hemoglobin molecules (Olivieri 1999; Rund and Rachmilewitz 2005). Secondary pulmonary hypertension in thalassemia is reported as the most common cardiovascular finding that can lead to heart failure (Gladwin, Sachdev et al. 2004; Farmakis and Aessopos 2011). Similar debates surrounding prevalence of PH exist in this group of patients with rates ranging from 10 percent to 93 percent, depending on screening protocols and echocardiography method employed. Investigators using magnetic resonance imaging report high right ventricular volumes and increased cardiac output correlating to degree of anaemia in thalassemia major

patients (Carpenter, Alpendurada et al.) Similar to SCD patients, a few right heart catheterisation studies have been performed in this subgroup of patients. Although prevalence and mechanism of pulmonary hypertension in thalassemia patients remain to be addressed, left ventricular diastolic dysfunction has been reported during invasive right heart catheterisation studies, however sample sizes have been too small to extrapolate these findings to the larger SCD population (Aessopos, Stamatelos et al. 1995; Aessopos, Farmakis et al. 2001).

Pulmonary hypertension diagnosed by a single echocardiography-screening tool, such as TR poses several limitations. One of which is it's inability to distinguish between pre- and post-capillary pulmonary hypertension. Pulmonary vascular diseases increase resistance in the pulmonary circulation that lead to pulmonary arterial hypertension and right heart failure (Bech-Hanssen, Lindgren et al. ; Tedford, Hassoun et al. ; Melikian, Seddon et al. 2009). Previous studies using pulmonary vascular resistance (PVR_{echo}) with a cut off value set at $\geq$ 2 Wood units have shown strong correlation with invasive right heart catheterisation hemodynamic measurements of pulmonary vascular resistance (PVR) (Abbas, Fortuin et al. 2003; Haddad, Zamanian et al. 2009).

To help identify SCD patients likely to develop pulmonary arterial hypertension and increase diagnostic sensitivity and specificity, the current study incorporated non-invasive calculations of PVR_{echo}. Interestingly when PVR_{echo} method was applied, the prevalence of pulmonary hypertension in our SCD patients reduced by two fold from 37% to 18%. Clinical and echocardiography features associated with raised PVR_{echo} were patients' age, female gender,

body surface area, systolic blood pressure and history of respiratory disease. Elevation in PVR_{echo} was not associated with left ventricular mass or diastolic dysfunction; however, left atrial size was significantly larger in patients with raised PVR_{echo}. The above study findings provide additional insights surrounding limitations faced when a single echocardiography method is used to screen for pulmonary hypertension. Non-invasive PVR_{echo} is not influenced by anaemia or high cardiac output. It is also encouraging to find that none of our patients had PVR_{echo} of >3 woods unit, which would indicate that PH in SCD patients is mild in nature. Multivariable analysis of increased PVR_{echo} with clinical and laboratory findings highlighted patient age to be an independent determinant.

The prevalence of pulmonary hypertension in our healthy black control subjects was found to be 10% when a TR cut-off value of 2.5m/s was applied; this prevalence rate was significantly higher than reported in the general population where 15 cases are seen in a million (McLaughlin and Suissa ; Hachulla, Gressin et al. 2005). However, none of the control subjects had raised pulmonary vascular resistance when PVR_{echo} was used. It could be argued that ethnicity can influence PH rates. Indeed, investigators have reported differences in the vascular biology of people from African descent that may contribute to increased incidences of pulmonary and systemic hypertension (Lang, Stein et al. 1995; Perregaux, Chaudhuri et al. 2000; Melikian, Wheatcroft et al. 2007). An ethnic disparity study among systemic sclerosis patients found diastolic dysfunction to be the main contributor to incidences of PH among African American subjects (Beall, Nietert et al. 2007). Other studies assessing

vascular function in young black African men found reduced endothelial dependent nitric oxide bioavailability and high incidence of cardiovascular disease (Lang, Stein et al. 1995; Melikian, Wheatcroft et al. 2007).

Accuracy of TR and PVR_{echo} screening techniques were determined when eighteen of our SCD patients were referred to have invasive right heart catheterisation studies performed. A moderately good correlation was found when echocardiography TR/PVR_{echo} measures were compared to gold standard invasive assessment of PVR. As hypothesised, TR cut-off value set at $\geq$ 2.5 m/s yielded a high proportion of 'false positives' rates. Non-invasive echocardiography based PVR_{echo} had a lower false positive rate, however, more data are required to judge accuracy of this method in the SCD population. Invasive results obtained so far suggest that only a small minority of SCD patients have true pulmonary arterial hypertension, with just two of eighteen patients catheterised showing PVR values greater than 2 woods units. Furthermore, in this study none of our SCD patients that underwent invasive diagnosis for PH required therapeutic intervention, as PVR was mild. Current study findings are in agreement with previous invasive reports where mild pulmonary hypertension and mild elevation in pulmonary vascular resistance have been documented (Gladwin, Sachdev et al. 2004; Machado, Martyr et al. 2005; Anthi, Machado et al. 2007; Parent, Bachir et al. 2011).

The question of left ventricular dysfunction in relation to increased pulmonary pressures through passive congestion of the pulmonary vascular tree was addressed in this study. In our catheterised SCD patients, 44% had elevated

pulmonary capillary wedge pressures greater than 15 mmHg and 11% were diagnosed with pulmonary arterial hypertension. These findings are in agreement with previous invasive reports where left ventricular diastolic dysfunction contributed to PHT in a significant proportion of patients (Anthi, Machado et al. 2007; Machado 2007). In contrast to previous reports, no significant mitral or aortic valve regurgitation was found in the current cohort of SCD patients to explain the increase in pulmonary capillary wedge pressure. Perhaps elevation in wedge pressure was due to increased preload preceding left ventricular diastolic dysfunction as highlighted in table 4.3. Raised plasma brain natriuretic peptide (BNP) has been shown to be effective in screening for left ventricular dysfunction and correlates well with severity symptoms of heart failure (Wang, Larson et al. 2004). Further assessment of biomarker BNP in our catheterised SCD patients would help to determine degree of left ventricular dysfunction particularly in patients that had raised pulmonary capillary wedge pressure.

In the current study, the overall rate of death over a follow-up period of 36 months was 4.9%, which is in agreement with a previous study by Gladwin et al where a 5.3% death rate was documented. Four of the six patients who died in our group had TR velocities >2.5m/s, and three had PVR_{echo} > 2 woods units. Comorbidities in deceased patients included lung pathology, vasculopathy, renal and liver dysfunction. Two of the six SCD patients underwent evaluations by right heart catheterisation and one was diagnosed with pulmonary arterial hypertension.

Respiratory diseases are common amongst SCD patients. Although in the current study, pulmonary function tests did not find significant differences in lung volumes between patients with and without pulmonary arterial hypertension, data acquired mostly revealed a pattern of restrictive lung pathology with some patients showing evidence of obstructive or mixed lung disease. Computer tomography studies found prominent pulmonary vessels, pulmonary fibrosis, ground glass opacification and evidence of lobular volume loss in a large percentage of our patients. However, no active thrombus was found insitu. These study findings support reported autopsy, CT and pulmonary function studies where restrictive lung disease, abnormal diffusing capacity and generalised pulmonary fibrosis are documented (Powars, Weidman et al. 1988; Machado and Gladwin 2005; Anthi, Machado et al. 2007).

Currently there is limited data on the effects of specific treatment for pulmonary hypertension in SCD patients. Therapy requires treatment of chronic hypoxia with vasodilators/ antiremodelling agents, thromboembolic disease, and identifying/ treating comorbid cardiopulmonary diseases (Vij and Machado ; Machado, Martyr et al. 2005). Oxygen therapy is routinely used in the management of vaso-occlusive crises, however there is lack of evidence to support its effectiveness in SCD (Embury, Garcia et al. 1984; Schulman 1984; Zipursky, Robieux et al. 1992; Yale, Nagib et al. 2000). A study looking at the effect of oxygen therapy on erythropoiesis and rheologic properties of sickled red blood cells found a rapid decline in erythropoietin levels and increased rise in the number of reticulocytes (Embury, Garcia et al. 1984). Another study looking at oxygen effect on the number of reversible sickled cells in SCD

patients found no association between oxygen therapy and incidence of crisis (Zipursky, Robieux et al. 1992).

The role of oxygen therapy in the context of pulmonary hypertension has not been established. In the current study, oxygen therapy was administered to patients with TR jet greater than 2.5m/s to help identity hypoxic vasoconstriction. Treatment with oxygen significantly reduced peripheral saturation; however, patient's heart rate and blood pressure remained unchanged. No significant reductions in tricuspid jet velocity, pulmonary vascular resistance and cardiac output were seen after oxygen treatment. Indeed more data and clinical trials are required to explore mechanisms contributing to hypoxia-induced vasoconstriction. It is likely that maximization of oxygen therapy may be of benefit in reducing pulmonary congestion.

4.5. Summary

In summary, these results in a large unselected cohort of SCD patients indicate that while greater than 30% of patients have TR $\geq$ 2.5m/s, this is largely driven by high cardiac output related to anaemia. Using a 2.5 m/s cut-off for TR results in high proportion of 'false positives' when compared to the gold standard invasive assessment of PVR. A non-invasive echo-based estimate of PVR has much lower false positive rate but more data are required to judge accuracy of this method in the SCD population. Study results so far suggests that only a small minority of SCD patients may have true pulmonary arterial hypertension, however, this is mild in nature and did not affect right ventricular function.

Chapter 5

Vascular dysfunction in adolescent patients with sickle cell disease

5.0 Introduction

The primary pathology in SCD is episodes of recurrent ischemia-reperfusion injury resulting from ruptured abnormal red blood cells occluding the microvascular circulation. Over a period of months and years, this can lead to multi-organ tissue damage and failure (Blum et al., 2005; Eberhardt et al., 2003; Gladwin et al., 2003). Although the primary genetic defect is identified in all HbSS patients, there is considerable heterogeneity in clinical presentation. It is possible that presentation of this maybe related to differences in endothelial/vascular function among patients. As discussed in previous chapters, a deficiency of NO maybe due to the features of SCD. However, what is not known is whether patients with SCD have evidence of significant vascular dysfunction at an early stage of their condition, before they have had multiple clinical complications or have acquired other risk factors for vascular diseases (eg hypertension, smoking, hypercholestrolimia or aging). Despite a considerable rise in observational data reporting cardiopulmonary complications, the contribution of cardiovascular abnormalities and endothelial dysfunction to SCD remains poorly understood.

High resolution B-mode ultrasonography of the carotid arteries is an imaging technique that is widely used to obtain measurements of the carotid artery lumen diameter, intima-media thickness and the presence and extent of plaque (O'Leary et al., 1999; O'Leary et al., 1991). Recent evidence suggests a link between carotid intima-media thickness and many cardiovascular outcomes, including cerebral and coronary events (Cao et al., 2007; del Sol et al., 2001). As such, carotid intima thickness maybe regarded as an early marker of

vascular disease (O'Leary et al., 1999; Raitakari et al., 2003; Simons et al., 1999). No study to date has reported on whether patients with SCD have abnormal carotid intima media thickness.

Endothelial dysfunction, including reduced nitric oxide release is a hallmark to many disease states such as, athesclerosis, pulmonary hypertension, cerebral ischaemia, hypercholesterolemia and diabetes (Benjamin et al., 2004; Katz et al., 2005; Lefer et al., 2001; Patti et al., 2005; Verma et al., 2003; Wolff et al., 2007). Ultrasound brachial artery flow mediated dilatation (FMD) is a non-invasive imaging technique that was first described in the late 1980s (Ludmer et al., 1986) and broadly used to evaluate endothelial function specifically, endothelial derived nitric oxide release through methods of shear stress (Anderson et al., 2000; Clarkson et al., 1997; Steffel and Luscher, 2009). Many investigators have associated impairment in FMD to endothelial dysfunction in the coronary circulation and systemic risk factors including the whole vascular system (Celermajer, 1997; Seddon et al., 2009; Vogel and Corretti, 1998). However, whether young patients with SCD have significant endothelial dysfunction is unclear.

5.2. Study aims

Therefore aim of the present study were to determine whether abnormal endothelial function and vascular disease exists in stable SCD adolescents independent of risk factors such as age, blood pressure, smoking or hypercholesterolemia.

5.3. METHODS

5.3.1. Study population and data collection

King's College Hospital is the biggest Sickle Cell Centre for children in the UK with approximately 400 children seen each year in the paediatric outpatient clinic (Deane et al., 2008). The study cohort was recruited from the paediatric outpatient clinic. The patient population consisted of twenty, stable patients with electrophoresis and chromatography diagnosis of sickle cell disease expressing only haemoglobin S phenotype. Potential participants with SCD were excluded if they had a vaso-occlusive crisis in the last two weeks. Fourteen healthy black subjects with no SCD were recruited from the general public as controls. All control subjects were matched for age, gender and ethnicity. The study was approved by the local ethics committee and written consent obtained from all parents or guardian before hand.

5.3.2. Echocardiography

Transthoracic echocardiography studies were performed using a portable Philips ultrasound (CX50) machine. Cardiac output was obtained by measuring the diameter of the left ventricular outflow tract in the parasternal long axis view. Spectral Doppler pulse wave measurements of left ventricular outflow tract forward flows were taken 1mm below the AV in the five chamber view (Baumgartner et al., 2009). Aortic valve forward flows were also obtained in the five chamber view at the tips of the aortic valve leaflets using continuous Doppler modality.

Images were digitally stored for off-line analysis using Xlera analysis software. Details of image analysis are described in the general methods chapter.

5.3.3. Common carotid intima-media thickness and FMD

Image acquisition and data analysis are described in the general methods chapter.

5.3.4. Transcranial Doppler ultrasonography

Transcranial Doppler studies were performed by trained technicians blinded to the study in the vascular laboratory at King's College Hospital using Duplex transcranial ultrasound scanners (Siemens Sequoia and Aspen) equipped with a 2 MHz transcranial transducer. The method of examination of intracerebral blood-flow velocity was similar to that previously described in the Stroke Prevention in Sickle Cell Disease Study (Adams et al., 1998b). Both sides of the middle cerebral, anterior cerebral, bifurcation, distal internal carotid artery and posterior cerebral arteries were studied. Maximum velocities were recorded as time-averaged maximum velocity (TAMMV) in centimetres per second.

5.4. RESULTS

5.4.1. Patients

All subjects tolerated the studies well. The demographic characteristics of the study patient population are shown in table 5.1. A total of 20 adolescent SCD patients with the homozygous sickle cell disease phenotype and 14 controls subjects were enrolled. Patients were aged between 6 and 18 years, and 55% were female. All study patients were from a black ethnic background. Therefore,

measures were taken to ensure controls were matched for ethnicity to reflect 'normal' parameters.

Interestingly, although SCD patients had lower body surface area than controls, no significant differences were observed in BSA. Blood pressure recordings of systole and diastole showed reduced readings in patients, however these were not statistically significant. Non-parametric analysis of pulse oximetry readings demonstrated that SCD patients had lower oxygen saturation ($P = 0.03$). Furthermore, comparisons of heart rate between patients and control subjects did not find any significant differences. The mean cardiac output in patients was significantly elevated (3.5 L/min vs 5.6 L/min).

Clinical characteristics in SCD patients demonstrated a history of systemic hypertension in 10% of study population. Twenty five percent of the patient cohort had previous history of proteinuria. Five percent of the study population were noted to have a history of priapism. A large number of patients (45%) were under hydroxy urea therapy. Five percent of the study population are undergoing regular transfusion therapy. None of the patients had a history of smoking. None of our patients had a history of respiratory disease, pulmonary embolism, renal dysfunction, liver disease, stroke or evidence of leg ulcers.

Table 5.1. Demographic, clinical, anthropometric and biochemical characteristics of SCD patients.

	Controls (n=14)	Patients (n=20)	P Value
Clinical			
Age — yrs	12 ±4	14 ±4	0.33
Female gender — no.(%)	9 (60)	11 (55)	0.6
Black ethnic origin —no. (%)	14 (100)	20 (100)	0.9
BSA — g/m^2	1.4 ±0.4	1.1 ±0.7	0.23
Blood Pressure — mmHg			
Systolic	106 ±9	108 ±13	0.65
Diastolic	65 ±6	62 ±11	0.28
Oxygen saturation — %	99 ±0	98 ±2	0.03
Heart rate — bpm	84 ±9	81 ±13	0.33
CO — L/min	3.5 ±1.0	5.6 ±1.9	<0.001
History of smoking — no.(%)	-	-	-
History of respiratory disease — no.(%)	-	-	-
History of systemic hypertension — no.(%)	-	-	-
History of acute chest syndrome — no.(%)	-	2 (10)	0.16
History of pulmonary embolism — no. (%)	-	-	-
History of renal dysfunction — no. (%)	-	-	-
History of proteinuria — no. (%)	-	5 (25)	0.02
History of liver disease — no. (%)	-	-	-
History of priapism — no. (%)	-	1 (5)	0.3
History of stroke — no. (%)	-	-	-
History of leg ulceration — no. (%)	-	-	-
Regular transfusion — no. (%)	-	1 (5)	0.3
Hydroxy urea therapy — no. (%)	-	9 (45)	0.001

Abbreviations: O_2, oxygen; BSA, body surface area; CO, cardiac output

5.4.2 Laboratory markers

Patient laboratory biomedical details are demonstrated in Table 5.2. None of the control subjects wanted their blood drawn therefore data comparisons could not be made. However, biochemical markers such as haemoglobin S and F in

patients were below normal range. Markers of haemolysis i.e. reticulocyte count and lactate dehydrogenase were above normal. The liver function marker asparate aminotranferase was elevated. Renal function marker, creatinine was below normal.

Table 5.2. Demographic biochemical characteristics in SCD patients and control subjects.

	Normal range	Controls (n=14)	Patients (n=20)
Laboratory			
Haemoglobin —g/dL	13-16.5	-	8.0 ±4.4
Haemoglobin F — %	<1.0	-	4.7 ±1.06
Lactate dehydrogenase — U/L	<240	-	462 ±190
Reticulocytes — U/L	50-150	-	255 ±106
Bilirubin — µmol/L	3-20	-	32 ±23
Creatinine — mg/dL	45-120	-	31 ±17
Alkaline phosphatase — U/L	58-237	-	132 ±79
Aspartate aminotransferase — U/L	3-35	-	46 ±14

5.4.3. Carotid ultrasound

Comparisons of carotid artery diameter were made between patients and control subjects (Figure 5.1.). Measurements were made by means of B-mode imaging and all analysed performed using Medical Imaging software. Interestingly the mean common carotid artery diameter in patients was significantly enlarged. Paired t-test analysis of common carotid intima-media thickness (Figure 5.2.) demonstrated significant wall thickness among patients (0.44 ±0.02 versus 0.50 ±0.04, P <0.01).

Figure 5.1. Comparison of common carotid diameter in SCD versus control subjects. SCD patient's common carotid arteries were significantly larger than control subjects.

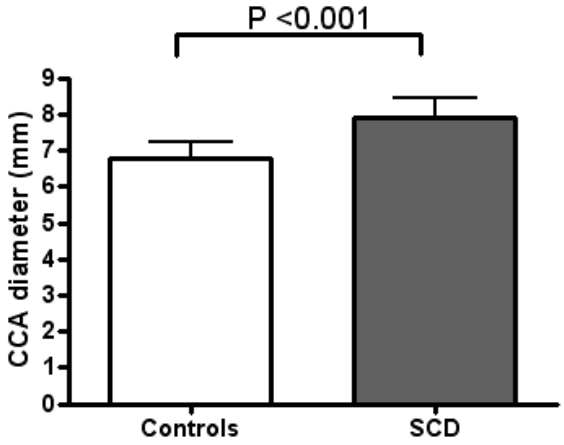
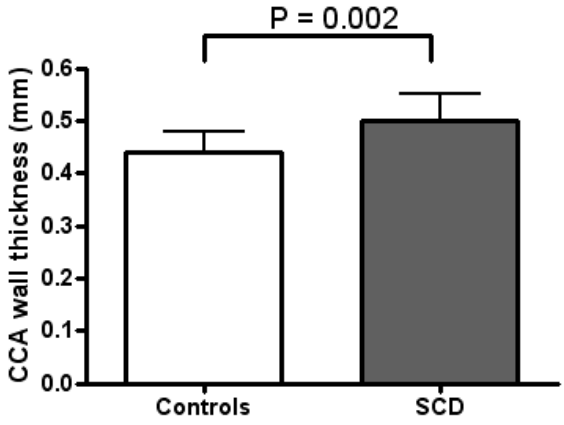


Figure 5.2. Comparison of common carotid intima-media wall thickness in SCD versus controls. There were statistically thicker common carotid artery walls in patients than control subjects.



5.4.4. Gender differences in common carotid artery parameters

Gender differences and hormonal effects of endothelial function are well documented in the literature (Corretti et al., 1995; Herrington et al., 2001; Jensen-Urstad and Johansson, 2001; Joannides et al., 2002). Endothelial function studies performed in healthy age matched subjects have demonstrated significantly greater FMD among females than males (Herrington et al., 2001). This increase in endothelial dependent vasodilatation among females have been reported to be augmented by the hormone estrogen (Hashimoto et al., 1995), hence resulting in varied FMD response to during the menstrual cycle.

Common carotid artery gender analyses were performed to determine whether gender contributed to differences observed in common carotid artery lumen size and wall thickness. To achieve this data was separated in two gender groups consisting of male or female subjects. Data presented in Figure 5.3 demonstrated that common carotid artery diameter were enlarged in both male and female SCD patients. Further analysis of vessel wall intima-media thickness showed that only female SCD patients had thickened common carotid arteries. No differences in wall thickness were demonstrated when male SCD patients were compared to control male participants.

Figure 5.3. Comparisons of common carotid artery diameter in male and female SCD patients. Significantly increased gender specific differences were demonstrated in common carotid artery diameter among SCD patients.

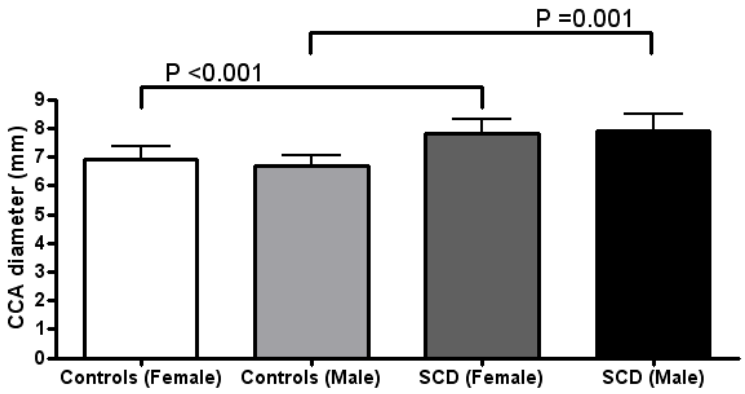
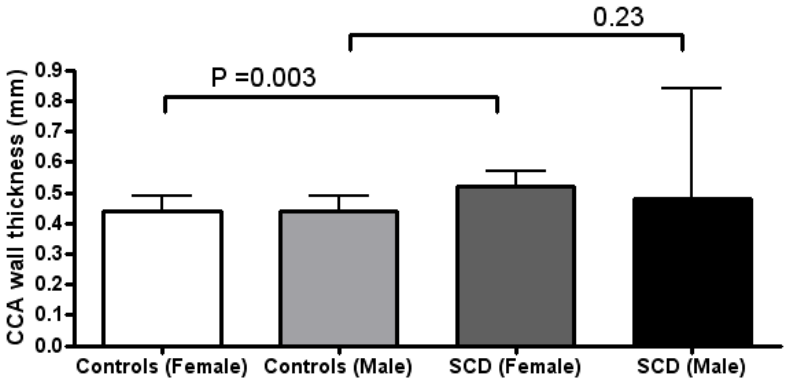


Figure 5.4. Comparisons of common carotid artery intima-medial wall thickness in male and female SCD patients. No difference was demonstrated between male SCD patients versus male control subject groups, however significant differences were seen in female SCD patients versus female control subjects.



5.4.5. Correlative analysis of common carotid artery parameters

Pearson correlative analyses of data were made to determine whether there was any correlation between changes in common carotid artery diameter and cardiac output as determined by Doppler echocardiography. Results demonstrated interesting findings. There was a strong positive correlation between carotid artery diameter and cardiac output (r=0.75, P<0.001). Data were further analysed to determine whether age (Figure 5.6) influenced common carotid artery diameter, a moderate correlation was found (r =0.64, P =0.01). No correlation was demonstrated between common carotid artery diameter and circulating haemoglobin S. Further association of common carotid artery lumen diameter with foetal haemoglobin concentration demonstrated a significantly inverse relationship (r= -0.57, P =0.01).

Figure 5.5. Correlation of common carotid artery diameter with cardiac output in SCD patients. A moderate positive correlation was seen; r =0.75, P <0.001.

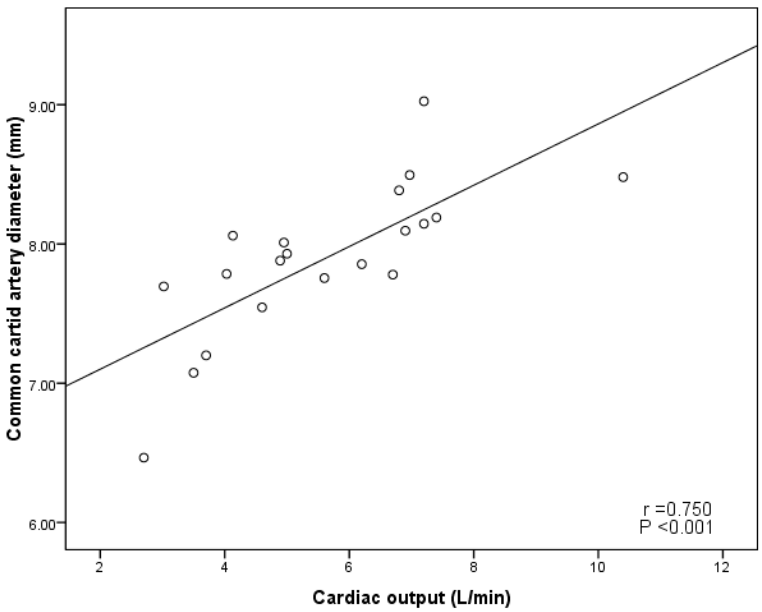


Figure 5.6. Correlation of common carotid artery diameter with age in SCD patients. A mild positive correlation was demonstrated; r =0.53, P =0.02.

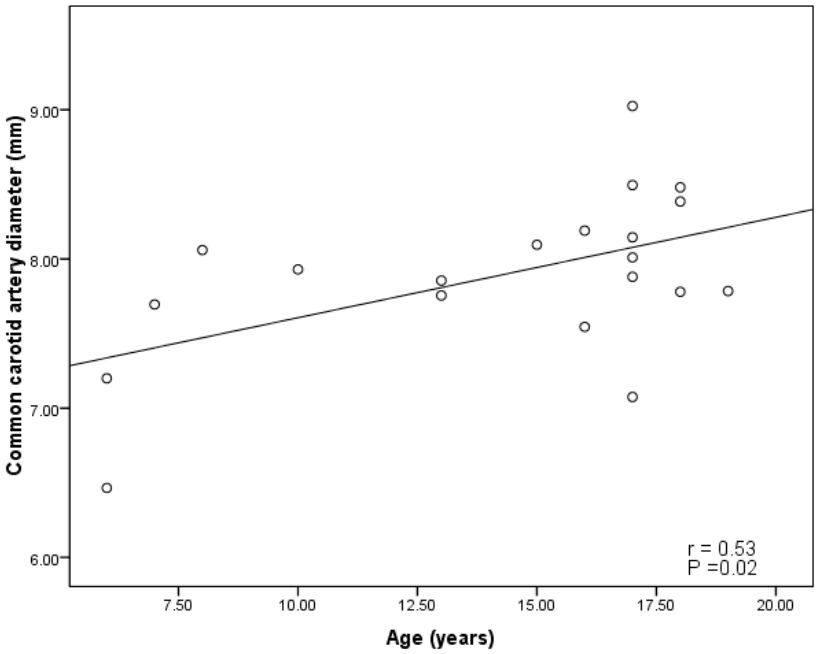


Figure 5.7 Correlation of common carotid artery diameter with haemoglobin S concentration. No correlation was demonstrated.

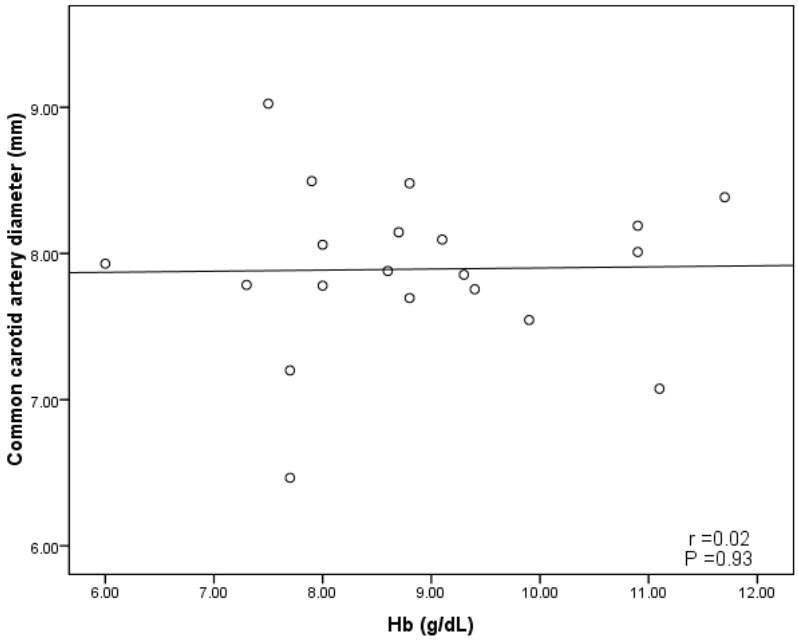


Figure 5.8. Correlation of common carotid artery diameter with foetal haemoglobin concentration in SCD patients. A significant inverse correlation was seen; r =-0.57, P =0.01.

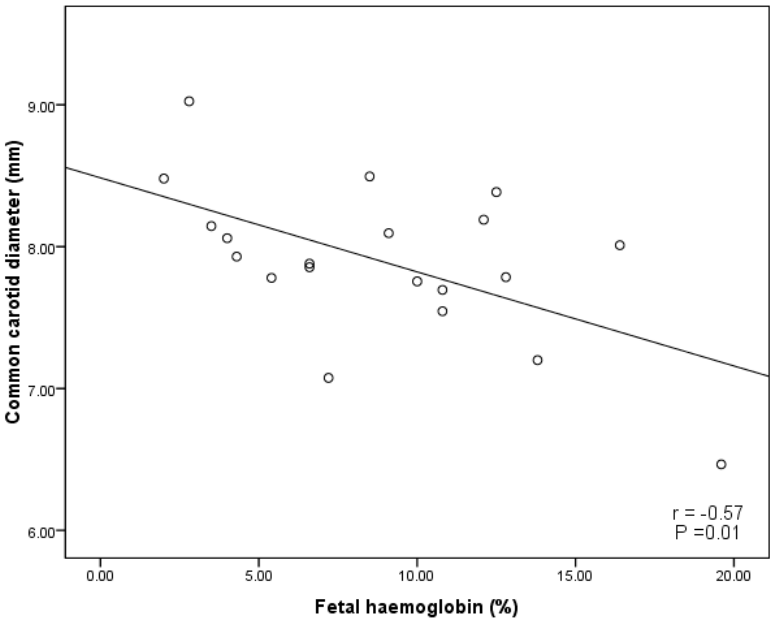
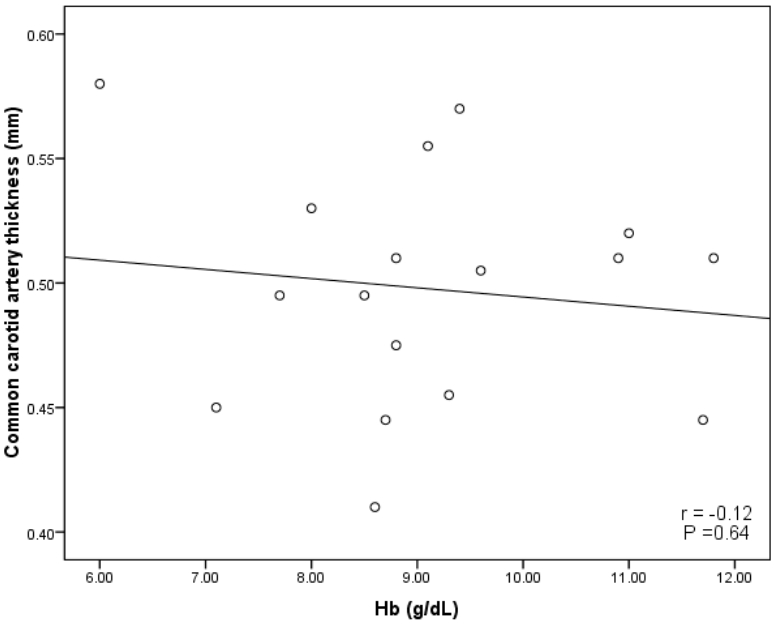


Figure 5.9. Correlation of common carotid artery intima-media wall thickness with haemoglobin concentration. No correlation was demonstrated.



Correlative analysis of common carotid artery thickness to cardiac output was performed to determine whether the hyperdynamic circulation in SCD influenced remodelling of the intima-media wall. Surprisingly, no association was found between cardiac output and carotid artery intima-media wall thickness (Figure 5.10.). Furthermore, no association was demonstrated between common carotid artery intima-media thickness and haemoglobin concentration.

Inflammatory marker, C-reactive protein has been linked to vasculopathy in many clinical studies (Cao et al., 2007; Szmitko et al., 2003). We carried out correlative analysis to determine whether inflammation influenced remodelling of vessel walls. Common carotid artery intima-media wall thickness correlation with C reactive protein demonstrated a moderate inverse association that did not make biological sense as data meant that large concentrations of inflammatory marker, C reactive protein would be associated with thinning of the intima-media walls, whereas low concentration of C reactive protein would be associated with thickening of walls. Further analysis of common carotid artery with inflammation markers such as white blood cell count and neutrophil did not demonstrate any association. No active thrombus was seen in our cohort of SCD patients.

Figure 5.10. Correlation of common carotid artery intima-media wall thickness with cardiac output in SCD patients. No association was found.

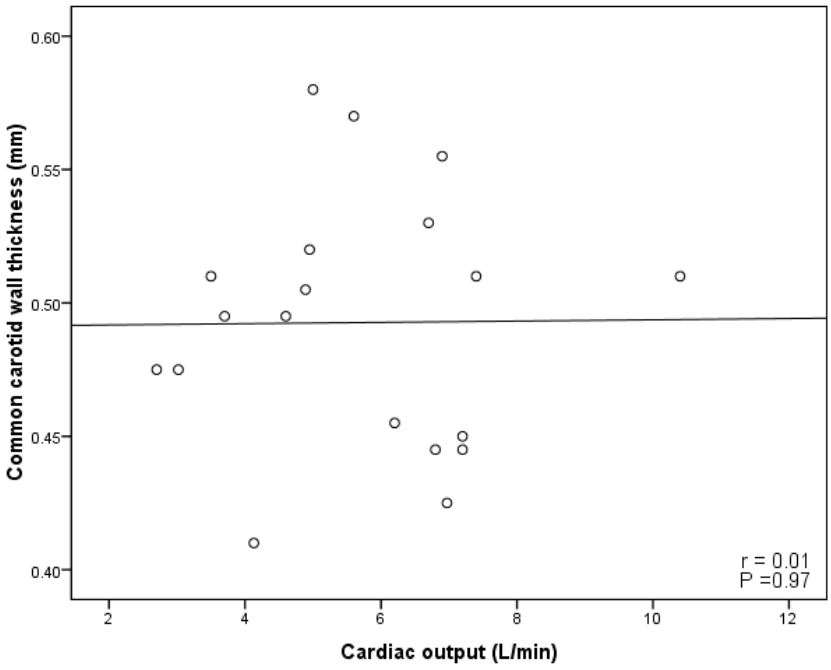


Figure 5.11. Correlation of common carotid artery intima-media wall thickness with C reactive protein in SCD patients. Moderate inverse correlation was demonstrated; r = -0.64, P =0.01.

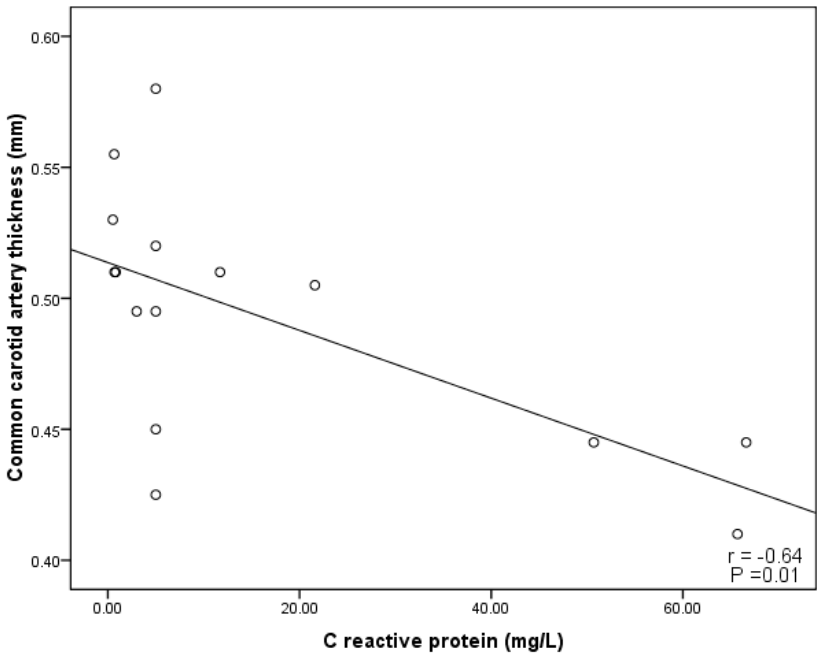


Figure 5.12. Correlation of common carotid artery intima-media wall thickness to white blood cell count. No association was demonstrated.

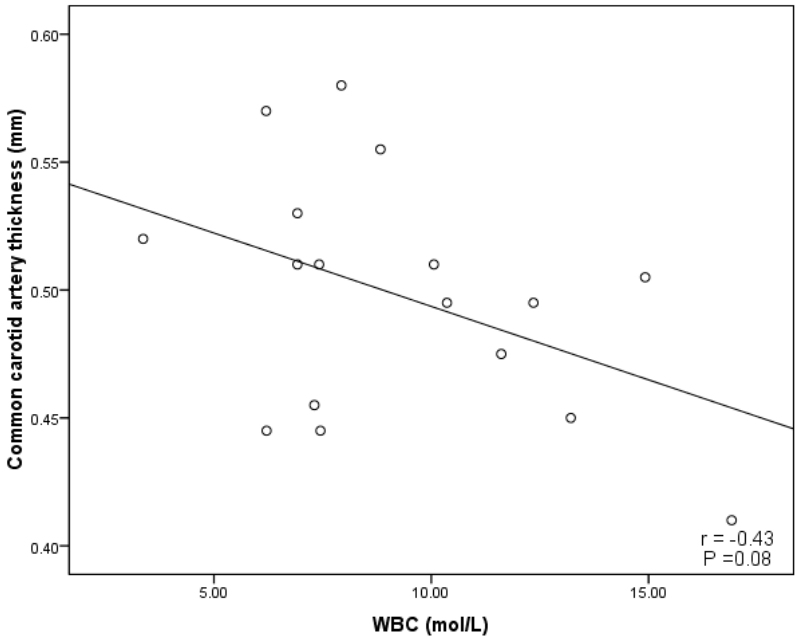
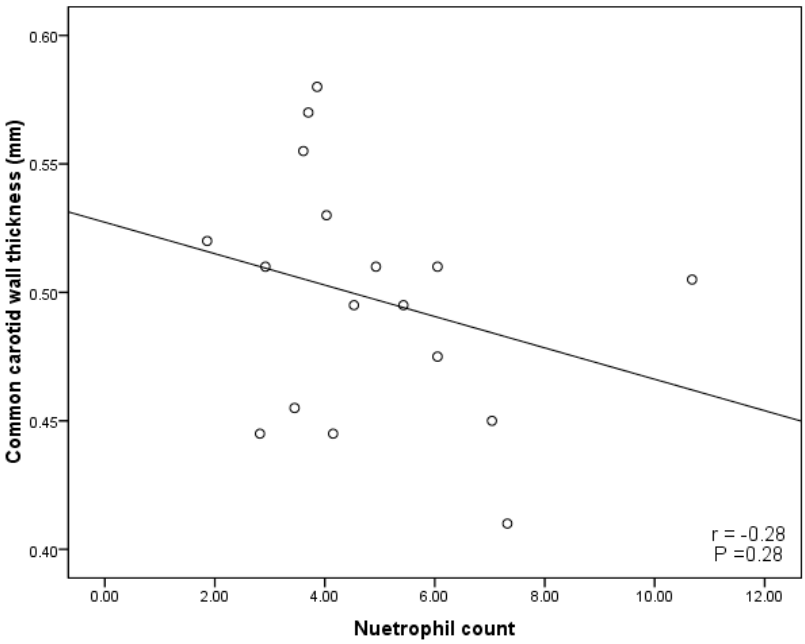


Figure 5.13. Correlation of common carotid artery intima-media thickness to neutrophil count. No association was shown (r = -0.28, P =0.3).



5.4.6 Effect of hydroxyurea on remodelling of common carotid artery

Paired t-test analyses were performed to determine whether there was any difference in common carotid artery architecture among patients on hydroxyurea therapy and patients not on therapy. No differences were found in common carotid artery diameter between the two groups. Furthermore no differences were demonstrated in common carotid artery thickness when patients prescribed with hydroxyurea treatment were compared to adolescent SCD not on treatment (P =0.8).

Figure 5.14. Comparisons of common carotid diameter in SCD patients not on or on treatment with hydroxlurea. No statistical difference was found between the two groups.

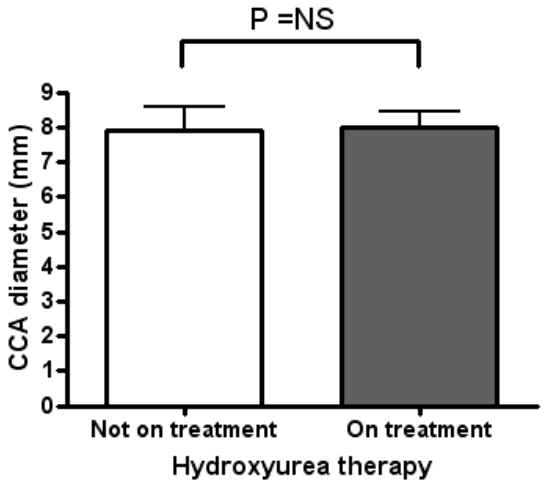
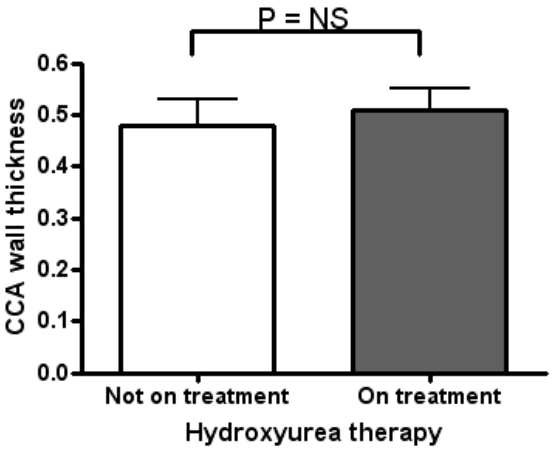


Figure 5.15. Comparisons of common carotid intima-media wall thickness in SCD patients on or not on treatment with hydroxlurea. Common carotid artery intima-media wall thickness was the same in both subject groups.



Multivariable model (Table 5.3.) of increased carotid artery intima-media thickness consisted of gender, age, oxygen saturation, haemoglobin S concentration, lactate dehydrogenase, and transcranial Doppler. Independent predictors were haemoglobin S concentration and transcarnial Doppler.

Table 5.3 Multivariable linear regression model demonstrating independent determinants of reduced intima-media wall thickness in HbSS patients.

	β, Estimated increase CCA thickness	*P*
Gender	-1.1	0.06
Age, yrs	0.15	0.08
Oxygen saturation	-0.35	0.07
Haemoglobin S, g/dL	1.8	0.02
LDH, U/L	-0.06	0.25
Transcranial Doppler, m/s	0.58	0.02

Variables entered into the model were gender, age, oxygen saturation, haemoglobin S, lactate dehydrogenase, C reactive protein, transcranial Doppler.

5.4.7 Flow mediated dilatation studies

Brachial artery flow mediated dilatation studies in SCD patients and controls were induced by means of shear stress as described in the general methods chapter. Initial measurements were performed on baseline brachial artery diameter (Figure 5.16), demonstrated no differences in brachial artery size between patients and controls. Further analysis of FMD was carried out to determine whether endothelial nitric oxide release was impaired in patients. Data in Figure 5.17 demonstrated a significant reduction in FMD among patients than controls (P =0.04). Paired t-test analysis of peak reactive hyperaemia flows did not show any differences between the two groups.

Figure 5.16. Comparison of baseline brachial artery diameter in SCD versus control subjects. No difference was seen in baseline brachial artery diameter.

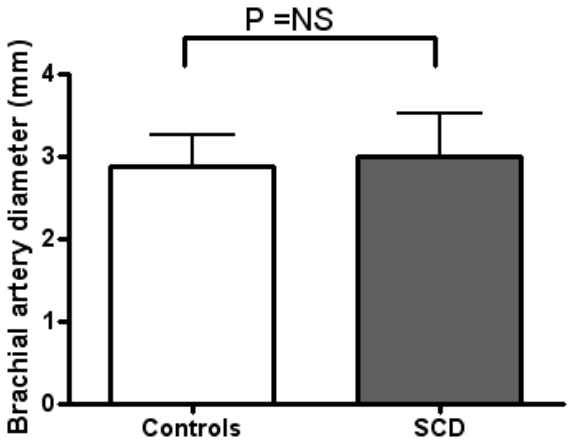


Figure 5.17. Comparison of flow mediated dilatation, expressed as percent change from baseline during hyperemia in SCD versus control subjects. SCD patients had impaired FMD function.

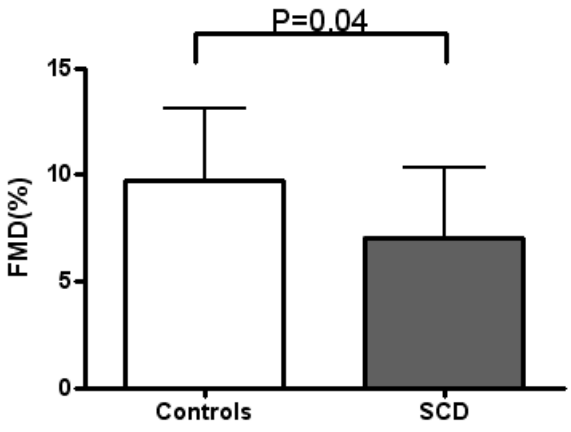
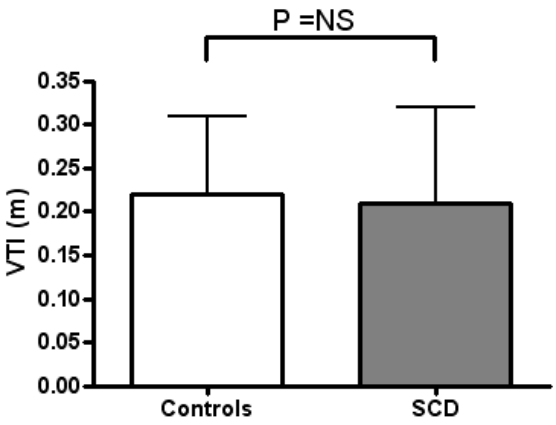


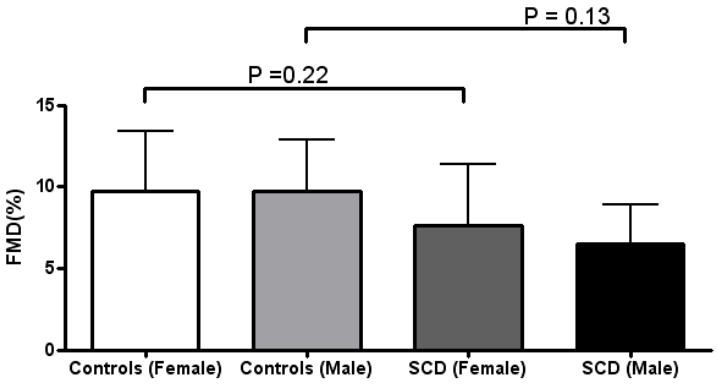
Figure 5.18. Comparison of brachial artery flow in SCD versus controls subjects. No significant differences were demonstrated in hyperemia flows.



5.4.8 Gender differences in FMD

In the current study gender influences in impaired FMD function were assessed by separating data into two groups consisting of male SCD patients versus male controls and female SCD patients versus female controls (Figure 5.19). Assessments made on FMD function between male patients and control subjects did not show any differences. There were no significant differences seen between female patients and female control groups. Female and male SCD patients' FMD function was not statistically different (7.6 $\pm$3.8% and 6.4 $\pm$2.5%). It is essential to point out that sample sizes in the current study were too low, which posed a major limitation in gender comparisons highlighted above.

Figure 5.19. Comparisons of brachial artery flow mediated dilatation in male and female SCD patients. No significant differences were demonstrated between male SCD and male control subjects. No differences were found between female SCD and female control FMD function.



5.4.9 Correlative analyses of FMD function with laboratory markers

Pearson correlation analysis of flow mediated dilatation were performed to determine whether inflammation played a role in impaired endothelial function. No association was found between inflammatory biomarker C reactive protein and FMD as demonstrated in Figure 5.20. No correlation was found between FMD and other markers of inflammation such as neutrophil count, levels of C reactive protein. The data was further analysed to determine whether remodelling observed in the common carotid intima-media wall was associated with FMD measurements. The data in Figure 5.22 demonstrated that brachial FMD did not correlate with common carotid artery thickness (r =0.14, P =0.56). Repeated correlation analysis of brachial FMD with transcranial Doppler parameters also found no association.

Figure 5.20. Correlation of FMD with C reactive protein in SCD patients. No correlation was demonstrated (r = -0.05, P =0.86).

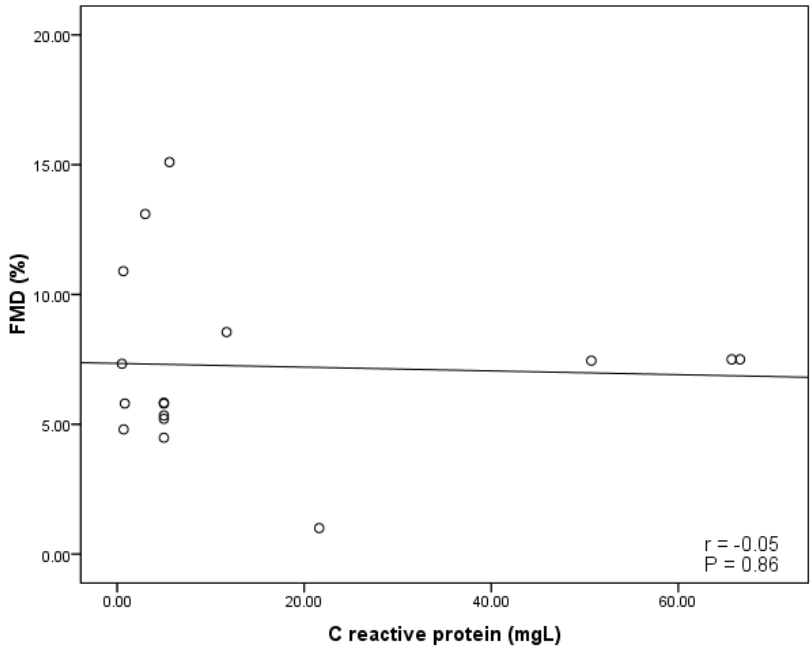


Figure 5.21. Correlation of FMD with Neutrophil count in SCD patients. No correlation was demonstrated (r =-0.20, P =0.4).

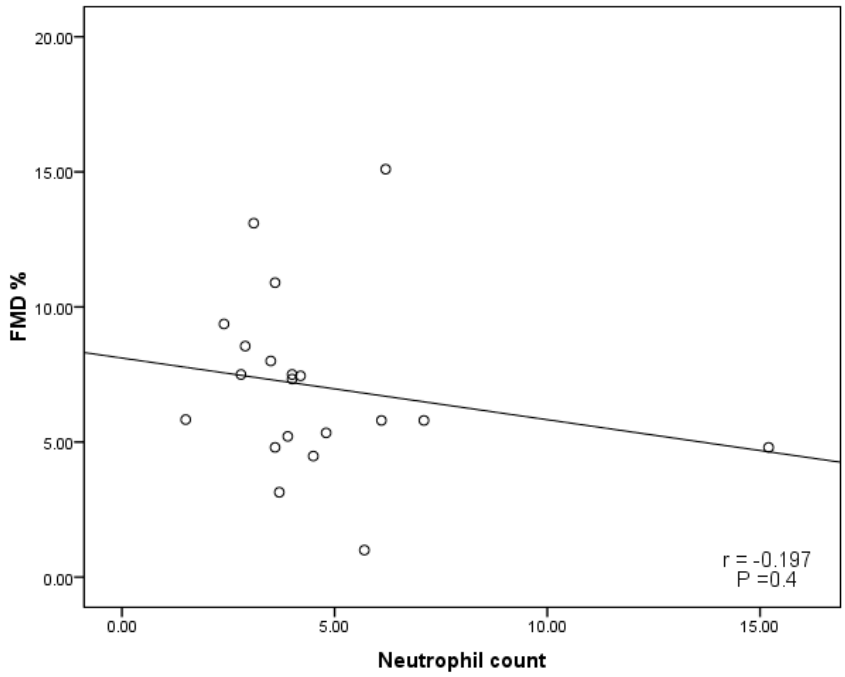
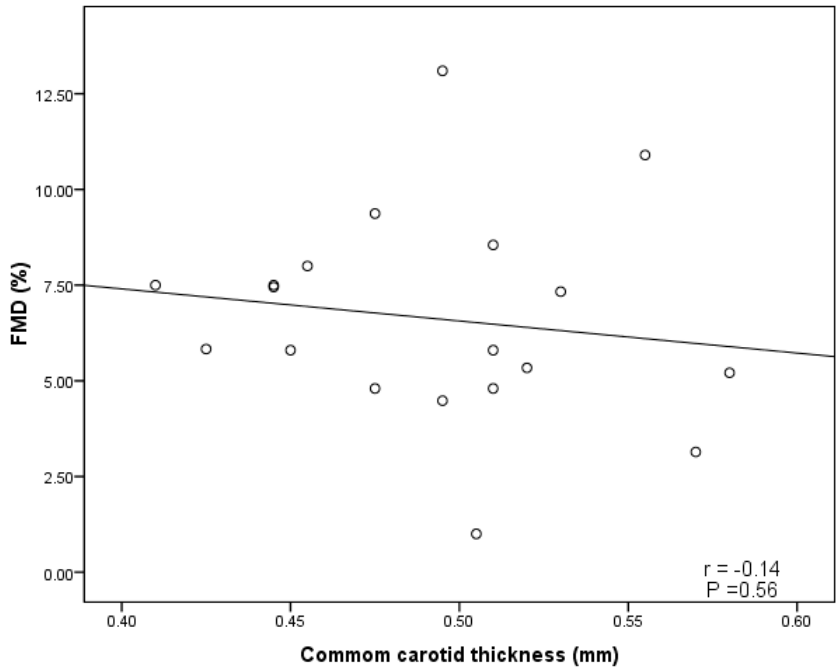


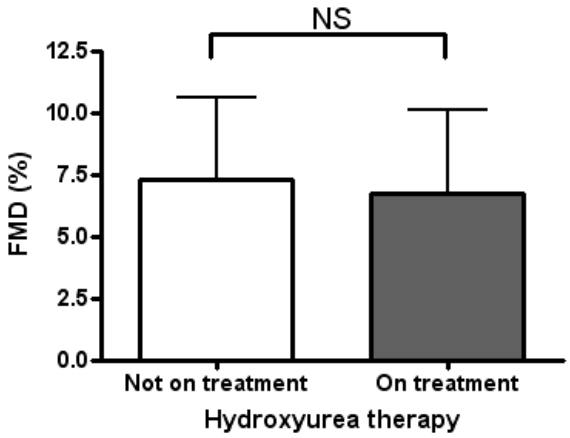
Figure 5.22. Correlation of common carotid artery wall thickness with flow mediated dilatation in SCD patients. No correlation was found.



5.4.10 Effect of hydroxyurea on FMD function in SCD patients

Comparisons were made in our cohort of patients to assess whether treatment with hydroxyurea had any effect on flow-mediated dilation, however no significant differences were found.

Figure 5.23. Comparisons of flow mediated dilatation in SCD patients on or not on treatment with hydroxyurea. No significant differences were shown in FMD function.



When multivariable linear regression analysis was performed for impaired FMD against covariates of gender, age, oxygen saturation, haemoglobin S concentration, lactate dehydrogenase, C reactive protein and transcranial Doppler, no independent determinants were found (Table 5.3).

Table 5.4. Multivariable linear regression model demonstrating independent determinants of FMD in HbSS patients.

	β, Estimated impaired FMD	*P*
Gender	3.7	0.34
Age, yrs	0.86	0.30
Oxygen saturation	-1.20	0.46
Haemoglobin S, g/dL	-1.16	0.42
LDH, U/L	-0.45	0.67
C reactive protein, mg/L	2.5	0.34
Transcranial Doppler, m/s	0.21	0.76

Variables entered into the model were gender, age, oxygen saturation, haemoglobin S, lactate dehydrogenase, C reactive protein, transcranial Doppler.

5.4.11 Transcranial Doppler

Transcranial Doppler measurements are clinically performed to identify patients at risk of having cerebral vascular accidents (Adams, 2005; Adams et al., 1998a; Bernaudin et al., 2011). In the current study associations of extracranial velocities were performed to determine whether circulating haemoglobin S concentration influenced velocities measured in the internal carotid artery, a significant correlation was shown ($r = -0.52$, $P = 0.03$). This association was investigated further, and results in Figure 5.25 demonstrated a significant inverse correlation between transcranial velocities and foetal haemoglobin concentration. A positive association was observed when marker of inflammation (C reactive protein) was correlated with transcranial velocity measurements, meaning that large amounts of C reactive protein resulted in high transcrainial Doppler velocities. A significantly positive correlation was shown when carotid artery intima-media thickness were associated with transcranial Doppler measurements ($r = 0.6$, $P = 0.02$).

Figure 5.24. Correlation of transcranial Doppler velocities with haemoglobin S. A significant positive inverse link was demonstrated (r =-0.52, P =0.03).

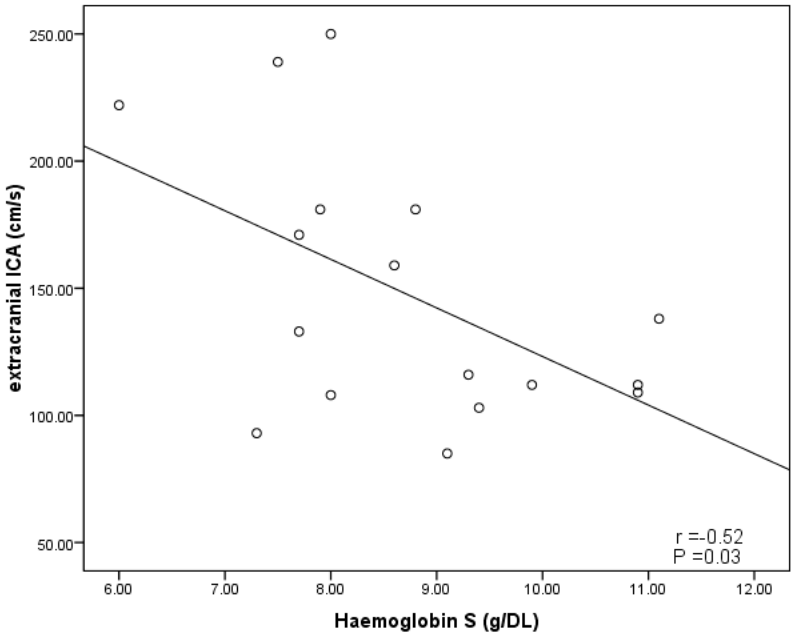


Figure 5.25. Correlation of transcranial Doppler velocities with foetal haemoglobin concentration in SCD patients. A significant inverse correlation was demonstrated (r =-0.51, P =0.03).

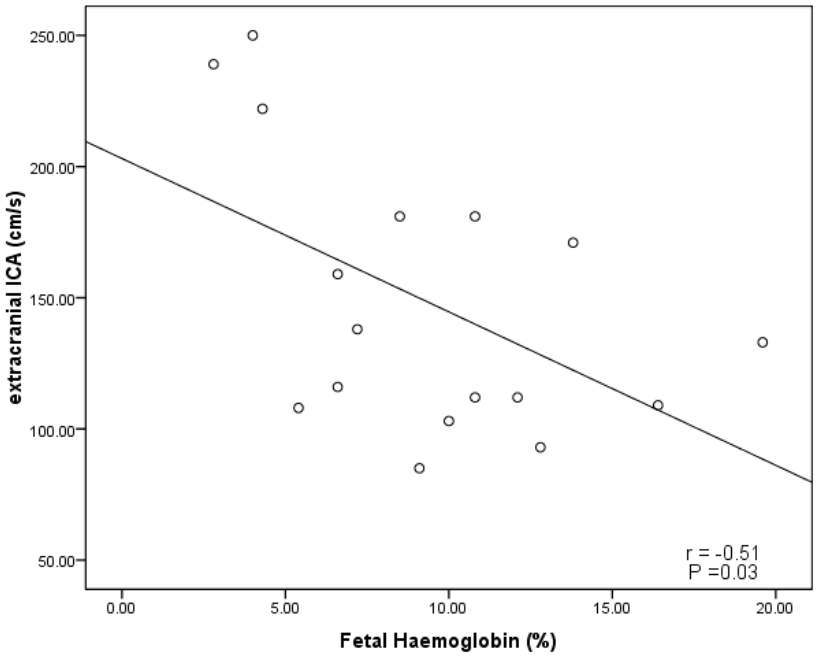


Figure 5.26. Correlation of C reactive protein with maximum extracranial ICA trancrainal doppler velocities in SCD patients. A significant positive correlation was demonstrated (r =0.56, P =0.05).

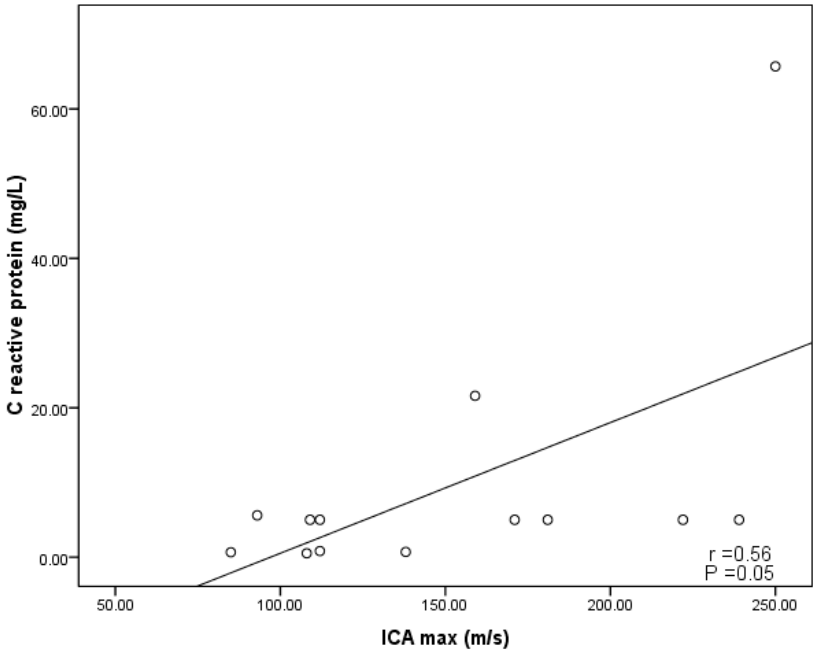
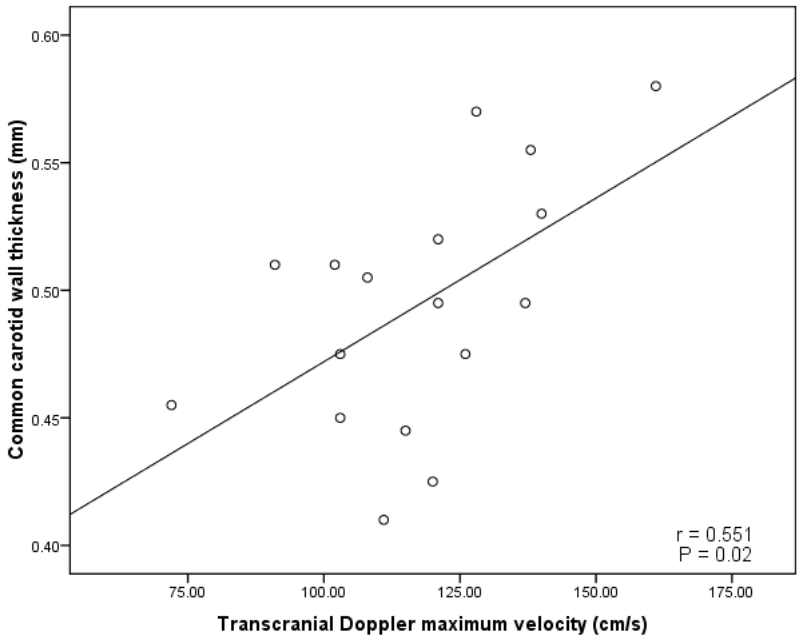


Figure 5.27. Correlation of common carotid artery intima-media wall thickness with maximum transcrainal Doppler flow in SCD patients. Significant correlation was demonstrated (r =0.55, P =0.02).



5.4.12 Effect on Vitamin D

Vitamin D deficiency is a common finding in young SCD patients (Rovner et al., 2008). Deficiency in vitamin D has been associated with cardiovascular diseases (Li et al., 2004), therefore we performed exploratory analysis to examine whether vitamin D levels in patients could enhance carotid artery intima-media thickness, however no association was found (Figure 5.28). Further associations between vitamin D and flow mediated dilatation did not demonstrate any association. Similarly, no association was shown between transcranial artery velocity measurements and vitamin D.

Figure 5.28. Correlation of vitamin D with common carotid artery intima-media wall thickness in SCD patients. There was no correlation found (r =0.03 and P =0.9).

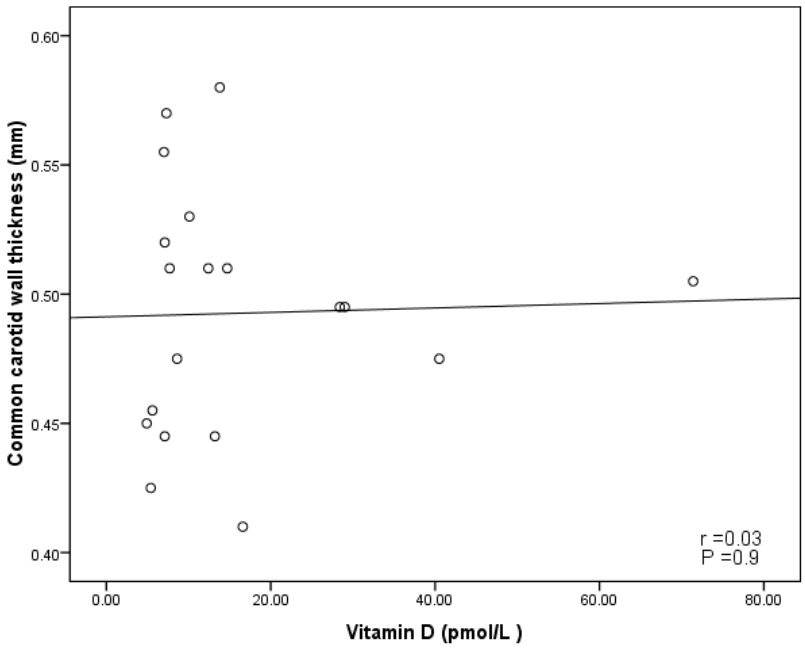


Figure 5.29. Correlation of FMD with vitamin D in SCD patients. No correlation demonstrated.

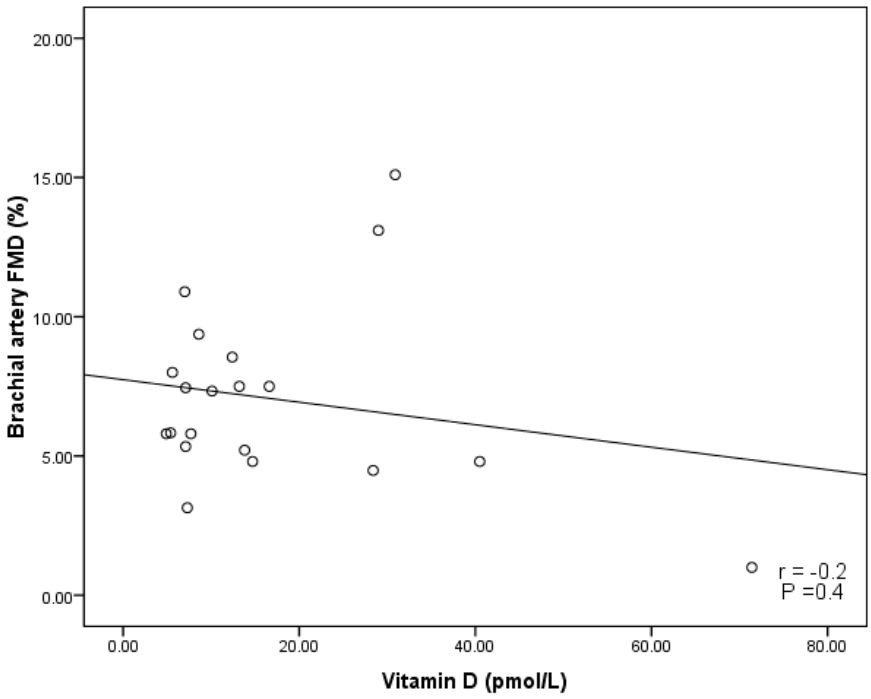
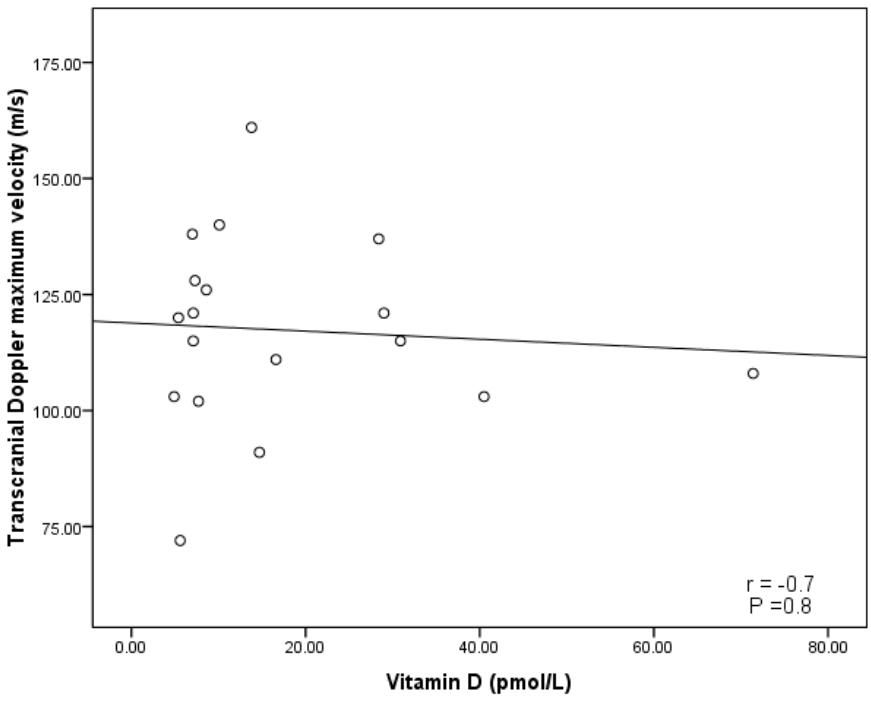


Figure 5.30. Correlation of vitamin D with transcranial Doppler velocity in SCD patients. No correlation was demonstrated (r =-0.7, P =0.8).



5.5 Discussion

The studies in this chapter were designed to determine whether adolescent SCD patients with no acquired vascular risk factors such as hypertension, smoking, hypercholestrolimia or aging have significant vascular dysfunction.

Previous clinical studies have reported carotid intima-media thickness as a strong predictor of cardiovascular disease and stroke and that the risk gradients are similar (Cao et al., 2007; Ebrahim et al., 1999; O'Leary et al., 1999; Taylor et al., 2009). In the current study we first investigated common carotid artery diameter and intima-media thickness to determine remodelling. The patients were recruited, scanned and analysed by one investigator. Although analysis of patient identification was anonymous, the investigator who carried out image data analysis (myself) was not blinded to the study, which poses as a study limitation. Both common carotid artery diameter and intima-media thickness were significantly increased in SCD patients compared to controls. Carotid artery diameter positively correlated with cardiac output and patient age. As previously discussed in chapter 3, increased HbF has been reported to lower morbidity and mortality rates in SCD patients (Charache et al., 1992). Although the exact mechanism is unclear and remains to be defined, it is postulated that HbF inhibits the polymerization of HbS molecules in red blood cells (Higgs and Wood, 2008). We initially correlated haemoglobin S with common carotid artery diameter, data did not show any significant association. Furthermore, correlative analysis of common carotid artery diameter with foetal haemoglobin concentration showed a significant inverse link ($r = -0.57$, $P = 0.01$). Meaning, individuals with smaller foetal haemoglobin concentrations tend to have larger

common carotid arteries. Perhaps foetal haemoglobin concentration has a protective role to play in the vasculature, however mechanisms remain to be explored further as our data did not demonstrate any link between haemoglobin S and common carotid artery diameter.

The present study is the first to demonstrate that adolescent SCD patients have intima-media wall thickening. In this study, common carotid artery intima-media thickness was associated with transcranial Doppler velocities. Data demonstrated a concomitant increase in transcranial Doppler maximum velocities with thickness of common carotid artery intima-media wall. The underlying cause of common carotid artery intima-media thickening remains unclear because no correlation was found between carotid artery intima-media thickness and cardiac output ($r = 0.01$, $P = 0.97$), nor was there any correlation found with degree of disease severity (i.e. Hb concentration).

Several studies have demonstrated that baseline inflammatory marker- C reactive protein is highly predictive of future events such as cardiovascular disease, stroke and peripheral arterial disease (Cao et al., 2007; Ridker, 2003; Ridker et al., 2002). In the current study, C reactive protein concentration inversely correlated with common carotid artery thickness. This finding did not make biological sense because data would essentially mean that low amounts of C-reactive protein resulted in thickened intima-media walls. We further determined whether inflammatory markers such as plasma neutrophil and white blood cell count influenced common carotid artery thickness in our young cohort of SCD patients, however no association was found. Certainly exploration with

more biomarkers of inflammatory such as low density lipoprotein, intracellular adhesion molecule 1, CD40 and E selectin would need to be assessed to confidently exclude inflammatory contribution to thickened intima-media.

Endothelial dysfunction, including reduced nitric oxide release is a hallmark to many diseases states such as coronary artery disease, cerebral ischaemia, hypercholesterolemia and pulmonary hypertension (Benjamin et al., 2004; Celermajer, 1997; Katz et al., 2005; Lefer et al., 2001; Patti et al., 2005; Seddon et al., 2009; Verma et al., 2003; Vogel and Corretti, 1998; Wolff et al., 2007). Ultrasound brachial artery flow mediated dilatation (FMD) is a non-invasive imaging technique that was first described in the late 1980s and broadly used to evaluate endothelial function, specifically endothelial derived nitric oxide release through increase in shear stress (Steffel and Luscher, 2009).

In the current study impaired FMD was observed in young SCD patients compared to controls (P =0.04). Our data suggests that reduced FMD is probably related to impaired endothelial function and could likely be present on a microvascular level that extends to large conduit vessels such as brachial artery from an early stage, however more investigations would need to be performed to justify microvascular impairment. Our FMD findings are in agreement with previous reports where endothelial nitric oxide release was reported to be impaired in adult SCD patients (Aessopos et al., 2007; Belhassen et al., 2001; Gladwin et al., 2003). It is important to emphasis that all young SCD subjects in the present study had no risk factors such as age, blood pressure, smoking or hypercholesterolemia. In contrast to other investigator

findings, our data demonstrated no differences in baseline brachial artery diameter or FMD flow velocities. Interestingly, in our study no association was found between LDH, CRP level and impaired FMD (r =0.05, P =0.86). However, there is only one study in the literature reporting FMD function in young SCD patients (de Montalembert et al., 2007). In their study of 21 SCD children, impaired FMD and preserved nitrate mediated vasodilatation was observed in patients. Similar to our findings, FMD data was not associated with patient haemoglobin S concentration or patient age. However, in their study no associations were made with markers of haemolysis i.e. LDH. Furthermore control subjects in their study carried the sickle cell gene.

Hydroxyurea is a drug that has been used to treat patients with SCD since the mid 1990s (Charache et al., 1995). Hydroxyurea (HU) has been shown to reduce the rates of vascular events by increasing endothelial nitric oxide production in umbilical embryonic endothelial cells and bone marrow endothelial cell lines (Cokic et al., 2007). Although still unclear, current evidence suggests that several possible mechanisms of action maybe in place, which when put together lead to increased production of HbF and general clinical benefits in SCD patients (Glover et al., 1999; Nahavandi et al., 2002; Ware, 2010). One proposed mode of action is that HU could be acting as a free radical by inhibiting ribonucleotide reductase- the enzyme essential for synthesis of deoxyribonucleic acid (DNA)- and eventually causing cellular cytotoxicity suppression of red blood cell progenitors (Halsey and Roberts, 2003; Lanzkron et al., 2008; Ware, 2010). The direct action of HU on DNA replication remains to be established, some investigators postulate that inhibition of DNA replication

increases production of foetal haemoglobin thereby activating soluble guanylate cyclase and eventually NO production (Cokic et al., 2003; Kolata, 1984). The rise in HbF concentration is also believed to alter the kinetics and thermodynamics of abnormal haemoglobin S molecules which may in turn lower the amount of HbS within cells, resulting in less polymerization of abnormal haemoglobin molecules (Lanzkron et al., 2008). Again, the mechanism by which hydroxyurea induces foetal haemoglobin remain unclear.

To our knowledge, the effects of HU on endothelial nitric oxide release using non-invasive FMD technique in SCD patients has not been explored. Our data found no significant differences in foetal haemoglobin concentration when patients on treatment were compared to those not receiving hydroxyurea treatment. Furthermore, brachial FMD, hyperemia flows, common carotid artery diameter and common carotid artery intima-media thickness were the same in patient groups on/off treatment, implying that HU did not reverse arterial remodelling (i.e wall thickness/dilatation) or improve endothelial NO release in SCD patients receiving regular treatment. However, further clinical trials would need to be conducted to support our findings.

Finally, vitamin D deficiency is highly prevalent worldwide, particularly among homozygous SCD children due to several factors such as the increased skin melanin concentration, reduced levels of physical exercise and poor vitamin D intake (Buison et al., 2004; Rovner et al., 2008; Wang et al., 2008). Emerging data suggests an inverse association between vitamin D deficiency and cardiovascular risk factors such as hypertension, increased vascular resistance

and left ventricular hypertrophy (Anagnostis et al.; Lee et al., 2008). The exact mechanism by which vitamin D protects the cardiovascular system remains unknown although mechanisms for lowering systemic blood pressure are postulated to act through the renin-angiotensin system via cyclic AMP signalling pathways (Li et al., 2002; Sugden et al., 2008). Clinical and laboratory studies have demonstrated a correlation between vitamin D and high blood pressure (Li et al., 2004). Investigators using vitamin D knockout murine models report increased incidence of hypertension, left ventricular hypertrophy and artherosclerosis (Simpson et al., 2007). In a small randomised trial consisting of thirty four diabetes patients, supplementary intake of vitamin D resulted in improved endothelial function (Sugden et al., 2008). In addition, studies report of down-regulation of inflammatory marker C-reactive protein in vitamin D-deficient individuals (Lee et al., 2008). In contrast to these studies, our data found no correlation between common carotid wall thickness, C reactive protein, brachial FMD and transcranial Doppler velocities.

Limitations to the current study were that the patient population was small. The study patients were recruited and data analysed in a non-blinded approach by one investigator. Therefore, study results in this chapter would need to be reanalysed by someone blinded to study protocol when data is presented for publication in a peer-reviewed journal.

5.6 Summary

To conclude, carotid intima-media thickening is increased in SCD patients, flow mediated dilatation is reduced and there is no association between these

parameters and C-reactive protein or vitamin D levels. Functional studies are needed to investigate whether detection of increased common carotid artery thickness or reduced FMD in young subjects predicts future complications. This would require a prospective follow-up study.

Chapter 6

General discussion

6.0 Overview

Sickle cell disease is a complex hereditary hemoglobinopathy that causes chronic complications due to repetitive vaso-occlusive events and hemolysis that can lead to multiorgan failure and shortened life expectancy. Among a spectrum of cardiovascular manifestations in these patients, pulmonary hypertension has been reported to pose the highest concern (Haque, Gokhale et al. 2002; Castro, Hoque et al. 2003; Gladwin and Kato 2005). The exact prevalence of pulmonary hypertension in sickle cell disease is controversial; clinical studies using echocardiography have suggested pulmonary hypertension to be highly prevalent and a major determinant of outcome (Anthi, Machado et al. 2007; Farmakis and Aessopos 2011). Furthermore, the contribution of cardiac and endothelial dysfunction to SCD remains poorly understood.

6.1 Characterisation of cardiac dysfunction in sickle cell disease

Echocardiography indexes of left ventricular volume and ejection fraction have been documented to predict clinical outcomes in many cardiovascular disease states and in several clinical trials (Cintron, Johnson et al. 1993; Dujardin, Enriquez-Sarano et al. 1999; Quinones, Greenberg et al. 2000; Wong, Staszewsky et al. 2002; Grayburn, Appleton et al. 2005). Recent advances in three dimensional echocardiography have led to better visualization of cardiac structures thereby proving to provide more accurate measures of ventricular volume and function than traditional two dimensional echocardiography (Soliman, Kirschbaum et al. 2008). In chapter 3, detailed 2D, 3D and Doppler echocardiography studies were performed in eighty-one unselected

homozygous SCD patients and 30 healthy age, gender and ethnically matched controls to characterise and determine cardiac function. Indexed left ventricular diastolic and systolic volumes were demonstrated to be significantly higher in patients as compared to controls. These findings were in agreement with previously published data reporting increased LV chamber size among SCD patients (Covitz, Espeland et al. 1995; Lamers, Ensing et al. 2006; Gladwin and Sachdev 2012). The increase in ventricular volume was also dependent on the patient's genotype. Results demonstrated that there is a change in LV shape among SCD patients suggesting probable pre-adaptation of the LV to a globular shape.

Inconsistencies in the literature regarding prevalence of LV diastolic dysfunction in SCD are apparent (Lewis, Maron et al. 1991; Kingue, Mbanya et al. 2000; Hankins, McCarville et al. 2010; Eddine, Alvarez et al. 2012). This could be due to limitations arising from the techniques used. These limitations can be due to loading conditions, heart rate, angle dependency or even patient age. Nonetheless, TDI techniques applied in the current study have been validated in the general population with invasive measurements of LV filling pressures such as pulmonary capillary wedge pressure (Nagueh, Mikati et al. 1998), although its accuracy has not been prospectively validated in haemolytic diseases such as sickle cell. Our data found that only a small proportion of SCD patients had left ventricular diastolic dysfunction (4%), these findings of diastolic dysfunction in adult SCD using TDI are in agreement with a study by Ahmed et al reporting a prevalence rate of 9% (Ahmed, Siddiqui et al. 2004). Indeed prospective studies comparing reliability of TDI are needed to further validate

findings in SCD patients, as figures of diastolic function still appear inconsistent.

Likewise, the left ventricular mass was also increased among patients. Evaluation of laboratory parameters provided interesting findings suggesting an association between increased left ventricular hypertrophy and remodelling with severity of anaemia and asparate aminotransferase. Assessment of left ventricular systolic function with multiple methods such as 3D echocardiography ejection fraction, stroke work index, systolic dyssynchrony index, tissue Doppler and strain rate imaging did not find evidence of systolic functional impairment but rather a hyperdynamic systolic function.

More so, exploration of contractile dysfunction in iron-overload patients undergoing regular long-term red cell transfusion found no associated with significant regional contractile dysfunction as assessed by speckle tracking echocardiology.

In summary, data in chapter 3 demonstrated that adults with SCD have eccentric (physiological) LV remodelling that is significantly associated with patient genotype and severity of anaemia and chronically higher cardiac output.

6.2 Prevalence and mechanism of pulmonary hypertension in sickle cell disease

In chapter 4, the effect of SCD on right ventricular structure and function was first investigated. Similar to the LV, right ventricular volumes and function were significantly increased in SCD patients. Enlarged cardiac dimensions were

associated with increased cardiac index that correlated with the degree of anaemia and patient genotype.

Detailed 2D, 3D and Doppler echocardiography studies were performed in 123 consecutive unselected SCD patients and 30 healthy age, gender and ethnically matched controls to determine the true prevalence of PHT. Two Doppler echocardiography methods namely, TR and PVR_{echo} were used. Interestingly, our study findings were able to demonstrate clearly that the prevalence of PHT in SCD varied significantly depending on echocardiography method applied. In agreement with previously published data (Gladwin, Sachdev et al. 2004; Sachdev, Machado et al. 2007), the traditional method of TR indicated that a high proportion (> 30%) of SCD patients have TR $\geq$ 2.5m/s. However, non-invasive estimation of pulmonary vascular resistance revealed that only a minority i.e. 5% had elevated readings.

Values of non-invasive TR and PVR_{echo} were further compared to gold standard invasive right heart catheterization measurements of PVR. As hypothesised, data confirmed that TR method yielded a high proportion of false positives, suggesting over diagnosis of PHT in SCD patients and that TR velocities in SCD maybe driven by a high cardiac output related to chronic anaemia than by pulmonary vascular resistance. In contrast to TR method, non-invasive echocardiogram based estimate of pulmonary vascular resistance yielded a much lower false positive rate but more data are required to judge the overall accuracy of this method. So far data from this study suggests that only a minority of SCD patients may have true pulmonary arterial hypertension. This

study is the first in the literature to directly compare prevalence of pulmonary hypertension using two different non-invasive echo techniques among SCD patients. As previously highlighted, PVR_{echo} methods have not been explored in the sickle cohort and certainly its validation in this patient group is critical if this method is to be applied during routine screening.

A study published by Gladwin and colleagues in 2004 was the first to arouse clinical interest surrounding PHT and its prognostic implications in SCD patients (Gladwin, Sachdev et al. 2004). In their study only a small subgroup (n =18) of patients were catheterised. Since their report, many investigators have cast their attention on determining the prevalence of PHT using only TR methods neglecting to question or take into account the technique limitations that could potentially lead to misdiagnosis, which is our main study objective.

Last year saw a turn around in non-invasive screening for PHT in SCD patients. A paper by a French group reported invasively diagnosed prevalence of PHT rate to be 6% as opposed to 30% when assessed by non-invasive TR, in a large (n =96) multicentre group of SCD patients (Parent, Bachir et al. 2011). Their study was able to demonstrate that echocardiography estimates of PHT by TR with a cutoff value set at 2.5m/s resulted in low positive predictive values. However, in an attempt made to increase TR thresholds to 2.9 m/s, large false negative rates were generated which led investigators to conclude that echocardiography is not suitable as a single screening tool for selecting patients to undergo right heart catheterisation.

In another most recent study, over diagnosis of PHT by TR method was mentioned but not explored further in a small subgroup (n =26) of SCD patients that underwent right heart catheterisation studies (Fonseca, Souza et al. 2012). In this study, pulmonary arterial hypertension was present in only 3 of the participating patients and pulmonary venous hypertension was found in 5 patients. It is therefore reassuring to discover that our data is in agreement with both the above-mentioned investigator findings. More so, our results will not only highlight the issue of misdiagnosis when TR method is used but also bring a breakthrough in screening algorithms by introducing an alternative reliable echocardiography method that can be incorporated with TR method during routine non-invasive screening for PHT. This should then give room to consider issues surrounding mechanisms such as left ventricular contribution or multi-organ failure and future therapeutic strategies for managing PHT in SCD patients.

We attempted to evaluate mechanisms underlying PHT in SCD by collaborating with staff from Computer Tomography and Lung Function Departments at Kings College Hospital. Data from CT studies found prominent pulmonary vessels, pulmonary fibrosis and ground glass opacification including volume loss in a large proportion of our SCD patients, which was further supported by lung function test findings revealing a mixed pattern consisting mainly of restrictive lung pathology with some evidence of obstructive lung disease. Both our CT and lung function findings of abnormal pulmonary function characterized by airway obstruction and restrictive lung pathology are in agreement with previous reports where pulmonary function abnormalities were present in up to 90% of

adults with the homozygous genotype (Santoli, Zerah et al. 1998; Klings, Wyszynski et al. 2006). Pathology of lung disease in SCD adults and children varies, hyper-reactive airway has been widely documented among young patients with SCD (Leong, Dampier et al. 1997; Siddiqui and Ahmed 2003; Boyd, DeBaun et al. 2009). One study consisting of 35 African American and 28 Hispanic HbSS children documented lung abnormalities in 43% of the study cohort (Koumbourlis, Zar et al. 2001). In contrast to our findings, a large percentage of children in their study had obstructive airway disease. Whether obstructive lung disease in SCD children precedes restrictive lung disease or if pulmonary complications contribute to the development of pulmonary hypertension remains to be explored further. Due to the small numbers of patients diagnosed with pulmonary arterial hypertension in our study contribution of lung disease to occurrence of pulmonary hypertension could not be fully investigated.

6.3 Vascular dysfunction in adolescent patients with sickle cell disease

Studies in chapter 5 were set out to determine whether a deficiency of NO exists in adolescent SCD patients. Vascular endothelial function was assessed by flow mediated forearm vasodilatation in twenty stable adolescent SCD patients without other risk factors and fourteen healthy age matched controls. Interestingly, data demonstrated impaired FMD function in the brachial artery of patients with preserved velocity flows. Changes in FMD were not associated with laboratory or clinical demographics. Taken together, current findings may suggest that impaired FMD in young SCD patients is present but mild in nature. Indeed, more data is required to support findings.

Analysis of common carotid artery showed intima-media wall thickening associated with severity of anaemia, increased cardiac output and increased transcranial Doppler flow velocities. Although current findings appear promising, it is important to note that this study was short in duration therefore longer term associations of intima-media to adverse cardiac or cerebral vascular events were not made, but are open to future exploration.

6.4 Clinical relevance

As mentioned previously, SCD is a complex disorder that can lead to multiorgan failure and shortened life expectancy. Patients with the homozygous genotype are faced with life long medical management and frequent hospitalization, which pose significant medical and socialeconomic burden. Improved medical care in SCD has seen a rising number of complications merging with time, particularly PHT, which has been linked to high mortality rate of upto 50%. Screening non-invasive echocardiography algorithms have been put in place to identify patients suspected of having PHT. The problem that is faced, is that traditional echocardiography methods for diagnosing PHT lead to false diagnosis of PHT as TR method is influenced by a high output state. This is why non-invasive method such as PVR_{echo} is important in this vulnerable group of patients as it allows for measurements of resistance within the pulmonary vasculature to be made. Most importantly comparisons of PVR_{echo} with gold standard right heart catheterization studies appear to show accuracy in PVR_{echo} at identifying patients with true PAH. However, more data are needed to test sensitivity and specificity of this method.

Furthermore, increased life expectancy in SCD patients as a result of improved medical management demands clarification of the type of cardiac remodelling involved to help identify patients likely to develop cardiac dysfunction. The data presented in chapter 3 of this thesis appears promising. Patients with SCD appear to have physiological rather than pathological cardiac remodelling. However, longer-term follow up studies in patients with increased cardiac volumes are essential to accurately rule out detrimental cardiovascular events.

Biomarkers play an important role in avoiding adverse events from occurring. Given that common carotid artery intima-media thickening was significantly increased with correlative transcranial Doppler velocities, it vital that prospective follow up studies are undertaken because intima-media thickening may serve as an important biomarker which could help identify patients likely to develop future cardiovascular or cerebral vascular events

6.5 Summary of implications and study design

Non-invasive echocardiography provides useful clinical information regarding prognosis and outcome. Evolving imaging techniques allow for better visualization and quantification of pathophysiological changes occurring within the heart. However, these novel echocardiography techniques still face limitations. Therefore when critical clinical decisions are needed, pressure-volume analysis by catheterization remains the gold standard method for assessing LV function.

In the current study, efforts made to associate echocardiography findings to

laboratory markers such as Hb, LDH and HbF found weak associations. I believed that this relationship would have been stronger if an average of patients' steady state readings were taken instead of using a single most recent set of blood results.

All the rhythm analysis in the current study were assessed by 12 lead ECG tracing, ambulatory 24 hr monitoring would be able to document any arrhythmia occurring over a long period of time. It would be useful to follow up the patients with larger hearts and document any cardiac events likely to develop with time.

Exploration of possible mechanisms underlying pulmonary hypertension in our SCD patients was made by examining the effects of systemic oxygenation by inhaling 5 L/min of oxygen for 20 minutes in patients with TR velocities $\geq$ 2.5m/s. So far this study managed to recruit a total of 18 SCD patients with raised TR >2.5 m/s. Reductions in TR, PVR and cardiac output were observed after oxygen therapy, however, these reductions were not statistically significant. More data are required to examine this mechanism further.

Although validation data for TR and PVR_{echo} support our hypothesis that TR yields high false positives, patient numbers remain low and therefore pose as a general limitation. Similarly, whilst the findings in the vascular study suggest significant FMD reduction in adolescent SCD patients, one limitation to this study was the relatively small sample size.

Finally, all of the patients enrolled in this study represent the out-patient clinic

population in our hospital. These findings may not be completely representative of the general sickle population. For instance all of the patients recruited in chapter 3 were clinically stable with no evidence of heart failure as there were non refered in our clinic. Also sample population did not include patients in the general public that do not attend frequent clinics i.e seen by general practitioners. Perhaps a larger multicentre study would add more information to my findings.

6.6. Outcomes study

The studies in this thesis have been instrumental in providing insight into non-invasive echocardiography methodological improvements when screening for pulmonary hypertension in SCD patients. The primary research aim for the project was “To determine the prevalence of pulmonary hypertension in the general outpatient SCD population and establish whether traditional non-invasive echocardiography TR method is effective as a screening tool at identifying patients with raised pulmonary arterial systolic pressure.” The outcomes are outlined as follows:

1. Prevalence of pulmonary hypertension varied greatly depending non-invasive echo method applied.
2. Traditional TR method yields high false positive rates when compared to invasive measures of pulmonary vascular resistance.
3. The left ventricle is enlarged in SCD. Remodelling appears physiological.
4. There is no left ventricular systolic dysfunction in the selected study cohort, however non of our SCD patients had established heart failure.

6.5.2 Summary of future research needed

As can be seen from aforementioned projects, the studies from this thesis have already made important contributions in terms of research development for later studies. The areas that should be of future research focus are:

1. A larger SCD patient sample will be recruited to undergo invasive right heart catheterisation studies in order to clarify accuracy of non-invasive, PVR_{echo} method.

2. Further analysis of LV function using strain rate in a broader cohort that includes SCD patients with established heart failure would provide better information on cardiac involvement.

6.6.1 Future idea on assessing effects of iron overload in SCD

Patients undergoing long-term regular transfusion program often develop iron-overload cardiomyopathy, which results in systolic and diastolic dysfunction secondary to increased deposition of iron in the heart independent of atherosclerosis or valve disease (Liu and Olivieri 1994). Regional longitudinal shortening abnormalities can be present despite there being preserved global function, which often makes it difficult to depict presence of dysfunction at an early stage (Vogel, Anderson et al. 2003). Speckle tracking echocardiography techniques have been valiated by cardiac MRI in assessing regional load-independent cardiac function (Sengupta, Tajik et al. 2008; Kim, Lee et al. 2009). An attempt was made in chapter 3 to look at regional contractions in our HbSS patients undergoing regular transfusion. Although no differences were found in

systolic function, future work is required in a broader cohort of SCD including those with established heart failure and also incorporating magnetic resonance imaging (MRI) to answer questions surrounding the relaxation phase in iron-overloaded patients. Cardiac MRI has been used to identify the presence of iron in the heart by non-invasively calculating image signal intensities among myocardial tissue during relaxation time (T2). The presence of iron overload normally causes diffraction in signals thereby decreasing tissue intensity on T2 images (Papakonstantinou, Maris et al. 1995). Cardiac MRI has been shown to be sensitive to T2 changes and therefore would be more effective at detecting abnormalities in ventricular relaxation phase. Furthermore, MRI can also detect fibrosis and subendocardial ischaemia.

6.6.2 Future idea on assessment of myocardial infiltration through tissue biopsy

Another possible future study proposal involves taking endomyocardial biopsy tissue in a selected number of patients with larger LV volumes as biopsy analysis would provide more accurate diagnosis of the type of pathophysiological processes involved in SCD. Although findings obtained would be highly informative, enrollment could prove to be challenging, as most patients are incredibly hesitant at taking part in studies involving invasive procedures.

6.6.3 Future idea on assessing left ventricular diastolic dysfunction by catheterisation

Earlier evaluation of left ventricular diastolic function with load independent

tissue Doppler demonstrated that only a small proportion of SCD patients had diastolic dysfunction. Interestingly, in our right heart catheteristion study, a large proportion of SCD patients showed increased left atrial pressure which could suggest increased preload or increased compliance. Since LV diastolic dysfunction has been associated with poor outcome in SCD patients presenting with PHT. It would be of value for further investigations be carried out to assess ventricular contractility. Gold standard pressure volume loop analyses of ventricular contractility, in SCD are needed to accurately determine the prevalence of diastolic dysfunction as these have not been reported among SCD patients.

6.6.4 Future idea on pulmonary vasoconstriction

Hypoxia pulmonary vasoconstriction is a protective physiological phenomenon responsible for maintaining ventilation-perfusion mismatch in the lungs (Wessel and Adatia 1995; Miller, Tang et al. 2000). Vasoconstriction is believed to protect localised under perfused areas of the lungs by constricting pulmonary arteries and diverting blood to better-ventilated areas. Sustained hypoxia perfusion vasoconstriction can lead to vascular remodelling, pulmonary hypertension and probable right heart failure. At the present moment treatment of pulmonary vasoconstriction remains experimental. Vasodilator drug treatments have been reported to be ineffective in hypoxic pulmonary vasoconstriction as it induces systemic vasodilatation. Nitric oxide treatment on the other hand appears to be potent and more selective for pulmonary vasodilatation in adults with severe pulmonary hypertension. Therefore another possible future project would look at effects of nitric oxide inhalation therapy in

SCD diagnosed with pulmonary hypertension. Although initial reports on treatment of pulmonary hypertension SCD patients with sidenifil seemed promising, the study by Gladwin et al was terminated early due to safety concerns (Machado, Barst et al. 2011).

6.7 Conclusions

In conclusion, the work that was undertaken during the course of this thesis have demonstrated that cardiomegaly in sickle cell disease is strongly associated with elevated cardiac index that correlated with severity of anaemia and is possibly physiological in nature. The results challenge the accepted notion that prevalence of pulmonary hypertension among SCD patient is high. Instead, our findings are able to clearly demonstrate that the traditional method of diagnosing PHT by TR is driven by high cardiac output and anaemia thereby resulting in large numbers of false positive diagnosis. Non-invasive estimates of pulmonary vascular resistance by echo appear promising when compared to gold standard right heart catheterisation, however more data are needed to determine its accuracy. Our results also demonstrate the presence of subclinical vascular endothelial dysfunction in adolescent SCD patients. Taken together, these results provide useful information regarding the underlying mechanisms involved in cardiovascular dysfunction and prevalence of pulmonary hypertension among sickle cell disease patients.

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Abstracts Arising From This Thesis

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MANUSCRIPT ONE

Pulmonary hypertension in sickle cell disease - The role of echocardiography derived pulmonary vascular resistance

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INTRODUCTION

Pulmonary hypertension (PH) is a well recognised complication, and an adverse prognostic marker, in sickle cell disease (SCD).[1-6] The prevalence of sickle cell induced PH, as defined by a tricuspid valve regurgitant velocity (TRV) ≥2.5 m/s, has been estimated to be as high as 30%.[4,7-9] However, in recent years the accuracy of these estimates have been questioned as Doppler-derived measures of pulmonary pressure in these original reports were not systematically confirmed by right heart catheterisation - a procedure that is recommended by international guidelines for diagnosis of PH as the standard of care.[10,11] In addition, an important weakness of Doppler echocardiography, in comparison to invasive measurements of pulmonary pressure, is the inability to distinguish between pre-capillary (arterial) and post-capillary (venous) PH. This is particularly important when considering a diagnosis of PH in patients with SCD, where both systolic and diastolic left ventricular (LV) dysfunction is common, and hence can influence pulmonary pressure in the absence of true pulmonary vascular pathology.

To identify the true prevalence of PH in SCD a number of recent studies have directly compared TRV-derived estimates of pulmonary pressure with invasive pulmonary vascular indices.[5,6,12] In the largest of the studies by Parent et al, the prevalence of PH on right heart catheterisation was significantly lower than in previous reports at 6% and the positive predictive value of TRV Doppler values for detection of PH as low as 25%. Furthermore, post-capillary PH was identified to be the most common cause for raised pulmonary pressures.[5] Similar results have been observed by two other groups where the prevalence of PH has been shown to be 10% and 12% in patients with stable SCD.[6,12]

Accurate diagnosis of PH in patients with SCD is important as it has significant implications on prognosis. However, repeated invasive measurement of pulmonary pressure with right heart catheterisation is not practical and remains an unattractive option for patients. The objective of this study is to determine the utility of echocardiography-derived estimates of pulmonary vascular resistance (PVR_{Echo}) as a tool for screening of PH in patients with SCD and to compare the accuracy of PVR_{Echo} estimates of PH against direct invasive pulmonary vascular indices in this group of patients.

METHODS

Selection of subjects and study overview

Between April 2008 and October 2010, 123 consecutive adult patients (≥18 years of age) with stable SCD, under long-term follow-up, were recruited to the study from the haematology outpatient clinic. The genotype on the basis of haemoglobin characteristics was haemoglobin SS in 80 (66%), haemoglobin SC 22 (18%), haemoglobin S-thalassaemia in 14 (12%) and haemoglobin SC-thalassaemia in 5 (4%) patients. Patients with a recent painful sickle crisis (within previous 6 weeks), on treatment for PH and evidence of left sided valvular / structural heart disease were excluded from the study. Our institutional ethics committee approved the study and all patients gave written informed consent prior to recruitment.

Baseline characteristics including a comprehensive clinical history, anthropomorphic measurements [weight (kg), height (m) and body surface area (BSA) (kg/m^2)] and steady state haematology and biochemistry profile were obtained for each patient. A

detailed echocardiographic assessment was performed including screening for presence / absence of PH using both Doppler-derived TRV values and estimation of PVR_{Echo}. A random subset of 18 patients with evidence of PH, as determined by a TRV ≥2.5 m/s, underwent further detailed evaluation with repeat echocardiography, right heart catheterisation, high resolution CT scanning of the lungs and lung function testing within a two weeks time-frame of one another.

Haematological and biochemical analyses were performed in the hospital laboratories. CT examination of the lung was performed according to the our imaging department protocol using a dual detector helical CT scanner (HiSpeed NX/I, GE Medical Systems, Milwaukee, WI, USA) and lung function tests according to American Thoracic Society guidelines[13] using a constant volume whole body plethysmograph (Morgan TLC, Morgan Medical, Rainham, UK). Radiological images were reported by two senior thoracic radiologists and lung function tests a respiratory technician - both blinded to the patients' condition.

Echocardiography

Trans-thoracic echocardiography was performed according to American Society of Echocardiography guidelines[14] with a Philips IE33 ultrasound machine using 2.5 MHz matrix array and stand-alone transducers. Images were obtained and stored digitally over an average of 3 cardiac cycles (5 cycles for patents in atrial fibrillation) in parasternal long- and short-axis, apical two-, four- and five-chamber and sub-costal views for off-line analysis using Xlera imaging software (Philips Medical Systems, The Netherlands). LV ejection fraction (LVEF %) was estimated from the biplane Simpson's method.[14] Right ventricular (RV) systolic function was

derived from measurement of tricuspid annular plane systolic excursion (TAPSE).[15] Mitral and tricuspid inflow recordings were obtained with the pulsed Doppler sample volume at the level of the valve leaflet tips during maximal opening in diastole. Two-dimensional colour coded tissue Doppler (TD) recordings were made with the sample volume at the level of lateral annulus of the mitral valve in the LV, and free wall annulus of the tricuspid valve in the RV. The ratio of early diastolic LV inflow (E) to lateral mitral annulus (e') velocity, and RV inflow to tricuspid free wall annulus velocity, was derived as an estimate of LV and RV filling pressures respectively (diastolic function).[16] TRV was measured with the continuous wave Doppler sample aligned parallel to the tricuspid regurgitant Doppler envelope. The highest recording obtained in one of the four standard views was used in the analysis. A TRV ≥2.5 m/s was accepted as an indicator of PH. PVR_{Echo} [Wood Unit (WU)] was derived from TRV and RV outflow tract (RVOT) time-velocity integral (TVI) as previously described (PVR_{Echo} = 10 x TRV / TVI_{RVOT}).[17] TVI_{RVOT} was obtained by placing the pulsed wave Doppler sample volume in the proximal RVOT just within the pulmonary valve in the parasternal short-axis view. A PVR ≥2 WU was accepted as a marker for PH. In the subset of 18 patients undergoing invasive assessment the mean values from the two consecutive echocardiographic studies was used for analysis.

Cardiac catheterisation

Right heart catheterisation was performed using standard techniques through the right femoral venous route. A 7.5F flow-directed pulmonary artery catheter (Arrow International Inc, Reading, PA, USA) was used to measure pulmonary artery (PAP in mmHg) and pulmonary capillary wedge (PCWP in mmHg) pressures and to

derive thermodilution cardiac out (CO_{Thermo} in L/min - in triplicate) and pulmonary vascular resistance (PVR_{RHCath} in WU). A PVR ≥2 WU was accepted as the standard for PH in this study.

Statistical analysis

Data was presented as mean ± SD or percentage as appropriate. Students t-test was used to compare continuous variables and Chi square test to compare dichotomous variables. Pearson's correlation coefficients (two-tailed) were calculated to compare the association between continuous variables. A multivariate regression model with backward elimination was created to determine independent determinants of PH as defined by TRV of ≥2.5 m/s and PVR_{Echo} ≥2 WU and corrected for age, gender, body surface area, mean blood pressure, oxygen saturation, a clinical history of regular blood transfusion, hypertension and pulmonary embolus, presence of proteinuria, renal function (eGFR), markers of haemolysis (including steady state haemoglobin and lactate dehydrogenase concentration and reticulocyte count) and LV / RV systolic and diastolic function. In patients who had undergone right heart catheterisation scatter plots were created to compare the distribution of TRV / PVR_{Echo} against PVR_{RHCath} values. κ Statistic was derived to investigate concordance between TVR / PVR_{Echo} and PVR_{RHCath} (a κ statistic of +1 indicating perfect agreement, 0 indicating agreement as expected by chance and -1indicating complete disagreement). Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) was calculated to determine the ability of PVR_{Echo} to identify PH in comparison to PVR_{RHCath}. Significance was accepted at $p<0.05$.

RESULTS

Patients were divided according to presence / absence of PH as defined by a TRV ≥2.5 m/s [53 patients (43%) had TRV ≥2.5 m/s] and PVR_{Echo} ≥2 WU [15 patients (12%) had PVR_{Echo} ≥2 WU] (Tables 1 and 2).

Patient characteristics according to TRV ≥2.5 or ≤2.5 m/s

Patients with a TRV ≥2.5 m/s had lower oxygen saturation (p=0.01), higher prevalence of proteinuria (p<0.01) and were more likely to have suffered a pulmonary embolism (p=0.01) or require a blood transfusion (p=0.02). This group also had a lower haemoglobin (p<0.01) and higher lactate dehydrogenase (p<0.01) concentration suggestive of greater haemolysis. In addition patients with TRV ≥2.5 m/s had impaired LV diastolic function (p=0.01) and a hyper-dynamic RV as measured by TAPSE (P=0.02) (Table 1).

Patient characteristics according to PVR_{Echo} ≥2 and ≤2 WU

Patients with PVR_{Echo} ≥2 WU were older (p=0.01), had a higher prevalence of hypertension (p<0.01) and were more likely to be male (p=0.03) or require a blood transfusion (p<0.01). There was no difference in laboratory markers of haemolysis between the two groups. Patients with a PVR_{Echo} ≥2 WU had a lower bilirubin concentration (p<0.01). LV / RV systolic and diastolic function was similar in both groups (Table 2).

Determinants of TRV and PVR_{Echo}

The correlation coefficients for the association between TRV / PVR_{Echo} and clinical, laboratory and echocardiographic parameters is summarised in Table 3. On

multivariate analysis the independent determinants of a TRV ≥2.5 m/s were haemoglobin concentration (P<0.01), a previous history of pulmonary embolism (p<0.01), impaired LV diastolic function (p=0.01) and regular blood transfusion (p=0.02) (Table 4). The only independent determinants of a PVR_{Echo} >2 WU were a previous history of pulmonary embolism (p<0.01) and advancing age (p=0.01) (Table 4).

Comparison of TRV and PVR_{Echo} with invasive pulmonary vascular indices

The distribution of TRV, PVR_{Echo} and PVR_{RHCath} values for the subset of patients undergoing invasive assessment, and the association between these parameters with presence of pulmonary fibrosis and restrictive pulmonary physiology, is summarised in Figures 1, 2 and 3. There was no association between presence of pulmonary fibrosis or restrictive pulmonary physiology and TRV or PVR_{Echo} values. Similarly, pulmonary fibrosis did not influence PVR_{RHCath} values. In contrast, patients with a restrictive physiology had a higher mean PVR_{RHCath} value (p=0.02).

On right heart catheterisation the mean PAP was 22±7 mmHg (range 12 to 37 mmHg), PCWP 13±4 mmHg (range 6 to 20 mmHg) with 8 patients having PCWP ≥15 mmHg , and CO 7.6±1.8 L/min (range 4.1 to 12.0 L/min). There was no correlation between TRV and CO_{Thermo} (p=0.34) / PCWP (p=0.96) or PVR_{Echo} and CO_{Thermo} (p=0.43) / PCWP (p=0.93).

Scatter plots were created to examine the association between TRV (r=0.58, p<0.01) / PVR_{Echo} (p=0.68, p<0.01) and PVR_{RHCath} (Figure 4). There was no concordance between TRV and PVR_{RHCath} values [κ = 0.0±0.08 (95% confidence

interval: 0.00 to 0.37), level of agreement = 0.33]. In contrast there was a moderately good concordance between PVR_{Echo} and PVR_{RHCath} values [κ = 0.76±0.22 (95% confidence interval: 0.32 to 1.00), level of agreement = 0.67]. The sensitivity, specificity, PPV and NPV of PVR_{Echo} for accurate detection of PH (as defined by PVR_{RHCath}) was 100%, 93%, 67% and 100% respectively.

DISCUSSION

Our study demonstrates that estimation of PVR_{Echo} can be a useful screening tool for detection of PH in patients with SCD. Using PVR_{Echo} (and a cut-off value of ≥2 WU as marker of PH) 12% of our cohort of unselected, stable, sickle cell patients were estimated to have PH. This estimate is similar in magnitude to recent studies where right heart catheterisation has been used to define the true prevalence of PH in SCD.[5,6,12] Furthermore, as seen in the invasive subset of our study, there was a good level of agreement between PVR_{Echo} and PVR_{RHCath} measurements.

Multiple recent reports, including our study, indicate that the prevalence of PH in SCD is likely to be significantly lower than previously estimated.[5,6,12] In addition, these studies further confirm that sickle cell patients with true PH (as determined by right heart catheterisation) have a worse short- to medium-term prognosis in comparison to sickle cell patients with normal pulmonary pressures.[5,6] There is also increasing information on the haemodynamic profile of the pulmonary circulation in SCD. In particular invasive right heart studies have shown that post-capillary (venous) PH is more is more common than pre-capillary (arterial) PH.[5,6] This is thought to be secondary to progressive worsening of both systolic and diastolic LV parameters in

patients with SCD. Furthermore, patients with SCD have markedly lower PVR and a higher cardiac output in comparison to other patient populations with PH.[2-4,6] In the current clinical classification of PH, sickle cell appears as a separate aetiological entity for development of pre-capillary (arterial) PH.[18]

Screening for PH and its subsequent accurate diagnosis in SCD remains important. Our study indicates that PVR_{Echo} can potentially be a robust substitute to the commonly used TRV for screening of PH in sickle cell patients - with only individuals with a PVR_{Echo} ≥2 WU going on to have invasive right heart evaluation for a conclusive diagnosis. The combination of PVR_{Echo} and right heart catheterisation has a number of advantages. The high NPV of PVR_{Echo} as seen in our study is likely to significantly reduce the number of false positive referrals for right heart catheterisation thus making the screening process for PH simpler, more cost-effective and acceptable to sickle cell patients. In turn invasive studies in appropriately selected patients will allow accurate differentiation between pre- and post-capillary PH - conditions which require different management strategies.[19] Preliminary results for the use of conventional treatments for pre-capillary PH in SCD (such as prostacyclin derivatives, phoshodiesterase inhibitors and endothelin receptor antagonists) have so far been inconclusive.[20,21] This has raised the question whether sickle cell associated pre-capillary PH is a distinct condition separate from other forms of pulmonary arterial hypertension with ongoing work directed towards development of novel therapeutic approaches. In contrast management of post-capillary PH is likely to be more predictable and directed towards treatment of sickle cell induced changes in left heart systolic and diastolic function.[19]

The pathophysiology of PH in SCD remains under debate.[22] There is increasing evidence to implicate deregulation of the nitric oxide (NO) signalling pathway in response to chronic haemolysis as the underlying mechanism for the development of PH.[22] A number of adult[4,7,8,11] and paediatric[23,24] studies have shown a close association between the development of PH and the severity of haemolysis. Nonetheless, these results have not been consistently replicated. In our study, as with previous reports, haemoglobin concentration was found to be an independent determinant of a TRV ≥2.5 m/s. However, haemoglobin concentration and other laboratory markers of haemolysis (such as reticulocyte count and lactate dehydrogenase concentration) were not independent markers PH as assessed by PVR_{Echo}. We did not use bilirubin concentration as a surrogate marker for haemolysis as liver function tests in SCD are often abnormal secondary to direct involvement of the liver in the sickling process. Interestingly patients with a PVR_{Echo} ≤2 WU in our study had a higher bilirubin concentration ($p<0.01$).

Study limitations

An important limitation of our study is that only a small number of patients underwent invasive assessment of pulmonary vascular parameters and thus validation of PVR_{Echo} against PVR_{RHCath} in the context of SCD remains limited. However, previous larger studies in non sickle cell patients have validated the accuracy of PVR_{Echo} against the current standard PVR_{RHCath} , and as indicated by our small invasive subset, it is unlikely that presence of SCD will interfere with this association.[17] Furthermore, PVR_{Echo}-derived estimates of PH in our study are in keeping with data from recent publications using more extensive right heart catheterisation data.[5.6.12]

In addition, comparison of TRV and PVR_{RHCath} in our study is incomplete as no patients with a TRV ≤2.5 m/s were included in the invasive subset. This precludes the calculation of specificity and PPV for diagnosis of PH based on a TVR ≤2.5 m/s in our study.

Our study did not demonstrate an association between pulmonary parenchymal disease or changes in respiratory physiology and PH in patients with SCD. These findings should be interpreted with caution, as our study was not adequately powered to investigate this association. Considering pulmonary pathology is common in patients with SCD larger future studies are required to address this important question.

Conclusion

Our study demonstrates that PVR_{Echo} can be a useful screening tool for PH in patients with SCD with the potential to significantly reduce the number of patients who require invasive right heart catheterisation for a definitive confirmation of PH. In addition, PVR_{Echo}-derived estimates of PH in our study are comparable to emerging data from invasive confirmation of PH in sickle cell patients.

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Table 1 - Clinical, laboratory and echocardiographic parameters for patients divided according to TRV ≤2.5 and ≥2.5 m/s.

	TRV ≤2.5 m/s (n = 70)	**TRV ≥2.5 m/s (n = 53)**	**p value**
Clinical parameters			
Age (years)	35±13	39±14	0.08
Female gender (%)	46 (66)	33 (64)	0.80
BSA (kg/m^2)	1.7±0.2	1.8±0.2	0.16
Mean blood pressure (mmHg)	85±11	83±11	0.50
Oxygen saturation (%)	97±3	94±5	0.01
Medical History			
History of smoking (%)	3 (4)	2 (4)	0.90
History of hypertension (%)	6 (7)	10 (19)	0.09
Proteinuria	12 (17)	22 (42)	<0.01
History of acute chest syndrome (%)	6 (7)	3 (6)	0.56
History of pulmonary embolism (%)	2 (3)	8 (15)	0.01
History of liver disease (%)	6 (9)	4 (8)	0.86
History of priapism (%)	3 (4)	4 (8)	0.42
History of embolic stroke (%)	4 (6)	5 (10)	0.42
History of leg ulceration (%)	3 (4)	6 (12)	0.13
Regular blood transfusion (%)	4 (6)	10 (19)	0.02
Hydroxyurea therapy (%)	8 (11)	10 (19)	0.23
Laboratory markers			
Haemoglobin (mg/dl)	9.9±2.0	8.3±1.6	<0.01
Reticulocyte count (%)	298±157	314±120	0.54
White-cell count (No/mm^3)	9.2±3.7	9.9±4.2	0.35
Platelet count (No/mm^3)	339±155	376±171	0.24
eGFR (ml/min)	87±29	90±28	0.54
Bilirubin (µmol/l)	49±38	53±33	0.64
Aspartate aminotransferase (U/l)	43±27	45±16	0.73
Alkaline phosphatase (U/l)	96±51	86±37	0.25
Lactate dehydrogenase (U/l)	337±122	427±149	<0.01
Echocardiographic parameters			
LV ejection fraction (%)	61±6	61±8	0.84
TAPSE (cm)	2.3±0.4	2.5±0.4	0.02
LV lateral wall E/e' ratio	6.9±2.2	7.9±2.5	0.01

RV free wall E/e' ratio	5.4±1.8	5.8±2.4	0.44
TRV (m/s)	1.9±0.4	2.9±0.5	<0.01

Table 2 - Clinical, laboratory and echocardiographic parameters for patients divided according to PVR_{Echo} ≤2 / ≥2 WU.

	PVR_{Echo} <2 WU (n = 101)	**PVR_{Echo} >2 WU (n = 22)**	**p value**
Clinical parameters			
Age (years)	35±13	47±15	0.01
Female gender (%)	73 (68)	6 (40)	0.03
BSA (kg/m^2)	1.7±0.2	1.8±0.2	0.04
Mean blood pressure (mmHg)	83±11	89±12	0.15
Oxygen saturation (%)	96±6	96±3	0.88
Medical History			
History of smoking (%)	5 (5)	0 (0)	0.39
Hypertension (%)	10 (9)	6 (40)	<0.01
Proteinuria (%)	31 (29)	3 (20)	0.47
History of acute chest syndrome (%)	9 (8)	0 (0)	0.24
History of pulmonary embolism (%)	8 ( )	3 (20)	0.08
History of liver disease (%)	9 (8)	1 (7)	0.82
History of priapism (%)	7 (7)	0 (0)	0.31
History of embolic stroke (%)	7 (7)	2 (13)	0.35
History of leg ulceration (%)	6 (6)	3 (20)	0.05
Regular blood transfusion (%)	9 (8)	5 (33)	<0.01
Hydroxyurea therapy (%)	16 (15)	2 (13)	0.87
Laboratory markers			
Haemoglobin (mg/dl)	9.2±2.0	9.4±2.0	0.71
Reticulocyte count (%)	309±142	271±136	0.40
White-cell count (No/mm^3)	9.2±4.0	8.7±2.6	0.29
Platelet count (No/mm^3)	356±163	352±128	0.94
eGFR (ml/min)	87±27	101±35	0.20
Bilirubin (µmol/l)	58±37	30±12	<0.01
Aspartate aminotransferase (U/l)	44±23	42±17	0.75
Alkaline phosphatase (U/l)	92±47	93±41	0.93
Lactate dehydrogenase (U/l)	374±140	357±169	0.77
Echocardiographic parameters			
LV ejection fraction (%)	61±6	59±10	0.47
TAPSE (cm)	2.4±0.4	2.4±0.4	0.99
LV lateral wall E/e' ratio	7.3±2.5	7.0±1.5	0.53

RV free wall E/e' ratio	5.6±2.1	5.2±2.1	0.52
PVR_{Echo} (WU)	1.3±0.4	2.4±0.4	<0.01

Table 4 - Pearson's correlation coefficients for clinical, laboratory and echocardiographic parameters with a potential influence on TRV and PVR_{Echo}.

	TRV (m/s)		**PVR_{Echo} (WU)**	
	r	**p value**	**r**	**p value**
Age	0.15	0.10	0.23	0.01
Female gender	0.02	0.83	− 0.17	0.07
BSA	0.10	0.28	0.11	0.23
Mean blood pressure	0.10	0.30	0.06	0.54
Oxygen saturation	− 0.27	<0.01	0.05	0.60
History of blood transfusion	0.23	0.01	0.22	0.02
History of hypertension	0.20	0.03	0.23	0.01
History of pulmonary embolus	0.33	<0.01	0.11	0.23
Proteinuria	0.16	0.08	0.01	0.94
eGFR	0.18	0.08	0.03	0.80
Haemoglobin	− 0.41	<0.01	− 0.002	0.98
Reticulocyte	0.05	0.60	− 0.18	0.06
Lactate dehydrogenase	0.29	<0.01	− 0.02	0.83
LVEF	0.02	0.81	− 0.21	0.03
TAPSE	0.15	0.10	− 0.02	0.86
LV lateral wall E/e' ratio	0.19	0.04	− 0.01	0.93
RV free wall E/e' ratio	− 0.01	0.87	− 0.03	0.73

Table 3 - Independent determinants of TRV ≥2.5 m/s and PVR_{Echo} ≥2 WU.

	B±SE	P value	95% confidence interval
Determinants of TRV ≥2.5 m/s			
Pulmonary embolism	0.64±0.20	<0.01	0.24 to 1.04
Haemoglobin	− 0.11±0.03	<0.01	− 0.17 to − 0.06
LV diastolic function	0.07±0.03	0.01	0.02 to 0.11
Blood transfusion	0.48±0.22	0.03	0.05 to 0.91
Determinants of PVR_{Echo} ≥2 WU			
Pulmonary embolism	0.51±0.17	<0.01	0.18 to 0.84
Age	0.01±0.004	0.01	0.002 to 0.02

Figure 1 - Distribution of TRV (Panel A), PVR_{Echo} (Panel B) and PVR_{RHCath} (Panel C) values for the subset of patients undergoing invasive assessment. In Panel A ● denotes patients with a TVR ≥2.5 m/s who have a PVR_{Echo} ≥2 WU and ♦ patients with TVR ≥2.5 m/s who have a PVR_{RHCath} ≥2 WU. In Panel B ● denotes patients who have a PVR_{Echo} and PVR_{RHCath} ≥2 WU.

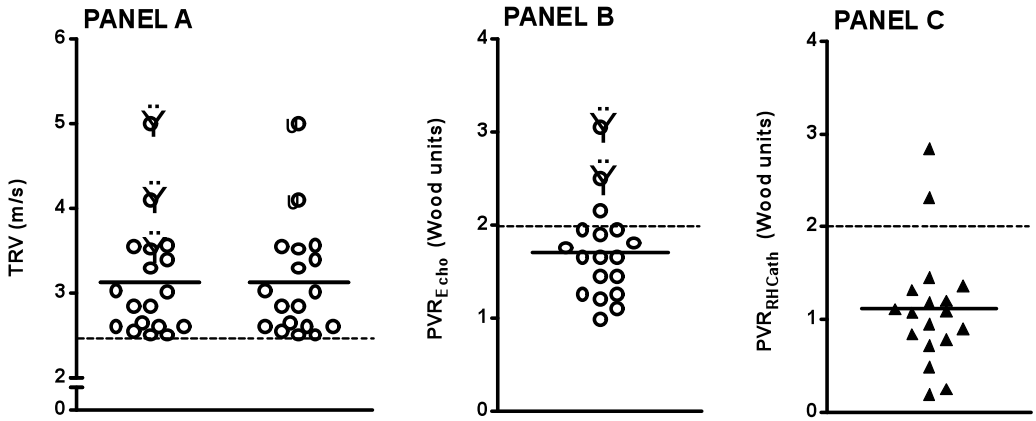


Figure 2 - Distribution of TRV (Panel A), PVR_{Echo} (Panel B) and PVR_{RHCath} (Panel C) values for the subset of patients undergoing invasive assessment according to presence / absence of pulmonary fibrosis.

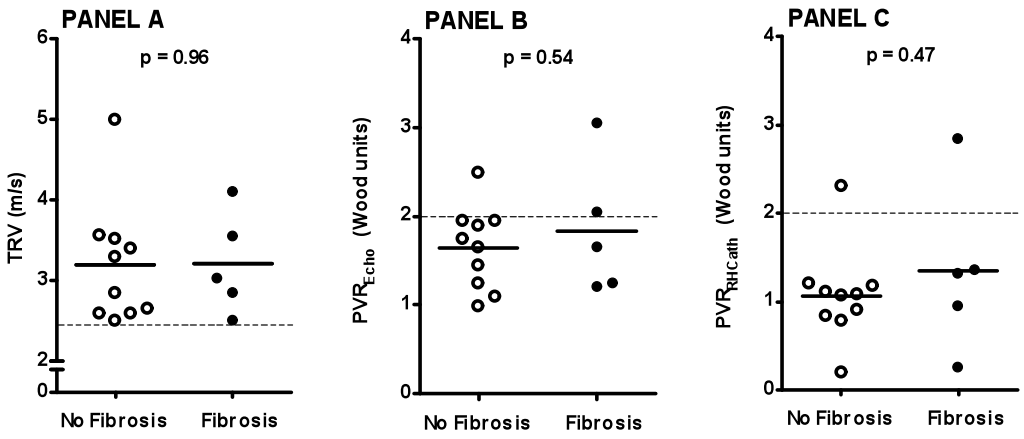


Figure 3 - Distribution of TRV (Panel A), PVR_{Echo} (Panel B) and PVR_{RHCath} (Panel C) values for the subset of patients undergoing invasive assessment according FEV1: FVC ration of ≥ or ≤ 70%.

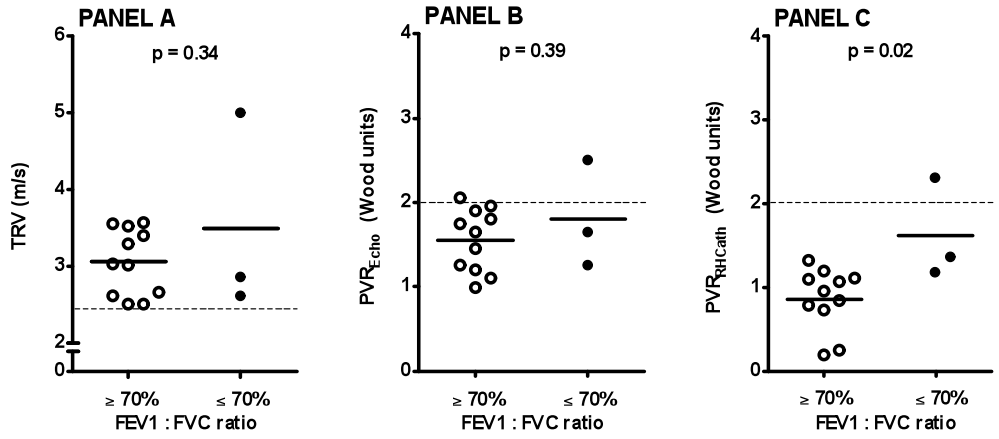
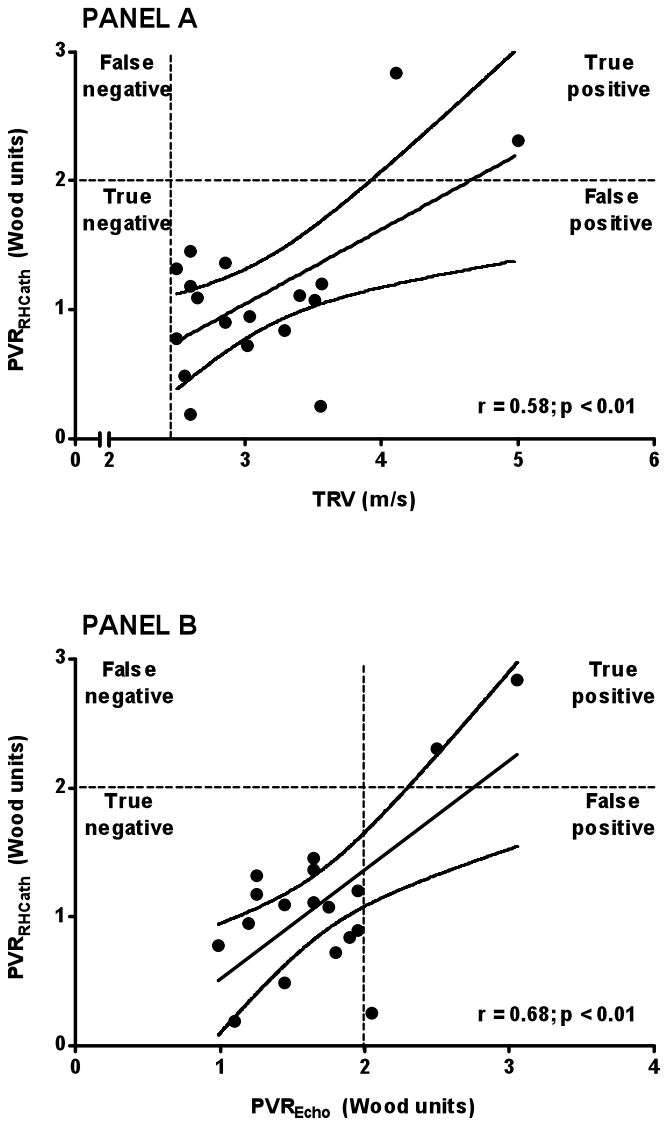


Figure 4 – Scatter plots to examine the association between TRV (Panel A) / PVR_{Echo} (Panel B) and PVR_{RHCath} with a superimposed 2x2 quadrant.



MANUSCRIPT TWO

Characterisation of cardiac dysfunction in sickle cell disease

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Abstract

Background— Sickle cell disease (SCD) has been reported to increase cardiac demands due to anaemia. Long-term cardiovascular consequences due to improved medical management remain unknown. Three dimensional (3D) echocardiography allows better analysis of cardiac structure and function and differentiates physiological from pathological remodeling.

Methods and Results— In this prospective randomized clinical study, we performed detailed 2D and 3D echocardiography studies in 81 homozygous SCD patients and thirty controls matched for age, gender and ethnic origin. Significantly larger LV volume, mass and stroke work index were found in HbSS patients than controls (P<0.005). Increased LV volumes were associated with degree of anaemia (r,0.3; P =0.03) and cardiac output (r,0.3; P =0.02). Patients had significantly higher LV sphericity index than controls (P<0.001). Fewer patients had left ventricular diastolic dysfunction (4%). No differences in LV mass index were found between male and female patients (P =0.1). In addition, independent variables associated with increased LV mass index were fetal heamoglobin concentration, body surface area, and aspartate aminotransferase. Compared with controls no differences were found in ejection fraction and remodelling index.

Conclusion— These data indicate that SCD patients have eccentric left ventricular remodelling probably related to anaemia and chronically high cardiac output. Only a small proportion of patients had left ventricular diastolic dysfunction.

Key words:

Three-dimensonal echocardiography, sickle cell, strain, left ventricular function, left ventricular remodelling.

Key words:

Speckle tracking, murine myocardial infarction, strain, strain rate, left ventricular function, left ventricular remodelling.

Abbreviations:

2D- Two Dimensional

3D- Three Dimensional

EDV- End diastolic volume

EF- Ejection fraction

ESV- End systolic volume

LV- Left ventricular

SDI- Systolic dyssynchrony index

STE- Speckle tracking echocardiography

Introduction

Although cardiac abnormalities are well recognized in sickle cell disease (SCD), the exact nature of cardiac dysfunction remains unclear (Johnson, Kirkham et al. ; Kato and Sachdev ; Sachdev, Machado et al. 2007; Westwood, Shah et al. 2007).

Sickle cell disease exists due to a genetic mutation on the hemoglobin S molecule (Lane 1993; Gladwin and Vichinsky 2008). Abnormal haemoglobin S molecules rupture easily and cause painful vaso occlusive crisis due to reperfusion injuries occurring in the microvasculature (Gladwin, Schechter et al. 2003). SCD can co-exist with other haemoglobin S mutations, such as thalasemia, haemoglobin C and haemoglobin SO_{arab} (Platt, Brambilla et al. 1994). The rate of haemolysis varies immensely among different SCD genotypes and between individuals expressing the same genotype (Nolan, Wyszynski et al. 2005; Kato, Gladwin et al. 2007). In the general sickle population, patients with the homozygous gene, HbSS often present with the severe form of anaemia, associated with high morbidity and mortality rates (Sangokoya, Telen et al.). The past decades have seen considerable improvement in the medical management of SCD patients that has led to prolonged life expectancy (Platt, Brambilla et al. 1994; Buchanan, DeBaun et al. 2004). However, a recent rise in the number of cardiopulmonary complications reports raises great concern. It is well recognised that aneamia in SCD increases cardiac output to compensate for the decreased oxygen tension in arterial blood (Manfredi, Spoto et al. 1960). However what remains unknown is whether long-term compensatory action may in turn inflict adverse demands on cardiac muscle cells.

Echocardiography measures of left ventricular (LV) volumes and systolic function provide meaningful predictors of prognosis in many cardiovascular diseases states (Macron, Lim et al. ; Dujardin, Enriquez-Sarano et al. 1999; Grayburn, Appleton et al. 2005), making quantification of LV parameters imperative during clinical decision-making. Recent advances in echocardiography technology have led to better visualization of ventricular chambers than traditional two-dimensional (2D) methods that relay on

geometrical assumptions. Owing to improved temporal resolution, three-dimensional (3D) echocardiography provides accurate detection of endocardial borders allowing accuracy in estimation of left ventricular volumes and mass. Furthermore, 3D echocardiography also allows for differentiation of pathophysiological disease states, thereby, identifying patients at risk of developing heart failure.

Indeed, if life expectancy is increasing with improved medical management, it is of great importance to clearly understand and describe pathophysiological changes that occur in the sickle cell disease heart. The aims of this study were to characterise in detail, global and regional functional changes occurring within the heart of stable homozygous SCD patients. To achieve this, novel 3D and speckle tracking echocardiography imaging techniques were employed.

Methods

Study Population

The study protocol was approved by the ethics committee at Kings College Hospital, London (UK's largest sickle cell department) and written consent obtained from all participants prior to recruitment. Subjects with the homozygous sickle cell hemoglobinopathy at steady state were eligible to take part in the observational study; a total of 84 patients were enrolled from April 2008 to October 2011. For comparison, thirty healthy volunteers matched for patients' age, gender and ethnic origin were enrolled from the general public.

All subjects underwent a comprehensive cardiological screening protocol that included physical examination, medical history, laboratory studies and transthoracic electrocardiography. Participants with intrinsic cardiac abnormalities such as significant valvular calcification or regurgitation, previous myocardial infarction (on echocardiogram) and uncontrolled hypertension were excluded from the study. Furthermore, participants were excluded if it was not possible to obtain adequate echocardiography images. Participants on cardiovascular medication at the time of enrolment were also excluded. One SCD patient was excluded due to poorly controlled systemic hypertension and another due to poor ultrasound imaging windows, bringing the final total of

HbSS patients in the study to eighty one; average age 34 ±12 years, 26 men.

Laboratory studies

Routine laboratory tests (full blood count, serum chemistry profile, reticulocyte count and lactate dehydrogenase) were obtained during patients' last clinic visit and blood samples analysed by the hospital's biochemistry laboratory. All study protocols were performed at the same sitting, apart from laboratory studies, these were obtained from patient's medical records at the most recent clinic visit.

Echocardiography examination

Detailed 2D and 3D echocardiography examinations were performed using a commercially available ultrasound imaging system (iE33, Philips Healthcare, Andover, MA, USA).

Real time 3D image acquisition and analysis

Full-volume real-time 3D digital images were obtained with the use of the phase array transducer (X3-1) from the apical 4-chamber window. The depth and size of sector were adjusted to a minimum level to allow for the highest temporal resolution. In each patient 3D datasets were acquired over 4 consecutive cardiac cycles using ECG triggers.

All transthoracic 3D datasets were analysed off-line on a commercially available workstation (Research Arena, TomTec analysis software) as previously described. Ventricular volumes were obtained by manually tracing the left ventricular endocardial borders at end-systolic and end-diastolic phases. Results were displayed as time-volume curves with maximum and minimum volumes corresponding to end-diastole and end-systole phases. Intra-observer variability of 3D left heart volumes were calculated from repeat measurements of 10 randomly selected subjects and inter-observer variability calculated from analysis of 3D LV volumes in the same subjects by a second investigator.

Two dimensional echocardiography

Conversional 2D echocardiography studies were performed in accordance with

the European Association of Echocardiography guidelines (Evangelista, Flachskampf et al. 2008). Digital 2D, colour Doppler and spectral Doppler images acquired with a transthoracic 2D transducer were stored onto a dedicated hospital database and data analysis performed off-line using Xlera analysis software.

Speckle tracking strain analysis

For speckle tracking analysis, standard grayscale 2D images were acquired in the short axis parasternal view, at the level of the papillary muscle and also in the 4-chamber apical view. Regions of interest were identified and divided into 6 segments that were colour coded as previously described (Puwanant, Park et al. ; Sengupta, Tajik et al. 2008). Endocardial borders were traced using a semi-automated tracking algorithm which followed myocardial motions throughout the cardiac cycle ensuring myocardial regions were included. Myocardial function was quantified depending on myocardial deformation i.e. distance travelled between tissue speckle deflections over time. Strain quantification was performed off line using commercially available software (QLab, Philips Healthcare).

Statistical analysis

Comparisons of continuous variables in HbSS patients and healthy controls were made using student *t-test* and data presented as mean $\pm$ SD. Non continuous variables were analysed using χ^2 or Mann-Whitney U test. After demonstration of significant differences, repeated measures with 2-way or 1-way ANOVA, including post hoc comparisons were made by use of Bonferroni-corrected t-tests. Associations between the continuous variables were done by linear regression using Pearson Correlation coefficient. To identify factors independently associated with LV remodeling, a linear multivariable regression model was constructed based on a selection of demographic and clinical variables. Odds ratios (B) and 95% confidence intervals (CI) were calculated for covariates in models. Data analyses were performed using SPSS statistical software (version 17, SPPSS Inc).

Results

A total of 81 eligible HbSS patients were randomly enrolled from the haematology outpatients department at Kings College Hospital, London between April 2008 and October 2011. Preliminary data were presented at the Euro Echo meeting in Madrid, Spain, on December 4, 2009.

Clinical characteristics

Table 1 depicts demographic and laboratory data of HbSS patients compared to thirty controls. Overall 55 (68%) of the patients were female, mean age was 34 $\pm$12 years and mean BSA 1.7 g/m^2. Patients and controls were similar at baseline in terms of age, gender and ethnic origin (table 1). However, HbSS patients had significantly lower body surface area ($P<0.001$) than control subjects. In addition, HbSS subjects had lower systolic and diastolic blood pressure ($P<0.05$), haemoglobin concentration [14 (g/dL)] and oxygen saturation. No statistical differences were found in the heart rates.

Structural changes by 3D echocardiography

Left ventricular chamber morphology and function

An initial assessment of left ventricular diastolic volume, uncorrected for patient body surface area was made. Left ventricular end-diastolic volume index was significantly higher in HbSS patients compared to controls ($P< 0.001$). These echocardiogram findings were then corrected for patients' body surface area to eliminated gender and growth relationships. Interestingly results still showed a statistically significant difference between HbSS patients and control group. Similar findings were also observed for left ventricular end-systolic volumes (table 1).

To evaluate the impact of increased left ventricular volumes, comparisons of left ventricular mass indices assessed by 3D echocardiography in HbSS patients and controls were made. Indeed, in the absence of cardiovascular abnormalities, left ventricular mass index in HbSS patients was higher ($P< 0.001$). We evaluated whether gender differences were apparent among HbSS patients. Although males had significantly larger end-diastolic, end-systolic volumes and cardiac output compared to female patients, we found no

significant differences in left ventricular mass index.

Assessment of Left ventricular systolic function was conducted by measuring ejection fraction. Although there were no differences in EF between HbSS and control groups, comparisons of left ventricular stroke volume (SV) and cardiac index (CI) showed significantly higher values. We also quantified LV stroke work index, which controls for differences in cardiac loading and therefore provides a better assessment of global contractile function. The data revealed that LV stroke work was significantly increased in HbSS patients compared to control ($P<0.001$).

Potential causes of left ventricular adaptation

Correlation analyses were made between echocardiographic parameters of LV chamber size and various clinical and laboratory markers of sickle cell disease. A significant negative association was found between LV end-diastolic index and Hb levels. Similar negative associations were evident between LV mass or cardiac index and Hb levels. This would be consistent with the hypothesis that anaemia leads to hyperdynamic circulation with increased cardiac output that in turn leads to increased LV mass.

We also found positive correlations when left ventricular mass index was associated to markers of haemolysis such as the reticulocyte count, and bilirubin and lactate dehydrogenase (LDH) levels (table 3). No correlation was found with other biomarkers of sickle cell such as white cell count, renal dysfunction (cystatin C, urea, creatinine, glomerular filtration rate) or hypercoagulability (platelet).

Multivariable linear regression analysis was performed for increased left ventricular mass against covariates of genotype, oxygen saturation, blood pressure, body surface area, haemoglobin F, aspartate aminotransferas and gender. Independent determinants of left ventricular mass were body surface area, haemoglobin S concentration and aspartate aminotransferase (table 3).

Left ventricular dysfunction

Diastolic dysfunction is a feature of pathological hypertrophy, tissue Doppler assessment of left ventricular diastolic function in our homozygous SCD patients showed the same prevalence rate as control group (4%, P>0.05). Interestingly, no correlation was found between left ventricular diastolic function and left ventricular mass index. However, we found a significant correlation between left ventricular diastolic function and patient age ($r = 0.4$, $P < 0.001$).

Left ventricular shape

Previous studies in failing hearts have demonstrated a shift in LV shape (i.e. from elliptical to spherical), which attributes to poor contractile performance and is a predictor of LV remodelling and outcome (van Dalen, Kauer et al. ; Hung, Papakostas et al. 2004; Wong, French et al. 2004; Monaghan 2006). To assess changes in global LV shape, 3D derived sphericity index was calculated as end-diastolic volume divided by volume of the sphere. A significantly ($P<0.001$) larger sphericity index was observed in HbSS patients compared to controls. Differences in sphericity index were present in both systolic and diastolic phases.

Left ventricular remodelling index (LVRI) serves as useful differentiator of ventricular adaptation processes, i.e. physiological versus pathological remodelling. It is calculated from 3D datasets as a ratio of LV mass and LV end-diastolic volume (De Castro, Caselli et al. 2007). Despite the increase seen in diastolic volume and mass indexes, comparisons of LVRI in HbSS patients and controls found no significant differences.

Systolic dyssynchony index (SDI) is a 3D echocardiography technique that analyses systematic propagation of electrical impulse in the heart (Kapetanakis, Kearney et al. 2005). Abnormalities in SDI help to explain why failing hearts are unable to eject blood effectively. This is usually a result of regional segments reaching peak ejection volumes at different times (Kapetanakis, Kearney et al. 2005; Marsan, Bleeker et al. 2008). In the current study comparisons of SDI in HbSS patients and controls showed no differences.

Analysis of left ventricular strain

Left ventricular strain can help distinguish between normal and abnormal muscle contraction and deformation. Detailed analyses of LV global, longitudinal, circumferential and radial strain were made in 3 standard LV regions namely, basal, mid and the apex. When looking specifically at peak global LV strain in a subgroup of HbSS patients (i.e. those with the most enlarged hearts) versus controls, no statistically significant differences were found. A similar pattern was observed in all LV segments when parameters of longitudinal, circumferential and radial strain were compared between patients and controls (table 4).

It is reported that iron overload arising from multiple blood transfusion therapy could potentially lead to cardiac hypertrophy, we therefore compared strain patterns in transfused patients versus non-transfused. The results did not show substantial differences between the two groups. However, these findings have to be interpreted with caution as the number of patients was small.

Discussion

Cardiopulmonary abnormalities are well-recognized complications in SCD (Fitzhugh, Lauder et al.). Although many researchers have attempted to understand the clinical manifestation, there still remains a lot of controversy around exact prevalence and impact of both cardiac and pulmonary diseases in this patient group (Johnson, Kirkham et al. ; Kato and Sachdev ; Sachdev, Machado et al. 2007; Westwood, Shah et al. 2007). More so, as medical support for patients increases life span (Platt, Brambilla et al. 1994), important questions arise regarding the type of cardiovascular abnormalities likely to appear with time and what long-term consequences they may have. The present study used 3D echocardiography to examine whether and to what degree cardiac abnormalities occur in SCD. Therefore this study focus was on stable SCD patients expressing the homozygous genotype associated with high morbidity and mortality rates, thus data provided may not be representative of the general sickle population. Overall clinical characteristics in this study were in agreement with previous reports (table 3-1). Significantly higher incidents of acute chest syndrome, respiratory and systemic hypertension were present in

our patients, including previous diagnosis of pulmonary emboli. Notably, HbSS patients had lower BSA, systolic and diastolic pressure when compared to control subjects. Low blood pressure in homozygous SCD patients has been associated with lower ventricular afterload and decreased viscosity (leading to decreased arteriolar tone) and renal dysfunction (Adams-Campbell, Nwankwo et al. 1993; Johnson 2005)

Left ventricular volume

It is well established that an increase in cardiac output is the haemodynamic consequence of reduced oxygen-carrying capacity in anaemia (Manfredi, Spoto et al. 1960). It is therefore not surprising that there was a significant increase in left ventricular diastolic volume and cardiac index amongst HbSS patients. Furthermore, increased left ventricular volume index was significantly associated with severity of anaemia (haemoglobin concentration), consistent with other reported studies (Johnson, Kirkham et al. ; Rees, Stefadouros et al. 1978; Covitz, Espeland et al. 1995). Although correlations cannot precisely identify drivers for the increased cardiac volumes and mass, we also looked at the possible impact of heamolysis, as it is the hallmark of SCD. In the current cohort of HbSS patients a significant association was found between steady state clinical markers of haemolysis (LDH, biliruibin and reticulocyte count) and left ventricular mass index, further supporting contribution of anaemia to cardiac remodeling.

Sickle cell disease is an example of balanced polymorphism. Generally individuals who are heterozygous (i.e. HbAS, HbSc/ HbSthal) at particular gene locus have fewer episodes of crisis and are resistant to the malarial parasite than those bearing the homozygous (HbSS) copy of the gene (Menzel, Qin et al. ; Elliott, Ashley-Koch et al. 2007). Here, the question of polymorphism was addressed as there are no articles in the literature focusing on how different SCD genotypes influence cardiac structure; hence left ventricular volumes in a total of 120 SCD patients recruited were grouped according to SCD genotype, i.e. HbSS, HbSthal and HbSC. The results demonstrated that remodelling of the left ventricle depend on the patients' genotype. Patients with the severe form of sickle cell (HbSS) have the largest left ventricular volumes compared to those

with a milder form of the disease (HbSC). If anything, the mildest form of SCD, HbSC, showed similar left ventricular chamber volumes as the control group.

Cardiac remodelling

Cardiac remodelling can occur due to volume/pressure overload, or even cardiac injury (e.g. myocardial infarction). Depending on the type of remodelling i.e. physiological or pathological, the clinical likelihood of developing heart failure can be greatly increased (Swynghedauw 1999; Cohn, Ferrari et al. 2000; Konstam, Udelson et al. 2003). Since current study findings demonstrated increased left ventricular volumes in HbSS patients, it was important to assess how the left ventricle adapts to this increase in wall tension. Even though there are many studies concerning cardiac function in SCD patients, it is difficult to compare data as previous studies employed traditional 2D or m-mode echocardiography techniques that have been proven to yield poor reproducibility in both inter- and intra observor data. Here, we investigated changes in left ventricular mass using novel 3D echo techniques, proven by postmortem and cardiac MRI studies to provide accurate volumetric and geometrical measurements (Brown, Javorsky et al. ; Monaghan 2006). In this study HbSS patients were found to have a significantly higher left ventricular mass index than control subjects. This increase in ventricular mass was significantly associated with circulating haemoglobin S levels ($r = 0.26$, $P = 0.05$).

Fetal haemoglobin (also known as hemoglobin F- HbF) is produced in large quantities during gestation because HbF has the ability to carry oxygen at low oxygen tension. Subsequently after birth, levels of HbF drop as the γ- gene is switched down and β gene is switched on to facilitate the production of normal haemoglobin A (Higgs and Wood 2008). Since HbF cells survive longer and inhibit polymerization of HbS red blood cells, researchers have reported high levels of HbF to low morbidity and mortality rates, (Charache, Dover et al. 1992; Steinberg, Lu et al. 1997; Koshy, Dorn et al. 2000; Higgs and Wood 2008). To date, no studies have assessed the effects of HbF on cardiac structure and function. Correlative analysis of left ventricular mass index and levels of HbF in our study indicate that patients with low levels of plasma HbF are likely to

develop heavier left ventricles. Although the mechanism by which HbF helps to maintain cardiac structure was not fully explored, it is possible that an increase in HbF percentage reduced ventricular workload by increasing overall oxygen carrying capacity. A study by Fabry et al found that increasing HbF concentration ameliorated SCD pathology particularly renal dysfunction (Fabry, Suzuka et al. 2001). Perhaps another possible mechanism could be that SCD patients with high HbF concentrations have smaller ventricles because their ventricular preload is reduced through maintained renal function.

In addition, to investigate independent determinants of LV mass (a marker of remodelling), a multivariable regression model was generated using variables known to increase left ventricular mass such as patient gender, blood pressure and age (Hammond, Devereux et al. 1986; Savage, Garrison et al. 1987; Levy, Garrison et al. 1990; Liebson, Grandits et al. 1993; Krumholz, Larson et al. 1995). In this model, body surface area and haemoglobin S levels and aspartate aminotransferase were the only variables independently associated with changes in left ventricular mass index.

The gold standard assessment of Left ventricular diastolic function requires invasive measurement of LV pressure and volume (Mirsky 1984; Little and Downes 1990). Conventional echocardiography Doppler measurements of the transmitral flow together with tissue Doppler of the mitral annulus (E/e' ratio) have been shown to provide useful clinical information concerning left ventricular filling pressures and outcome (Ommen, Nishimura et al. 2000; Geske, Sorajja et al. 2007). Tissue Doppler E/e' in current HbSS cohort found prevalence of LV diastolic dysfunction to be the same as controls i.e. 4%. The only parameter that correlated with left ventricular diastolic function (Figure 3.21) was patient age (r 0.4, $P< 0.001$), which is well known to relate to diastolic dysfunction in the normal population (Miller, Grossman et al. 1986; Villari, Vassalli et al. 1997). Previous studies have suggested left ventricular diastolic dysfunction to be related to poor prognosis in patients with SCD (Lewis, Maron et al. 1991; Sachdev, Machado et al. 2007; Caldas, Meira et al. 2008). Published data by other researchers has shown varying levels of diastolic dysfunction. For example Arslankoylu et al's study reported no diastolic

abnormalities (Arslankoylu, Hallioglu et al. 2009) whereas Sachdev and Machado's study, reported diastolic dysfunction as present in 18% of the patient population tested (Sachdev, Machado et al. 2007). However, it is important to highlight that the traditional echocardiography diastolic methods used in these studies depend on multiple interrelated factors, such as heart rate and mitral valve incompetence, including atrial and ventricular compliance.

Left ventricular systolic function

There is great controversy regarding left ventricular systolic function in SCD. A study by Rees *et al* (Rees, Stefadouros et al. 1978) reported presence of LV systolic dysfunction in a significant proportion of young SCD patients who had dyspnea and fatigue as assessed by 2D echo derived EF and velocity of shortening of myocardial fibres during ejection. Other contradictory studies, including those performed by Sachdev *et al* (Sachdev, Machado et al. 2007), show no impairment in LV systolic function as assessed by 2D echo derived EF. In the present study, several approaches were taken to identify systolic dysfunction in our patients. Initially we found no differences in EF between sickle cell patients and control group as assessed by 3D echocardiography. Although EF is a widely used clinical measure of cardiac systolic function, care has to be taken when interpreting findings because EF is highly influenced by loading conditions. Stroke work index controls for differences in cardiac loading, thereby provides a better assessment of contractile performance. Here, non-invasive comparisons of stroke work index were calculated using mean arterial pressure and 3D derived stroke volume index. Results demonstrated that a significantly higher stroke work index was present in HbSS patients compared with controls ($P< 0.001$).

Speckle tracking is a new echocardiography imaging technique that can provide useful information on the presence, location and extent of segmental scarring, fibrosis and regional contractile function independent of loading conditions. To date, there have not been any studies published in SCD patients on ventricular function by speckle tracking technology. Regional strain rate data was evaluated in twenty two HbSS patients compared to thirteen controls. Results did not reveal any difference between HbSS patients and control group

suggesting normal contractile function.

Red-cell transfusion therapy serves to correct for low haemoglobin levels, thereby reducing the number of circulating sickle cells and thus improving oxygen carrying capacity of blood. It has been documented that transfusion reduces incidents of cardiac stroke and improves morbidity and mortality (Switzer, Hess et al. 2006; Josephson, Su et al. 2007). However long-term therapy has potential adverse effects, such as iron overload (Switzer, Hess et al. 2006), because humans are not capable of effectively excreting excessively high non physiological levels of iron (Brittenham ; Inati, Khoriaty et al.). It has been reported that iron complications in sickle cell anaemia often develop later as a result of iron entering multiple specific cells such as cardiomyocytes, pituitary, hepatic and pancreatic cells therefore leading to cellular dysfunction, apoptosis and necrosis (Brittenham ; Finch, Lee et al. 1982; Darbari, Kple-Faget et al. 2006). Little is known about the impact of myocardial iron-overload in long-term transfused patients. Results in this study show no statistical differences between patients receiving regular longterm transfusion therapy (n=7) and those not on transfusion (n=15).

Left ventricular shape

Tumkosit *et al,* 2007 demonstrated that patients with dilated LV often develop globular/spherically shaped ventricles that exhibit both reduced contractility and filling (Tumkosit, Martin et al. 2007). Until recently, spherical indices measured by 2D echocardiogram failed to characterise changes in regional LV geometry. Three dimensional echocardiography has been shown to visualise LV endocardial walls better and also validated to accurately quantify LV parameters (Mor-Avi, Sugeng et al. 2009). As there is no data published on the shape of the LV in SCD and how ventricular adaptation affects contractility, we further evaluated LV shape by assessing spherical remodelling using 3D echocardiography. The results of this study demonstrated significantly larger LV sphericity index, both in diastole and systole among HbSS patients, suggesting probable pre-adaption of the LV to a globular shape.

In addition, 3D echocardiography derived left ventricular remodelling index

(LVRI) expresses the relationship between LV mass and LV end-diastolic volume. A simple ratio of LV mass/volume can serve as a useful differentiator of physiological and pathological adaptation (De Castro, Caselli et al. 2007). Patients with dilated cardiomyopathy have been shown to have lower left ventricular remodelling index (LVRI) ~1.03 g/ml, while patients with hypertensive cardiomyopathy have higher LVRI values (mean 2.4 g/ml). In the HbSS group, we could not show a significant difference in LVRI compared to control subjects. It may well be that in HbSS patients the left ventricle adapts to increased demands by remodelling physiologically. However, presence of pathological remodelling cannot be ruled out completely without definitive tissue biopsy examinations or cardiac magnetic resonance (MRI).

Different segmental activation times within the LV are a common and problematic phenomenon in heart failure patients. Left ventricular dyssynchrony leads to reduced efficiency because blood does not synchronously move around the heart. Systolic left ventricular dyssynchrony index (SDI) derived from 3D data on time to peak segmental contraction is a widely used clinical index for assessing ventricular contraction in heart failure. The SDI in the current study failed to show differences between HbSS patients and control subjects indicating that during the systolic phase of contraction all segments are activated at the same time.

Limitations

In the current study, efforts made to associate echocardiography findings to laboratory markers such as Hb, LDH and HbF found weak correlations that were statistically significant. It is believed that this relationship would have been stronger if an average of patients' steady state readings were taken instead of using a single most recent set of blood results. Non-invasive echocardiography provides useful clinical information regarding prognosis and outcome. Indeed evolving imaging techniques allow for better visualization and quantification of pathophysiological changes occurring within the heart. However, novel echocardiography techniques still face limitations. Therefore when critical clinical decisions are needed, pressure-volume analysis by catheterization remains the gold standard method for assessing LV function. Patients

undergoing regular transfusion program often develop iron overload, although an attempt was made to look at regional contractions in our HbSS patients using speckle-tracking techniques, a clear limitation was in the small number of patients analysed, as this might bias the results. Furthermore MRI would be more effective at depicting areas of myocardial fibrosis/scar tissue. In addition, LV biopsy tissue in a selected number of patients with larger LV would also provide accurate diagnosis of the type of pathophysiological involved in SCD. Finally, all of the patients enrolled in this study represent clinic population; therefore these findings may not be completely representative of the general sickle population.

Conclusion

To our knowledge, the present study is the first non-invasive 3D echocardiography report providing important insights into underlying cardiac pathophysiological mechanisms likely to arise in SCD patients. Based on these findings, patients with SCD have eccentric (physiological) LV remodelling that is probably related to anaemia and chronically higher cardiac output. Novel 3D echocardiography may allow for early detection and minimizing risk, especially in SCD patients diagnosed with post capillary pulmonary hypertension because this is associated with poor outcome.

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Table 1. Demographic and clinical characteristics of HbSS patients compared with control subjects matched for age, gender and ethnicity.

Patient characteristics	Controls (n=30)	Patients (n=81)	P value
SCD phenotype	-	80 (66)	
Haemoglobin SS	-	14 (12)	
Sβthalassemia	-	22 (18)	
Haemoglobin SC	-	5 (4)	
Haemoglobin SC thalassemia			
Black Ethnic origin, no.(%)	30 (100)	81 (100)	
Age, yrs	38 ± 9	34 ±12	0.64
Female, no.(%)	18 (60)	55 (68)	0.7
BSA, g/m^2	2.0 ±0.2	1.7 ±1.2	<0.001
Blood pressure, mmHg			
Systolic	123 ±14	113 ±14	<0.02
Diastolic	76 ±22	66 ±18	<0.001
Mean arterial Pressure	92 ±11	82 ±10	<0.001
Oxygen saturation, (%)	98 ±2	95 ±5	<0.0001
>2 A&E visits in past 5 yrs, no.(%)	-	7 (7)	-
History of acute chest syndrome, no.(%)	-	8 (10)	-
History of respiratory disease, no. (%)	-	16 (22)	-
Controlled systemic hypertension, no. (%)	-	8 (10)	-
History of liver disease, no.(%)	-	8 (10)	-
History of pulmonary embolism, no.(%)	-	7 (7)	-
History of stroke, no.(%)	-	8 (10)	-
History of renal dysfunction, no.(%)	-	10 (12)	-
History of leg ulcers, no.(%)	-	9 (11)	-
History of priapism, no.(%)	-	7 (7)	-
History of MI, no.	-	-	-
Hydroxylurea therapy, no.(%)	-	15 (18)	-
Regular transfusion pogramme, no.(%)	-	14 (17)	-
Haemoglobin, g/dL	13 ±1.1	8.5 (1.6)	<0.0001
White cell count, mm^2	7.4 ±3.1	10.4 ±2.0	0.04
Platelet count, mm^2	252 ±66	395 ±157	<0.0001
3D Echocardiography parameters			
Left ventricular end-diastolic volume index, mL/m^2	47 ±12	78 ±23	<0.0001
Left ventricular end-systolic volume index, mL/m^2	20 ±8	30 ±9	<0.0001
Left ventricular mass index, g/m^2	51 ±11	81 ±18	<0.0001
Left ventricular ejection fraction, %	59 ±6	61 ±7	0.2
Stroke work index, g/m	27 ±1	48 ±15	<0.0001
Cardiac index, L/min/m^2	1.9 ±0.5	3.6 ±1.2	<0.0001
Left ventricular remodelling index, g/mL	0.94 +0.3	1.0 +0.3	0.4
Left ventricular sphericity index diastole, %	26 ±4.6	34 ±5.9	<0.0001
Left ventricular sphericity index systole, %	22 +4.6	27 +5.3	<0.0001
Left ventricular SDI, %	1.6 +0.3	1.1 +0.3	0.2

Abbreviations: yrs, years; BSA, body surface area; BP blood pressure; SDI, systolic dyssynchrony index; MI, myocardial infarction.

Fig 1. Left ventricular diastolic volume index in three SCD genotype compared to control group.

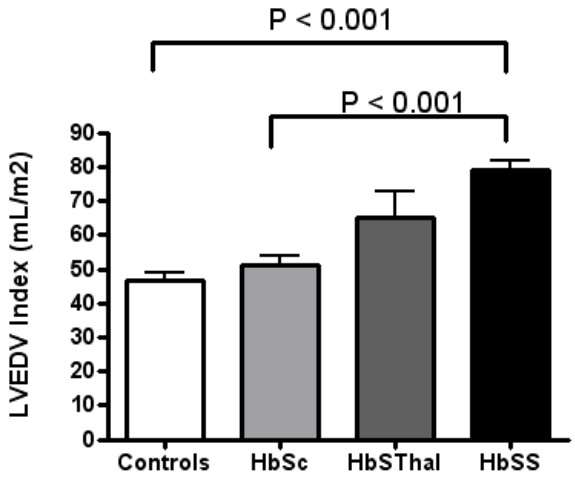

Figure 2. Adjusted LV mass in male and female HbSS patients.

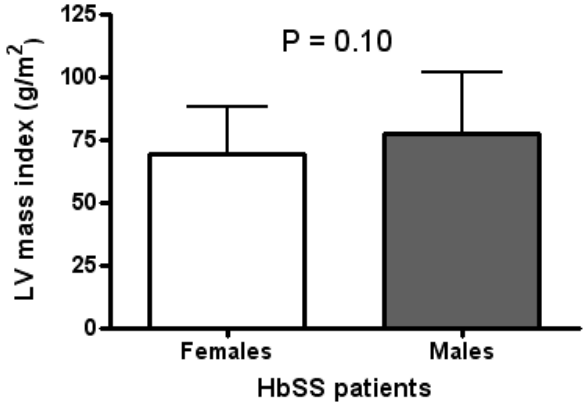

Figure 3. Correlation of left ventricular mass index with Haemoglobin F in HbSS patients.

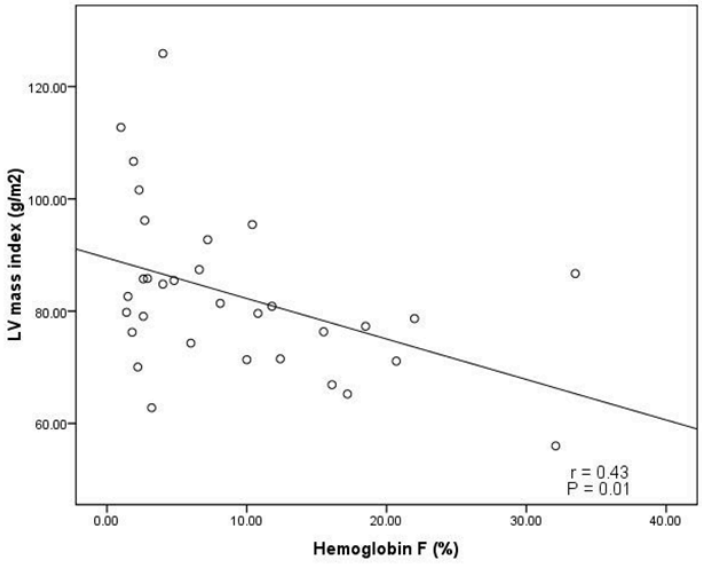

Table 2. Pearson correlation coefficients (r) and 2-tailed P values showing correlation of left mass diastolic index and laboratory biomarkers of sickle cell disease.

	R Value	P Value
Haemolysis		
Bilirubin, μmol/L	0.28	0.03
LDH, UL	0.28	0.03
Reticulocyte, UL	0.31	0.02
Inflammation		
White blood cell count	0.002	0.98
Renal dysfunction		
Cystisin C	0.19	0.12
Creatinine	0.08	0.52
eGFR	0.16	0.23
Hypercoagulation		
Platelet	-0.06	0.64
Liver		
Asparate Transaminase	0.23	0.07

Table 3. Multivariable linear regression model demonstrating independent determinants of left ventricular mass index in HbSS patients.

	β, Estimated increase in LV mass index (±SE)	*P*
Gender	-14 (8)	0.10
Body surface area	74 (21)	0.002
Haemoglobin F, g/dL	-6.8 (2.3)	0.01
Aspartate Aminotransferase, U/L	0.5 (0.2)	0.01

Variables entered into the model were oxygen saturation, blood pressure, body surface area, age, haemoglobin F, aspartate aminotransferase, gender and genotype.

Table 4. Peak systolic longitudinal strain rate of left ventricular segments in HbSS and control groups.

Region of interest	Controls	Patients	P value
Longitudinal strain			
Basal Anterior Lateral	-26.0 ±11.3	-22.0 ±7.9	0.32
Basal Interventricular septum	-14.4 ±5.3	-15.5 ±6.0	0.61
Mid-Anterior Lateral	-15.0 ±6.2	-11.9 ±5.7	0.17
Mid-Interventricular septum	-15.6 ±5.2	-14.7 ±4.2	0.62
Apical Anterior Lateral	-10.7 ±5.5	-17.6 ±3.6	0.56
Apical Interventricular septum	-15.5 ±8.2	-20.4 ±2.0	0.40
Circumfrecial strain			
Basal septal	-18.0 4.3	-19.6 ±5.5	0.44
Basal Inferior Lateral	-19.3 ±6.5	-16.9 ±3.15	0.36
Basal Anterior	-14.0 ±5.9	-18.7 ±3.47	0.16
Basal Inferior	-14.7 ±2.9	-16.6 ±3.1	0.18
Basal Anterior Lateral	-16.9 ±4.7	-17.9 ±4.8	0.76
Basal Inferior Septal	-19.3 ±4.0	-19.7 ±4.3	0.85
Radial strain			
Basal Anterior septal	23.0 ±6	25.2 ±15	0.65
Basal Inferior Lateral	25.7 ±16.6	26.8 ±11.0	0.88
Basal Anterior	22.7 ±10.3	18.9 ±16.4	0.75
Basal Inferior	22.1 ±8.7	25.9 ±14.3	0.47
Basal Anterior Lateral	18.3 ±5.7	16.9 ±16.9	0.83
Basal Inferior Septal	15.5 ±8.2	20.4 ±2.0	0.59

APPENDIX 1 **STUDY PROTOCOL**

Title: Echocardiographic markers of cardiac dysfunction in patients with sickle cell disease

Project Reference No: 08/H0808/196

Chief Investigator: Prof Ajay M Shah

BACKGROUND

Sickle cell disease (SCD) is an autosomal recessive haemoglobinopathy. It occurs throughout the world, but is particularly common amongst African Americans and people from sub-Saharan Africa, Mediterranean countries, and Southern India. It is estimated that worldwide around 200,000 to 300,000 people with SCD are born every year.

The genetic defect responsible for SCD is a point mutation resulting in a single amino acid substitution (valine to glutamic acid) in position 6 in each of the two haemoglobin β chains. The resultant abnormal haemoglobin S (HbS) is much less soluble than normal haemoglobin (HbA) and tends to readily form elongated crystals inside red blood cells in particular when exposed to low oxygen tension. HbS crystals alter the normal shape of red cells preventing their passage through the micovasculature. This results in aggregation of red cells and in turn occlusion of pre-arterioles and arterioles, thus leading to regular cycles of tissue ischaemia and reperfusion (vascular-occlusive crisis). These cycles account for the painful, vascular-occlusive crises suffered by patients with SCD and potentially can affect any organ in the body. Furthermore, HbS crystals rupture red cells leading to anaemia and release of HbS into the circulation (haemolytic anaemia).

In addition to acute symptoms of pain, secondary to microvascular occlusion, patients with SCD develop chronic complications primarily affecting the brain, retina, lungs, heart and kidneys. The mechanisms leading to chronic organ damage are complex and multi-factorial. A reduction in the bioavailability of NO and the ensuing endothelial dysfunction is thought to play a pivotal role in this process. Regular cycles of ischaemia and reperfusion during vascular-occlusive crises lead to release of reactive oxygen species [such as the superoxide anion radical ($O_2^{-}\cdot$)], which promote endothelial dysfunction through pro-oxidant and pro-inflammatory stress. In addition, intra-vascular haemolysis releases HbS and red-cell arginase into plasma, which also interfere with production of NO. Red-cell arginase hydrolyses L-arginine, the main substrate for the production of NO, into urea and ornithine. In turn ornithine competitively inhibits the transport of L-arginine into endothelial cells. Furthermore, the interaction of HbS with pro-oxidants propagates a chain of oxidative reactions resulting in the formation of potent pro-oxidants [such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH\cdot$)]. In conjunction with endothelial dysfunction, micro-infarction secondary to microvascular occlusion is another important source of tissue damage and inflammation. With time regular cycles of injury lead to a proliferative vasculopathy, which as outlined above can affect multiple organs throughout the body.

Title: Echocardiographic markers of cardiac dysfunction in patients with sickle cell disease

Project Reference No: 08/H0808/196

Chief Investigator: Prof Ajay M Shah

Pulmonary hypertension and ventricular dysfunction are the two most important cardiac complications of SCD and continue to remain a major cause of morbidity and mortality. Pulmonary hypertension is defined as a sustained elevation in pulmonary arterial pressure [(PAP), systolic PAP ≥ 30mmHg, mean PAP ≥ 25mmHg] and resistance (>3 Wood units). The exact prevalence of pulmonary hypertension in SCD remains unclear. Echocardiographic studies that between 20-30% of all sickle cell patients may have pulmonary hypertension. However, recent autopsy studies suggest that up to 75% of all sickle cell patients may have histological evidence of pulmonary hypertension at the time of death. An important consequence of pulmonary hypertension is right ventricular dilatation and failure. Patients with sickle cell also exhibit signs of myocardial hypertrophy and impaired diastolic function. However, sickle cell does not appear to cause an overt cardiomyopathy leading to significant systolic dysfunction. Both pulmonary hypertension and diastolic dysfunction are independent determinants of mortality with patients who exhibit both abnormalities having an extremely poor prognosis.

The exact mechanisms for pulmonary hypertension and diastolic dysfunction are not fully understood. Proliferative vasculopathy affecting the pulmonary arterial circulation and/or the myocardial microcirculation is one potential cause. Pathological findings in patients with SCD and pulmonary hypertension have shown evidence of vascular medial hypertrophy, intimal fibrosis and microvascular occlusion within the pulmonary vasculature. However, recent studies also indicate that in a significant proportion of patients, pulmonary hypertension may be secondary to left ventricular (LV) hypertrophy and dysfunction.

2-dimensional (2-D) echocardiography has played an important role in investigating the cardiac manifestations of SCD. However, it has important limitations. Accurate quantification of right ventricular (RV) function and volume using 2-D echocardiography is challenging as its complex crescent shaped geometry only allows limited visualisation of this chamber. In addition accurate regional assessment of myocardial contractile indices requires perfect alignment of the Doppler beam with the ventricle, which is often difficult to achieve. Estimation of the pulmonary vascular resistance (PVR) from pulmonary arterial pressure, which is derived from the peak tricuspid valve (TV) velocity profile, may be inaccurate in SCD for 2 reasons. Firstly, the severe anaemia that is commonly seen in sickle cell patients leads to an increase in cardiac output and hence a peak TV velocity and pulmonary pressure that overestimates PVR. Secondly, hypoxia is SCD patients may cause significant pulmonary vasoconstriction. There is increasing evidence that many of the limitations of 2-D echo can be overcome using novel real time 3-dimensional (3-D) echocardiography. This technique allows acquisition of superior images of the heart,

including that of the RV, for precise quantitative analysis. In addition speckle tracking of the myocardium has opened further avenues to accurately quantify regional RV and LV myocardial function using velocity changes, strain and strain rate that is angle independent – these methods may provide a better measure significantly elevated PVR. 3-D echo also allows estimation of cardiac output which can then be used to correct estimated PVR. Although, these techniques have entered clinical practice they have not been used to provide a detailed assessment of the cardiac manifestations of SCD.

STUDY AIM

The aims of this study are to:
1) Assess in detail the differences in: a) the presence and level of pulmonary hypertension, b) effects of pulmonary hypertension on RV function and c) global and regional RV and LV function in patients with SCD compared with a healthy age-, gender- and ethnicity-matched control group using real-time 3-D echocardiography and speckle tracking of the myocardium.
2) To compare 2-D and 3-D echocardiographic parameters of pulmonary hypertension and ventricular function.

METHODS

Using real-time 3-D echocardiography, we propose to characterise in detail the cardiac influences of SCD and compare these findings with a group of matched healthy controls. Each participant will undergo a detailed conventional 2-D and 3-D echocardiogram (single sitting). A venous sample of blood will be taken and stored for future analysis of biochemical markers of vascular function.

Patient selection and recruitment

Patients with SCD

Patients with SCD attending the haematology clinic (Prof SL Thein) who are being referred for an echocardiogram either as a baseline screening test or to rule out possible cardiac disease will be invited to take part in the study. Potential participants will be approached in the outpatient clinic. The rationale for the study and the study protocol will be explained by one of the study investigators and an information leaflet outlining the study details will be provided before obtaining written consent for the study.
Inclusion criteria: Unselected group of male and female patients with a diagnosis of SCD and over 18 years of age.
Exclusion criteria: 1) Patients less than 18 years of age, 2) Patients with a cardiac disorder known to be caused by an alternative diagnosis.

Healthy volunteers
Healthy volunteers will recruited through general advertisement or from friends and relatives of SCD subjects. Volunteers will be asked to contact the researchers. The rationale for the study and the study protocol will be explained by one of the study investigators and an information leaflet outlining the study details will be provided before obtaining written consent.

STUDY PROTOCOL

Title: Echocardiographic markers of cardiac dysfunction in patients with sickle cell disease

Project Reference No: 08/H0808/196

Chief Investigator: Prof Ajay M Shah

Inclusion criteria: Unselected group of healthy volunteers over 18 years of age, who have no known medical conditions and are on no regular medication.
Exclusion criteria: 1) Volunteers less than 18 years of age, 2) Presence / previous diagnosis of any known medical condition, 3) Presence of conventional cardiovascular risk factors (current smoker, smoker over past year, hypertension, abnormal lipid profile, diabetes), 4) Regular medication.

Patient numbers:
Working on power calculation estimates were as follows, 30-50 controls and 70-100 patients.

Echocardiography protocol
Images will be taken in the Cardiology Department, King's College Hospital, London. A Philips IE33 machine equipped with both 2-D and real-time 3-D software will be used to acquire images. The entire echocardiographic protocol will last between 35-40 minutes. Echocardiography will be undertaken by a research echocardiographer.

2-D echocardiographic images
Each subject will initially undergo a full real time 2-D echocardiogram according to the guidelines of the British Society of Echocardiography. A 5mHz phase array transducer will be used to acquire images. Images will be obtained in the 4 conventional views (parasternal long and short axis, apical and subcostal views) and in brief this will involve: 1) 2-D imaging of all 4 cardiac chambers, 2) 2-D and M-mode assessment of RV / LV systolic function [expressed as % ejection fraction (% EF)], 3) Colour, Pulse wave (PW) and Continuous wave (CW) Doppler analysis of valve haemodynamics and 4) Tissue Doppler analysis of regional myocardial contractility. Maximum RV systolic pressure (RVSP) will be estimated from the CW Doppler signals of the peak TV regurgitant jet velocity using the Bernoulli equation [RVSP ~ 4 x (peak TV regurgitant jet velocity)^2]. Estimates of pulmonary artery systolic pressure will in turn be derived from the sum of RVSP and right atrial (RA) pressure [RA pressure will be determined by size and degree of inferior vena cava (IVC) collapse during inspiration: A) 5mmHg

= IVC collapse of at least 50%, B) 15mmHg = IVC collapse of <50%, C) 20mmHg = if IVC is >2cm in diameter and fails to collapse].

STUDY PROTOCOL

Title: Echocardiographic markers of cardiac dysfunction in patients with sickle cell disease

Project Reference No: 08/H0808/196

Chief Investigator: Prof Ajay M Shah

3-D echocardiographic images and speckle tracking

3-D echocardiography

A X4 matrix array ultrasound transducer will be used to obtain real-time 3-D images. Images of all cardiac chambers and colour Doppler analysis of all valves will be obtained in the 4 conventional views (see above). Acquisitions will be stored and analysed off-line at the end of the study using TOMTEC imaging systems. Detailed functional and volumetric quantifications of the RV and LV will be performed. End-diastolic volume (EDV), end-systolic volume (ESV) and % EF [% EF = 100 x (EDV – ESV) / EDV] will be calculated manually by tracing ventricular endocardial borders in short axis and longitudinal planes for both RV and LV. Two blinded independent observers will perform measurements without knowledge of results obtained.

Speckle tracking

Speckle tracking analysis of regional strain and strain rate will be performed using high rate 2-D echo images. Multiple analysis will be performed in different planes in both the RV and LV. This will facilitate derivation of numerical strain data plus colour coded parametric images of regional strain patterns. Images will be analysed off-line at the end of the study using QLab V6 software (Philips Medical Systems). Unlike tissue Doppler images, this technique is independent of angulation of the ultrasound beam.

Blood-based biomarkers

A venous blood sample will be drawn under standardised conditions. Samples will be stored at −80°C and analysed in a blinded manner at the end of the study. The following biochemical markers of endothelial function / oxidative stress will be assessed: 1) Markers of systemic inflammation [C-reactive protein (CRP)]. 2) Oxidative stress [isoprostance F2α (isoF2α)] and 3) Soluble adhesion molecules (ICAM-1 and VCAM-1).

Co-investigators: Narbeh, Sitali, Prof SL Thein, Dr Mark Monaghan

APPENDIX 2 **STUDY PROTOCOL**

Title: Vascular function in adolescents with sickle cell disease

Project Reference No: 09/H0808/20

Chief Investigator: Prof Ajay M Shah

BACKGROUND

Sickle cell disease (SCD) is an autosomal recessive haemoglobinopathy[1]. It occurs throughout the world, but is particularly common amongst African Americans and people from sub-Saharan Africa, Mediterranean countries, and parts of India[1]. It is estimated that worldwide around 200,000 to 300,000 people with SCD are born every year with a significantly higher number of people being carriers for the condition. South London has a large population with SCD who suffer significant morbidity and mortality from the condition.

The genetic defect responsible for SCD is a point mutation resulting in a single amino acid substitution (valine to glutamic acid) in position 6 in each of the two haemoglobin β chains[2,3]. The resultant abnormal haemoglobin S (HbS) is much less soluble than normal haemoglobin (HbA) and tends to readily form elongated crystals inside red blood cells in particular when exposed to low oxygen tension[2,3]. HbS crystals alter the normal shape of red cells preventing their passage through the micovasculature. This results in aggregation of red cells and in turn occlusion of pre-arterioles and arterioles, thus leading to regular cycles of tissue ischaemia and reperfusion (vascular-occlusive crisis)[2,4]. These cycles account for the painful, vascular-occlusive crises suffered by patients with SCD and potentially can affect any organ in the body. Furthermore, HbS crystals rupture red cells leading to anaemia and release of HbS into the circulation (haemolytic anaemia)[2,4].

In addition to acute symptoms of pain, secondary to microvascular occlusion, patients with SCD develop chronic complications primarily affecting the brain, retina, lungs, heart, kidneys and the skin[5,6]. The mechanisms leading to chronic organ damage are complex and multi-factorial[7,8]. However, there is increasing evidence from experimental models of SCD and sickle cell patients that vascular endothelial dysfunction may play an important role in this process[7-15]. In transgenic murine models of SCD endothelial function has been shown to be impaired in both conduit vessels as well as the microcirculation[9-11]. In patients with SCD flow mediated dilatation (FMD, a marker of conduit vessel endothelial function) is reported to be reduced in comparison to healthy controls[12-14], with the level of endothelial dysfunction being greatest during a vascular-occlusive crisis[15]. Some studies have also demonstrated microvascular endothelial dysfunction in patients with SCD[12,13]. In addition there is also indirect evidence of endothelial dysfunction in this group of patients. Blood plasma levels of L-arginine [the substrate for synthesis of nitric oxide (NO), which is the main determinant of endothelial function (see below)] are depressed in patients with SCD, in particular during vascular-occlusive crises[16,17]. Furthermore, endothelium-independent vascular function may also be affected, with nitroglycerin-induced vascular dilatation being impaired in some patients with SCD[13].

STUDY PROTOCOL

Title: Vascular function in adolescents with sickle cell disease

Project Reference No: 09/H0808/20

Chief Investigator: Prof Ajay M Shah

Reduction in the bioavailability of NO is pivotal to endothelial dysfunction in patients with SCD[8,18]. The main mechanisms which contribute to this process include, inactivation of NO by reactive oxygen species, reduction in plasma concentration of L-arginine and direct interaction of NO with cell-free plasma haemoglobin[8,18]. Regular cycles of ischaemia and reperfusion during vascular-occlusive crises lead to release of reactive oxygen species [such as the superoxide anion radical ($O_2^{-\cdot}$)], which promote endothelial dysfunction through pro-oxidant and pro-inflammatory stress[8,18]. In addition, intra-vascular haemolysis releases HbS and red-cell arginase into plasma, which also interfere with production of NO by reducing the concentration of L-arginine[6,8,19]. Red-cell arginase hydrolyses L-arginine, the main substrate for the production of NO, into urea and ornithine. In turn ornithine competitively inhibits the transport of L-arginine into endothelial cells. Furthermore, the interaction of HbS with reactive oxygen species propagates a chain of oxidative reactions resulting in the formation of further potent pro-oxidants [such as hydrogen peroxide (H_2O_2) and the hydroxyl radical ($OH^{\cdot}$)]. Cell-free plasma haemoglobin also rapidly scavenges NO contributing to a further reduction in its bioavailability[1,8]. Over time the ensuing chronic state of endothelial dysfunction leads to a proliferative vasculopathy, which as outlined above can affect multiple organs throughout the body.

While a correlation between endothelial dysfunction and SCD in adult patients has been reported in several studies[7-15], particularly in those with significant morbidity, it is unclear whether endothelial dysfunction is merely a consequence of repeated crises in such patients or whether it precedes and promotes the development of chronic disease. This question is quite difficult to address in adult patients with SCD since the majority of these have several other risk factors for endothelial dysfunction (such as hypertension, smoking, hyperlipidaemia and the metabolic syndrome), and may already have significant complications. In contrast, adolescents with SCD are generally free of other risk factors for endothelial dysfunction and also have less advanced disease. This age group therefore provides an appropriate population in which to investigate the role of endothelial dysfunction in the pathophysiology of SCD. Furthermore, the study of adolescents who are heterozygous carriers for SCD but are unaffected by disease complications may also be informative in establishing the mechanistic relationship between SCD and endothelial dysfunction. Stroke due to large-vessel vasculopathy is an important, but uncommon, complication of paediatric SCD. A better understanding of endothelial dysfunction in this group of adolescents, especially in comparison to adolescents with SCD and carriers, will provide additional information about the role of endothelial function in SCD and may potentially be of value to prevent stroke in adolescents with SCD.

STUDY PROTOCOL

Title: Vascular function in adolescents with sickle cell disease

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Chief Investigator: Prof Ajay M Shah

STUDY AIM

The aims of this study are to:
1) Assess endothelial function and its determinants in adolescent patients with SCD who have no other classical risk factors for vascular disease as compared to a matched group of healthy adolescent controls.
2) To correlate measurements of endothelial function with other clinical and laboratory parameters in adolescents with SCD.
3) To compare endothelial function in adolescents with SCD and a matched group of adolescents who are heterozygous carriers for SCD.

METHODS

Study overview

35 adolescent patientswith known SCD (homozygous for HbS mutation - SCD group) (25 with no complications of SCD and 10 who have had a stroke), 25 adolescent carriers for SCD (heterozygous for HbS mutation - carrier group) and 25 healthy matched control adolescents (control group) will be invited to take part in the stugroups will be matched for age, gender and ethnicity. Each individual will undergo detailed characterisation on the basis of anthropomorphic measurements, vascular function (both endothelium-dependent and –independent vascular function) and biochemical markers of systemic inflammation, oxidative stress and endothelial function. In adolescents with SCD routinely measured laboratory clinical data will also be used in the characterisation process. A high-resolution non-invasive ultrasound technique will be used to assess vascular function. A sample of venous blood will be obtained and stored for biochemical analysis at the end of the study period from all participants. All measurements will be performed in the morning after an overnight fast. The full study protocol is described below.

Selection and recruitment of study subjects

Patients with SCD and carriers for SCD will be identified and approached in the paediatric haematology clinic at King's College Hospital (Dr D Rees, Senior Lecturer in Paediatric Haematology). Healthy controls will be identified from relatives or friends of either patients with known SCD or carriers for SCD.otential participants will be approached during a routine outpatient visit. The rationale for the study and the study protocol will be explained by one of the study investigators to the potential participant and his/her parents and/or legal guardian. An information leaflet outlining the study details will be provided to both participant and parent/guardian. Written consent will be obtained from both participant and parent/guardian.

STUDY PROTOCOL

Title: Vascular function in adolescents with sickle cell disease

Project Reference No: 09/H0808/20

Chief Investigator: Prof Ajay M Shah

Inclusion criteria: Adolescent, non smoker, male/female subjects between ages 13 to 18 years of age with no known conventional risk factors for vascular disease who belong to one of the following groups: A) Known SCD (homozygous for HbS mutation), B) Carriers for SCD (heterozygous for HbS mutation) and C) Healthy adolescent subjects and D) Known SCD with stroke.

Exclusion criteria: Any adolescent (between ages of 13 to 18 years of age) with a known conventional risk factor for vascular disease (for example, diabetes, smoking, abnormal lipid profile), a history of an inflammatory disorder, regular treatment with any medication (except stroke patients), blood transfusion in the last 4 months (apart form stroke patients) and any acute illness and/or complication of sickle cell disease requiring treatment in last 4 months.

Patient / healthy volunteer numbers: The primary aim of this study is to compare endothelial function, as represented by % FMD in the brachial artery, in adolescents with SCD and a matched group of healthy adolescent controls. Previous studies of endothelial function in young adults with SCD (using an identical method) have demonstrated differences of between 2.2% to 7.1% in FMD between patients with SCD and healthy controls. Based on this information to demonstrate a mean incremental difference of 4% in FMD between the two proposed groups (power 80%; $P<0.05$; alpha 0.10) we estimate a total of 25 adolescent subjects will be required in each group.

Anthropomorphic measurements

Weight (kg), height (m), body mass index (BMI, kg/m^2), waist and hip circumference (cm) and waist to hip ratio will be measured according previously published guidelines immediately prior to assessing vascular function[20].

Biochemical measurements and determinants of endothelial function

A venous blood sample will be drawn under standardised conditions after an overnight fast in the morning for analysis of haemoglobin concentration, full fasting lipid profile [total cholesterol, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides concentrations (all in mmol/L)], simultaneous glucose (mmol/L) and insulin concentrations (IU) to derive insulin resistance, L-arginine concentration and markers of systemic inflammation [C-reactive protein (CRP)], oxidative stress [isoprostane F2α (isoF2α)] and endothelial activation (soluble VCAM-1 and ICAM-1) using previously published methods. All blood samples will be stored at −80°C and analysed in a blinded manner at the end of the study. Insulin resistance will be quantified using the homeostasis model assessment (HOMA) index as described by Matthews e*t al*[21].

In adolescents with known SCD a number of routinely acquired clinical laboratory

data will also be used for characterisation detailed haematology profile [to include Hb F%, full white blood cell count (WBC), neutrophil count], lactate dehydrogenase concentration, G6PD status, alpha globin genotype, urine albumin:creatinine ratio and trans-cranial measurements of intra-cranial blood velocity. All this information is routinely recorded for adolescents with SCD in their clinical notes.

Assessment of vascular function

A non-invasive, high-resolution ultrasound technique will be used to assess endothelium-dependent [by measuring % flow-mediated dilatation (FMD)] and -independent [by measuring % glyceryl trinitrate (GTN) dilatation] vascular function in the brachial artery[22]. This is a well-established non-invasive technique for assessment of vascular function. We have used this technique successfully in multiple previous studies[20,23,24].

The brachial artery will be scanned longitudinally and a stereotactic clamp used to maintain the position of the transducer throughout the study. Ultrasound settings will be set to optimise images of the lumen-arterial wall interface and magnified using a high-resolution box. The distance between opposite lumen-arterial interfaces will be used to calculate vessel diameter. Electrocardiographically (ECG)-gated images will be acquired with every cardiac cycle.

The detailed ultrasonographic protocol has been described previously and in brief consists of one-minute baseline imaging of the brachial artery diameter and Doppler blood flow velocity prior to occlusion of blood flow using a pneumatic cuff around the right forearm (inflated to above systolic pressure) for 5 minutes. A second recording is made immediately following cuff deflation and continued for 2 minutes.

FMD (a measure of endothelium-dependent vascular response) will be expressed as the % change in the brachial artery diameter from baseline to 45-60 seconds (maximal vessel diameter) after deflation of the forearm cuff. Fifteen minutes will then allowed for vessel recovery after which a second baseline scan will be performed as described. GTN (400μg) will then administered sublingually. This will be followed by a further scan 4 minutes later to assess maximal GTN-induced vasodilatation (endothelium-independent vascular response). Scans were recorded and analysed off-line.

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