THE EFFECT OF HARVESTING TECHNIQUES AND CARDIOVASCULAR RISK FACTORS ON ENDOTHELIAL FUNCTION OF HUMAN CORONARY ARTERY BYPASS GRAFTS

by

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2-VP	2-Vinylpyridine
ACE	Angiotensin converting enzyme
ARB	Angiotensin receptor blocker
AT-1	Angiotensin II type I
ATP	Adenine triphosphate
Ca^{2+}	Calcium
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
$CaCl_2$	Calcium chloride
cGMP	Cyclic guanosine monophosphate
CHAOS	Cambridge Heart Antioxidant Study
CO_2	Carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
Cu	Copper
CVD	Cardiovascular disease
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DMSO	Dimethyl sulfoxide solution
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EDHF	Endothelium-derived hyperpolarising factors
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase

ET-1	Endothelin-1
ET_A	Endothelin-A
GSH	Reduced glutathione
GSSG	Oxidised glutathione
HDL	High density lipoprotein
HCl	Hydrochloric acid
H_2O	Water
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
IMA	Internal mammary artery
iNOS	Inducible nitric oxide synthase
K^+	Potassium ion
KCl	Potassium chloride
KH_2PO_4	Potassium dihydrogenphosphate
LDL	Low density lipoprotein
LNMMA	NG-monomethyl L-arginine
LSV	Long saphenous vein
$MgSO_4$	Magnesium sulphate
MIVH	Minimally invasive vein harvesting
MPA	metaphosphoric acid
mRNA	Messenger ribonucleic acid
M_2VP	1-Methyl-2-vinyl-pyridium trifluoromethane sulfonate
NaCl	Sodium chloride
NAD(P)H	Nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate

$NaPO_4$	Sodium phosphate
NEM	N-ethylmaleimide
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
O_2	Oxygen
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
phox	Phagocyte oxidase
ROS	Reactive oxygen species
SEM	Standard error of the mean
SOD	Superoxide dismutase
TAC	Total antioxidant capacity
TOVH	Traditional open vein harvesting
TXA_2	Thromboxane A_2
VEGF	Vascular endothelial growth factor
VLDL	Very low density lipoprotein
VSMC	Vascular smooth muscle cell

SUMMARY

Endothelial dysfunction is a common pathophysiological feature which develops in the evolution of cardiovascular diseases. Strategies to maintain a healthy endothelium or to reverse endothelial dysfunction are crucial for the normal function of the cardiovascular system and the maintenance of cardiovascular health. Endothelial dysfunction is observed both in the coronary and peripheral vasculature.

Studies have demonstrated that surgical preparation of coronary artery bypass grafts can cause endothelial dysfunction and influence the viability and patency of these grafts. An important consideration in the improvement of surgical techniques is to prevent damage to the endothelium during harvesting and implantation. The relative influence of the Mayo stripper minimally invasive long saphenous vein (LSV) harvesting technique and the influence of internal mammary artery (IMA) pedicle width in preserving the integrity of endothelial function are uncertain.

Increased production of reactive oxygen species, in particular, superoxide and radicals derived from superoxide, has been associated with endothelial dysfunction in animal models of disease, and there is increasing evidence of a link between oxidative stress and endothelial dysfunction in humans. It has been reported that endothelial dysfunction and increased oxidative stress may predict future events in patients with coronary artery disease. However, concurrent and comparative data on endothelial function, direct measures of superoxide in human vessels, and biomarkers of oxidative stress are not available simultaneously in patients with coronary artery disease nor in control subjects with no documented cardiovascular disease. Circulating biomarkers of oxidative stress have been investigated in patients with essential hypertension and in control subjects, but the relationship between these markers and endothelial function has not been examined. In addition, although the degree of endothelial

function has been consistently linked to the number of risk factors present in patients with coronary artery disease, the relative importance of individual risk factors in determining levels of oxidative stress and endothelial function remains uncertain.

To address these questions, this thesis studied the influence of harvesting techniques and cardiovascular risk factors on endothelial function of human blood vessels commonly used in coronary artery bypass grafting.

The aim of the first study was to compare endothelium-dependent vasorelaxation in LSV harvested from patients undergoing coronary artery bypass grafting (CABG) by traditional open and Mayo stripper minimally invasive techniques. The experimental approach was to determine calcium ionophore A23187 mediated relaxation and sodium nitroprusside mediated relaxation of LSV rings from patients undergoing CABG by traditional open and minimally invasive techniques. This study found no significant difference in calcium ionophore A23187 mediated vasorelaxation or sodium nitroprusside mediated vasorelaxation in LSV harvested by traditional open and minimally invasive techniques. These results demonstrate no evidence that the increased manipulation of the LSV, consequent upon using the minimally invasive technique, affected functional trauma to both endothelial-dependent and -independent function any more than the traditional open harvest method.

The aim of the second study was to determine the effect of IMA pedicle width on endothelium-dependent vasorelaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG. The experimental approach was (1) to measure IMA pedicle width and IMA diameter post harvest, (2) to measure calcium ionophore A23187 mediated relaxation and sodium nitroprusside mediated relaxation of IMA rings from patients undergoing CABG and (3) to record cardiovascular risk

factors and current drug therapy. This study demonstrated the predominant role of IMA pedicle width in determining calcium ionophore A23187 mediated relaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG. This study demonstrated that a wider IMA pedicle harvested with monopolar electrocautery better preserves IMA nitric oxide-dependent endothelial function. Total cholesterol level was also a significant determinant of calcium ionophore A23187 mediated relaxation.

The aim of the third study was (1) to study endothelium-dependent and -independent vasorelaxation of LSV in a group of patients with severe coronary artery disease compared to controls, (2) to study superoxide production levels in LSV in a group of patients with severe coronary artery disease compared to controls, and (3) to study circulating indicators of oxidative stress in a group of patients with severe coronary artery disease compared to controls. The experimental approach was to measure relaxation of LSV rings to calcium ionophore A23187 (endothelium dependent vasodilatator), sodium nitroprusside (endothelium independent vasodilatator), allopurinol (inhibitor of xanthine oxidase) and apocynin (reduced nicotinamide adenine dinucleotide phosphate (NAD(P)H) oxidase inhibitor) from patients undergoing CABG and age- and sex-matched control patients with no documented cardiovascular disease who were undergoing surgery for removal of varicose veins, (2) to measure circulating indicators of oxidative stress: reduced to oxidized glutathione ratio and total antioxidant capacity from patients undergoing CABG and age- and sex-matched control patients with no documented cardiovascular disease who were undergoing surgery for removal of varicose veins and (3) to measure superoxide production using lucigenin chemiluminescence and oxidative fluorescent microtopography with hydroethidine from patients undergoing CABG and

age- and sex-matched control patients with no documented CVD who were undergoing surgery for removal of varicose veins. This study showed severe depression of relaxation to calcium ionophore A23187 in veins from patients with severe coronary artery disease despite normal responses to sodium nitroprusside, consistent with a specific defect in endothelium-dependent nitric oxide pathways. Superoxide levels, measured directly within the vessel wall using the 2 methods, were elevated in blood vessels from patients with severe coronary artery disease compared with control patients. In addition, in the organ bath studies, inhibition of xanthine oxidase and NAD(P)H oxidase with allopurinol and apocynin, respectively, caused significantly greater relaxation in vessels from patients with severe coronary artery disease compared with control patients. These findings indicate excess superoxide production as an important cause of the attenuation of endothelium-dependent relaxations in patients with severe coronary artery disease and suggest that both xanthine oxidase and NAD(P)H oxidase contribute to superoxide production in these patients.

The aim of the final study was to determine which cardiovascular risk factors influence calcium ionophore A23187 mediated relaxation of LSV in patients with severe coronary artery disease. The experimental approach was (1) to measure relaxation of LSV rings to calcium ionophore A23187, sodium nitroprusside, allopurinol and apocynin from patients undergoing CABG, (2) to measure circulating indicators of oxidative stress (reduced to oxidized glutathione ratio and the total antioxidant capacity) from patients undergoing CABG, and (3) to record cardiovascular risk factors and current drug therapy from patients undergoing CABG. The study showed a significant relationship between low density lipoprotein (LDL) - cholesterol levels and calcium ionophore A23187 mediated relaxation. LDL-

cholesterol level was also a significant determinant of circulating indicators of oxidative stress (reduced to oxidized glutathione ratio and total antioxidant capacity), but, here, other risk factors, including diastolic blood pressure, age, and smoking, also played a role. These data indicate that there is a significant relationship between LDL cholesterol and vascular oxidative stress across the entire range of LDL cholesterol concentrations. This provides a mechanistic explanation in support of intensive LDL cholesterol-lowering therapy as suggested by recent clinical trials

In conclusion, this thesis proposes that a multifactorial strategy aimed at prevention of endothelial dysfunction and graft failure should include improved surgical techniques and use of specific pharmacologic agents, including statins, as a form of intervention. The successful application of this strategy may impact on long-term graft patency.

Chapter 1

Introduction

1.1 Cardiovascular disease

Cardiovascular disease (CVD) is a major cause of morbidity and mortality in the world today and is set to be the leading cause of death worldwide by 2020 (Levenson et al. 2002). According to the World Health Organization, 16.7 million, or 29.2% of total global deaths result from CVD, of these 7.2 million deaths are due to coronary artery disease (CAD) (Beaglehole 2004). The diseases constituting its range of fatal expression (end organ CVD) include myocardial infarction and acute coronary syndrome secondary to CAD. In the United Kingdom in 2000, the calculated annual direct costs of the care of patients with CAD were £1.8 billion, with the indirect costs of loss of productivity three to four times greater at £6.3 billion (Shearer et al. 2004). All forms of CVD are associated with some degree of endothelial dysfunction (Feletou and Vanhoutte 2006).

1.2 Coronary artery bypass grafting

Coronary artery bypass grafting (CABG) was developed in the 1960s and in the 1970s, utilizing the long saphenous vein (LSV) and internal mammary artery (IMA) grafts, and has dramatically changed the management of patients with ischemic heart disease.

The goal of CABG is to provide long-term patent grafts in the native coronary arterial system (Ogus et al. 2007; Kleisli et al. 2005; Sirivella et al. 2005; Eagle et al. 2004). The main reasons for CABG are: a) to relieve ischemia resistant to medical treatment, b) to prevent myocardial infarction and c) to increase life expectancy (Ogus et al. 2007; Kleisli et al. 2005; Eagle et al. 2004; Barra et al. 2000; Canver 1995; Bell et al. 1992; Myers et al. 1989; Rahimtoola et al. 1977). Although CABG has achieved these goals, the degeneration of grafts with time is a major problem (Rosamond et al.

2006). There is increasing interest for the use of arterial conduits in CABG (Ferrari and von Segesser 2006). This is due to the well-documented long-term failure of vein conduits (Schachner 2006), which is the main cause of reoperation and is even more common than the progression of native CAD (Eagle et al. 2004; Weintrab et al. 1994; Salomon et al. 1990). Despite the undoubted success and benefits of CABG with the use of autologous LSV, the occlusion rate of LSV grafts in the first year is between 15% and 26% and by 10 years up to 59% of LSV grafts are occluded (Cho et al. 2006; Eagle et al. 2004; Eagle et al. 1999; Favaloro 1998; Fitzgibbon et al. 1999; Mortwani 1998). The vein grafts that remain patent at 5 and 10 years frequently show angiographic luminal irregularity or narrowing (Cho et al. 2006; Eagle et al. 2004; Eagle et al. 1999; Grondin et al. 1984). However, the ready availability of LSV grafts still accounts for its use in over 70% of CABG (Hata et al. 2007; Eagle et al. 2004).

With 427,000 coronary bypass graft operations now performed annually in the United States in 2004 alone (Rosamond et al. 2007), the growing number of degenerated grafts presents an increasing clinical dilemma. In spite of the fact that LSV graft failure remains a significant clinical and economic burden (Rosamond et al. 2006), the majority of bypass procedures continue to use LSV (Rosamond et al. 2006). Thus, for grafts to remain useful as stable and long-lasting coronary bypass conduits, the most important pathologic changes causing graft failure, which are graft thrombosis and atherosclerosis, must be delayed or prevented (Jeremy et al. 2007; Akowuah et al. 2003; Shuhaiber et al. 2002).

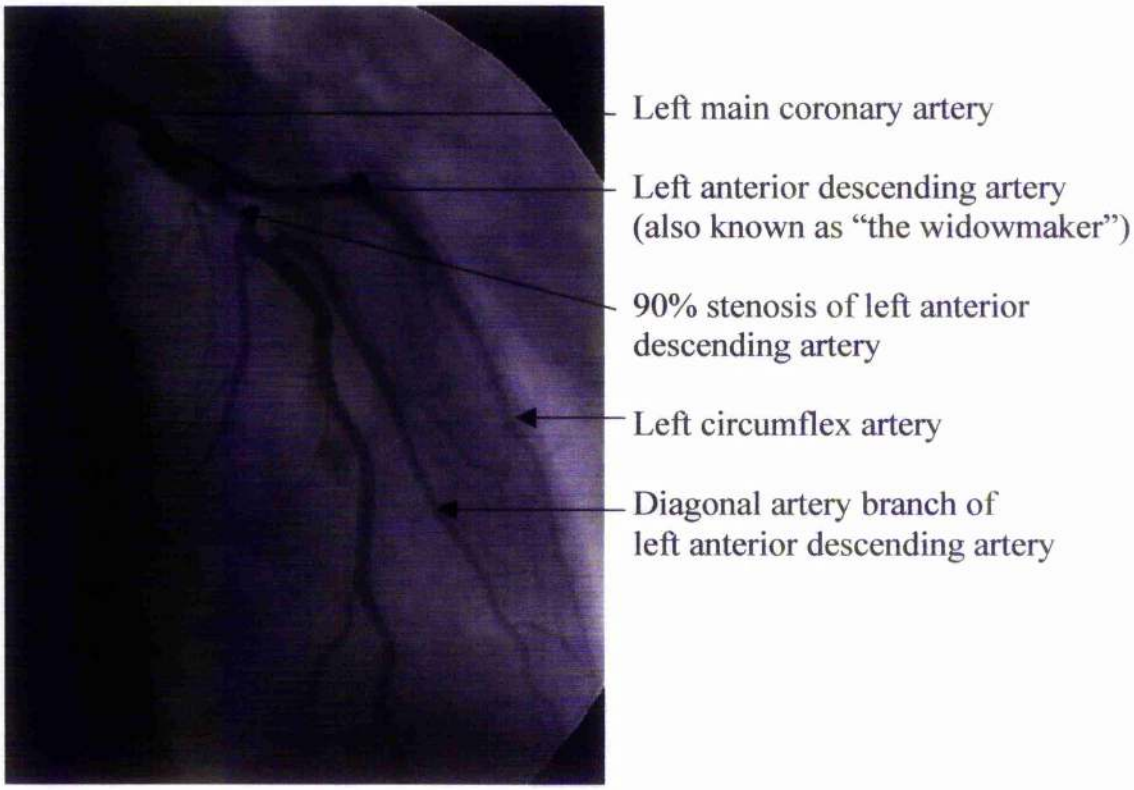

Figure 1.1 Left anterior descending artery stenosis. (Courtesy of Medical Illustration Services, Glasgow Royal Infirmary). A stenotic artery obstructs blood flow from moving through it normally. CABG involves the palliative bypassing of blockages or narrowings in the coronary vessels with a conduit from the aorta to a point on the coronary vessel beyond the major disease. The nature of CAD lends itself to bypass surgery since it tends to be both proximal and focal as shown in the figure with 90% stenosis of the left anterior descending artery. The left main coronary artery arises from the posterior aortic sinus. It gives off the left anterior descending artery which descends to the apex of the heart in the anterior interventricular groove. The vessel provides diagonal branches to the left ventricular free wall. Both the anterior descending artery and its diagonal branches are important vessels to graft if diseased. The left main vessel passes posteriorly towards the diaphragmatic surface of the heart in the atrioventricular groove where it is known as the circumflex artery. The circumflex artery is another important vessel to graft if diseased.

A principal cause of early graft failure following CABG is surgical trauma during graft harvesting which can cause endothelial and smooth muscle injury that has important implications for conduit longevity (Poston et al. 2006; Manchio et al. 2005; Thatte and Khuri 2001). In fact, endothelial disruption in LSV used for CABG has been associated with the risk of developing early thrombosis (Poston et al. 2006; Manchio et al. 2005) and 70% of early graft occlusions in CABG are caused by thrombi overlying areas of endothelial loss (Alrawi 2001). Therefore modifications to surgical technique must be investigated to ensure endothelial damage is minimised to optimise graft survival and function or there will be significant mortality and cost implications.

1.3 Historical review of myocardial revascularization

In the 1890s, as surgeons were discovering that blood vessels were lined with cells with a capacity for active repair, they hypothesized the possibility of performing vascular anastomosis (Morris 1935). In 1896, Mathieu Jaboulay, introduced a technique of intima to intima anastomosis of vessels using interrupted everting mattress sutures (Jabouley and Briau 1896). Morris suggested the possibility of performing vascular anastomosis to the surgeon James Murphy and in 1897, James Murphy published the first report on vascular anastomosis in human patients (Murphy 1897). In 1902, Alexis Carrel a student of Jabouley, moved to work with Charles Guthrie and further refined vessel anastomosis by the trifurcation technique (Carrel and Guthrie 1905). In 1905, Charles Guthrie, Jose Goyanes and Alexis Carrell reported the first experimental use of autologous vein grafts (Carrel and Guthrie 1905). Jose Goyanes, following Alejandro San Martin Y Sastrustigui's advice,

performed the first successful vein autograft of popliteal vein to bypass a popliteal aneurysm (Goyanes 1906).

Surgical treatment for CAD began in the early 1900s. Experimental techniques by Alexis Carrel for directly anastomosing the aorta with the left anterior descending coronary artery failed (Carrel 1910) and indirect methods of myocardial revascularization by Claude Beck (Beck 1935; Beck and Leighninger 1954) and later by Arthur Vineberg (Vineberg and Miller 1951) only marginally increased the blood supply to the myocardium. Development of the first selective coronary angiography by Mason Sones at The Cleveland Clinic led to the application of direct myocardial revascularization and demonstrated the angiographic patency of the Vineberg procedure (Sones and Shirey 1962).

In 1962 Donald Effler (Effler et al. 1964), was able to repair a tight narrowing of the left main trunk of the coronary artery (Figure 1.1) by using the patch graft technique developed by Ake Senning (Senning 1961). However, the mortality when applying this technique was extremely high. In 1962 David Sabiston performed the first CABG on a beating heart, from the aorta to the right coronary artery using an autologous LSV as the conduit (Sabiston 1963). In 1965 Kolessov performed the first anastomosis between the left IMA and the left anterior descending through a left thoractomy (Kolessov 1967).

In 1967 Rene Favaloro successfully reconstructed the right coronary artery by interposing a segment of LSV and later on he started using LSV graft direct from the aorta to the coronary arteries. Afterwards, significant progress occurred when Favaloro and his group were able to perform double bypass, emergency revascularisation and even combined operation (Favaloro 1970). The results of these efforts compiled by Sheldon et al. (Sheldon et al. 1969; Sheldon et al. 1970) were

immediately obvious to the medical community. The same year, George Green and Bailey and Hirose separately published reports in which the IMA was used for CABG in patients (Green et al. 1968; Bailey and Hirose 1968).

In the 1980s studies demonstrated that severe atherosclerotic deterioration of LSV graft occurs between 6 and 11 years post CABG (Bourassa et al. 1984; Campeau et al. 1984; Grondin et al. 1984; Campeau et al. 1983). They proposed that aortocoronary surgery, with the use of LSV grafts, should be reconsidered and suggested that modifications of harvesting techniques and pharmacological intervention might improve the patency of aortocoronary vein grafts. At the same time Floyd Loop, in a great number of patients, showed a clear advantage of using IMA over the LSV (Loop et al. 1986). A few years later the benefit from expanded internal thoracic artery grafting techniques by using bilateral, free and sequential anastomoses was suggested (Loop et al. 1989). Until 1980, only 13% of surgeons were using IMA grafts (Miller et al. 1981). Since the mid 1980s the number of surgeons has increased steadily and today most surgeons employ them (Eagle et al. 2004).

Due to the excellent results of the IMA grafts, surgeons looked for other sources of arterial conduits. In 1987, Pym et al. (1987), Suma et al. (1987) and Attum et al. (1987) reported the first studies on the use of the gastroepiploic artery for direct myocardial revascularization. In 1990, Puig et al. (1990) introduced the use of inferior epigastric artery. In 1971, Carpentier introduced the radial artery as an alternative conduit for CABG; however, the initial results were disappointing (Carpentier et al. 1973). It was reintroduced after modification of the harvesting technique and the use of preoperative calcium channel blockers, which significantly improved the mid-term angiographic results (Gardner 2007; Acar et al. 1998; Acar et al. 1992; Acar et al. 1991). Two randomised studies have provided no evidence of its superiority to the

LSV. (Desai et al. 2004; Buxton et al. 2003) Therefore, the most prudent choice for a high proportion of patients continues to be the use of a single left IMA and multiple vein grafts (Souza et al. 2006).

Autologous IMA are the graft of choice as this conduit is the single most important factor in improved survival and freedom from angina (Hata et al. 2007; Ferrari and von Segesser 2006; Sirivella et al. 2005; Tatoulis et al. 1999; Acinapura et al. 1989). Despite the widespread use and superior patency of the IMA and other arterial conduits, the LSV continues to be the most commonly used conduit for CABG (Hata et al. 2007; Eagle et al. 2004). LSV graft failure after CABG surgery may be as high as 5% to 10% in the first postoperative week (Manchio et al. 2005). Up to 26% of LSV grafts occlude within the first year after bypass surgery (Cho et al. 2006; Eagle et al. 2004; Fitzgibbon et al. 1999; Eagle et al. 1999; Favaloro 1998; Mortwani and Topol 1998; Fitzgibbon et al. 1996; Bourassa 1991; Campeau et al. 1984). Thereafter, for the next 5 years, the attrition rate is 1% to 2% per year, accelerating to up to 4% per annum as the graft ages further (Cho et al. 2006; Eagle et al. 2004; Fitzgibbon et al. 1999; Eagle et al. 1999; Favaloro 1998; Mortwani and Topol 1998; Fitzgibbon et al. 1996; Bourassa 1991; Campeau et al. 1984). As a result, by 10 years after surgery, up to 59% of LSV grafts are occluded and the grafts that remain patent frequently show angiographic luminal irregularity or narrowing (Cho et al. 2006; Eagle et al. 2004; Fitzgibbon et al. 1999; Eagle et al. 1999; Favaloro 1998; Mortwani and Topol 1998; Fitzgibbon et al. 1996; Bourassa 1991; Campeau et al. 1984).

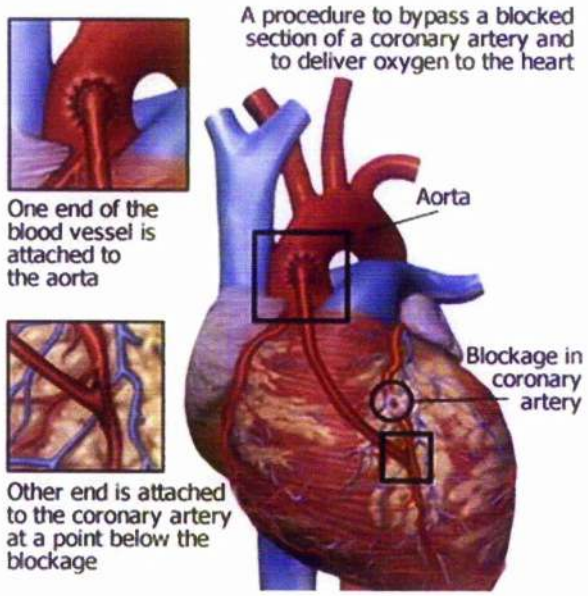

Figure 1.2 A picture of a coronary artery bypass graft: It involves a procedure to bypass a blocked section of coronary artery and deliver oxygen to the heart. One end of the blood vessel is attached to the aorta and the other end is attached to the coronary artery at a point below the blockage thereby bypassing the blockage. (From: North Glasgow University Hospitals NHS Trust Coronary Artery Bypass Grafting Patient Information Leaflet. Courtesy of Medical Illustration Services, Glasgow Royal Infirmary).

The superior long-term patency rate of IMA grafts compared with LSV grafts is well documented (Ferrari and von Segesser 2006; Cho et al. 2006; Loop et al. 1986). Up to 5% of IMA grafts occlude within the first year and patency rates of the IMA implanted into the left anterior descending artery are as high as 83% at 10 years (Sirivella et al. 2005; Eagle et al. 2004; Schroeder et al. 2000; Lamm et al. 2000; Tatoulis et al. 1999; Mehta et al. 1997; Verrier et al. 1996; Izzat et al. 1994; Zilla et al. 1993; Lytle et al. 1985) but again the grafts that remain patent frequently show angiographic luminal irregularity or narrowing. (Eagle et al. 2004; Eagle et al. 1999).

Reflecting this graft attrition and progression of native CAD, angina recurs in up to 20% of patients during the first year after LSV grafting and in 4% of patients annually during the ensuing 5 years (Cameron et al. 1995). Indeed, further revascularization is required in 4% of patients by 5 years, 19% of patients by 10 years, and 31% of patients by 12 years after initial bypass surgery (Weintrab et al. 1994).

With the exception of lipid lowering therapy (Campeau 2000; Datani et al. 2000; The Post Coronary Artery Bypass Graft Trial Investigatiors 1997), no intervention has hitherto proved clinically effective in preventing late vein graft failure (Jeremy et al. 2007). Therefore, every effort should be made to optimise the patency rate of the grafts. Preparation of the graft is important since there is direct evidence that surgical injury during vessel preparation (Dashwood and Loesch 2007; Cunningham 1996) causes severe intimal loss as well as biochemical and functional changes of the grafts (Dashwood et al. 2007; Poston et al. 2006; Schmid et al. 2006; Manchio et al. 2005; Dashwood et al. 2005; Deja et al. 2003; Thatte and Khuri 2001; Mills and Bringaze 1989; Mills and Everson 1995).

1.4 Pathophysiology of graft failure associated with graft preparation

Bypass grafts undergo changes that are divided into two chronological phases. In post-CABG days 1-3, nondenuding endothelial injury, inflammation, limited fibrin deposition and platelet aggregation occur (Davies and Hagen 1995; Davies et al. 1994; Davies et al. 1993). In post-CABG days 14-28, the inflammatory infiltrate resolves, the functional integrity of the endothelium is restored, and vascular smooth muscle cell (VSMC) proliferation results in intimal hyperplasia (Mitra et al. 2006; Davies et al. 1994; Bulkley and Hutchins 1977).

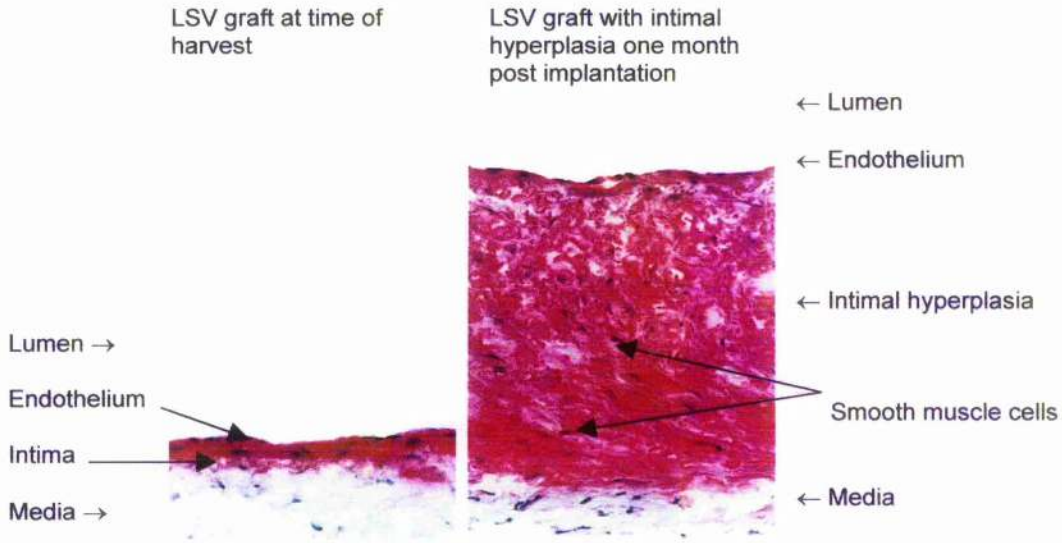

Figure 1.3 Haematoxylin and eosin-stained sections of transected normal LSV graft (left) and LSV graft with intimal hyperplasia one month after implantation (right) (Courtesy of Dr MG Davies). Intimal hyperplasia is the universal response of a vessel to injury (Mitra et al. 2006). It involves the coordinated stimulation of smooth muscle cells by mechanical, cellular and humoral factors to induce a program of cellular activation that leads to proliferation, migration and extracellular matrix deposition (Mitra et al. 2006). As intimal hyperplasia slowly builds up significant restenosis may develop, as the neointima renders the graft susceptible to atherogenesis with macrophages infiltrating this layer to develop into foam cells and then plaque (Mitra et al. 2006), such that as many as 59% of grafts will occlude within a decade after the procedure.

1.5 Early graft occlusion

There are multiple mechanisms underlying graft failure (Jeremy et al. 2007; Schachner 2006; Poston et al. 2006; Sarjeanta and Rabinovitch 2002; Shuhaiber et al. 2002; Tsui and Dashwood 2002; Sessa et al. 2001; Davies et al. 1995; Angelini et al. 1992). This failure results from two processes (Schweiger et al. 1992; Campeau et al. 1983). First, acute thrombosis occurs early in the postoperative period in at least 5% of all vein grafts (Goldman et al. 1989). Second, late graft failure occurs at a rate of 5% to 10% per year per vein graft due to a progressive atherosclerosis-like process. The substrate for the rapid development of atherosclerosis in venous bypass grafts is intimal hyperplasia resulting from VSMC migration/proliferation, a characteristic feature of the accelerated atherosclerosis syndrome (Schwartz et al. 1996; Davies and Hagen 1995; Ip et al. 1990; Neitzel et al. 1986).

Maintaining structural and functional integrity of the endothelium of the graft is an important feature of any procurement technique, as a principal cause of early graft failure following CABG is surgical trauma during graft harvesting which can cause endothelial and smooth muscle injury that has important implications for conduit longevity (Dashwood and Loesch 2007; Poston et al. 2006; Manchio et al. 2005; Dashwood et al. 2004b; Thatte and Khuri 2001). In fact, endothelial disruption in LSV used for CABG has been directly correlated with the risk of developing early thrombosis (Poston et al. 2006; Manchio et al. 2005) and 70% of early graft occlusions in CABG are caused by thrombi overlying areas of endothelial loss (Alrawi 2001).

It has been demonstrated that early graft occlusion, thrombosis or stenosis (reduced graft patency) is related to the inevitable vascular trauma, especially a result of endothelial damage and/or dysfunction and/or loss that occurs during harvesting

and surgical manipulation for CABG (Dashwood and Loesch 2007; Dashwood et al. 2007; Souza et al. 2006; Dashwood et al. 2005; O'Regan et al. 1997; Soyombo et al. 1993; Dilley et al. 1988; Angelini et al. 1987b; Unni et al. 1974). Preparation techniques have demonstrated the importance of minimal manipulation of the vein graft on the integrity of the endothelium, intima, and media (Dashwood and Loesch 2007; Dashwood et al. 2007; Souza et al. 2006; Dashwood et al. 2005; Gundry et al. 1980). Studies have implicated harvest techniques, graft preparation and storage media as sources of endothelial trauma and dysfunction (Dashwood and Loesch 2007; Dashwood et al. 2007; Souza et al. 2006; Dashwood et al. 2005; Perrault et al. 2005; Alamanni et al. 2002; Soyombo et al. 1993; Liu et al. 2001) and intraoperative endothelial integrity relates directly to subsequent graft patency (Soyombo et al. 1993; Dries et al. 1992; Angelini et al. 1987b; Lawrie et al. 1990; Roberts et al. 1984; Gundry et al. 1980). This trauma has been demonstrated to occur during (Dashwood et al. 2007; Dashwood and Loesch 2007; Souza et al. 2006; Dashwood et al. 2005; Angelini et al. 1989; Gundry et al. 1980) and after surgical preparation, when distending (Hasse et al. 1981; Bonchek et al. 1980) or stretching (Bush et al. 1986) the graft. It has been suggested that standardized vein harvest techniques may preserve LSV morphology and reduce the incidence of graft failure (Souza et al. 2006; Dries et al. 1992). Furthermore, a relationship between early endothelial damage and late graft atherosclerosis has also been postulated but not clearly established (Poston et al. 2006; Manchio et al. 2005; Thatte and Khuri 2001).

Biological endothelial integrity is required for NO production in the LSV and IMA (Dashwood et al. 2005; Lüscher et al. 1988), and inhibition of platelet deposition (Radomski et al. 1991; Radomski et al. 1987). Few studies have assessed vascular reactivity and surgical techniques (Barker et al. 1994 Dhein et al. 1991), or compared

different techniques (Chester et al. 1990), especially minimally invasive techniques (Black et al. 2001; O'Regan et al. 1997; O'Neil et al. 1993). The activity of endothelium-dependent NO is sensitive to preparation techniques in human LSV segments (Lawrie et al. 1990).

Impairment of biological endothelial integrity exposes sub-intimal tissue to circulating blood, enhances platelet and fibrin deposition (Baumgartner et al. 1976), initiates a chronic injury-repair process, and often may be followed by fibrous and myo-epithelial proliferation endothelial layer (Catinella et al. 1982; Furchgott and Zawadzki 1980) which affects short- and long-term graft performance (Dhein et al. 1991; He et al. 1989). However, damage to the tunica media and surrounding tissue of the vessel also occurs and observations indicate that adventitial remodelling and subsequent effects on microvessels (vasa vasorum) and vascular nerves may also be important in the pathophysiology of graft narrowing and occlusion (Dashwood et al. 2007; Dashwood and Loesch 2007; Souza et al. 2006 Dashwood et al. 2004a; Gutterman 1999; Barker et al. 1994).

Dashwood and Loesch (2007) state that "damage to segments of the LSV during harvesting as a graft is inevitable." This damage to the LSV graft is aggravated by subsequent exposure to raised arterial pressure (Dobrin et al. 1989; Svendsen et al. 1986; Brook 1975). One of the earliest findings is the insudation of blood constituents including fibrin, erythrocytes, and neutrophils into the graft intima. This is attributed to increased permeability of the endothelium and is observed consistently in all grafts (Vlodaver and Edwards et al. 1971). In an animal study (Wyatt and Taylor 1966), the autologous vein grafts used as arterial replacement have shown considerable damage to the endothelial cells during the process of grafting. The vessels were still partly denuded of endothelium after four weeks. By 12 weeks and possibly as early as six

weeks, a complete endothelial covering was present. Another study found that surgical preparation by performance of the proximal venous anastomosis first, allowing the patient's own blood pressure to distend the vein, preserved both endothelial and medial function (Angelini et al. 1987a).

Graft failure is not only dependent on the quality of the conduit used and poor surgical technique but also poor distal run-off of the coronary arteries (Catinella et al. 1982; Gundry et al. 1980).

1.6 Fibrointimal hyperplasia

Fibrointimal hyperplasia is the formation of scar tissue by smooth muscle proliferation and is believed to be a major contributor to the occlusion of LSV bypass grafts following placement of vein grafts into the aortocoronary circulation (Schwartz et al. 1996; Davies and Hagen 1995; Neitzel et al. 1986). Fibrointimal hyperplasia is also caused by endothelial injury and invariably occurs to some degree (Schwartz et al. 1996; Davies and Hagen 1995; Soyombo et al. 1993; Cox et al. 1991; Adcock et al. 1984). Endothelial removal also results in the loss of vasculoprotective systems that prevent inflammation and thrombosis, these principally being nitric oxide (NO) and prostacyclin (PGI_2) systems (Wu and Thiagarajan 1996; Gryglewski et al. 1995; Angelini et al. 1989; Angelini et al. 1987c). Subsequent platelet activation may release platelet-derived growth factor and basic fibroblast growth factor, which stimulate the smooth-muscle cells of the media to migrate to the intima and to proliferate and synthesize fibrous tissue, including collagen and proteoglycans (LoGerfo et al. 1983). The latter process causes the intimal thickening or hyperplasia that generally results in a 25% decrease in the luminal vessel diameter (Fuster and Chesebro 1986). Evidence shows that basic fibroblast growth factor released from injured endothelial cells

(Nguyen et al. 1994) is important in fibrointimal hyperplasia. Recent data from a canine model showed that the density of receptors for this growth factor is increased when the vein graft is distended at 200 mmHg (McNeil et al. 1989). The majority of grafts that do not completely occlude exhibit some degree of fibrointimal hyperplasia by one month (Angelini 1992). Intravascular ultrasonography has demonstrated that significant fibrointimal hyperplasia occurs by 1 year (Hozumi et al. 1996).

1.7 Ischaemic damage

It has been suggested that hypoxia may be important in mediating LSV graft disease (Jeremy et al. 2004; Dashwood et al. 2004a; Jeremy et al. 1997a). Surgical harvest of the LSV will results in a loss of continuity of the vasa vasorum, a microvascular network responsible for the exchange of gases and supply of nutrients to the vessel wall, which in turn would result in hypoxia of the tissue (Jeremy et al. 2007; Dashwood et al. 2004a). Interference of the vasa vasorum has been associated with CVD (Barker et al. 1993; Martin et al. 1991; Batson and Sottiurai 1985; McGeachie et al. 1981). It has been suggested that damage to the vasa vasorum during LSV distension with resultant ischemia, may be an important mechanism promoting the subendothelial changes (Corson et al. 1985; Spray and Roberts 1977). There is evidence demonstrating that medial fibrosis is induced when grafts are made ischaemic by interruption of vasa vasorum and this is independent of intraluminal pressure (Brody et al. 1972). Pig LSV becomes rapidly hypoxic after excision and remains so after implantation for at least a month (Jeremy et al. 2007). As the LSV graft thickens rapidly, the graft is probably subject to an increase in oxygen demand which may also increase hypoxia (Jeremy et al. 2007). Hypoxia promotes superoxide formation via activation of NAD(P)H oxidase, xanthine oxidase and mitochondrial

respiratory chain (Muzaffar et al. 2005). This prolonged hypoxia upregulates the expression of many proteins that promote LSV graft disease (Jeremy et al. 2004; Jeremy et al. 2002).

1.8 Altered local haemodynamics

Following implantation, the vein graft, which has been subjected only to an internal pressure of 10 mmHg, is immediately subjected to arterial pressure (100 mmHg) (Dobrin et al. 1989). These pressures cause increased circumferential and radial stresses to the vein grafts) (Dobrin et al. 1989). Haemodynamic forces, principally, excessive high wall shear stresses have been postulated as promoters of intimal hyperplasia since studies have demonstrated that the ratio of luminal radius to wall thickness in grafts tends to adapt to the same value as that in the grafted artery, which suggests that wall thickening occurs to normalise tangential wall stress (Dobrin et al. 1989). Hydraulic distension was associated with mural thinning and endothelial damage (Kennedy et al. 1989a), but the implications of these findings remain, unclear. Increased flow velocity is associated with reduced development of fibrointimal hyperplasia and atherosclerosis, and may thereby be a factor of the higher long-term patency rate for IMA grafts (Fujiwara et al. 1988). There is evidence supporting a localized autoregulatory mechanism that senses shear stress and transduces that information into a message to regulate luminal diameter (Zwolak et al. 1987). Further data suggests that this mechanism is dependent on the presence of the endothelium that acts as the transducing element (Langille and O'Donnell 1986). Since the LSV is much thinner than the artery and lacks connective tissue layers that characterizes arteries it adapts to arterial environment by thickening or remodelling. This effectively involves the proliferation of VSMCs and a concomitant deposition of

matrix proteins (Berk 2001; Gibbons and Dzau 1994). The process of LSV graft remodelling also intrinsically alters intra-graft haemodynamics. Both high and low shear stress are associated with enhanceed platelet adhesion (Kawano et al. 2002; Turitto et al. 1998; Kroll et al. 1996). The asymmetric growth of the LSV graft may also promote chaotic blood flow patterns (Caro et al. 2002) which promote platelet and leucocyte adhesion and thrombosis (Caro et al. 2002). This in turn would augment the initial triggering of neointima formation and autoregulatory malfunction which may conceivably lead to excessive fibrointimal hyperplasia with obstruction of the lumen (de Souza 2002).

1.9 Atherosclerosis

Initially vein grafts were thought to be resistant to the development of atherosclerosis (Manderson and Campbell 1986), but subsequent reports showed that atherosclerotic changes occur as early as three to six months postoperatively (Grondin 1986; Barboriak et al. 1974). The incidence of these lesions increases with time and may be as high as 30% after three years (Sarjeant and Rabinovitch 2002; Fuchs et al. 1978). A detailed morphological study of vein grafts suggests that virtually all LSV grafts older than one year show atherosclerotic plaque formation (Kalan et al. 1990). The majority of LSV grafts that occlude in the late postoperative period, that is five years or more after grafting, develop a rapidly progressive form of atherosclerosis (Sarjeant and Rabinovitch 2002; Ratliff and Myles 1989). There is evidence that endothelial damage stimulates migration and proliferation of VSMCs into the intima, which is a key event for development of atherosclerosis (Sarjeant and Rabinovitch 2002; Ross 1973).

1.10 Vasospasm

Reports of coronary bypass spasm have been published in the literature (Fukui et al. 2005; Myers et al. 2003; Reddy et al. 2001; Shapira et al. 2000; Taggart et al. 2000; Caputo et al. 1999; Massa et al. 1991; Dye et al. 1984; Walinsky 1982; Singh et al. 1982; Singh et al. 1981; Baduini et al. 1981). It has been demonstrated that LSV are capable of spasm (Zerkowski et al. 1982; Waddell et al. 1973). Spasm may be a clinically important mechanism in the early and/or late occlusion of vein bypass grafts (Mann et al. 1987; Victor et al. 1981; Singh et al. 1981; Waters et al. 1980; Buxton et al. 1981). Spasm of arterial and venous graft conduits may occur both during harvesting and after the graft has been implanted (Mann et al. 1987). The cause of spasm of a vein during harvesting is not well understood. The most likely cause is the response of smooth muscle to mechanical stimulation during handling and dissection (Mann et al. 1987). Thus, unless specific pharmacologic measures are taken, the LSV is often in spasm after harvesting (Rosenfeldt et al. 1999; Mann et al. 1987). In general, spasm of vascular graft conduits is best managed by prevention by good harvest technique rather than treatment after spasm has occurred (Rosenfeldt et al. 1999).

1.11 Potential role of the adventitia in grafts

The tunica adventitia is the outermost layer of blood vessels and since it gradually merges with the loose connective tissue surrounding the vessel, this region is usually removed during conventional harvesting of LSV and during IMA skeletonisation for use as bypass conduits (Dashwood and Loesch 2007; Boodhwani et al. 2006; Matsumoto et al. 2006; Deja et al. 2005; Ueda et al. 2003; Gaudino et al. 1999). In medium sized vessels, such as the LSV and IMA, the adventitia consists of

longitudinally oriented bundles of VSMCs, collagen fibres and networks of elastin fibres (Wheater et al. 1987). Fibroblasts, macrophages and unmyelinated nerves are also found within the adventitia of medium and large sized vessels (Wheater et al. 1987).

The removal of the adventitia may play an important part in the subsequent processes involved in graft occlusion because the adventitia does not merely provide structural support for the media, but contains the vasa vasorum (Dashwood and Loesch 2007). The vasa vasorum of veins penetrate much closer to the intima than the vasa vasorum of arteries and this is particularly evident in the LSV (Wheater et al. 1987). The vasa vasorum has been shown to be associated with the long-term development of neointimal hyperplasia in vein grafts (McGeachie et al. 1989), and also with tissue healing around LSV grafts (O'Brien et al. 1997). Damage to vasa vasorum in LSV grafts may result in vessel wall hypoxia with subsequent neointima formation, similar to the observation in arteries where occlusion of vasa vasorum leads to neointima formation and atherosclerosis (Barker et al. 1993; Martin et al. 1991). This may also occur when skeletonising the IMA.

Although the LSV graft is "disconnected" from its vascular bed, the supply of blood to medial and adventitial structures will be maintained by the retrograde blood flow observed through the vasa vasorum in "no-touch" vein grafts at implantation (Souza et al. 2006). Furthermore, the connective tissue surrounding the LSV graft when the "Souza no-touch" technique is used may act as a buffer against coronary arterial haemodynamics, "protecting" the graft in very much the same way that has been suggested with the use of experimental external stents (Mehta et al. 1997) and by retaining its surrounding connective tissue cushion during harvesting, perivascular sources of NO are maintained that contribute further to the improved patency rate in

CABG patients receiving LSV prepared with minimal vascular damage (Dashwood et al. 2007).

The vascular nerves located within the tunica adventitia of blood vessels are disrupted during bypass surgery (Dashwood et al. 1998a). The innervation of veins differs from that of arteries, so there will be major differences between the LSV graft and the host artery with respect to neurogenic factors (Dashwood et al. 1998a). Arteries remain under a degree of vascular tone that is influenced by the autonomic nervous system. Apart from classical compounds, such as acetylcholine, noradrenaline and serotonin, there is an increasing list of vasoactive transmitters including NO, which affect vasomotor tone of arteries and veins. The autonomic vascular nerves within the adventitia penetrate the adventitia tunica media to release neurotransmitters that regulate vasomotor tone.

Adventitial fibroblasts have also been suggested to be involved in neointimal formation and vessel occlusion (Shi et al. 1996a; Shi et al. 1996b). There is evidence for adventitial remodelling following vascular damage, such as that caused during graft surgery. An increase in adventitial mass, due mainly to the proliferation of fibroblasts has been described. More recently, it has been suggested that fibroblasts from the adventitia are the progenitors of neointima (Shi et al. 1996a). The neointima may then progress, resulting in atherosclerotic changes in the vessel wall, causing the neointima to be called "soil for atheroma" (Schwartz et al. 1995).

1.12 Storage solution for graft preservation

Several solutions have been used as a storage medium for the preservation of grafts after harvesting (Alamanni et al. 2002). The important role of the temperature and the type of solution used for graft preservation is well recognized (Karnsz et al.

1981). However, there is no ideal solution for rinsing, distending and storing the grafts prior to their implantation (Alamanni et al. 2002). Although, as there is yet no universal agreement on this subject, the superiority of blood over crystalloid solutions for the preservation of endothelial and medial VSMC morphology has been suggested (Gundry et al. 1980; Sottiurai et al. 1983). It has been demonstrated that the preservation of endothelium was better in autologous heparinised blood than in heparinised saline solution (Schaeffer et al. 1997). However, the best preservation of the endothelium was observed with Bret Schneider's solution compared to all other solutions tested (Schaeffer et al. 1997). Plasma-Lyte solution at 37°C has been recommended as a venodilating storage solution during coronary bypass operations to optimise vein graft relaxation before implantation (Sanchez 1994). A study showed that vessel storage in heparinised blood maintained good contractility the first 24 hours and NO dependent vasodilation was effective for a period of up to 12 hours, University of Wisconsin solution and Perfadex gave good preservation for 24 hours but Euro-Collins solution was not a suitable solution for long-term preservation of blood vessels (Ingemansson 1995). Vessels stored in Krebs solution, the only solution containing calcium, manifested no reduction in contractility throughout the 36 hour test period (Ingemansson 1995). Considering the excellent results obtained with Krebs solution regarding contractility, it was suggested that the addition of calcium to University of Wisconsin solution and Perfadex would improve their ability to preserve VSMC function during prolonged storage (Ingemansson 1995). However, another study has shown specific potentially detrimental effects of University of Wisconsin solution on the endothelium and smooth muscle function of isolated LSV (Anastasiou et al. 1997). A more recent study demonstrated that incubation with Celsior and University of Wisconsin solutions substantially preserved endothelial viability and

proliferative capability (Alamanni et al. 2002). Conversely, a prolonged incubation in either Euro-Collins or St. Thomas solutions caused severe and potentially irreversible damage referable to the induction of, respectively, apoptotic or necrotic changes (Alamanni et al. 2002).

1.13 Methods of reducing graft failure

Various methods have been attempted to reduce graft occlusion (Schachner 2006; Shuhaiber et al. 2002). In addition to established adjuvant therapy, modifications of the surgical technique for the preparation of grafts and emerging strategies are currently being used. All these methods are aimed to maintain endothelial integrity, reduce early platelet activation, prevent excessive neointimal proliferation and avoid lipid deposition.

1.14 Vasoactive substances

The use of arterial rather than venous grafts guarantees better long-term results after CABG (Ferrari and von Segesser 2006), mainly because these conduits show a well-preserved endothelial layer, which leads to an enhanced adaptability to acute and chronic flow changes, and to a preserved antithrombotic state (Yang and Luscher 1993). Preferential NO-mediated relaxation (Hamilton et al. 1999; Luscher et al. 1990) and PGI_2 (Sala et al. 1994) release appear have been proposed to contribute to the better patency rate of arterial grafts as compared with venous grafts as they resist vasoconstriction, development of intimal thickening and thrombus formation (Pearson et al. 1992). The wall of the IMA is more resistant to arteriosclerosis than the wall of the venous conduit, because of differences in muscular layers and in the lamina elastica interna (Del Campo 2003). Surgical preparation of the LSV graft itself results

in a significant reduction in NO as compared with the freshly isolated vein and is associated with marked denudation of the endothelium (Angelini et al. 1989). In free harvested vein grafts, endothelium lost to sloughing (in both human beings and experimental animals) requires up to 3 months to be re-populated by blood elements (LeMaitre 1989). Preservation of endogenous NO levels, by minimising endothelial damage during harvesting, may prevent spasm when using atraumatic techniques (Dashwood et al. 2005). This "protective" mechanism may also be maintained during the early postoperative period, counteracting any effects of endogenous vasoconstrictors to which the graft is highly sensitive (Tsui and Dashwood 2002; Chester et al. 1998). As long as 1 week after CABG, surgical preparation that results in endothelial injury causes platelet aggregation at the exposed intimal surface and thrombotic occlusion (Shuhaiber et al. 2002; Tsui and Dashwood 2002). As an inhibitor of platelet adhesion and activation (Moncada and Higgs 2006), NO is beneficial in reducing early graft failure (Dashwood et al. 2005). In addition, NO promotes endothelial cell migration and proliferation (Murohara et al. 1999), which may lead to a more rapid re-endothelialization of the graft, thus reducing the period during which it is vulnerable to thromboses (Tsui et al. 2002).

1.15 Surgical techniques

The normal healthy vascular endothelium of autologous grafts is antithrombogenic (Simionescu 2007) and its preservation will minimise early graft thrombosis (Dashwood et al. 2005). Platelet aggregation, coagulation, vasospasm, occlusive intimal hyperplasia, and accelerated arteriosclerosis occur as a result of endothelial denudation (Hinokiyama et al. 2006; Shuhaiber et al. 2002; Tsui and Dashwood 2002; Thatte and Khuri 2001; Hickethier et al. 1999). Light and scanning

electron microscopy have shown complete loss of endothelium with electrocautery (Lehtola et al. 1989; Yoshida et al. 1995; Sparmann et al. 1992; Gaudino et al. 2000; Noera et al. 1993). Modifications to surgical technique which minimise endothelial damage should improve graft survival and function will have significant mortality and cost implications.

Routinely, vein graft preparation includes dissection of the vein from its bed, ligation of side branches, flushing and distension of the lumen to overcome spasm and to identify leaks (Gundry et al. 1980; Hofer et al. 1981). A recent paper demonstrated that standard surgical handling of vein grafts induces inflammation in the vessel wall and impairs vascular function and suggested that this may potentially contribute to both early and late graft occlusion (Hinokiyama et al. 2006).

Endothelial injury results from direct mechanical trauma (Catinella et al. 1982) and stretching as a result of luminal distension (Adcock et al. 1984; Haudenschild et al. 1981). Impaired fibrinolytic activity, caused by uncontrolled distension of LSV prior to its use as a vascular conduit, may contribute to early vein graft thrombosis, and can be avoided by using controlled distension to <120 mmHg (Underwood 1993).

The technique of harvesting the IMA for CABG influences the fate of artery grafts. Skeletonisation of the IMA (Keeley et al. 1987) has been advocated to provide greater length, probable reduction in sternal wound and pulmonary complications (Cunningham et al. 1992; Matsumoto et al. 1997), and superior free flow (Choi and Lee 1996; Gaudino 1999; Takami and Ina 2002; Wendler et al. 1999; Deja et al. 1999), but its effect on vascular function has not been studied. Cunningham (1996) and Yacoub (1996) state that avoiding the use of electrocautery near the IMA can (at least in theory) prevent or minimize endothelial function. A more friendly surgical handling also plays a determinant role in favour of arterial grafts, especially when

managed *in situ*, as free grafts can undergo significant endothelial damage, due to preparation and storage (Lehmann et al. 1989).

In 1977, an endothelium preserving technique without touching the vein during the anastomosis was described (Gottlob 1977). In 1980 it was recommended that the vein should be harvested by a "no touch" technique to minimize manipulation, side branch ties should be placed away from the LSV wall, veins should be immersed in cold blood and distension above 100mmHg was to be avoided (Gundry 1980). Prevention of endothelial damage in veins was demonstrated by applying pharmacologic relaxation of the vessel that allows a gentle dilatation with low, controlled pressure (Haudenschild et al. 1981). Based on this principle, it was suggested that the LSV preparation should incorporate subcutaneous and perivenous infiltration with papaverine, atraumatic dissection, controlled gradual distension, and storage of the vein in cold heparinised blood (Adcock et al. 1984). The use of glyceryl trinitrate-verapamil solution both topically and intraluminally was introduced in 1993 as a very effective and safe combination to promote relaxation of the vein during preparation (He et al. 1993).

The no-touch technique of LSV harvesting provides high short- and long-term patency rates for vein grafts and is comparable to the patency rate of the IMA graft (Souza et al. 2006). This technique provides better structural, functional, and mechanical protection of the vein wall, thereby resulting in a nonthrombogenic graft immediately after surgery and long-term improvement due to reduced late intimal and medial hyperplasia (Souza et al. 2006). Dashwood et al. (2007) demonstrated a reduction in endothelial nitric oxide synthase (eNOS) and NO release in LSV harvested by conventional surgical methods compared with those prepared atraumatically by the no-touch technique. An improvement in LSV graft surgery may

have a significant impact on graft usage, reduced graft pathology, improved graft patency leading to improved patient care and survival.

1.16 Established adjuvant medical therapy

Adjuvant pharmacological interventions have been introduced in an attempt to improve graft patency (Schachner 2006). Post-CABG, anti-platelet agents and lipid-lowering agents are the established strategies for reducing graft occlusion (Schachner 2006). A multicentre study showed that aspirin improved graft patency at one year post CABG surgery, and the major benefit occurred in LSV grafts placed to smaller vessels (Goldman et al. 1989). The same authors (Goldman et al. 1990) found in a prospective, randomised double-blind, placebo study that both IMA grafts and LSV grafts had excellent patency rates at one year. Aspirin did not alter this at one year, and there were no differences in patency between IMA grafts and LSV grafts when they were anastomosed to the left anterior descending artery. Consistent beneficial effects of other anti-thrombotic agents have yet to be established (Stein 1995). In 1997, The Post Coronary Artery Bypass Graft Trial Investigators demonstrated that aggressive reduction of LDL (low density lipoprotein) -cholesterol with lovastatin significantly reduces the rate of vein graft occlusion assessed by angiographic follow-up (The Post Coronary Artery Bypass Graft Trial Investigators 1997).

1.17 Emerging strategies: pharmacological agents

There is evidence that NO synthesis is impaired at sites of vascular injury and that the NO system is involved in graft failure (Tsui et al. 2002; Tsui et al. 2001; Jeremy et al. 1998). Particularly, early vasospasm and thrombotic occlusion may be due to reduced endothelial NO activity in vein grafts. NO donors, such as S-

nitrosoglutathione have been investigated in vein grafts and were found to cause vasodilation (Sogo et al. 2000) and inhibit platelet deposition. A recent study demonstrated that NO-releasing aspirins induced vasodilation of LSV grafts (Lorusso et al 2006).

Therapies that reduce neointimal hyperplasia are also being investigated as potential pharmacological approaches for preventing vein graft occlusion. Thapsigargin is a substance that increases cytosolic calcium^{2+} (Ca^{2+}) by its action as an irreversible inhibitor of Ca^{2+}-adenine triphosphatase. Intracellular calcium pools are important in regulating vascular smooth muscle migration, a prerequisite for neointimal hyperplasia. Pre-treatment with thapsigargin *ex vivo* reduces neointima formation in cultured LSV (George et al. 1997a) although the effects of exposing LSV to thapsigargin prior to implantation have not been studied *in vivo*. Oral and intramuscular administration of rapamycin, a macrolide antibiotic with anti-mitotic properties, has also been found to reduce neointimal formation after balloon-induced vascular injury in porcine models (Burke et al. 1999; Gallo R et al. 1999).

There is evidence from animal studies, that endothelin-A (ET_A) receptor antagonists reduce neointimal formation (Dashwood et al. 1998b), therefore these agents may possess therapeutic potential in patients undergoing bypass surgery. If so, the timing of antagonist administration may be an important consideration. Used as an adjunct to CABG ET_A antagonists may be effective at reducing early stages of graft occlusion (which normally occurs in the first month) by reducing subintimal VSMC proliferation caused by intraluminal distension and surgical trauma (Dashwood et al. 2004b).

Whilst these approaches have been shown to reduce neointima formation in experimental models, their clinical potential has yet to be demonstrated.

1.18 Emerging strategies: gene transfer

Vascular gene therapy is a new area of investigation and the use of gene transfer to reduce intimal hyperplasia and subsequent graft failure is receiving considerable attention (George et al. 2006; Baker et al. 2006; Conti et al. 2005). The use of gene transfer is attractive since it may potentially produce long-term therapeutic benefit without systemic side effects (George et al. 2006; Baker et al. 2006; Conti et al. 2005. Promising genes currently being evaluated include genes for NOS (nitric oxide synthase) and vascular endothelial growth factor (VEGF) (George et al. 2006; Baker et al. 2006; Conti et al. 2005; Miller et al. 2005; Dominiczak et al. 2005; McBride et al. 2005). NOS gene transfer may prevent vein graft failure by locally increasing NO synthesis in grafts.

Several animal studies using liposomal, adenoviral or retroviral delivery of eNOS to injured arteries have demonstrated a reduction in intimal hyperplasia (Chen et al. 1998; Janssens et al. 1998). iNOS (inducible NOS) gene transfer also inhibits intimal hyperplasia in rat and pig models (Shears et al. 1998), and since iNOS produces greater levels of NO compared to eNOS, the viral load required with iNOS gene transfer to achieve the same levels of NO should be reduced, which may be advantageous in clinical settings.

VEGF is a potent angiogenic factor, which promotes endothelial cell regrowth following vascular injury via a NO dependent mechanism. Early vascular gene transfer studies using VEGF have focused on its potential in therapeutic angiogenesis in both myocardial and lower limb ischemia (Chen et al. 1998; Janssens et al. 1998). In a rabbit model of arterial injury, VEGF reduced intimal thickening and macrophage influx into the vessel wall (Rutanen et al. 2005) but in another rabbit model of arterial

injury local transfer of the VEGF gene did not inhibit neointima formation (Dulak et al. 2005).

Direct intravascular delivery of target genes to vein grafts is possible when using endovascular techniques (Yla-Herttuala and Martin 2000). It is not clear if intravascular delivery of genes to the intima or extravascular targeting of the adventitia will be more effective. For example, an animal model using LSV grafts interposed in femoral artery has shown that *ex vivo* adventitial liposomal transfection of the eNOS gene is more effective at inducing NOS activity than transfection of the intimal surface (Kalra et al. 2000). Miller et al. highlighted the importance of vector targeting to achieve therapeutic gain and presented the first such study in cardiovascular gene therapy (Miller et al. 2005).

Whilst new developments are being made in this area, results are mainly limited to data from animal studies and there is little information from controlled trials, and long-term effects remain unknown.

1.19 Emerging strategies: external stenting

External stenting procedures have been introduced as a strategy to prevent late graft failure. Placement of a non-restrictive external stent around a porcine vein graft prevents early neointima formation and medial thickening (Mehta et al. 1998; Violaria et al. 1993). The mechanisms underlying this effect are not clear and are under investigation. Initially, it was believed that the external stent reduces the pulsatile stretch and shear forces to which the arterialised LSV graft is exposed. Circumferential stretch rather than pressure itself may provide a partial explanation for the beneficial effect derived from external stents (Okon et al. 2004; Mehta et al. 1998). Protection of veins against overstretching is perhaps an important reason for

long-term patency seen in LSV grafts where the LSV is harvested with the surrounding tissue (Souza et al. 2002). This would reduce the generation of endothelium- and tissue-derived vasoactive factors with prothrombotic and proliferative properties. Placement of an external stent also promotes angiogenesis in the adventitial region of the graft (Mehta et al. 1997) and this may improve oxygenation to otherwise hypoxic regions of the graft. Effects on the levels of the adventitial vasoactive factors, such as NO, PGI_2 and ET-1 may also be potential mechanisms (Dashwood et al. 1998b; Jeremy et al. 1998; Jeremy et al. 1997b). There is evidence that PGI_2 and cGMP are conserved at intimal and medial regions of stented LSV grafts and that adventitial formation of these compounds is increased (Jeremy et al. 1998). NOS content and activity within the adventitia of stented grafts is also higher than in unstented grafts (Jeremy et al. 1998).

1.20 The vascular endothelium

More than a trillion endothelial cells line the inner surface of the cardiovascular system, thereby representing a critical strategic interface between blood and tissue (Félétou and Vanhoutte 2006; Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Cockcroft 2005). The endothelium of an average-sized adult human contains 6×10^{13} cells, constitutes a mass of almost 1 kg and covers an area approximately 7000 m^2 (Simionescu 2007). It is the largest regulatory organ present in the human body (Simionescu 2007; Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Cockcroft 2005). The location and the enormous surface area enable the endothelium to interact very effectively with blood components and also with adjacent vascular smooth muscle cells (VSMCs) (Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Cockcroft 2005). Endothelial cells, strategically

located in the interface between the blood and vascular smooth muscle cells, release a number of vasoactive substances and play a major role in the control of vascular structure and function and platelet aggregation (Moncado and Hicks 2006; Yetik-Anacak and Catravas 2006; Félétou and Vanhoutte 2006; Vane et al. 1990). Under physiological conditions, the predominant effect of substances released by the endothelium is vasodilator, antiproliferative and antiaggregant, limiting the raise in blood pressure, regulating tissue blood flow and maintaining blood fluidit (Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Félétou and Vanhoutte 2006). Endothelial-derived substances with vasodilator and antiproliferative effects include NO, PGI_2 and endothelium-derived hyperpolarising factors (EDHF), and substances with vasoconstrictor and mitogenic effects include endothelin-1 and prostaglandin H_2 (PGH_2).

1.21 Endothelial dysfunction

The equilibrium between vasodilator and vasoconstrictors is shifted in CVD, where vasoconstrictor and proliferative effects predominate, leading to hypertension, atherosclerosis, platelet aggregation and ischemia (Simionescu 2006; Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Cockcroft 2005). Endothelial dysfunction has been defined as an imbalance between endothelial relaxing and contracting factors, but also between anti- and pro-coagulant factors, and between growth inhibiting and growth promoting factors (Feletou and Vanhoutte 2006; Endemann and Schiffrin 2004). With respect to the functional investigation the term endothelial dysfunction is generally used for an impaired maximal dilative response and/or an impaired sensitivity to endothelium dependent vasodilators such as calcium ionophore A23187, under conditions of a preserved response to endothelium-

independent dilators such as sodium nitroprusside (Feletou and Vanhoutte 2006; Endemann and Schiffrin 2004). Endothelial dysfunction is an early and independent predictor of poor prognosis in most forms of CVD (Endemann and Schiffrin 2004; Schachinger et al. 2000; Widlansky et al. 2003; Heitzer et al. 2001). Thus, alterations in endothelial function have been consistently found in hypertension, atherosclerosis, coronary heart disease, diabetes, sepsis, obesity and aging (Endemann and Schiffrin 2004; Kawashima and Yokoyama 2004; Widlansky et al. 2003; Heitzer et al. 2001; Schachinger et al. 2000).

1.22 Nitric oxide

NO is a freely diffusible molecule formed from L-arginine by the endothelial isoforms of NOS which requires Ca^{2+}/calmodulin, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin as cofactors (Moncada and Higgs 2006; Libby 2006). eNOS is activated upon increases in cytosolic Ca^{2+} which occurs in response to multiple stimuli such as shear stress, hormones, platelet derived substances and several drugs (Moncada and Higgs 2006). NO diffuses to the adjacent smooth muscle cells where it activates soluble guanylyl cyclase and induces vasodilatation (Simionescu 2007; Moncada and Higgs 2006; Warner et al. 1994). The vascular endothelium and NO play important roles in vascular homeostasis (Simionescu 2007; Moncada and Higgs 2006).

NO causes vasodilation, prevents thrombus formation, suppresses smooth muscle proliferation and decreases leucocyte attachment to the vascular wall (Moncada and Higgs 2006; Yetik-Anacak and Catravas 2006; Cockcroft 2005; Shimosawa et al. 2002). All these events have been implicated in the initiation and progression of atherosclerosis, therefore NO is considered an anti-atherosclerostic

agent (Simionescu 2007; Moncada and Higgs 2006; Libby et al. 2006; Stary et al. 1995).

NO plays a fundamental role in controlling blood pressure, tissue flow and blood fluidity (Moncada and Higgs 2006). Loss of endothelial NO bioavailability is therefore a maladaptive event and is one common manifestation of "endothelial dysfunction" (Lahera et al. 2007; Yetik-Anacak and Catravas 2006). The most characteristic pathophysiological feature of endothelial dysfunction is this diminished bioactivity of endothelium-derived NO resulting in impaired vascular homeostasis (Endemann and Schiffrin 2004; Kawashima and Yokoyama 2004; Widlansky et al. 2003; Heitzer et al. 2001; Schachinger et al. 2000) and is an early event in the atherosclerotic process that predicts future cardiovascular events (Papaharalambus and Griendling 2007).

Endothelial NO bioavailability can be assessed in animals and humans by the measurement of endothelial-dependent relaxation (Halcox and Deanfield 2004; Widlansky et al. 2003; Verma et al. 2003). The classical approach to analyse endothelial function, both in vitro and in vivo, is to stimulate endothelial NO release with agonists such as acetylcholine (Verma et al. 2003).

Decreased endothelial-dependent relaxation is an early, yet reversible feature of atherosclerosis that strongly predicts the risk of future cardiovascular events (Endemann and Schiffrin 2004; Widlansky et al. 2003; Gokce et al. 2002; Heitzer et al. 2001; Al Suwaidi et al. 2000; Schachinger et al. 2000). These findings have prompted speculation that endothelial dysfunction plays a causal role in the progression from stable to unstable atherosclerosis (Lahera 2007; Cockcroft 2005). The important role of NO in both the development and clinical expression of atherosclerosis is supported by many experimental and clinical studies which have

found decreased NO bioavailability in the setting of pre-atherothesclerotic factors such as diabetes mellitus (Guzik et al. 2002), hypertension (Rajagopalan et al. 1996a), hypercholesterolaemia (Warnholtz et al. 1999), and smoking (Zeiher et al. 1995).

The mechanisms involved in the decreased endothelial-derived NO responses have been extensively studied and include (Moncada and Higgs 2006; Félétou and Vanhoutte 2006; Endemann and Schiffrin 2004; Heitzer et al. 2001; Cai and Harrison 2000; Miwa et al. 1997):

(1) inhibition of the signal transduction from receptor activation to eNOS activation,

(2) changes in the activity and/or expression of eNOS,

(3) changes in the vascular levels of superoxide and, thus, superoxide driven NO inactivation and,

(4) changes in the sensitivity to the NO-cyclic guanosine monophosphate (cGMP) pathway in vascular smooth muscle cells.

Derivatives of oxygen [reactive oxygen species (ROS)] decrease NO bioactivity and interfere with NO signalling processes and thus represent a potential target for the treatment of atherosclerotic vascular disease (Valko et al. 2007; Yung et al. 2006). Reduced NO synthesis associated to endothelial dysfunction may be caused by reduced expression of eNOS, postransductional modification of the enzyme (e.g. phosphorylation or fatty acid modifications), interactions with calmodulin or caveolin, suboptimal concentrations of the substrate L-arginine or the cofactor tetrahydrobiopterin, or the presence of endogenous NOS inhibitors such as asymmetric dimethylarginine and N-monometylarginine (Endemann and Schiffrin 2004). The isoprenoid geranylgeranyl pyrophosphate, an intermediate factor in the cholesterol synthesis pathway, also inhibits the activity of eNOS. In addition, hyperhomocysteinemia, which is associated with increased risk of stroke, ischemic

heart disease, peripheral vascular disease and venous thrombosis, leads to reduced eNOS activity (Lentz et al. 2003).

1.23 Cyclooxygenase

Activation of endothelial cyclooxygenase leads to the transformation of arachidonic acid into PGH_2 which is metabolized by several enzymes into different products (Terlain et al. 1995). In endothelial cells, under physiological conditions, the major metabolite is PGI_2 (Moncada and Vane 1978). The vasodilator activity of PGI_2 is determined by the expression of specific receptors on vascular smooth muscle cells that are coupled to adenylyl cyclase (Narumiya et al. 1999). Cyclooxygenases exist in two isoforms, COX-1 (cyclooxygenase-1) and COX-2 (cyclooxygenase-2), which are constitutively expressed or inducible, respectively (Mitchell and Warner 1999; Fitzgerald 2002). Some of the cyclooxygenase by-products are endothelium-derived contracting factors (Matz et al. 2000; Taddei et al. 1997; Koga et al. 1988). The use of inhibitors of cyclooxygenase, like indomethacin, pointed out an increased participation of endothelial cyclooxygenase-derived vasoconstrictor metabolites in conductance and resistance arteries in animal models of CVD and in humans (Matz et al. 2000; Taddei et al. 1997; Koga et al. 1988). The cyclooxygenase metabolite(s) involved in the increased endothelium-dependent vasoconstriction are those who can activate the thromboxane A_2 (TXA_2) / endoperoxide receptor such as PGH_2, TXA_2 or prostaglandin $F_{2\alpha}$ (Matz et al. 2000; Heymes et al. 2000; Taddei et al. 1997; Kung and Luscher 1995; Koga et al. 1988). Hence, the use of an antagonist of the TXA_2/endoperoxide receptor improves endothelial dysfunction in certain circumstances (Matz et al. 2000; Heymes et al. 2000; Taddei et al. 1997; Kung and Luscher 1995; Koga et al. 1988). The increased participation of vasoconstrictor

factors derived from cyclooxygenase has been associated with an increased expression of COX-1 and COX-2 proteins in the vessel wall (Matz et al. 2000). Regarding the cyclooxygenase isoform responsible for the release of vasoconstrictor prostanoids, no consensus can yet be established. The change in the pattern of products released by cyclooxygenase might be related to an alteration in the lipid substrates. Indeed, an increase in lipid peroxidation was associated with an increased participation of cyclooxygenase-derived vasoconstrictors in endothelium-dependent relaxation in the rat. This may be a consequence of an increased oxidative stress on the cyclooxygenase-dependent response for example in the course of aging. Finally, it should be noted that NO can chemically interact with PGH_2 and decrease the availability of NO (Auch-Schwelk et al. 1992; Ito et al. 1991).

1.24 Endothelium-derived hyperpolarising factor

Another important endothelium-derived relaxing factor, especially in resistance arteries, is EDHF. The nature of EDHF, depending on the type of artery considered, has been proposed to be epoxyeicosatrienoic acid, potassium ions (K^+), anandamide and hydrogen peroxide (H_2O_2) (Busse et al. 2002; Dora and Garland 2001; Campbell and Harder 1999). The responses mediated by EDHF are resistant to NO synthase and cyclooxygenase inhibitors but are sensitive to the combination of the SKC_a and IKC_a channel inhibitors apamin plus charibdotoxin. EDHF-mediated responses are initiated by the activation of endothelial SKC_a and IKC_a channels which leads to the hyperpolarisation of endothelial cells (Busse et al. 2002). The consecutive hyperpolarisation of smooth muscle cells involves the spread of an electric current through myo-endothelial gap junctions (Busse et al. 2002). Endothelial dysfunction is associated in some cases with a reduced EDHF-component of the relaxation,

independently or in addition to NO. Possible reasons to explain the differences observed in the magnitude of the alteration in EDHF-mediated relaxation could be species or anatomical heterogeneity of vasomotor regulation conductance vs. resistance arteries (Marijic et al. 2001). Also changes might occur downstream of EDHF release such as a decreased expression of voltage- and Ca^{2+} -activated K^{+} channels (Marijic et al. 2001). Under physiological conditions, these channels serve as a hyperpolarising force that oppose contraction. Thus, their reduced expression could lead to a decreased vasodilatory capacity. However, this change is not uniform among different vascular beds and species.

1.25 Endothelin-1

An increased participation of the potent vasoconstrictor endothelin-1 (Jeremy et al. 2005) could also explain reduction of endothelium-dependent vasodilatation. Indeed, endothelial dysfunction is associated with an upregulation of mRNA expression of the precursor of endothelin-1, preproendothelin-1 and endothelin-1 protein (Brunner et al. 2006; Barton 2000). Numerous conditions characterized by an impaired availability of NO have been found to be associated with enhanced synthesis of endothelin-1 and vice-versa, thereby suggesting that these two factors have a reciprocal regulation (Rossi et al. 2001). Endothelin-1 has been described to exert a bidirectional effect by either enhancing NO production via endothelin-B receptors located in endothelial cells or blunting its effect via endothelin-A receptors prevalently located in the vascular smooth muscle cells (Brunner et al. 2006). Conversely, NO was found to inhibit endothelin-1 synthesis in different cell types (Brunner et al. 2006). Several factors affect in opposite direction the transcription of prepro-endothelin-1 and NOS genes, peroxisome proliferator-activated receptors

playing a key role in these regulatory mechanisms (Rossi et al. 2001). All these data suggest that endothelial dysfunction is also characterized by this dual effect on the NO and endothelin-1 pathways.

1.26 Free radicals, reactive oxygen species, oxidative stress and antioxidants

A free radical is any species that contains one or more unpaired electrons, that is, electrons singly occupying an atomic or molecular orbital (Halliwell and Whiteman 2004). ROS is a collective term that includes both oxygen radicals and certain nonradicals that are oxidizing agents and/or are easily converted into radicals (Halliwell and Whiteman 2004).

Oxidative stress is a condition in which cells are exposed to excess levels of oxygen or ROS, principal among which is $^{\bullet}O_2^-$ (Seifried 2007; Young and Woodside 2001; Betteridge 2000; Sies 1999; Kodja and Harrison 1999). Protective systems that remove $^{\bullet}O_2^-$ include superoxide dismutase (SOD; breaks down $^{\bullet}O_2^-$ to H_2O_2) and catalase [breaks down H_2O_2 to water (H_2O)] (Young and Woodside 2001; Betteridge 2000; Kodja and Harrison 1999). Thus, oxidative stress can be considered to be the up-regulation of $^{\bullet}O_2^-$ generating systems coupled with down-regulation of protective systems leading to potential damage (Griendling and FitzGerald 2003).

Halliwell and Gutteridge (1999) defined an antioxidant as "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate." The term "oxidizable substrate" includes every type of molecule found in vivo (Halliwell and Gutteridge 1999). This definition emphasizes the importance of the damage target studied and the source of reactive species used when antioxidant actions are examined.

1.27 Reactive oxygen species in the vasculature

Atherosclerosis and its risk factors such as diabetes, hypertension and hypercholesterolaemia are characterised by excess vascular production of ROS (Harrison et al. 2003; Cai and Harrison 2000). Over the last two decades considerable effort has been devoted to identify sources of ROS in vascular disease. All layers of the vascular wall have enzyme systems that produce ROS (Yung et al. 2006). In general, ROS are formed by one or two electron reduction of molecular oxygen, yielding either the $^{\bullet}O_2^-$ or H_2O_2, respectively (Halliwell and Gutteridge 1999). Among the important ROS sources in the vasculature are NAD(P)H oxidases, mitochondria, xanthine oxidase and eNOS (Landmesser et al. 2007; Yung et al. 2006).

1.28 NAD(P)H oxidases

Considerable investigation indicates that a major source of $^{\bullet}O_2^-$ is an NAD(P)H dependent oxidase (Griendling 2004; Lassegue and Clempus 2003). Originally, NAD(P)H oxidases were described and characterised in phagocytic immune cells (Babior et al. 2002). Neutrophils and other phagocytic cells produce ROS during phagocytosis through activation an a NAD(P)H oxidase, also called the respiratory burst oxidase, which catalyses the production of $^{\bullet}O_2^-$ (Babior et al. 2002). Then $^{\bullet}O_2^-$ can dismutate to form H_2O_2, that supports the production of hypochlorous acid (Babior et al. 2002). Together, these oxygen-derived species are largely responsible for the bactericidal activity of neutrophils (Babior et al. 2002).

The phagocytic NAD(P)H oxidase is a multicomponent enzyme, consisting of two main components, cystolic and membranous (Bedard and Krause 2007). The cystolic component is composed of $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the small G protein Rac (phox stands for phagocyte oxidase) (Moldovan et al. 2006). Together $gp91^{phox}$

and $p22^{phox}$ form an integrative membrane complex, termed cytochrome b_{558}, whose catalytic subunit is $gp91^{phox}$ (Bedard and Krause 2007). Upon activation of the enzyme, the GDP on the G-protein Rac is exchanged for GTP, and $gp47^{phox}$ is phosphorylated by protein kinase C (Moldovan et al. 2006; Sauzeau et al. 2000). These two events trigger a conformational change in cytosolic components, which facilitates the association of the cystolic components with cytochrome b_{558} in the membrane, forming a functional enzyme. NAD(P)H, the reduced substrate of the enzyme, binds to the catalytic subunit, $gp91^{phox}$, on the cytoplasmic side of the membrane and releases two electrons, which are passed subsequently to two successive molecules of oxygen on the opposite side of the membrane to produce molecules of $^{\bullet}O_2^-$ (Griendling et al. 2000) Once activated, phagocytes produce large quantities of $^{\bullet}O_2^-$ during the oxidative burst (Bedard and Krause 2007;).

Recently it has become evident that the expression of the major phox units is not restricted to phagocytic cells, but many vascular cell types exhibit oxidases similar to the neutrophil enzyme. Since the enzyme activity requires $gp91^{phox}$, $p22^{phox}$,$p47^{phox}$, $p67^{phox}$, and the G-protein Rac, considerable effort has been devoted to determining the vascular distribution of these NAD(P)H oxidase components (Moldovan et al. 2006; Griendling et al. 2000). The $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ components of the NAD(P)H oxidase were found to be ubiquitously distributed among the major cell types of the vascular wall (Bedard and Krause 2007; Cai et al. 2003; Griendling et al. 2000; Jones et al. 1996). In contrast, the catalytic subunit $gp91^{phox}$ was not ubiquitously distributed, suggesting that other isoforms of $gp91^{phox}$ might exist in these cells. Indeed, we now know that there is a family of $gp91^{phox}$ isoforms found in all cells and this family is termed the NOX family (NAD(P)H oxidase) (Bedard and Krause 2007; Sorescu et al. 2002). Thus far, the NOX family includes 5 members,

named NOX1-5 (Bedard and Krause 2007). They are thought to transfer electrons from the reduced and the small G protein Rac (phox stands for phagocyte oxidase) (Moldovan et al. 2006). They are thought to transfer electrons from the reduced NAD(P)H substrate to molecular oxygen in a way similar to the gp91phox (named as NOX2) (Bedard and Krause 2007; Lambeth 2004: Lambeth et al. 2000).

Expression of the NOX isoforms varies among different vascular cells. Endothelial cells have been shown to express all the essential subunits of the enzyme (Jones et al. 1996). These results were confirmed by cloning of endothelial complementary deoxyribonucleic acids almost identical to the corresponding phagocytic sequences and demonstration of the proteins by Western blotting and immunocytochemistry (Zeiher et al. 1995). Moreover, it has now been shown that endothelial cells express mostly the NOX4 isoform and a lesser amount of the NOX2 (Sorescu et al. 2002). The situation in vascular smooth muscle cells (VSMC) appears to be more complicated. In aortic VSMC, NOX2 levels are very low or undetectable. The absence of functional NOX2 was supported by experiments showing that basal and growth factor-induced oxidase activity of aortic vascular smooth muscle cells were unaffected by disruption of this gene (Barry-Lane et al. 2001; Souza et al. 2001). Recently it was discovered that VSMC derived from large vessels, like aortic VSMC, mostly express NOX4 and to a lesser extent NOX1 with negligible amounts of NOX2, while small resistance vessels predominatly express NOX2 (Touyz et al. 2002).

Since the products of the vascular NAD(P)H oxidase are potentially cytotoxic (Griendling 2004; Cai et al. 2003; Li and Shah 2004; Touyz and Schiffrin 2004; Brandes 2003), one would expect the expression of the vascular oxidases to be more tightly regulated. Moreover, if one considers ROS as mediators of vascular cell signaling, one must account for both the physiological and pathophysiological role(s)

of these species. In particular, any normal response can be rendered pathophysiological simply by removing normal control mechanism(s). The regulatory role of NAD(P)H oxidase has been investigated by exposing cultured cells to various stimuli known to increase ROS generation. Endothelial cells exposed to angiotensin II exhibited elevated expression of $p22^{phox}$,$p47^{phox}$, $p67^{phox}$ and NOX2 (Lassegue and Clempus 2003; Patterson et al. 1999). Conversely treatments that decrease ROS production (e.g. activators of peroxisone proliferators-activated receptor and statins) downregulate mRNA of these subunits (Du et al. 2001; Matthews et al. 1999). Exposure of VSMC to stimuli such as platelet-derived growth factor and tumour necrosis factor α also increase the expression of $p22^{phox}$,$p47^{phox}$, $p67^{phox}$, and NOX1, the latter representing an abundant NAD(P)H oxidase catalytic subunit found in smooth muscle cells (DeKeulenaer et al. 1998). In summary, there seems to be a relationship between ROS production in the vascular wall and expression of NAD(P)H oxidase subunits, suggesting that cells in the vascular wall express functional NAD(P)Hoxidase(s).

Apocynin is a methoxy-catechol (4-hydroxy-3-methoxyacetophenone), originally extracted from the root of the medicinal herb Picrorhiza kurroa, from the Himalayas, and has shown to possess anti-inflammatory properties (Ximenes et al. 2007; Engels et al. 1992). Apocynin has been used as an efficient inhibitor of NAD(P)H oxidase in many experimental models involving phagocytic and non-phagocytic cells (Ximenes et al. 2007; Zhang et al. 2005; Lafeber et al. 1999). The use of apocynin as an inhibitor of the activation of the NAD(P)H oxidase complex is based on the inhibition of the assembly process, as the migration of the p47phox component to the membrane is impeded in its presence (Ximenes et al. 2007; Barbieri et al. 2004). The vasodilator effect of apocynin is endothelium mediated as it can be

reversed by the NOS inhibitor N^G-nitro-L-arginine-methyl ester (Hamilton et al. 2002).

1.29 Regulation of NAD(P)H oxidase in atherosclerosis

NAD(P)H oxidase is relevant to human atherosclerosis (Griendling and FitzGerald 2003). For example, early atherosclerosic lesions exhibit upregulation of the $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$ subunits of the vascular NAD(P)H oxidase compare to non-atherosclerotic vessels (Barry-Lane et al. 2001; Szocs et al. 2002; Shi et al. 2001). Upregulation of NAD(P)H oxidase appears to be of importance as a loss of $p47^{phox}$ in hyperlipidaemic $ApoE^{-/-}$ mice results in a dramatic reduction in aortic lesion formation (Barry-Lane et al. 2001). This effect was mainly observed in early lesions of the descending aorta, rather than advanced lesions in the aortic sinus, suggesting a greater role for ROS-mediated events at that site. Furthermore, disruption of the $p47^{phox}$ gene results in decreased superoxide production in the vessel wall (Hsich et al. 2000). These results establish the significance of NAD(P)H oxidase and $p47^{phox}$ in particular, in the development of atherosclerotic lesions.

The expression of $gp91^{phox}$, however, appears elevated only in established lesions, not in early stages of atherosclerosis. The latter is characterized by the proliferation of VSMC, which express NOX1 rather that $gp91^{phox}$ (Szocs et al. 2002). The data do not suggest a role for $gp91^{phox}$ in developing atherosclerosis and are consistent with data in $gp91^{phox}$ knockout mice that have not demonstrated a significant effect of this gene product on murine models of atherosclerosis (Kirk et al. 2000).

The role of NOX4, the most abundant isoforms in vascular cells, is not clear at the moment, although it is upregulated in the neointima after balloon injury and in advanced atherosclerotic lesions (Szocs et al. 2002).

1.30 Regulation of NAD(P)H oxidase in diabetes mellitus

It is well established that diabetes mellitus is associated with accelerated rate of atherosclerosis and disproportionately increased risk of cardiovascular events. Several *in vitro* studies have demonstrated that high glucose levels and advanced glycalation end products increase NAD(P)H oxidase-induced superoxide production in the vascular wall (Weidig et al. 2004; Ülker et al. 2004). Furthermore, the expression of $p22^{phox}$ is also increased in diabetes (Christ et al. 2002). In blood vessels from genetically diabetic rats (a model of type 2 diabetes mellitus), streptozotocin-treated rats, and patients with type 2 diabetes mellitus, there is an increase in NAD(P)H oxidase activity (Kanic et al. 2002; Kim et al. 2002). This increased superoxide production in diabetic vessels correlates with reduced vascular NO bioactivity (Guzik et al. 2002). The specific reasons for increased NAD(P)H oxidase activity are not yet clear, although several putative mechanisms exist. For example, hyperglycaemia increases protein kinase C activity, a well known stimulus for NAD(P)H oxidase activation (Lee et al. 1989). In addition, the mRNAs of the major phagocytic NAD(P)H oxidase subunits $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ were upregulated in vessels from diabetic animals (Kanic et al. 2002; Kim et al. 2002; Hink et al. 2001). Therefore, these data indicate an association between vascular NAD(P)H oxidase activity and diabetes mellitus.

1.31 Xanthine oxidase

Xanthine oxidoreductase exists in two different forms in mammals (Parks and Granger 1986). *In vivo*, the most abundant form is xanthine dehydrogenase, whereas the xanthine oxidase form constitutes only a minor fraction (Frederiks and Bosch 1996). Xanthine dehydrogenase can be reversibly transformed into xanthine oxidase by sulfhydryl compounds or it can be irreversibly converted by proteolytic cleavage. Both forms of xanthine oxidoreductase catalyse the reaction of the purine metabolite xanthine to hypoxanthine and urate. However, only xanthine dehydrogenase employs nicotinamide adenine dinucleotide as an electron acceptor (Harrison 2002). In contrast xanthine oxidase transfers electrons to molecular oxygen, yielding significant amounts of superoxide and H_2O_2 that may contribute to vascular disease (Meneshian and Bulkley 2002).

The subcellular location of xanthine oxidase in vascular cells is still a matter of controversy. Initial reports found the enzyme in an exclusively cytosolic location in endothelial cells (Jarasch et al. 1981), whereas other publications showed xanthine oxidase can bind to the outer surface of endothelial cells (White et al. 1996). Xanthine oxidase can also be released into the circulating blood, thereby increasing the amount of xanthine oxidase in plasma, another compartment with xanthine oxidase activity. Since serum proteases can cleave xanthine oxidoreductase, it is noteworthy that most of the enzyme in the circulation is in the oxidase form. Information about the location of xanthine oxidase is of particular importance, as low substrate availability may limit xanthine oxidase activity in the tissues.

Xanthine oxidase has been identified as a major endothelial source of superoxide (Landmesser et al. 2007; Berry and Hare 2004; Spiekermann et al. 2003; White et al. 1996; Ohara et al. 1993) that is activated in experimental atherosclerosis

(White et al. 1996; Ohara et al. 1993). Initial studies that suggested a role for xanthine oxidase in vascular disease found that ROS produced by xanthine oxidase contribute to ischaemia/reperfusion associated vascular injury (Zweier et al. 1988; Korthuis et al. 1985). Activation of xanthine oxidase was also linked to early stages of atherosclerotic disease, as superoxide generation and endothelial dysfunction in hypercholesterolaemic rabbits is abolished by the xanthine oxidase inhibitor, oxypurinol (Ohara et al. 1993). Several subsequent studies have demonstrated increased xanthine oxidase activity in heart failure (Landmesser et al. 2002) and atherosclerosis (Spiekermann et al. 2003). Moreover, it has been demonstrated that endothelial xanthine oxidase activity and protein levels are substantially increased in patients with coronary disease or carotid stenosis, (Guzik et al. 2006; Spiekermann et al. 2003; Patetsios et al. 2001) and inversely related to endothelium dependent vasodilation (Spiekermann et al. 2003). In addition, serum levels of uric acid, the product of xanthine oxidase, have been suggested as a predictor of CVD mortality (Niskanen et al. 2004; Fang et al. 2000). The mechanisms, however, leading to increased endothelial xanthine oxidase activation in atherosclerosis remain to be determined (Landmesser et al. 2007).

1.32 Mitochondria

Mitochondrial respiration is an essential energy producing organelle in eukaryotic cells (Chandel and Budinger 2007; McFarland et al. 2007; Saks et al. 2006). Enzymes of the inner mitochondrial matrix space use the electron transfer chain to generate a proton gradient that is used by adenine triphosphate (ATP) synthetases to generate ATP (Chandel and Budinger 2007; McFarland et al. 2007; Saks et al. 2006). However, even under normal conditions, up to 1–2% of the

electrons may react with molecular oxygen to form superoxide and H_2O_2 (Chandel and Budinger 2007; McFarland et al. 2007; Saks et al. 2006). This "leakage" is exacerbated by very high mitochondrial membrane potentials (Chandel and Budinger 2007; McFarland et al. 2007; Saks et al. 2006). Eukaryotic cells have two major means to limit electron loss and mitochondrial ROS production Chandel and Budinger 2007). First, several uncoupling proteins at the mitochondrial membrane reduce mitochondrial ROS production (Casteilla et al. 2001). These proteins protect against excess electron loss while they decrease the inner mitochondrial membrane potential. Moreover, a mitochondrial form of SOD (manganese SOD) eliminates superoxide which has been produced in this cellular compartment (Casteilla et al. 2001).

Mitochondrial ROS production in vascular cells is particularly important in hypoxia (Chandel and Budinger 2007; Chandel et al. 2000) and diabetes mellitus (Nishikawa et al. 2000). During hypoxia, ubisemiquinone, a part of complex III, contributes to vascular ROS production and thereby causes increased endothelial cell permeability and interleukin-6 release (Pearlstein et al. 2002). In diabetes mellitus, mitochondrial-derived ROS have been implicated in other processes known to promote oxidative stress such as protein kinase-C activation, advanced glycation end product formation and increased aldose reductase activity (Pearlstein et al. 2002). Thus, mitochondrial ROS generation in diabetes mellitus appears to be an early event involved in oxidant injury.

1.33 eNOS Uncoupling

Pritchard et al. (1995) discovered that eNOS can switch from a NO producing to a superoxide producing enzyme and this phenomenon has been termed eNOS uncoupling. In this setting, electron flux through the enzyme results in reduction of

molecular oxygen rather than production of NO (Stuehr et al. 2004). Several mechanisms have been identified that are able to initiate NOS-derived ROS production (Stuehr et al. 2004). Decreased availability of the NOS substrate L-arginine has been shown to account for ROS production in the neuronal isoforms of the enzyme (Pou et al. 1992; Heinzel et al. 1992). As proper eNOS activity requires cofactors, their limited availability may also cause eNOS derived ROS production. Indeed, oxidation of the eNOS cofactor tetrahydrobiopterin is a well established mechanism that leads eNOS uncoupling (Laursen et al. 2001; Landmesser et al. 2003). Recent data implicates post-translational protein modification in eNOS uncoupling (Sampaio et al. 2007). For a catalytically active enzyme, eNOS must be a homodimer, linked by a zinc thiolate cluster. Oxidation of the zinc thiolate cluster results in zinc release and eNOS monomerization, which leads to uncoupling of the enzyme (Zou et al. 2002). With either tetrahydrobiopterin or zinc thiolate oxidation, a strong oxidant is necessary and current evidence implicates peroxynitrite in this process, an oxidant that is formed by the reaction of NO with superoxide (Laursen et al. 2001; Zou et al. 2002). Several animal studies have confirmed that eNOS-derived ROS occur *in vivo*. In particular, this ROS source has been shown to contribute to endothelial dysfunction observed during hypercholesterolaemia (Laursen et al. 2001), nitrate tolerance (Munzel et al.2000), diabetes mellitus (Guzik et al. 2002; Hink et al. 2001) and hypertension (Landmesser et al. 2003; Mollnau et al. 2002).

1.34 How do ROS affect vascular homeostasis?

Oxidative stress plays a major role in the development of endothelial dysfunction by direct and indirect mechanisms (Lahera et al. 2007; Cai and Harrison 2000). An important mechanism of endothelial dysfunction is inactivation of

endothelial-derived NO by form superoxide. NO reacts with form superoxide with a biomolecular rate that approaches the diffusion limit ($k = 1.9 \times 10^{10}\ M^{-1}s^{-1}$) to form peroxynitrite (Kissner et al. 1997). The reaction of superoxide + NO → peroxynitrite is axiomatic in CVD since this effectively diminishes NO levels which in turn is associated with angina, atherogenesis, hypertension, diabetic angiopathy and vein graft disease (Jeremy et al. 2004; Napoli et al. 2001; Napoli et al. 2001; Cai and Harrison 2000; Jeremy et al. 1997b). Unlike NO, that readily activates guanylyl cyclase and increases cyclic GMP formation in vascular smooth muscle, peroxynitrite is a much weaker agonist for guanylyl cyclase (Tarpey et al. 1995). Thus, any reaction of NO with superoxide will impair NO-induced relaxation. Peroxynitrite may also alter other enzyme systems that are important in vascular homeostasis (Zou et al. 1996). For example, peroxynitrite causes tyrosine nitration of the prostacyclin synthase leading to inactivation of this enzyme (Zou et al. 1996). Since endothelial PGI_2 release causes vascular relaxation, loss of PGI_2 will contribute to endothelial dysfunction under increased conditions of oxidative stress (Klumpp et al. 2005). There is evidence that prostacyclin synthase tyrosine nitration is particularly important in diabetes mellitus, as peroxynitrite formation is induced in endothelial cells by hyperglycaemia and this is responsible for inactivation of the enzyme (Zou et al. 2002). Another detrimental action of peroxynitrite relates to its potent oxidant properties. Peroxynitrite oxidizes tetrahydrobiopterin (Milstein et al. 1999) or the zinc thiolate cluster of eNOS (Zou et al. 2002), processes that may cause eNOS uncoupling, which further limits eNOS derived NO production (Landmesser et al. 2003; Laursen et al. 2001). Thus, an increased superoxide flux not only inactivates endothelial-derived NO, but also decreases production of PGI_2 via peroxynitrite

formation and both mechanisms contribute to endothelial dysfunction in the setting of increased oxidative stress.

In addition, increased ROS production may affect vascular homeostasis by indirect mechanisms (Clempus and Griendling 2006). A large body of evidence shows that increased oxidative stress may promote atherosclerotic disease through the oxidation of LDL. This phenomenon is germane for endothelial dysfunction as consequences of LDL oxidation include endothelial cell toxicity (Negre-Salvayre 1993), adhesion of leucocytes to the endothelium and migration of leucocytes into the subendothelial space (Navab et al. 1991). Components of oxidised LDL also inactivate NO (Chin et al. 1992) and impair receptor induced endothelial NO production (Kugiyama et al. 1990). Thus, ROS produced in the vasculature have a number of effects on both atherosclerosis and vascular homeostasis.

1.35 Antioxidants in the prevention of endothelial dysfunction and CVD

As discussed above, ROS may cause endothelial dysfunction by a number of mechanisms. Since antioxidants have been implicated in both atherosclerosis and endothelial dysfunction, it is not surprising that a number of antioxidant compounds have been tested as agents to restore endothelial function and treat atherosclerosis (Wassmann et al. 2006).

1.36 Lipid-soluble antioxidants: Vitamin E

Vitamin E is a potent lipid-soluble antioxidant compound that inhibits LDL oxidation, thus, it has drawn considerable attention as a potential treatment for vascular disease. In accordance with this concept, vitamin E has shown beneficial effects on endothelial function in various animal models. For example, vitamin E was

able to reverse endothelial dysfunction caused by hypercholesterolaemia (Keaney et al. 1993), diabetes mellitus (Cinar et al. 2001), and heart failure (Bauersachs et al. 2001). However it is noteworthy that these positive effects in experimental studies might depend on the chosen vitamin E concentration. In hypercholesterolaemic rabbits, high concentrations of vitamin E worsened endothelial function while a lower dose improved endothelial function (Keaney et al. 1994). This observation may explain why the results of vitamin E treatment in patients with endothelial dysfunction were mixed. Studies that have investigated the effect of vitamin E on endothelial function in patients with hypercholesterolaemia, diabetes mellitus, smoking, advanced age, previous myocardial infarction or vasospastic angina, have demonstrated a partial benefit (Fang et al. 2002; Skyrme-Jones et al. 2000; Heitzer et al. 1999; Motoyama et al. 1998; Green et al. 1998), whereas others showed no benefit for vitamin E in these settings (Gazis et al. 1999; Simons et al. 1999; Chowienczyk et al. 1998; Elliott et al. 1995; Gilligan et al. 1994). These mixed results on endothelial function parallel larger clinical studies that examined the effect of vitamin E on atherosclerotic progression and cardiovascular events. Two smaller clinical trials have shown a benefit for vitamin E supplementation in patients with prevalent CVD. The "Secondary prevention with antioxidants of CVD in endstage renal disease trial showed that vitamin E in haemodialysis patients with known vascular disease, decreased the risk for myocardial infarction (Boaz et al. 2000). The "Cambridge Heart Antioxidant Study" (CHAOS) showed similar results in patients with CAD (Stephens et al. 1996). In the CHAOS study, patients had a lower risk of myocardial infarction if they were treated for one year with vitamin E (Stephens et al. 1996). Despite these two positive results, the majority of larger prospective clinical trials failed to show any benefit for vitamin E intake in preventing CVD, in particular three large scale trials, the "Heart

Outcomes Prevention Evaluation" Study (Yusuf et al. 2000), the "Heart Protection Study" (Heart Protection Study Collaborative Group 2002) which used an antioxidant vitamin "cocktail" that also included vitamin E and the "Heart Outcomes Prevention Evaluation - The Ongoing Outcomes" (Lonn et al. 2005).

Thus, although experimental data from animal studies suggest that vitamin E improves endothelial function in different pathophysiological setting, vitamin E treatment in patients has yielded equivocal results. In accordance with these results on endothelial function, trials with vitamin E to reduce atherosclerotic complications have not been uniformly successful. There are several potential explanations for these discrepant results. First, vitamin E potently protects against lipid peroxidation in vitro, but the relevance of LDL oxidation for atherosclerotic progression in vivo is much less clear (Thomas and Stocker 2000). Second, vitamin E is not a strong scavenger of superoxide, and superoxide and its reaction product with NO, peroxynitrite accounts for many deleterious effects of ROS on endothelial function. Thus, vitamin E does not seen to be the antioxidant of choice to prevent endothelial dysfunction. Since endothelial dysfunction is a predictor of future cardiovascular events (Endemann and Schiffrin 2004; Widlansky et al. 2003; Gokce et al. 2002; Heitzer et al. 2001; Al Suwaidi et al. 2000; Schachinger et al. 2000), one might speculate that this failure of vitamin E to improve endothelial function might explain its inability to prevent clinical manifestations of atherosclerosis.

1.37 Lipid-soluble antioxidants: Probucol

Probucol is a synthetic antioxidant that effectively inhibits LDL oxidation (Kita et al. 1987) and limits atherosclerosis in animal models (Kita et al. 1987; Tawara et al. 1986; Wissler and Vesselinovitch 1983). Since decreased NO

bioavailability is a feature of atherosclerosis and oxidative stress, the effects of probucol on NO bioavailability have been studied. In animal models of hypercholesterolaemia (Simon et al. 1993; Keaney et al. 1995) and hyperglycaemia (Tesfamariam and Cohen 1992), probucol restored NO bioavailability. The mechanism for this effect is related to vascular ROS levels as probucol treatment reduces the ROS flux in hypercholesterolaemic rabbits (Keaney et al. 1995; Inoue et al. 1998). Since probucol is only a weak superoxide scavenger, these two studies suggest that probucol interferes with signals involved in stimulating the superoxide flux within the arterial wall (Keaney et al. 1995; Inoue et al. 1998), perhaps by reventing accumulation of lysophosphatidylcholine (Keaney et al. 1995). In patients, probucol seems to have similar protective effects regarding endothelial function when combined with lipid lowering (Anderson et al. 1995).

1.38 Lipid-soluble antioxidants: β-Carotene

β-Carotene is another lipid soluble antioxidant that has been tested with regard to endothelial function (Constans et al. 1998). Rabbits that were fed with a high cholesterol diet showed a marked degree of endothelial dysfunction, and concomitant treatment with β-Carotene was able to improve endothelial function, although the responsible mechanism remains obscure, as β-Carotene did not affect LDL resistance to ex vivo oxidation (Keaney et al. 1993). A similar study demonstrated a reduction in atherosclerosis in cholesterol-fed rabbits treated with β-Carotene (Shaish et al. 1995). However, human trials with β-Carotene have failed to demonstrate a benefit with regard to clinical CVD (The Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group 1994).

1.39 Water soluble antioxidants: Vitamin C

Lipid peroxidation is not the only important feature of oxidative stress in vascular disease. With regard to water-soluble antioxidant protection, vitamin C is the most effective water-soluble antioxidant in human blood plasma (Frei et al. 1989). and it is able to scavenge a variety of ROS including superoxide, hydroxyl radical ($^{\bullet}OH^{-}$) and H_2O_2 (Frei et al. 1989; Nishikimi 1975; Bodannes and Chan 1979). Its antioxidant activity has prompted its investigation as an agent to improve endothelial function. There is evidence from animal as well as human studies that vitamin C can improve endothelial function in a number of settings characterised by excess oxidative stress such as CAD (Levine et al. 1996; Gokce et al. 1999), diabetes mellitus (Ting et al. 1996), hypercholesterolaemia (Ting et al. 1997), hypertension (Taddei et al. 1998; Duffy et al. 2001), heart failure (Hornig et al. 1998), and in smokers (Heitzer et al. 1996; Fennessy et al. 2003).

The specific mechanisms responsible for the effect of vitamin C on endothelial function are not straightforward. Originally, it was speculated that superoxide scavenging was the principal means of improving NO bioactivity by preventing the formation of peroxynitrite. However the reaction of NO with superoxide proceeds at the diffusion limit ($k = 1.9 \times 10^{10}\ M^{-1}s^{-1}$), whereas the reaction of vitamin C with superoxide occurs at a much lower rate of $\sim 10^{5}\ M^{-1}s^{-1}$ (Sherman et al. 2000; Kissner et al. 1997). As a consequence, direct superoxide scavenging by vitamin C can only occur at concentrations of the antioxidant that are higher than 1mmol/L, and data to support this direct effect in humans is available (Sherman et al. 2000). However, several other factors of vitamin C have been discovered that may contribute to the improvement of endothelial dysfunction at lower concentrations of the compound. For example, vitamin C can enhance eNOS derived NO production by increasing and

stabilizing cellular tetrahydrobipterin levels (d'Uscio et al. 2003; Heller et al. 2001; Huang et al. 2000) and it might also decrease ROS production by inhibiting NAD(P)H oxidases (Ulker et al. 2003).

1.40 Water soluble antioxidants: Glutathione

Glutathione (GSH) is an important intracellular antioxidant and some studies have investigated its effects on endothelial cell NO bioactivity (Ford et al. 2006). GSH (L-γ-glutamyl-L-cysteinylglycine) is a naturally occuring ubiquitous tripeptide present in virtually all animal cells, whose nucleophilic and reducing properties play a central role in metabolic pathways, as well as in the antioxidant system of most aerobic cells (Sies 1999; Fahey and Sundquist 1991; Dolphin 1989; Meister et al. 1983). It is usually the most abundant intracellular small molecular weight thiol, present in the 1-10 millimolar range in mammalian cells (Sies 1999; Anderson 1997). The peptidic γ-linkage between glutamic acid and cysteine is thought to protect the tripeptide from degradation by aminopeptidases (Sies 1999). GSH is also less prone to oxidation than cysteine, making it an ideal compound for maintaining intracellular redox potential (Sies 1999). GSH exists either in reduced (thiol, GSH) or oxidized (disulfide, GSSG) form (Chakravarthi et al. 2006; Sies 1999). GSH is the predominant form, and GSSG content is usually less than 1% of GSH (Sies 1999). In the cell, almost 90% of GSH is in the cytosol, 10% in the mitochondria and a small percentage in the endoplasmic reticulum and in the nucleus (Sies 1999; Meister 1991). Mitochondria appear to have a distinct pool of GSH that is resistant to GSH depletion (Meister 1991). Mitochondria do not synthesize GSH themselves but import it through an, as yet unidentified, ATP-dependent mechanism (Fernandez-Checa et al. 1998). As mitochondria do not contain catalase, GSH-dependent reactions are thought

to be the main mechanism by which mitochondria dispose of H_2O_2 (Meister 1991), although the recent characterization of the thioredoxin-redox system implies that these could be involved as well (Pedrajas et al. 1999).

Studies in endothelial cells that manipulated intracellular GSH levels yielded some contradictory results regarding NO bioactivity. For example, a study addressing the association generation was impaired after intracellular depletion of GSH in bovine endothelial cells (Hecker et al. 1992). However, one of the agents used, 2,2'-dithiodipyridine, had no significant effect on intracellular GSH levels making an interpretation of the results problematic. Another study found no effect on endothelial NO production, after intracellular GSH levels were decreased by 90% with butathione sulfoximine (Mugge et al. 1991).

The effects of GSH manipulation on eNOS have been examined in detail, and there seems to be no association despite the finding that thiol modulating agents have GSH-independent effects on endothelial NO bioactivity (Huang et al. 2001). In contrast, a deficiency of glutathione peroxidase, an important cellular defence against oxidative stress by utilising GSH, elicits endothelial dysfunction in mice (Forgione et al. 2002). Consistent with these experimental data, GSH showed an improvement of endothelial function in human studies. Treatment with oxo-4-thiazolidine, an agent that selectively increases intracellular GSH levels, improved endothelial function in patients with CAD (Vita et al. 1998). Other clinical studies showed similar effects of GSH on endothelial function in patients with atherosclerotic risk factors (Prasad et al. 1999) or vasospastic angina (Kugiyama et al. 1998). A recent paper demonstrated that glutathione depletion in vivo enhances contraction and attenuates endothelium-dependent relaxation of isolated rat aorta (Ford et al. 2006). This functional effect was associated with elevations in aortic ROS release and elevated H_2O_2 levels and with a

slight compensatory upregulation of the components of the signaling pathways controlling NO bioavailability in the aorta (Ford et al. 2006).

The mechanisms leading to some of the observed beneficial effects of increasing intracellular GSH are not entirely understood. Although thiol groups are essential for eNOS activity (Patel et al. 1996; Hofmann and Schmidt 1995), some of the beneficial effects of GSH might be unique to this molecule, as other thiol containing compounds such as cysteine have much less impact on NO bioactivity (Komori et al. 1995). One potential explanation for these data is the fact that GSH is a cofactor for GSH peroxidase, whereas cysteine is not.

1.41 Angiotensin converting enzyme inhibitors and angiotensin receptor blockers

Activation of the renin angiotensin system is known to play a deleterious role in the initiation and progression of atherosclerosis (Mazzolai and Hayoz 2006). Initially designed as antihypertensive drugs, angiotensin converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARB) are today commonly used in the therapy of heart failure and CAD. A large body of evidence shows that these drugs strongly reduce cardiovascular morbidity and mortality in patients with hypertension (Dahlof et al. 2002), heart failure (The SOLVD Investigators 1991; The Acute Infarction Ramipril Efficacy (AIRE) Study Investigators 1993), and CAD (The Heart Outcomes Prevention Evaluation Study Investigators 2000). Experimental data from recent years suggests these effects may be due to reduced oxidative stress. ACE inhibitors block angiotensin II formation from its precursor angiotensin I. This results in stimulation of several angiotensin II receptor subtypes, the best characterised being the angiotensin II type I receptor (AT-1 receptor). Angiotensin receptor blockers

inhibit the effects of angiotensin through direct interaction with this receptor. The AT-1 receptor mediates classical angiotensin II effects such as vascular contraction, smooth muscle cell hypertrophy (Geisterfer et al. 1988), extracellular matrix synthesis (Kato et al. 1991), increased platelet aggregation (Feener et al. 1995), monocyte adhesion (Hernandez-Presa et al. 1998) and activation (Hahn et al. 1994), and release of inflammatory cytokines (Kranzhofer et al. 1999). All of these events are crucial steps in both atherosclerosis and the control of vascular homeostasis. More recent data suggests that many effects of angiotensin II are mediated by ROS. For example, rats rendered hypertensive with angiotensin II infusion show a marked elevation in vascular ROS production, whereas norepinephrine infusion in these rats causes a similar degree of hypertension, but no change in ROS generation (Rajagopalan et al. 1996a). Angiotensin II, but not norepinephrine, causes endothelial dysfunction in this model, a condition that is known to increase the chance for future cardiovascular events (Endemann and Schiffrin 2004; Widlansky et al. 2003; Gokce et al. 2002; Heitzer et al. 2001; Al Suwaidi et al. 2000; Schachinger et al. 2000). NAD(P)H oxidases are a particularly important ROS source in the vasculature, and it is now known that angiotensin II is a potent stimulus for the activation of NAD(P)H oxidases (Griendling et al. 2004; Griendling and FitzGerald 2003).

Given all the deleterious effects of angiotensin II discussed above, inhibition of renin angiotensin system would be expected to reduce vascular ROS generation and prevent many of its consequences. In fact, treatment of hypercholesterolaemic rabbits with angiotensin receptor blockers reduced vascular ROS generation, improved endothelial function, and slowed progression of atherosclerotic plaque formation (Warnholtz et al. 1999). In addition, an increase in SOD expression observed after treatment with ACE inhibitors as well as angiotensin receptor blockers

might also improve NO bioactivity (Hornig et al. 2001). ACE inhibitors exert their beneficial effects mostly by inhibiting angiotensin II action, but other mechanisms may contribute. For example, ACE inhibitors inhibit bradykinin breakdown via inhibition of kinase II (Linz 1992; Busse and Lamontagne 1991; Clozel 1991). Increased bradykinin levels cause vasorelaxation and NO release. As NO may scavenge some of the superoxide being produced, this effect may lower the ambient ROS levels in the vasculature. Since angiotensin receptor blockers do not affect kinase II activity, their effects are independent of bradykinin mediated NO release.

The well described beneficial actions of ACE inhibitors in experimental studies are consistent with clinical studies that compared the effects of ACE inhibitors to other antihypertensive drugs. Despite similar blood pressure lowering effects of β-blockers, diuretics or calcium channel anagonists, only ACE inhibitors improved vascular compliance and reduced intima/media ratio in patients (London et al. 1994; Thybo et al. 1995; Breithaupt-Grogler et al. 1996; Chen et al. 1995; Schiffrin and Deng 1995). Furthermore, the ACE inhibitor quinapril improved endothelial function in normotensive patients with CAD (Mancini et al. 1996) and the ACE inhibitor, enalaprilat, enhanced NO-mediated endothelial function in type I diabetic patients (O'Driscoll et ak. 1997a). Angiotensin receptor blockers showed similar effects in hypercholesterolaemic patients (Wassmann et al. 2002). As endothelial dysfunction predicts the prognosis for patients with CVD (Endemann and Schiffrin 2004; Widlansky et al. 2003; Heitzer et al. 2001; Schachinger et al. 2000), the improvement of this parameter by ACE inhibitors may account for their beneficial effects observed in secondary prevention trials (MacMahon et al. 2000).

A recent study suggested that angiotensin II induced endothelial xanthine oxidase activation and therefore angiotensin II blockade in patients with CAD could

reduce endothelium-bound xanthine oxidase activity which would contribute to improved endothelial function (Landmesser et al. 2007). A small clinical study attempted to link the beneficial effects of angiotensin II blockade to a decrease in oxidative stress (Wassmann et al. 2002). The angiotensin II receptor blocker candesartan improved endothelial function in hypercholesterolaemic individuals and concominantly decreased levels of oxidative stress, monitored by serum concentrations of 8-isoprostane (Wassmann et al. 2002). In contrast, the calcium antagonist felodipine did not affect endothelial dysfunction or 8-isoprostane levels (Wassmann et al. 2002).

Taken together, ROS generation by the renin angiotensin system plays a role in the development of endothelial dysfunction (Lonn 2002). Both ACE inhibitors and angiotensin receptor antagonists decrease NAD(P)H oxidase activity (Cai et al. 2003; Griendling et al. 2003), a major vascular ROS source in experimental studies. Therefore, the limitation of oxidative stress by these compounds may contribute to the improved prognosis for patients with CVD, although data from large clinical trials to support this link are not yet available.

1.42 Statins

Among the more important findings in the last century has been the link between circulating cholesterol and atherosclerosis. This link is also supported by data demonstrating that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors, or "statins", which inhibit the rate limiting step of cholesterol biosynthesis, have a profound effect on atherosclerosis (Lahera et al. 2007; Shepherd et al. 1995; The Scandinavian Simvastatin Survival Study (4S) Investigators 1994; Sacks et al. 1996; The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study

Group 1998; Downs et al. 1998). A post-CABG trial has shown that aggressive lowering of LDL is effective in reducing the progression of atherosclerosis in LSV grafts; low-dose warfarin, on the other hand, had no effect (The Post Coronary Artery Bypass Graft Trial Investigators 1997). Statins are known to have many properties that are independent of cholesterol lowering (Lahera et al. 2007; Duffy et al. 2001). This has prompted speculation that other mechanisms might contribute to the overall effect of statins (Lahera et al. 2007). Support for this hypothesis is derived from experimental studies revealing that statins exert antioxidant effects in the vascular wall by increasing eNOS activity (Kureishi et al. 2000; Laufs et al. 1998) and decreasing ROS production (Wassmann et al. 2001a; Wagner et al. 2000). In particular, statins are able to prevent lipid oxidation (Suzumura et al. 1999; Giroux et al. 1994; Aviram et al. 1998) and they decrease AT-1 receptor dependent ROS generation (Wassmann et al. 2001b). Statins inhibit NAD(P)H oxidase expression (Mason et al. 2004; Wassmann et al. 2001a), activity (Christ et al. 2002), and assembly by preventing isoprenylation of the small p21 rac protein (Moldovan et al. 2006; Wagner et al. 2000). In accordance with these data, statin withdrawal increase oxidative stress and elicits endothelial dysfunction in mice (Vecchione and Brandes 2002). Interestingly, these effects are absent in $gp91^{phox}$ knockout mice, suggesting that NAD(P)H oxidase dependent ROS production is the causal event (Vecchione and Brandes 2002).

All the aforementioned effects will increase NO bioavailability by preventing its reaction with superoxide, but there is evidence that statins also directly increase eNOS activity (Lahera et al. 2007). For example, statins increase levels of the eNOS cofactor tetrahydrobiopterin (Hattori et al. 2002) and also stabilize eNOS mRNA (Laufs and Liao 1998) that may lead to enhanced expression of eNOS protein. Statins

may also promote NO production by preventing LDL or oxidized LDL mediated downregulation eNOS protein expression (Lahera et al. 2007; Martinez-Gonzalez et al. 2001; Laufs et al. 1998; Hernandez-Perera et al. 1998). Another mechanism of eNOS activation by statins involves a decrease in caveolin abundance, the latter can bind to eNOS and directly inhibit its activity (Feron et al. 2001). Statins are also able to increase Akt phosphorylation and activity, which is an important pathway of eNOS activation via phosphorylation, that may play a role in NO dependent angiogenesis (Kureishi et al. 2000).

In summary, statins strongly prevent ROS generation, either by directly decreasing cholesterol levels or due to their well-established pleiotropic effects. Moreover, they also directly increase eNOS activity. As a consequence, statins have been shown to improve endothelial function (Fichtlscherer et al. 2006; Tsunekawa et al. 2001; Anderson et al. 1995; John et al. 2001; John et al. 1998; O'Driscoll et al. 1997b). Given the important prognostic significance of endothelial dysfunction, part of the beneficial statin effects may be explained by their antioxidant and NO elevating properties, although direct evidence for this hypothesis is not yet available.

1.43 Summary I: Surgical damage of the IMA and LSV and endothelial dysfunction

The long-term success of CABG depends on continued patency of the bypass conduits (Dashwood et al. 2007; Souza et al. 2006; Eagle et al. 2004). Many strategies have been used to improve the short- and long-term graft patency rates. Apart from established adjuvant medical therapy (Goldman S, et al. 2004.; Goldman et al. 1990; Goldman et al. 1989; FitzGibbon et al. 1987; Voors et al. 1996), new pharmacologic agents (Schachner 2006; Shukla et al. 2003), gene therapy (George et al. 2006; Baker

et al. 2006; Conti et al. 2005; Tanner et al. 2004) and the use of mechanical devices (Jeremy et al. 2007) are presently undergoing evaluation. Many of these strategies show promise in experimental bypass models, but few have successfully been transferred into clinical practice. An important consideration in the improvement of surgical techniques is to prevent damage to the endothelium during harvesting and implantation (Dashwood et al. 2007; Dashwood and Loesch 2007; Souza et al. 2006; Cunningham 2006; Deja et al. 2005; Cunningham 1996; Cunningham et al. 1992). Damage to the bypass conduits during conventional CABG is a major contribution to graft failure and improved patency may be achieved simply by reducing endothelial damage with the use of less traumatic harvesting techniques (Dashwood and Loesch 2007; Cunningham 2006; Cunningham 1996).

1.44 Summary II: CVD and endothelial dysfunction

Endothelial dysfunction occurs in conjunction with CVD (Feletou and Vanhoutte 2006). It is observed both in the coronary and peripheral vasculature (Takiuchi et al. 2004; Drexler 1997; Anderson et al. 1995). Moreover, risk factors for CVD have been almost universally associated with a degree of endothelial dysfunction in humans (Landmesser et al. 2004; Drexler 1999). Increased production of reactive oxygen species, in particular, superoxide and radicals derived from superoxide, has been associated with endothelial dysfunction in animal models of disease, and there is increasing evidence of a link between oxidative stress and endothelial dysfunction in humans (Touyz et al. 2004; Taniyama et al. 2003; Harrison et al. 2003; Hamilton et al. 2002; Alexander et al. 2000). It has been reported that endothelial dysfunction and increased oxidative stress may predict future cardiovascular events in patients with CVD (Heitzer et al. 2004; Al Suwaidi et al.

2000). However, concurrent and comparative data on endothelial function, direct measures of superoxide in human vessels, and circulating indicators of oxidative stress are not simultaneously available in patients with CVD and in control subjects with no documented CVD. Circulating indicators of oxidative stress have been investigated in patients with essential hypertension and in control subjects (Simic et al. 2006; Redon et al. 2003), but the relationship between these indicators and endothelial function has not been examined. In addition, although the degree of endothelial function has been consistently linked to the number of risk factors present in patients with CVD, the relative importance of individual risk factors in determining levels of oxidative stress and endothelial function remains uncertain (Guzik et al. 2000; Huraux et al. 1999).

1.45 Hypotheses

1 LSV harvested from patients undergoing CABG by traditional open vein harvesting (TOVH) have better calcium ionophore A23187 mediated relaxation than LSV harvested by minimally invasive vein harvesting (MIVH).

2 The width of pedicles harvested by monopolar electrocautery influences calcium ionophore A23187 mediated relaxation of IMA harvested from patients undergoing CABG.

3 Patients with severe CAD have endothelial dysfunction and increased levels of oxidative stress compared to healthy control subjects with no documented CVD.

4 Cardiovascular risk factors modify endothelial function.

1.46 Aims

1 To compare calcium ionophore A23187 mediated relaxation in LSV harvested from patients undergoing CABG by TOVH and MIVH.

2 To determine the effect of pedicle width on calcium ionophore A23187 mediated relaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG.

3.1 To study endothelium-dependent and -independent relaxation of LSV in a group of patients with severe CAD compared to controls.

3.2. To study superoxide production levels in blood vessels in a group of patients with severe CAD compared to controls.

3.3. To study circulating indicators of oxidative stress in a group of patients with severe CAD compared to controls.

4 To determine which cardiovascular risk factors influence calcium ionophore A23187 mediated relaxation of LSV in patients with severe CAD.

2

Methods

2.1 Summary

This chapter provides a detailed description of how and from whom blood and blood vessels were obtained, and secondly the laboratory and clinical techniques used in the studies described in this thesis. Chapters 3, 4, 5 and 6 detail the studies. Within each individual chapter, a description particular to that study will be given. In this present chapter are described the materials, apparatus, experimental technique and physiological basis behind each of the main techniques used in the studies.

Laboratory studies were undertaken within the British Heart Foundation Glasgow Cardiovascular Research Centre, Division of Cardiovascular and Medical Sciences, University of Glasgow.

2.2 Ethics Committee Approval

All the studies contained within this thesis were fully approved by the West Ethics Committee of the Western Infirmary [Reference 01/67(2)] and the East Ethics Committee of the Glasgow Royal Infirmary (Reference 01SC006), on behalf of the North Glasgow Hospitals University NHS Trust (ethical approvals letters appended in chapter 9). All subjects gave written informed consent, the forms being approved by the Ethics committee.

2.3 Materials used in Experimental Procedures

2.3.1 Chemicals

The following chemicals (all purchased from Sigma Company Limited, Poole, United Kingdom) were used for the studies in this thesis:

- 4',6-diamidino-2-phenylindole dihydrochloride (DAPI);
- Allopurinol;

- Apocynin;
- Calcium chloride ($CaCl_2$);
- Calcium ionophore A23187;
- Carbachol;
- Lucigenin;
- Dimethyl sulfoxide solution (DMSO);
- Ethylenediaminetetraacetic acid disodium salt solution (EDTA);
- Glucose;
- Hepes;
- Hydroethidine;
- Indomethacin;
- Magnesium sulphate ($MgSO_4$);
- Phenylephrine;
- Potassium chloride (KCl);
- Potassium dihydrogenphosphate (KH_2PO_4);
- Sodium bicarbonate ($NaHCO_3$);
- Sodium chloride (NaCl);
- Sodium nitroprusside.

The following chemical was purchased from Dow Corning (Midland, Michigan, United States of America) for the studies in this thesis:

- Sylgard 3140RTV (clear silicone).

2.3.2 Buffers

The following buffer solutions which were prepared fresh each day were used for the studies in this thesis:

- Krebs buffer solution (composition in mmol/L: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, glucose 11.1, EDTA 0.023 and indomethacin 0.02, dissolved in DMSO, which gives a pH of 7.4 ± 0.22);
- Krebs / Hepes buffer solution (composition in mmol/L: NaCl 118.4, KCl 4.7, $MgSO_4 \cdot H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.9, $CaCl_2$ 2.5, glucose 11.1, EDTA 0.023, indomethacin 0.02, dissolved in DMSO and Hepes 10, which gives a pH of 7.4 ± 0.22).

2.3.3 Instruments

The following instruments were used for the studies in this thesis:

- Mercian Basic Micro Instrument Set BMS-1 (Mercian Surgical Supply Co Ltd, Birmingham, United Kingdom);
- Swann–Morton surgical steel disposable scalpel (LIG Supplies Ltd., Cambridge, United Kingdom);
- Fixed position, through-the-lens surgical telescopes (3.5X; Designs for Vision, Ronkonkoma, New York, United States of America);
- Grass force-displacement transducer FT03 (Grass Instruments, Quincy, Massachusetts, United States of America);
- ETH-400 amplifiers (CB Sciences, Dover, New Hampshire, United States of America);
- MacLab 8/s data acquisition system (Apple Computer, Cupertino, California, United States of America);

- Power Macintosh 7200/90 (Apple Computer, Cupertino, California, United States of America);
- Chart recording software version 4/s (ADInstruments, Milford, Massachusetts, United States of America);
- Pye Unicorn SP8-500 UV/VIS spectrophotometer (Spectronic Unicam, Cambridge, Cambridgeshire, United Kingdom);
- Tricarb 2100TR liquid scintillation counter (Hewlett Packard, Meriden, Connecticut, United States of America);
- SPECTRAmax Plus 384 Microplate Reader (Molecular Devices Corporation; Sunnyvale, California, United States of America);
- MRC 1024 laser scanning confocal microscope (Bio-Rad Laboratories; Hercules, California, United States of America);
- Scion Image software version 4.0 (Scion Corporation Inc., Frederick, Maryland, United States of America);
- Hettich Rotofix 32A centrifuge (Hettich-Zentrifugen GmbH & Co., Tuttlingen, Germany);
- Caliper ruler (Vicarey, Davidson & Company Limited, Glasgow, United Kingdom);
- Theatre ruler (Vicarey, Davidson & Company Limited, Glasgow, United Kingdom);
- Scintillation vials (Packard Instruments Co., Downers Grove, Illinois, United States of America);
- Capped universal containers (Packard Instruments Co., Downers Grove, Illinois, United States of America);

- Microcentrifuge tubes (Packard Instruments Co., Downers Grove, Illinois, United States of America).

2.3.4 Assay kits

The following assay kits were used for the studies in this thesis:

- Bioxytech® AOP-490™ assay (Oxis Research, Portland, Oregon, United States of America).
- Bioxytech® GSH/GSSG-412™ assay (Oxis Research, Portland, Oregon, United States of America).

2.3.5 Laboratory system

The following laboratory system was used for the studies in this thesis:

- Bayer® ADVIA 1650 chemistry system (Bayer Diagnostics, Newbury, Berkshire, United Kingdom).

2.4 Human volunteers

The University of Glasgow is located adjacent to the Western Infirmary; part of the North Glasgow University Hospitals NHS Trust, which is a tertiary healthcare centre. Coronary artery bypass surgery is a common procedure performed at the Western Infirmary and Glasgow Royal Infirmary, with approximately 2000 operations being performed per annum. Usually, distal segments of left IMA and proximal segments of LSV are surplus to requirement and are consequently discarded. This situation therefore presents an opportunity to obtain human blood vessels from patients with atherosclerotic vascular disease. All study participants underwent

elective first time CABG for isolated CAD. These patients gave consent to join the study 5-10 days prior to the day of their operation.

Healthy volunteers were identified from those patients who were undergoing elective varicose vein surgery at Gartnavel General Hospital, which is part of the North Glasgow University Hospitals NHS Trust. Varicose vein surgery (saphenofemoral ligation, stripping of the long saphenous vein with multiple avulsions of varicosities) is a common procedure performed at the Gartnavel General Hospital, with approximately 200 operations being performed per annum. These patients gave consent to join the study 5-10 days prior to the day of their operation. Clinical details were recorded from case note examination.

A history of current cigarette smoking, hypertension (defined as either current anti-hypertensive treatment or a blood pressure > 140/90 mmHg), diabetes mellitus (insulin treated or non-insulin treated), hypercholesterolaemia (plasma cholesterol > 5.4 mmol/L), peripheral vascular disease, cerebrovascular disease, family history, body mass index and left ventricular function were considered as risk factors for CHD. Information on current medication was also documented at this point. Blood for measurement of circulating indicators of oxidative stress and LDL-cholesterol was taken from the CABG patients one week prior to surgery.

2.5 Cardiopulmonary bypass technique

All patients underwent elective primary coronary surgery for the treatment of multivessel coronary artery disease. Cardiopulmonary bypass with moderate hypothermia (28-30°C) and cold blood intermittent anterograde cardioplegia (4°C) was the technique of choice for myocardial protection.

2.6 Handling of human vessels

After sternotomy, the IMA was dissected as a pedicle with its venae commitantes from the thoracic wall by a no-touch technique, leaving the vessels in their anatomic environment surrounded by internal thoracic fascia, using electrocautery and surgical clips (Deja et al. 2005) (IMA pedicle ≡ internal thoracic fascia surrounding the IMA). The discarded distal end (1-2cm) of the IMA was transferred to the laboratory as described below. Relaxation of IMA which have been harvested in this no-touch manner has been studied in our laboratories for fourteen years (Hamilton et al. 2002a; Hamilton et al. 2002b; Berry et al. 2000; Hamilton et al. 2002; Hamilton et al. 1999; Hamilton et al. 1998; Hamilton et al. 1997; Thorin-Trescases et al. 1995; Thorin-Trescases et al. 1993) and light microscopic examination of haematoxylin and eosin stained paraffin sections of IMA harvested by this no-touch technique at our unit has shown the vessels to be muscular arteries with an average external diameter of 1.9 mm (Hamilton et al. 1999). Importantly, our unit has previously found that IMA harvested with this technique had intact endothelium, which appears to be closely applied to the luminal aspect of the internal elastic lamina, on light microscopic examination of haematoxylin and eosin stained paraffin sections (Hamilton 1997) (Figure 2.1a).

LSV was obtained from veins in excess on completion of the last proximal anastomosis by traditional open or minimally invasive techniques (Mahmood et al. 2006). Relaxation of LSV harvested by the traditional open technique has been studied at our laboratories for fourteen years (Hamilton et al. 2002; Hamilton et al. 1999; Berry et al. 2000; Hamilton et al. 1997; Thorin-Trescases et al. 1995; Thorin-Trescases et al. 1993), and histological examination has confirmed the endothelial integrity of the harvested LSV (Hamilton 1997) (Figure 2.1b).

IMA and LSV were collected from the operating theatre and placed in ice-cold Krebs / Hepes buffer-solution on ice and immediately taken to the laboratory (Berry et al. 2000). The time delay from operating theatre to the laboratory was 0.5 - 2 h.

2.6.1 Vessel preparation

The vessels were pinned down at either end in a clear glass dissecting dish, where the floor was covered with a layer of clear silicone (Sylgard 3140RTV; Dow Corning, Midland, Michigan, United States of America) and filled with cool Krebs / Hepes solution. The dish lay on a bed of ice. The loose connective tissue was carefully removed under a light source using fixed position, through-the-lens surgical telescopes (3.5X; Designs for Vision, Ronkonkoma, New York, United States of America) and fine surgical grade instruments (Mercian Basic Micro Instrument Set BMS-1; Mercian Surgical Supply Co Ltd, Birmingham, United Kingdom). At this point, the ring segments were transferred into cold Krebs / Hepes buffer solution in a capped universal container (Packard Instruments Co., Downers Grove, Illinois, United States of America). and maintained in atmospheric conditions and stored in a fridge at 4°C overnight for study the next day (Hamilton et al. 1997). Storage of rings of IMA and LSV in Krebs / Hepes buffer solution overnight under these conditions has been shown to have no effect on endothelial responses when compared to rings studied immediately (Hamilton et al. 1997). The following morning, the vessel was straightened out by slightly stretching and pinning at either end before cutting transverse rings of length precisely known, 2 mm for IMA, and 3 mm for LSV (Thorin-Trescases et al. 1995; Thorin-Trescases et al. 1993), with a Swann–Morton surgical steel disposable scalpel (LIG Supplies Ltd., Cambridge, United Kingdom). Subjectively, all vessel segments appeared normal and were free of abnormalities.

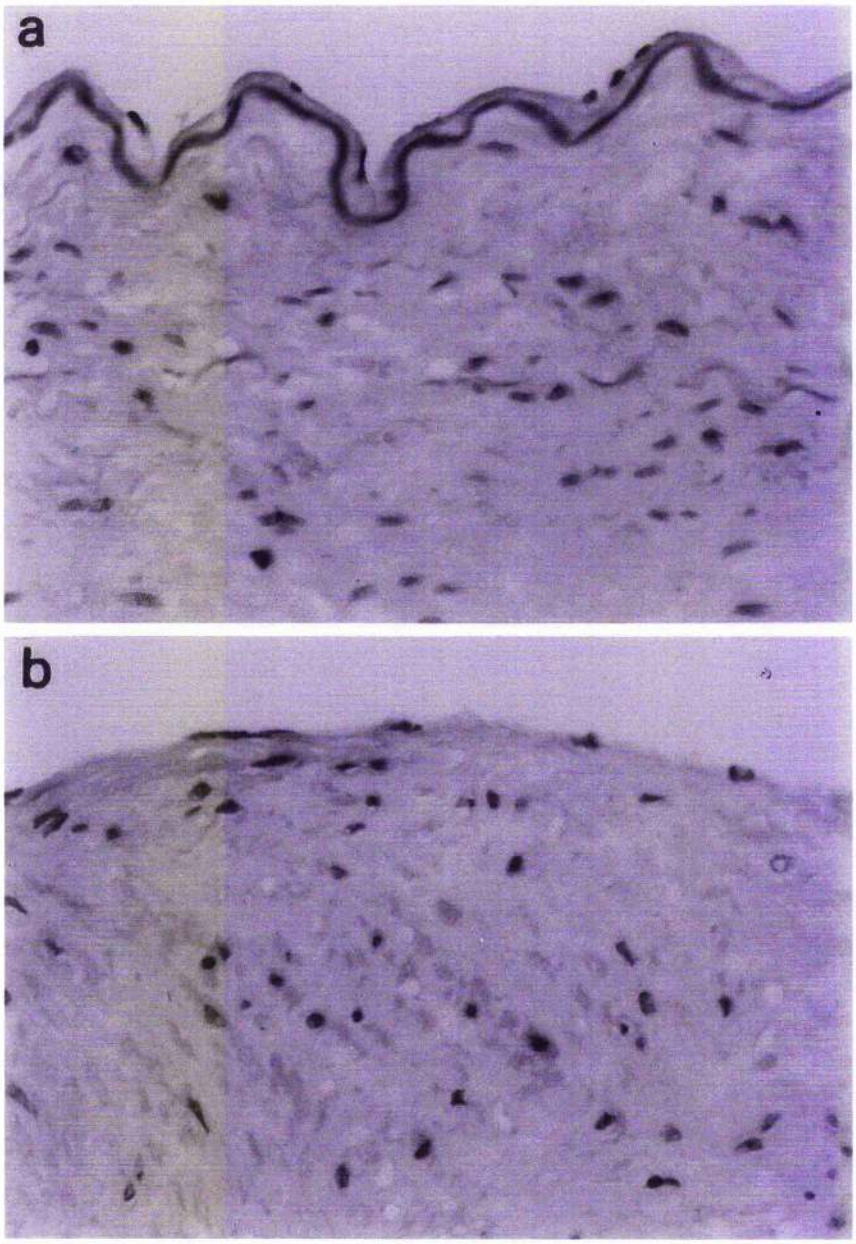

Figure 2.1 Photomicrographs of bypass conduits (a) haematoxylin and eosin stained section of 150× human IMA demonstrating a normal arterial wall with a continuous intact endothelial layer lining the vessel lumen; (b) haematoxylin and eosin stained section of a 150× human LSV demonstrating a normal vein wall with a continuous intact endothelial layer lining the vessel lumen. The luminal surfaces are at the top with an intact endothelial lining. (From Hamilton et al. 1997; reproduced with permission from Elsevier, Amsterdam, The Netherlands).

2.6.2 Principles of normalisation technique for setting resting tension

Vessels with circumferentially arranged smooth muscle must be distended to bring the actin–myosin fibres into alignment for optimal force development (Angus and Wright 2000). The preferred response when measuring blood vessel reactivity is to stretch the vessel radially to its optimal length for development of active force and measure changes in isometric tension (Angus and Wright 2000). The amount of passive force (stretch) applied to a segment of artery or vein in an organ bath should be relevant for the amount of muscle present and its geometry (Angus and Wright 2000). That is why techniques have been developed to determine the passive length–tension relationship for each vessel segment, cut to a precise length (Angus and Wright 2000). This normalisation procedure attempts to set the passive distension to correspond with that caused by transmural pressure experienced in vivo vessels (Angus and Wright 2000).

2.6.3 Isometric tension studies in organ baths

The 2mm long IMA and 3 mm long LSV segments (Thorin-Trescases et al. 1995; Thorin-Trescases et al. 1993) were suspended between two L-shaped 40-µm-diameter stainless steel wires. One wire was permanently attached to a tissue bath hook, and the other was directly coupled to lengths of wire to an isometric force-displacement transducer (FT03; Grass Instruments, Quincy, Massachusetts, United States of America). The output from force-displacement transducers was fed to analogue signal ETH-400 amplifiers (CB Sciences, Dover, New Hampshire, United States of America). The voltage signals were converted to digital signals and recorded with MacLab 8/s (Apple Computer, Cupertino, California, United States of America) and computer-based recorders on a Power Macintosh (7200/90, Apple Computer,

Cupertino, California, United States of America). Chart recording software (ADInstruments, AD Instruments, Milford, Massachusetts, United States of America) was used for data acquisition.

2.6.4 Krebs buffer-solution

Krebs buffer-solution is the standard salt solution used in our laboratories. The 10 mL glass-jacketed organ baths were filled with Kreb's solution, pre-heated and maintained at 37°C and saturated by vigorous gassing with 95% oxygen (O_2) and 5% carbon dioxide (CO_2).

The Krebs buffer-solution also contained the cyclo-oxygenase inhibitor indomethacin (0.02 mmol/L). Previous experiments performed at our laboratories studied relaxation to carbachol in the absence and then in the presence of indomethacin (0.02 mmol/L dissolved in DMSO) (Hamilton et al. 1999). Indomethacin did not attenuate relaxation in blood vessels used for CABG suggesting that prostanoids do not contribute to endothelium-dependent relaxation to carbachol (Hamilton et al. 1999). Indeed, there was a tendency for relaxation to improve in the presence of indomethacin and it is possible that carbachol caused contraction by the stimulated release of prostanoids such as PGH_2 (Hamilton et al. 1999). Based on the results from this study, indomethacin was added to the Krebs buffer-solution to inhibit prostanoid synthesis (Hamilton et al. 1999).

2.6.5 Drugs and solutions used

Stock solutions of phenylephrine (0.01 mol/L), carbachol (0.001 mol/L), potassium chloride (2 mol/L), allopurinol (0.001 mol/L) and sodium nitroprusside (0.01 mol/L) were prepared and frozen in aliquots at -20°C. Stock solutions of

apocynin (1 mol/L) and calcium ionophore A23187 (0.01 mol/L) were prepared daily in 0.5% dimethyl sulfoxide solution. Vehicle doses of dimethyl sulfoxide solution at the concentration present in the organ bath have been previously documented to evoke no vascular effects (Luscher et al. 1988). Serial dilutions were performed with distilled water or Krebs solution.

Calcium ionophore A23187-mediated vasorelaxations occur via an endothelium-dependent receptor-independent pathway and represent the final capacity of the endothelium to release NO after endothelial NO synthase (eNOS) stimulation (Perrault et al. 2005; Yang et al. 2004; Deckert et al. 2002). This is in contrast to acetylcholine which activates endothelial NO synthase through a receptor-dependent mechanism (Deckert et al. 2002). Therefore, calcium ionophore was used in preference to acetylcholine to elicit receptor-independent and endothelium-dependent vasorelaxations.

2.6.6 Organ bath protocol

The conditions and normalisation protocol in the next paragraph have been established in our laboratories (Hamilton et al. 2002a; Hamilton et al. 2002b; Berry et al. 2000; Hamilton et al. 1997; Thorin-Trescases et al. 1995; Thorin-Trescases et al. 1993).

Rings were allowed to equilibrate for 60 min with changes of buffer every 15 min and with several adjustments of length until baseline tension stabilized at 1 g. In previous studies at our laboratories, it was found that 1 g of resting tension are optimal for these types of experiments (Thorin-Trescases et al. 1995). This tension has been shown to be on the optimal part of the tension-response curve for both human IMA and LSV (Thorin-Trescases et al. 1995) and our laboratories have used

this protocol as a routine normalisation procedure for both human IMA and LSV (Hamilton et al. 2002a; Hamilton et al. 2002b; Berry et al. 2000; Hamilton et al. 1997). When tension was stable, the experiments were initiated.

Figure 2.2 A typical concentration response curve. This original recording shows the response of a ring of LSV precontracted with phenylephrine (pe in figure) and vasorelaxed with increasing concentrations of calcium ionophore A23187 (Cal in figure). The x axis measures time in minutes and the y axis measures tension in grams.

2.6.7 Organ bath initial protocol

The rings were contracted with phenylephrine (3 μmol/L). Then, vasorelaxation in response to carbachol (0.3 μmol/L) was studied. Next the organ bath was flushed thoroughly and the process of vasocontraction and vasodilation repeated. The organ bath was again flushed thoroughly. The rings were then exposed to a high (0.1 mol/L) concentration of KCl until maximal contractions were achieved. After a plateau contraction had been attained with KCl, rings were excluded if contractions were less than 0.5 g or showed no relaxation to carbachol (and were therefore considered to have no functionally intact endothelium). The vessels were then incubated for a further 30 minutes in Krebs solution prior to the commencement of the cumulative concentration response curves.

2.6.8 Main study protocol in order of drug addition

Rings were precontracted with phenylephrine (3 μmol/L) and vasorelaxation was evaluated by cumulative addition of apocynin, a NAD(P)H oxidase inhibitor, (0.01 to 0.3 mmol/L). Next the organ bath was flushed thoroughly and the vessels allowed to re-equilibrate.

Rings were precontracted with phenylephrine (3 μmol/L) and endothelium-dependent derived relaxation was evaluated by cumulative addition of calcium ionophore A23187 (0.01 to 10 μmol/L). Next the organ bath was flushed thoroughly and the vessels allowed to re-equilibrate.

Rings were precontracted with phenylephrine (3 μmol/L) and vasorelaxation was evaluated by cumulative addition of allopurinol, a xanthine oxidoreductase inhibitor, (0.01 to 0.3 mmol/L). Next the organ bath was flushed thoroughly and the vessels allowed to re-equilibrate.

Rings were precontracted with phenylephrine (3 μmol/L) and endothelium-independent derived relaxation was evaluated by cumulative addition of sodium nitroprusside (0.001 to 10 μmol/L).

For each dose of drug added, continuous tension measurements were made over 5 minutes. The final tension measurement of these continuous readings was used as the tension measurement (g) for that drug concentration. This allowed concentration response curves for each vessel ring of each subject to be constructed. Relaxation to apocynin, calcium ionophore A23187, allopurinol and sodium nitroprusside was expressed as a percentage of the contractile response to phenylephrine.

2.7 Lucigenin chemiluminescence and oxidative fluorescent microtopography

All the lucigenin chemiluminescence and oxidative fluorescent microtopography experiments were performed by Dr CA Hamilton (Senior Lecturer in Pharmacology at University of Glasgow) and Mrs EJ Jardine (Laboratory Technician at University of Glasgow).

Superoxide was measured using lucigenin chemiluminescence as described by Berry et al. (2000). 3 × 5 mm lengths of vein were blotted, weighed and placed in scintillation vials (Packard Instruments Co., Downers Grove, Illinois, United States of America) containing 2ml of Krebs buffer at pH 7.4 ± 0.22 and maintained in atmospheric conditions at room temperature. Next, either Krebs buffer, allopurinol (0.1 mmol/L) or apocynin (0.1 mmol/L) were added and after 60 minutes incubation at room temperature, lucigenin (0.25 mmol/L) was added and counts were recorded every 10 seconds for 3 minutes in a liquid scintillation counter (Tricarb 2100TR, Hewlett Packard; Hercules, California, United States of America) switched to the out-of-coincidence mode. Absolute counts were quantified with a xanthine / xanthine oxidase calibration curve for superoxide generation and reported as nmol min^{-1} mg of tissue. In all experiments, superoxide production was measured in paired samples with a matched control from the same vessel in every case.

Hydroethidine, a sodium borohydride-reduced form of ethidium bromide was used for oxidative fluorescent microtopography. Hydroethidine, a specific and sensitive indicator of superoxide (Rothe and Valet, 1990) is freely permeable to cells and, in the presence of superoxide, is oxidized to red-fluorescent ethidium bromide, where it is trapped by intercalation with deoxyribonucleic acid. (Carter et al. 1994). Ethidium bromide is excited at 488 nm with an emission spectrum of 610 nm. In cell-

free assays, addition of H_2O_2 to hydroethidine does not significantly increase ethidium bromide fluorescence (Miller et al. 1998; Carter et al. 1994).

For oxidative fluorescent microtopography with hydroethidine, Unfixed frozen LSV ring segments were cut into 30 µm thick sections and placed on a glass slide and incubated with the nuclear marker DAPI (20 µmol/L for 2 minutes) followed by hydroethidine (2 µmol/L for 20 minutes). The oxidative fluorescent dye hydroethidine was used to evaluate the in situ formation of superoxide as described by Mollnau et al. (2005), Hink et al. (2001) and Miller et al. (1998). Fluorescence was detected using a laser scanning confocal microscope (MRC 1024, Bio-Rad Laboratories; Hercules, California, United States of America) with an optical band pass filter of 470 ± 30 nm for DAPI (2-photon excitation 750 nm) and a band pass of 580 ± 16 nm for hydroethidine (single-photon excitation 488 nm). Vessels from CAD and control patients were analyzed in parallel under identical laser settings. Relative superoxide levels within vessel segments were quantitated by determining relative fluorescence in the red (hydroethidine) and blue (DAPI) channels over a defined area, using Scion Image version 4.0 software (Scion Corporation Inc., Frederick, Maryland, United States of America) (Chamseddine and Miller 2003).

2.8 Collection of human blood samples

Whole blood samples were collected 5-10 days prior to CABG surgery or varicose vein surgery from the chapter 5 and 6 patients. Patients were fasted overnight and rested in the supine position for 30 minutes prior to donating blood. Whole blood samples were obtained from vena cubitalis of the subjects; 5 to 10 ml was drawn into two tubes, one containing EDTA and the other containing lithium heparin and immediately chilled in ice.

For the GSH sample, 50 μL of whole blood was added to a microcentrifuge tube (Packard Instruments Co., Downers Grove, Illinois, United States of America). For the GSSG sample, 100 μL whole blood was mixed with 10 μL of M2VP (1-Methyl-2-vinyl-pyridium trifluoromethane sulfonate in hydrochloric acid (HCl), 2 mL) to scavenge GSH in a microcentrifuge tube. The GSH and GSSG samples were then stored frozen at –70°C. This freezing step lysed the red blood cells to maximize the concentration of GSSG in the sample.

The heparinised blood samples were centrifuged (Hettich Rotofix 32A; Hettich-Zentrifugen GmbH & Co., Tuttlingen, Germany) at 4700 revolutions per minute for 10 min at 4°C and the supernatant plasma was collected for measurement of total antioxidant capacity and cholesterol. All samples were frozen, and stored at -70°C until assay.

Within individuals, changes in circulating lipids and circulating indicators of oxidative stress occur in response to a variety of factors (Dikalov et al. 2007; Seifried et al. 2007; Choi et al. 2006; Kamuren et al. 2006; Kato et al. 2006; Bairaktari et al. 2005; Cole et al. 2005; Watson et al. 2005; Nedeljkovic et al. 2005; Redon et al. 2003; Anderson et al. 2001; Bae et al. 2001; Nourooz-Zedeh et al. 2001). Several steps within the methodology help reduce this variability:

- maintenance of a quiet environment at a fixed temperature;
- all subjects were studied in the morning after an overnight fast of at least 12 hours;
- subjects were asked to avoid caffeine, alcohol and smoking tobacco for at least 24 hours prior to the study and were rested supine for 30 minutes prior to taking blood.

2.9 Measurement of circulating indicators of oxidative stress in human blood

No single component of the antioxidant complex exists that fully reflects the protective capacity of whole blood. Thus, we assessed two different circulating indicators of antioxidant capacity: total antioxidant capacity (TAC) was assessed in plasma, and GSH and GSSG content in whole blood.

2.10 Total Antioxidant Capacity

Individual antioxidant moieties play specific roles in combating oxidative stress and measurement of single antioxidants may be beneficial to some studies (Kaur et al. 2006; Scheibmeir et al. 2005; Maritim et al. 2003; Polidori et al. 2001; Sies 1997). However, individual results may not be indicative of the overall effect of multiple antioxidants working in concert with one another (Kaur et al. 2006; Scheibmeir et al. 2005; Maritim et al. 2003; Polidori et al. 2001; Sies 1997). Therefore, an important index in oxidative stress studies may be measurement of the total antioxidant capacity of the biological system (Kaur et al. 2006; Polidori et al. 2001; Sies 1997). Copper (Cu) has advantages over iron for total antioxidant assays in that all classes of antioxidants, including thiols, are detected with little interference from reactive radicals and the copper reaction kinetics are faster than iron (Prior et al. 2005).

Total antioxidant capacity was evaluated by the established Bioxytech® AOP-490™ assay kit (OxisResearch™, Portland, Oregon, United States of America) (Huffman et al. 2006a; Huffman et al. 2006b; Vitalini et al. 2006; Cole et al. 2005; Prior et al. 2005; Roberts et al. 2005; Hermo et al. 2005; Chyu et al. 2004; Zeidler et al. 2004a; Zeidler et al. 2004b; Zeidler et al. 2003; Calo et al. 2003; Caruso et al. 2001; Visioli et al. 2001). The Bioxytech® AOP-490™ product insert stated that this

assay has a coefficient of variation of 2.2 % for intra-assay and 4.2 % for inter-assay (OxisResearch 2002). Oxis International Inc. correspondence states that they do not release formulation information about their kits (appended in appendix 9.2). Therefore concentrations of chemicals in their kits are not available.

2.10.1 Principles of the total antioxidant capacity assay

The Bioxytech® AOP-490™ assay is based upon the reduction of Cu^{2+} to Cu^{+} by the combined action of all antioxidants present in the plasma sample (OxisResearch 2002; Yamashita et al. 1998). A chromogenic reagent, bathocuproine (2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline), selectively forms a stable 2:1 complex with Cu^{1} which has a maximum absorbance at 490 nm (Huffman et al. 2006; Vitalini et al. 2006; Cole et al. 2005; Prior et al. 2005; Roberts et al. 2005; Hermo et al. 2005). The rate of this reaction is followed by the bathocuproine complexation of the Cu^{+} produced (Calo et al. 2003). The results are compared with a standard calibration curve obtained from samples of known concentration of uric acid (a water soluble antioxidant), used as a reductant (Bellomo et al. 1998). This dilution curve generated by uric acid standards is used to convert sample absorbance to uric acid equivalents. The results of the assay may be expressed either as these "mol/L uric acid equivalents" or as "mol/L copper reducing equivalents." The conversion between the two units is based upon the reduction of 2189 mmol/L Cu^{2+} to Cu^{+} by 1 mmol uric acid.

2.10.2 Total antioxidant capacity assay standard preparation

The uric acid standard was dissolved in 1.0 mL of 10 % sodium hydroxide solution and 2 mL of deionized water was added. The pH value was adjusted to 7.4 by

adding concentrated HCl dropwise. Next, deionized water was added to make a total volume of 100 ml to make a 2 mmol/L uric acid. The uric acid standard was diluted with deionized water to obtain 6 concentrations of uric acid: 0.063, 0.125, 0.25, 0.5, 1.0 and 2.0 mmol/L.

2.10.3 Total antioxidant capacity assay

Samples and standards were then diluted in 1/40 with R1 buffer, mixed, and 200 µL laced into wells, on a 96 well plate. 50 µL of R2 solution was added to each well, mixed, and incubated for three minutes at room temperature. 50 µL of stop solution was then added to each well and mixed. The plate was then read on a SPECTRAmax Plus 384 microplate reader (Molecular Devices Corporation; Sunnyvale, California, United States of America) set at 490nm. A R1 buffer blank and set of standards was run on each plate and a standard curve was constructed. The uric acid equivalent concentration of each sample was determined using the standard curve.

2.11 Measurement of reduced/oxidised glutathione (GSH/GSSG) ratio in whole blood

The GSH/GSSG molar ratio was determined by using the established Bioxytech® GSH/GSSG-412™ assay (Oxis International Inc., Portland, Oregon, USA) (Griguer et al. 2006; Kamuren et al. 2006; Liang et al. 2006; Berson et al. 2006; Sandstrom et al. 2006; Shao et al. 2006; Berson et al. 2006; Andziak et al. 2006; Liu et al. 2006; Liang et al. 2006; Waypa et al. 2006; Martirosyan et al. 2006; Franco et al. 2006; Choi et al. 2006; Nedeljkovic et al. 2005; Hsu et al. 2005; Bianchi et al. 2005; Watson et al. 2005; Hochscheid et al. 2005; van Rensburg et al. 2005; Sanz-

Rosa et al. 2005; Hino et al. 2005; Robin et al. 2005; de Moffarts et al. 2004; Felix et al. 2004; Bowler et al. 2003; Yamamoto et al. 2003; Belinky et al. 2003).

This was measured in whole blood by a colorimetric reaction using Ellman's reagent. Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid in sodium phosphate ($NaPO_4$) with EDTA, with ethanol, 40 mL) reacts with GSH to form a spectrophotometrically detectable product with an absorption maximum at 412 nm. GSSG was determined using glutathione reductase and NAD(P)H to reduce GSSG to GSH, followed by reaction with Ellman's reagent. The concentration of GSH can then be calculated from a calibration curve (0 to 3 μmol/L GSH). The Bioxytech® GSH/GSSG-412™ product insert stated that this assay has a coefficient of variation of 3.0 % for intra-assay and 3.9 % for inter-assay (OxisResearch 2001). As stated previously, Oxis International Inc. do not release formulation information about their assay kits, so the concentrations of chemicals in this kit was not available.

The accurate measurement of GSSG levels has proved very difficult due to the low amount of GSSG in tissues and because of the absence of effective methods to prevent oxidation of GSH to GSSG during sample preparation. To measure GSSG in tissues, Guntherberg and Rost (1966) first introduced N-ethylmaleimide (NEM) to eliminate the GSH. Although NEM can react with GSH to form a stable complex and prevent the participation of the reduced form in the enzymatic assay, NEM also inhibits GR. For this reason, Griffith (1980) first introduced 2-vinylpyridine (2-VP), which does not inhibit GR significantly, to derivatize GSH. However, the 2-VP reaction is relatively slow and the reagent has little solubility in an aqueous medium. 2-VP at 10 mmol/L, usually takes 60 minutes to remove 70 % of the GSH in the sample during which time oxidation of GSH may occur, resulting in significant overestimation of the GSSG concentration. The Bioxytech® GSH/GSSG-412™ assay

uses the thiol-scavenging reagent, M2VP at a level that rapidly scavenges GSH but does not interfere with the GR assay (OxisResearch 2001). Using M2VP, complete scavenging of GSH is accomplished in less than one minute (OxisResearch 2001).

2.11.1 GSH/GSSG ratio assay

When assessment was performed, the samples were thawed and immediately mixed and incubated at room temperature for 2-10 minutes. Both samples were prepared in 5% metaphosphoric acid (MPA) to remove proteins.

350 µL of 5% MPA was mixed with the GSH sample and 290 of 5% MPA wase mixed with the GSSG samples. A GSSG blank was prepared by mixing 50 µL MPA with to 700 µL GSSG buffer.

For measurement of GSH and GSSG, 50 µl MPA supernatant was mixed with 3 ml assay buffer (assay buffer: $NaPO_4$ with EDTA), and for GSSG estimation, 50 µl of the MPA supernatant with M2VP was mixed with 700 µl GSSG assay buffer. For both assays, the samples were mixed with 200 µl of chromagen, glutathione reductase and 3.8 µmol NAD(P)H, and 4.5 min later the absorbance (reduction of dithiobis-2-nitrobenzoic acid at 412 nm) was measured in a spectrophotometer (Pye Unicorn SP8-500 UV/VIS; Spectronic Unicam, Cambridge, Cambridgeshire, United Kingdom). The spectrophotometric detection was recorded at 412 nm every 10 s for 3 min. Reduced glutathione at concentrations from 0 to 3 µmol/L was used as the standard to calibrate the curves. The GSH/GSSG ratio was calculated as follows: GSH/GSSG = [GSH - (2 GSSG)]/GSSG.

2.12 Measurement of cholesterol in human blood

2.12.1 Background

The association between cholesterol and the risk of atherosclerotic disease is well established by many studies (Larosa et al. 2005; Nissen et al. 2005; Cannon et al. 2004; Castelli et al. 1986). LDL particles are the main carriers of the circulating cholesterol and they play a key role in cholesterol transfer and metabolism (Bairaktari et al. 2005). Increased superoxide production has been reported in hypercholesterolemia and it is well established that free radical damage contributes to the aetiology of CVD (Collin et al. 2007; Ohara et al. 1993). Free radicals can cause oxidation of LDL and oxidised LDL has been demonstrated to accelerate endothelial damage, macrophage recruitment and increased uptake of LDL-cholesterol by foam cells, which gradually develops into fatty streaks (Collin et al. 2006; Ohara et al. 1993). The executive summary of the National Cholesterol Education Program identifies LDL-cholesterol as the primary target of cholesterol-lowering therapy (National Cholesterol Education Program 2001) and recent clinical trials show that LDL-lowering therapy reduces the risk for CAD (Larosa et al. 2005; Nissen et al. 2005; Cannon et al. 2004).

2.12.2 Friedewald equation

Most clinical laboratories and clinical trials use the Friedewald equation for the calculation of LDL-cholesterol levels (Bairaktari et al. 2005; Sniderman 2003; Bairaktari et al. 2001; Yu et al. 1997; Friedewald et al. 1972). The Friedewald et al. (1972) article has more than 3,000 citations (Bruns 1998; Gotto and Cooper 1998).

The principle of the Friedewald equation is based on the assumptions that: (1) total cholesterol is distributed among the three major lipoprotein classes [high-density

lipoprotein (HDL), LDL and very low-density lipoprotein (VLDL)], and (2) VLDL carries most of the circulating triglycerides and therefore VLDL-cholesterol can be estimated reasonably well from measured total triglycerides (triglycerides / 2.2 for mmol/L units). LDL-cholesterol is then calculated as (Friedewald et al. 1972):

LDL-cholesterol = Total cholesterol – HDL-cholesterol – Triglycerides / 2.2

The advantages of the Friedewald calculation are that there is extensive experience of its use in many clinical studies (Bairaktari et al. 2005; Nauck et al. 2002; Bairaktari et al. 2001), it has well established clinical significance (Bairaktari et al. 2005) and is convenient and inexpensive when total cholesterol, triglycerides and HDL-cholesterol are measured (Bairaktari et al. 2005).

The well-known limitations of the calculation concern mainly three circumstances in which the equation cannot be used:

1. In the presence of chylomicrons, which contain proportionately less cholesterol relative to triglycerides than VLDL and therefore leads to overestimation of VLDL-cholesterol and underestimation of LDL-cholesterol. As postprandial specimens often contain traces of chylomicrons, a fasting specimen (8–12 hours) is required (Nauck et al. 2002);

2. In patients with Type III hyperlipoproteinimia or dyshetalipoproteinemia, characterized by the accumulation of remnant lipoproteins with an increased proportion of cholesterol relative to triglycerides, leading to underestimation of VLDL-cholesterol and overestimation of LDL-cholesterol (Warnick et al. 1990);

3. In increased triglyceride concentration when the proportion of cholesterol to triglycerides in VLDL decreases, giving rise to errors (Bairaktari et al. 2005; Nauck et al. 2002).

For this reason, calculation was recommended only for specimens with triglyceride levels of less than 4.5 mmol/L. However, its reliability is considerably decreased even at triglyceride concentrations of 2.25 to 4.5 mmol/L. Specimens with triglyceride concentrations below 2.25 mmol/L give the best agreement with β-quantification (86%–92% of the samples show deviations of less than 10%), whereas in specimens with triglyceride concentrations of 2.25 to 3.37 mmol/L and 3.37 to 4.5 mmol/L, only 75% and 61% of the samples show deviations of less than 10%, respectively (Warnick et al. 1990; McNamara et al. 1990). Many studies have focused on determining alternative triglyceride multipliers to improve the reliability of the Friedewald equation (Fujimoto 1988; Hata and Nakajima et al. 1986; Lippi et al. 1986; Wilson et al. 1985; Wilson et al. 1981; Castelli et al. 1977; Tyroler et al. 1975) but (1) the use of alternative triglyceride multipliers gives only marginally better LDL-cholesterol values and may not apply equally to all populations anyway, and (2) the Friedewald equation triglyceride multiplier seems to distribute the error about equally on both sides of zero and thus, it is concluded that there is little advantage in using a modified Friedewald equation (Rifai et al. 1992).

The application of the Friedewald formula was also investigated in patients with secondary hyperlipidemia, such as diabetes mellitus, renal disease, hepatic failure, and hormone replacement therapy (Bairaktari et al. 2001; Legault et al. 1999; Branchi A et al. 1998; Johnson et al. 1997; Rubies-Prat et al. 1997; Whiting et al. 1997; Nauck et al. 1996; Hirany et al. 1996; Matas et al. 1994; Winocour et al. 1989). As these conditions are characterised predominantly not only by increased triglyceride levels but also by lipoprotein alterations, the acceptable reliability of the calculation at triglyceride concentrations of 2.25 to 4.5 mmol/L needs special concern in patients with secondary hyperlipidemia. The most recent executive summary of the National

Cholesterol Education Program did pragmatically acknowledge continued use of the Friedewald calculation, despite these known concerns, until well validated methods become available (Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults 2001). Most clinical laboratories and clinical trials continue to use the Friedewald equation for the calculation of LDL-cholesterol levels (Bairaktari et al. 2005).

2.12.3 ADVIA 1650 chemistry analyser for measurement of cholesterol in plasma

Venous blood (10 ml) was collected into serum specimen tubes and centrifuged at 4700 revolutions per minute (Hettich Rotofix 32A; Hettich-Zentrifugen GmbH & Co., Tuttlingen, Germany) for 10 min. A 1.0 ml aliquot of the supernatant plasma was withdrawn, and total cholesterol, HDL-cholesterol, and triglyceride levels were measured by enzymatic methods on a Bayer® ADVIA 1650 chemistry analyser with standard reagents (Bayer Diagnostics, Newbury, Berkshire, United Kingdom).

The Bayer® ADVIA 1650 chemistry analyser (Bayer Diagnostics, Newbury, Berkshire, United Kingdom) is the routine method to measure total cholesterol, HDL-cholesterol and triglycerides at the North Glasgow University Hospitals NHS Trust. Dr Ian Louden (Biomedical Scientist, North Glasgow University Hospitals NHS Trust) and the Department of Biochemistry staff at Gartnavel General Hospital (North Glasgow University Hospitals NHS Trust) carried out all the cholesterol measurements.

The ADVIA 1650 method has been certified to measure total cholesterol, HDL cholesterol, LDL-cholesterol and triglyceride levels by the NHS Purchasing and Supply Agency Centre in the United Kingdom and total cholesterol, HDL cholesterol

and LDL-cholesterol levels by the Cholesterol Reference Method Laboratory Network on behalf of the Centers for Disease Control and Prevention in the United States of America (NHS Purchasing and Supply Agency 2005; Centers for Disease Control and Prevention 2007a; Centers for Disease Control and Prevention 2007b; Centers for Disease Control and Prevention 2007c).

For the total cholesterol method (Bayer Diagnostics, Newbury, Berkshire, United Kingdom), the cholesterol esters were hydrolysed by the enzyme cholesterol esterase to yield free cholesterol and fatty acids. The free cholesterol was then oxidised to cholestene-3-one and H_2O_2 by another enzyme, cholesterol oxidase. Peroxidase then catalysed the reaction of H_2O_2 with 4-aminoantipyrine and phenol to produce a coloured quinoneimine dye (Trinder's reaction) (Trinder 1969). The absorbance of the coloured dye was measured spectrophotometrically as an endpoint reaction at 505nm.

The direct HDL Cholesterol method (Bayer Diagnostics, Newbury, Berkshire, United Kingdom) measures HDL cholesterol without prior separation, based on procedures developed by Izawa et al. (1997). A synthetic polymer with a polyanion selectively blocked the non-HDL fractions by forming complexes with LDL, VLDL particles and chylomicrons which were then eliminated. The cholesterol in HDL particles was then released by detergent and measured by Trinder's reaction (Trinder 1969) in a similar manner to the total cholesterol method.

The triglycerides method (Bayer Diagnostics, Newbury, Berkshire, United Kingdom) was based on the Fossati three-step enzymatic reaction (Fossati and Prencipe 1982) with a Trinder endpoint (Trinder 1969). The triglycerides were converted to glycerol and free fatty acids by lipoprotein lipase. The glycerol was then converted to glycerol-3-phosphate by glycerol kinase in the presence of glycerol-3-

phosphate-oxidase to form H_2O_2. Next, a red quinoeimine dye was formed from H_2O_2, 4-aminophenazone and 4-chlorophenol under the catalytic influence of peroxidase. The absorbance of the complex was measured as an endpoint reaction at 505 nm.

Measurement of the total cholesterol, HDL cholesterol and triglyceride levels from the patients in chapters 5 and 6 permitted the estimation of LDL-cholesterol concentrations, based upon the Friedwald formula (Friedwald et al. 1972). For chapters 3 and 4, total cholesterol level was obtained from reviewing the medical record of each patient, for the most recent result, as described by Raggi et al. (2004).

2.13 Statistical analysis

All of the statistical analyses within this thesis were performed using Minitab version 13.1 software package (Minitab for Windows 13.1, Minitab Inc., State College, Pennsylvania, United States of America). Dr Niall Anderson (Senior Lecturer in Statistical Genetics, University of Edinburgh) performed the statistical analyses for chapters 3 and 4. Dr Ian Ford (Senior Lecturer in Biostatistics, University of Glasgow) and Dr John McClure (Lecturer in Biostatistics, University of Glasgow) performed the statistical analyses for chapters 5 and 6.

All of the comparisons of vasorelaxant responses in blood vessels, circulating indicators of oxidative stress and superoxide production between CAD and control patients were made in age- and sex-matched samples. Age matching was performed using subjects at most two years apart from each other (Goodman et al. 2000; Terán-Santos et al. 1999; Cassidy et al. 1999; Strom et al. 1998; Duggan et al. 1998; Jick et al. 1998). This was done to create similar age/sex profiles for the 2 groups, because CAD patients tended to be older and have a higher male/female ratio than patients undergoing varicose vein surgery.

Relaxation to apocynin, calcium ionophore A23187, allopurinol and sodium nitroprusside was expressed as a percentage of the contractile response to phenylephrine. For each group, concentration response curves were constructed with mean ± standard error of the mean (SEM) values, allowing easy visual comparisons. Where appropriate, unpaired Student t tests were used for the comparisons of relaxation at different concentrations to calcium ionophore A23187, sodium nitroprusside, apocynin, and allopurinol between the 2 groups. Statistical analyses of vascular superoxide concentrations were undertaken by use of the unpaired Student t test. Normal distribution of this data was examined by the Kolmogorov-Smirnov test and the Mann-Whitney U test was used for comparison of non-normally distributed data between the groups (TAC, GSH, GSSG, GSH:GSSG ratio).

To investigate which risk factors were independently related to vasorelaxation, multiple linear regression analysis was performed using data from the 264 CABG patients in chapter 4 and 188 CAD patients in chapter 6, and estimated effects, 95% confidence intervals, and p values were tabulated. Multiple correlation coefficients were calculated as an overall measure of the relationship between risk factors and vasorelaxation. Data not following normal distributions (TAC and GSH/GSSG ratio) were log transformed for this analysis. β coefficients and their confidence intervals for these 2 analyses were back transformed from the log scale, and they indicate the estimated proportional increase in the response associated with a unit increase in the predictor. Where appropriate, Pearson correlation coefficients are displayed.

Unless otherwise indicated, results are shown as mean ± standard error of the mean (mean ± SEM), including 95% CIs where appropriate, and $p < 0.05$ was considered significant.

Chapter 3

Comparison of calcium ionophore A23187 mediated relaxation of LSV harvested by traditional open vein harvesting and minimally invasive vein harvesting techniques

3.1 INTRODUCTION

The traditional open vein harvesting (TOVH) method of the LSV for CABG requires an extended longitudinal lower extremity incision from the ankle to the groin (Dashwood et al. 2007). This long incision is associated with significant intra-operative and postoperative complications in up to 25% of patients (infection, cellulitis, drainage, dehiscence, delayed healing, lymphangitis, sepsis, and limb amputation), creating an important clinical and economic burden (Aziz et al. 2006; Cheng et al. 2005; Cheng et al. 2005; Nickum 2005; Bonde et al. 2004; Bitondo et al. 2002; Bitondo et al. 2002).

As an alternative, minimally invasive vein harvesting (MIVH) has been reported to reduce leg wound complications and improve patient satisfaction (Aziz et al. 2006; Bonde et al. 2005; Aziz et al. 2005; Cheng et al. 2005; Cheng et al. 2005; Nickum 2005; Furnary 2005; Yun et al. 2005; Cook et al. 2004; Bitondo et al. 2002). Recent data show that MIVH is performed in the 65% of CABG cases in the United States of America (Burris et al. 2006). The rapid adoption and acceptance of this technology is the consequence of a dramatic reduction in leg wound complications (Bonde et al. 2004; Schurr et al. 2002). Several techniques are available for minimal invasive vein harvesting (MIVH), but all necessitate traction on the LSV to maximize surgical visibility and enable side branch ligation (Aziz et al. 2006; Aziz et al. 2005; Nickum 2005; Yun et al. 2005; Cook et al. 2004; Bitondo et al. 2002; Carpino et al. 2002; Kiaii et al. 2002; Griffith et al. 2000; Meyer et al. 2000). Excessive surgical manipulation of saphenous vein impairs endothelial cell function and reduces the bioavailability of NO (Lawrie et al. 1990; Angelini et al. 1989; Bush et al. 1986). Light microscopy and scanning and transmission electron microscopy have been used to compare the histological quality of the LSV conduit with MIVH versus TOVH and

found no significant difference between veins harvested using the two techniques (Bonde et al. 2004; Bonde et al. 2002; Lamm et al. 2002; Kiaii B, et al. 2002; Alrawi et al. 2001; Fabricius et al. 2000; Griffith et al. 2000; Meyer et al. 2000; Fabricius et al. 2000; Lancey et al. 2001). However, concerns remain, that minimally invasive manipulation of the LSV graft could traumatise the functional integrity of the harvested and prepared LSV and compromise immediate and long-term graft patency (Cook et al. 2004; Black et al. 2001).

3.1.1 Hypothesis

LSV harvested from patients undergoing CABG by TOVH have better calcium ionophore A23187 mediated relaxation than LSV harvested by MIVH.

3.1.2 Aim

To compare calcium ionophore A23187 mediated relaxation in LSV harvested by TOVH and MIVH techniques.

3.1.3 Experimental Approach

Endothelium-dependent relaxation to calcium ionophore A23187 and endothelium-independent relaxation to sodium nitroprusside will be determined in organ baths in rings of LSV from patients undergoing CABG by TOVH and MIVH techniques.

3.2 METHODS

3.2.1 Patient selection and characteristics

Thirty-one consecutive patients scheduled for elective first time CABG at the Department of Cardiothoracic Surgery, Glasgow Royal Infirmary were recruited into the study. Study participants had isolated CAD that required at least part of their revascularisation to be done using the LSV. Exclusion criteria included patients undergoing emergency CABG (MIVH equipment was electively ordered) and those with severe varicose veins. The consultant cardiac surgeons (Udim Nkere and Andrew Murday) would choose radial artery in preference to severe varicose veins as the mismatch in diameter between the coronary artery recipient (1-3 mm) and varicose veins (~1 cm) would give a poor anastomosis. The study was approved by the Local Research and Ethics Board at Glasgow Royal Infirmary. Before enrolment and randomisation each participant provided written informed consent.

3.2.2 Power calculations

Pilot data from 19 patients undergoing CABG with TOVH at the Glasgow Royal Infirmary (Age 59-72) demonstrated that after precontraction of LSV rings with phenylephrine (3 μmol/l), calcium ionophore A23187 (10 μmol/L) mediated vasorelaxation was 20.9% ± 3.1 % (mean ± standard deviation). Based on a study with 90% power at a level of significance of $p < 0.01$, it is estimated that to identify a difference of 1.5 standard deviations from the mean in MIVH patients by a 2-sample t-test 15 TOVH patients and 15 MIVH patients would be required. To detect the same difference in vasorelaxation with 80% power, 12 patients would be required and hence we would conservatively aim to recruit 15 TOVH patients and 15 MIVH patients.

Table 3.1 Power calculations. This table illustrates the power calculations used and from it 15 individuals per group would give us at least 90% power at a level of significance of $p < 0.01$ to detect differences as small as 1.5 standard deviations. We thus would seek to recruit between 15 and 20 TOVH and MIVH patients to ensure that the study is adequately powered.

Difference in multiples of standard deviations	Number of Patients Required Per Group for Power	Power
1.0	25	80%
1.0	29	85%
1.0	34	90%
1.5	12	80%
1.5	13	85%
1.5	15	90%
2.0	7	80%
2.0	8	85%
2.0	9	90%
2.5	5	80%
2.5	5	85%
2.5	6	90%

In the only study to show a difference in vasodilation in MIVH patients and TOVH patients, there was no net relaxation in LSV rings from MIVH group after exposure to 10 μmol/L acetylcholine (Cook et al. 2004). In contrast, rings from the TOVH group demonstrated relaxation of -0.32 ± 0.09 g after exposure to 10 μmol/L acetylcholine (Cook et al. 2004). Hence our aim to detect a difference of 1.5 standard deviations in vasodilation is an appropriate one.

3.2.3 Surgical techniques

Two consultant cardiac surgeons (Udim Nkere and Andrew Murday) carried out all of the operative procedures reported in this study.

3.2.3.1 Traditional open technique for LSV harvest

The incision was commenced just above the medial malleolus. The vein was identified and cleared of all adventitia and connective tissue using sharp and blunt dissection. The skin was incised over the whole length of the vein to the required length and careful dissection was used to isolate the vein in situ, with attention given to avoid unnecessary trauma to the vein or its tributaries. Side branches were ligated with 4/0 ethibond ligatures on the vein side and metal clips on the patient side. The leg wound was closed in layers and a full length pressure dressing was applied.

3.2.3.2 Minimal invasive technique for LSV harvest

A 2 cm longitudinal incision was made above the medial malleolus. The LSV was identified and cleared of all adventitial and connective tissue using sharp dissection. The distal end of vein was tied. The proximal part was also tied and about 6–8 cm of thread was left with that end. The vein was then divided between the two

tied ends. The end with a thread was passed through the ring in the Mayo vein stripper and forward pressure was applied to the vein stripper in the direction of usual vein anatomy while applying traction on the vein through the length of thread. Whenever resistance was felt on the vein stripper, a small incision (2 to 3 cm) at the area where resistance was felt was made. With a combination of sharp and blunt dissection any branch of the LSV at that site was isolated and ligated using a 4/0 ethibond ligature on the vein side and a metal clip on the patient side. The same process was repeated with multiple short incisions until the required length of vein was obtained. The skin incisions were closed with skin staples and full length pressure dressing applied.

In common to both techniques of harvest, the vein was inflated with heparinised blood to check for any unidentified side branches or tears in the vein. Any that were identified were either ligated with a 4-0 ethibond suture or closed with a 6-0 prolene suture.

3.2.4 Minimisation

Randomisation is the most effective and efficient method to remove selection bias between two groups of patients (Pocock 1983). The primary objective of randomisation is to ensure that all other factors that might influence the outcome will be equally represented in the two groups, leaving the treatment under test as the only dissimilarity (Pocock 1983).

Supposing one group has more elderly women with diabetes and symptoms of heart failure. It would then be impossible to attribute a better outcome in the other group to the beneficial effects of treatment since poor left ventricular function and age at outset are major determinants of survival in any longitudinal study of heart disease,

and women with diabetes, as a group, are likely to do worse. At this point the primary objective of randomisation, exclusion of confounding factors, has failed.

The way to avoid this is by minimisation. It was first described by Taves (1974) and shortly after by Pocock and Simon (1975) and Freedman and White (1976). With this method the group allocation does not rely solely on chance but is designed to reduce any difference in the distribution of known or suspected determinants of outcome, so that any effect can be attributed to the treatment under test (Treasure and MacRae). The trialists determine at the outset which factors they would like to see equally represented in the two groups (Treasure and MacRae).

At the point when it is decided that a patient is definitely to enter a trial, these factors are listed. The treatment allocation is then made, not purely by chance, but by determining in which group inclusion of the patient would minimise any differences in these factors. Thus, if group A has a higher average age and a disproportionate number of females, other things being equal, the next elderly female is likely to be allocated to group B. The allocation may rely on minimisation alone, or still involve chance but "with the dice loaded" in favour of the allocation which minimises the differences. The theoretical validity of the method of minimisation was shown by Smith (1984). Recent examples of the use of minimisation are found in Capell et al. (2007), Houslay et al. (2006), Struijs et al. (2006) and Robinson et al. (2006).

In this study the following patient characteristics were employed for minimization: age, gender, diabetes mellitus and peripheral vascular disease.

3.2.5 Vascular reactivity of veins

LSV segments that were surplus to CABG requirements were collected from the operating theatre and placed in ice-cold Krebs / HEPES buffer-solution on ice and

immediately taken to the laboratory. All rings were stored in Krebs-Hepes solution overnight at 4°C for study the next day. 3 mm long rings of vein were studied in organ chambers according to methods described in Chapter 2. Vessels were constricted with phenylephrine (3 μmol/l), a relative α_1-selective adrenoreceptor agonist which has β-adrenergic effects at high doses. Endothelium-dependent relaxation was evaluated by cumulative addition of calcium ionophore A23187 (0.1 - 10 μmol/L) and endothelial-independent relaxation was evaluated by cumulative addition of sodium nitroprusside (0.001 - 0.1 μmol/L). The LSV taken and prepared traditionally, served as a control group. No patient from MIVH group was converted to the traditional open technique. Relaxation was expressed as a % of the constriction to phenylephrine.

3.2.6 Statistical analyses

When the comparison was made between MIVH veins and TOVH veins, statistical analysis of maximal vasorelaxation was undertaken using unpaired Student's t test. A probability value of less than 0.05 was considered statistically significant. Results are shown as means ± SEM.

3.3 RESULTS

3.3.1 Patient characteristics

The study population consisted of 31 consecutive patients with CAD who underwent elective CABG at the Glasgow Royal Infirmary from September 2002 until March 2003. Two patients who died within the first 48 hours were excluded from further analysis on the instructions of the consultant cardiac surgeons. Clinical variables known to be important in vascular function were recorded. Data on age, sex, risk factors, and drug therapy are given in Table 3.2. Patient age ranged from 53 to 79

years. 100 % of patients had ≥ 1 risk factor for CAD, and 100 % of patients were on ≥ 1 types of anti-anginal therapy. The TOVH and MIVH groups were closely matched in demographics (Table 3.2). There were no statistical significant differences between the patient characteristics.

3.3.2 Intra-operative Findings

The total operative time taken to harvest vein was divided into 3 parts; stage 1: start of skin incision to removal of vein, stage 2: incision closure and stage 3: vein preparation. Adding the 3 gives the total vein operation time (Table 3.3).

Table 3.3 demonstrates that MIVH was significantly quicker than TOVH, whilst requiring no increase in the time required to prepare the vein. Time was saved both during removal of the vein and during skin closure. There was no statistically significant difference in length of vein harvested but as expected total wound length and number of incisions were significantly reduced in MIVH (Table 3.3).

3.3.3 Endothelial-dependent relaxations to calcium ionophore A23187

After contraction induced by phenylephrine (3 μmol/L), the NO-dependent endothelial vasodilator calcium ionophore A23187 (0.1 – 10 μmol/L) caused similar endothelial-dependent relaxations in TOVH (n = 14) and MIVH groups (n = 15) (calcium ionophore A23187, 10 μmol/L; 18.4 ± 1.9 % versus 17.6 ± 1.9 % respectively; p = 0.446, CI -5.4 % - 2.4 %) (Figure 3.1).

3.3.4 Endothelial-independent relaxations to sodium nitroprusside

After contraction induced by phenylephrine (3 μmol/L), the NO donor sodium nitroprusside (0.001 - 0.1μmol/L) caused similar endothelial-independent relaxations

in relaxations in TOVH (n = 14) and MIVH groups (n = 15) (sodium nitroprusside, 0.1 μmol/L; 37.6 ± 3.2 % versus 40.1 ± 3.4 % respectively; p = 0.647, CI -7.4 % - 1.4 %) (Figure 3.2).

Table 3.2 General characteristics of the TOVH and MIVH study groups.

Variables	TOVH	MIVH
Number	14	15
Age, y	62 ± 6	61 ± 4
Gender (M/F)	9 / 5	10 / 5
Body Mass Index	31±7	30±6
Systolic Blood Pressure, mmHg	136±4	137±4
Diastolic Blood Pressure, mmHg	86±4	85±4
Total cholesterol, mmol/L	5.7 ± 0.7	5.9 ± 1.2
Diabetes mellitus, %	14	13
Smokers % (active / stopped / none)	8 / 3 / 3	8 / 4 / 3
Peripheral vascular disease %	14	13
ACE inhibitor/ARB medication, %	29	20
New York Health Association class (I / II / III / IV)	0 / 2 / 4 / 8	0 / 2 / 6 / 7
Aspirin %	100	100
Beta Blockers %	57	67
Calcium Channel Antagonists %	57	60
Insulin (%)	7	7
Oral hypoglycaemics (%)	7	7
Nitrate %	57	60
Statin medication, %	50	47

Results are shown as mean ± SEM or %.

Table 3.3 Intra-operative data on the TOVH and MIVH study groups.

	TOVH **n = 14**	**MIVH** **n = 15**	**p**
Cardiopulmonary bypass time (min: mean ± SEM)	85 ± 2.9	78 ± 1.9	0.471
Theatre time (min: mean ± SEM)	200 ± 4.0	192 ± 3.0	0.722
Vein harvest site:			
Calf only	35	35	
Calf and thigh	3	4	
Total no of grafts (mean ± SEM)	3.3 ± 0.2	3.2 ± 0.1	0.342
No of vein grafts (mean ± SEM)	1.9 ± 0.2)	1.6 ± 0.1	0.302
Length of vein harvested (cm: mean ± SEM)	31 ±3.5	25 ± 2.3	0.018
Length of wound (cm: mean ± SEM)	35 ±3.5	16 ± 1.5	< 0.001
Number of incisions (mean ± SEM)	1.2 ±0.1	6.9 ± 0.7	< 0.001
Vein harvest time			
(min: median + interquartile range)			
Stage 1	16 ± 10	11 ± 14	0.01
Stage 2	9 ± 6	2 ± 1	<0.001
Stage 3	2 ± 2	2 ± 4	0.513
Total	26 ± 16	15 ± 22	0.002

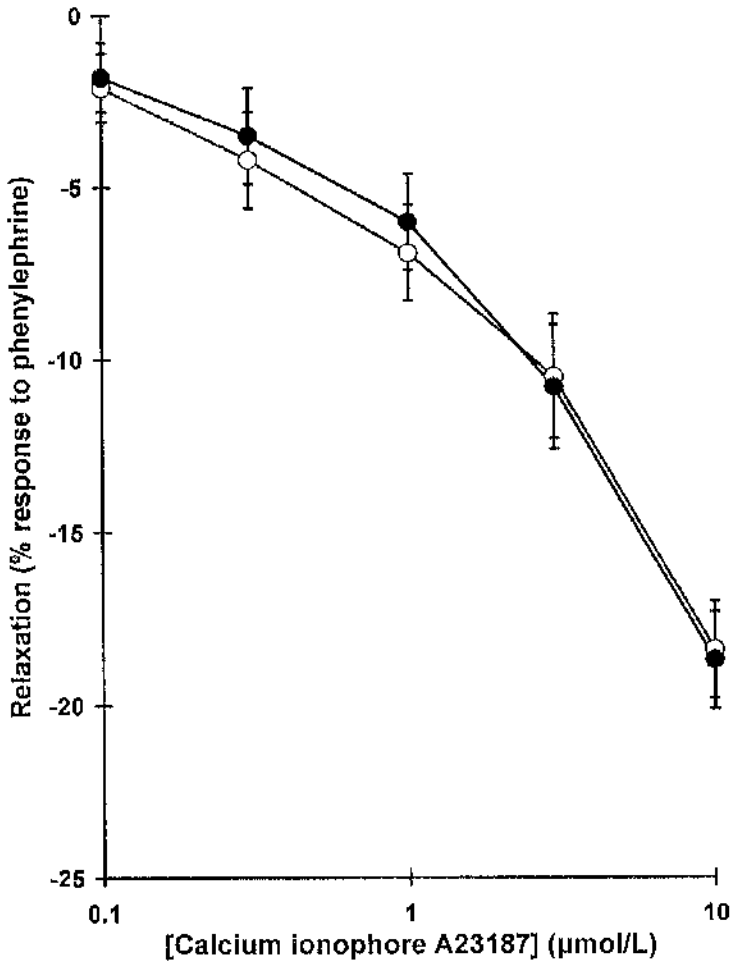

Figure 3.1 Vasorelaxation to calcium ionophore A23187 in rings of LSV from TOVH (n = 14) and MIVH (n = 15) patients. The x-axis is on a logarithmic scale. ○ TOVH ● MIVH

Results are shown as mean ± SEM.

There was no significant difference in relaxation mediated by calcium ionophore A23187 between TOVH and MIVH groups were noted using unpaired t-tests.

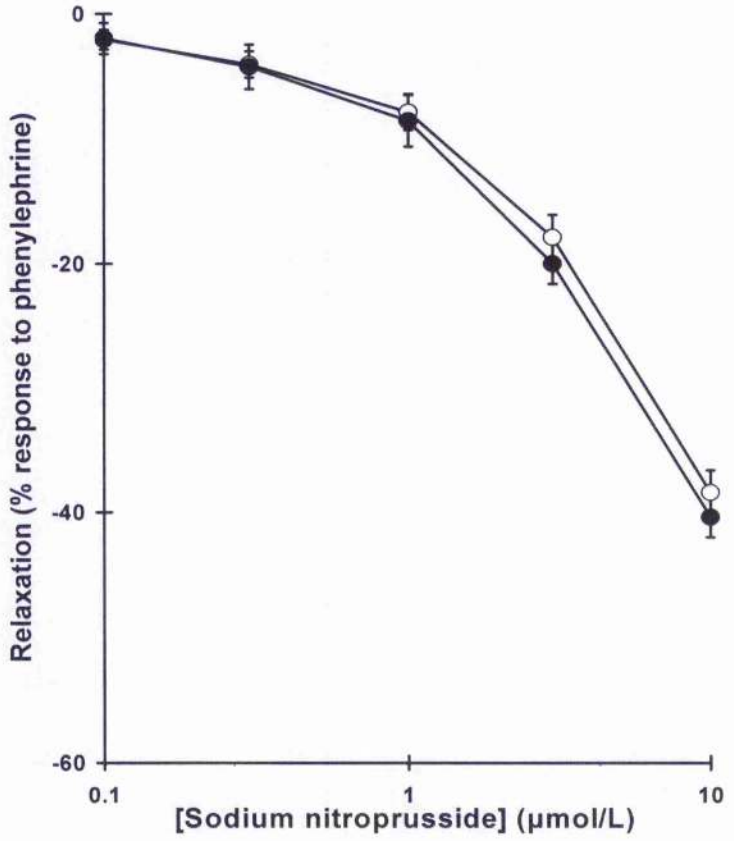

Figure 3.2 Vasorelaxation to sodium nitroprusside in rings of LSV from TOVH (n = 14) and MIVH (n = 15) patients. The x-axis is on a logarithmic scale.

○ TOVH ● MIVH

Results are shown as mean ± SEM.

There was no significant difference in relaxation mediated by sodium nitroprusside between TOVH and MIVH groups were noted using unpaired t-tests.

3.4 DISCUSSION

The study demonstrates similar endothelial-dependent and -independent relaxation of LSV harvested by minimally invasive and traditional open techniques and proposes that concerns over surgical trauma may be exaggerated. There was no difference in vascular function between these two groups of segments. Within the limitations of our experimental set-up, the results were surprising, as the mechanical force applied to veins prepared by MIVH seemed to be higher than in the TOVH group.

The data from this study are concordant with the observations of five studies who found no significant differences in the vascular reactivity of LSV harvested by traditional open or minimally invasive techniques (Black et al. 2001; Fabricius et al. 2000a; Rinia-Feenstra et al. 2000; O'Regan et al. 1997). In contrast, Cook et al. (2004) found significantly impaired acetylcholine-mediated endothelium-dependent relaxation in LSV segments isolated using a minimally invasive technique compared to traditional open technique.

A functional method was used to evaluate and compare the viability of the LSV used in CABG operations harvested by means of MIVH and TOVH because the presence of an intact wall, as obtained by means of morphologic studies, does not necessarily imply normal function of the tissue. As loss of endothelial function is a highly sensitive indicator of vascular injury, this study sought to evaluate responses in LSV rings to endothelial-dependent relaxation.

These results demonstrate no evidence that the manipulation of the LSV, consequent upon using the Mayo stripper, affected functional trauma to both endothelial-dependent and -independent function any more than the traditional open harvest method. In order to eliminate the effect of surgical expertise, the study used 2

very experienced surgeons who were equally comfortable with either technique to carry out all of the vein harvesting. However, there is no reason why the less invasive technique cannot be taught to surgical trainees and surgical assistants, or why they should not be able to carry it out to an equally high standard. These data indicate that MIVH veins should perform as well as TOVH veins.

In this randomised study it was found that compared to the traditional open method of harvesting LSV, a less invasive technique employing a Mayo vein stripper was quicker. There are other instruments available but the Mayo stripper has the advantage of being reusable and is therefore less expensive than disposable devices.

As the surgeons' practice is to use the left IMA and usually a radial artery as the conduits of choice, the number of vein grafts per patient in this study is an average of 1.7 per patient. This means that the majority of patients only have vein harvested from the calf rather than the thigh. The practice of these two surgeons is to commence vein harvesting at the ankle whereas others commence at the groin. This study cannot necessarily extrapolate its findings to those who choose such a practice.

This study has several noteworthy limitations that might influence interpretation of results. The number of patients and therefore the number of LSV samples is relatively small and statistical power is thereby limited. Although a type II error may be present with the small sample size in the present study, the similarity in the two groups suggests a larger sample size would, at most, only detect a small difference that, although interesting, would not likely be clinically relevant. Also, sampling only two rings from harvested veins may miss areas of injury at other sites within the vein. Finally, although significant functional differences were not seen, more studies evaluating clinical outcomes are necessary to further evaluate the importance of preservation of endothelial function and to determine whether MIVH

has an effect on patency rates of LSV grafts used in CABG surgery. For long-term quality control, post-operative assessment of graft patency by angiography may give further insight into possible differences between conventional and minimally invasive harvesting techniques.

Extrapolations from the results of this study to the in vivo situation must be made with caution, as additional factors may cause endothelial injury of vein grafts, aside from harvest trauma. These factors include high-pressure distention, nonphysiologic pH of the preservation solution, and transient loss of luminal flow with resultant ischemia (Dashwood and Loesch 2007; Dashwood et al. 2007; Souza et al. 2006; Dashwood et al. 2005; Perrault et al. 2005; Alamanni et al. 2002; Liu et al. 2001; Sessa et al. 2001; O'Regan et al. 1997; Soyombo et al. 1993; Dilley et al. 1988; Angelini et al. 1987b; Unni et al. 1974). The present study did not examine all of these variables as the purpose was limited to comparison of injury to endothelium-dependent function.

In this study, the cardiac surgeons at Glasgow Royal Infirmary inflated the LSV to check for any unidentified side branches or tears in the vein. Manual distension of the LSV changes its biomechanical properties (Zhao et al. 2006); it can structurally and functionally damage the endothelium (Chello et al. 2003; Johnson et al. 2001; Thatte and Khuri 2001; Mills and Everson 1995; Chester et al. 1998; Sayers et al. 1992; Dhein et al. 1991; Angelini et al. 1989; Angelini et al. 1987a; Angelini et al. 1987b; Angelini et al. 1987c; Svendsen et al. 1986; Hasse et al. 1981; Bonchek et al. 1980), smooth muscle (Cornelissen et al. 2004; O'Brien et al. 1998; Angelini et al. 1987b; Angelini et al. 1987c; George et al. 1997; Angelini et al. 1985), and interstitium (Kennedy et al. 1989a and Kennedy et al. 1989b). The patients in chapters 4, 5 and 6, had operations by a different set of cardiac surgeons at a different hospital,

the Western Infirmary Glasgow, from the patients in this chapter. Importantly, the LSV studied in chapters 5 and 6 was not distended prior to study. Therefore the patients in this chapter were not included in further studies.

Chapter 4

Effect of IMA pedicle width harvested by monopolar electrocautery on calcium ionophore A23187 mediated relaxation of IMA obtained from CABG patients

4.1 INTRODUCTION

During CABG, the IMA is traditionally harvested as a pedicle together with concomitant veins, lymphatics, sympathetic plexus and internal thoracic fascia (Deja et al. 2005). In contemporary cardiac surgical practice, however, certain surgeons practice the IMA skeletonisation technique. (Deja et al. 2005; Raja and Dreyfus 2005). Skeletonisation involves harvest of only the IMA without adventitia or any other surrounding tissue (Deja et al. 2005; Raja and Dreyfus 2005).

Skeletonisation of the IMA has been proposed as a solution to many of the problems associated with IMA harvesting (Deja et al. 2005; Raja and Dreyfus 2005; Athanasiou et al. 2004). The skeletonisation technique allows for longer availability of the graft (Deja et al. 1999), hence opportunity for more distal coronary artery anastomoses and easier sequential grafting (Raja and Dreyfus 2005). The increased early blood flow through the skeletonised IMA (Takami et al. 2002) has been implied as a factor to decrease the risk of hypoperfusion syndrome (Takami and Ina 2002). Also, chest wall injury is minimized, sternal blood supply is preserved, the sternal infection rate is decreased (De Paulis et al. 2005; Cohen et al. 1999), pain is reduced and there is better postoperative respiratory function (Boodhwani et al. 2006; Peterson et al. 2003; Matsumoto et al. 1997).

Despite these advantages, skeletonisation is time consuming, technically more demanding and may theoretically induce mechanical and physical damage to the vessel wall (Deja et al. 2005; Raja and Dreyfus 2005; Athanasiou et al. 2004; Ueda et al. 2003). This may result in detrimental effects on the functional integrity of the IMA (Deja et al. 2005; Raja and Dreyfus 2005; Athanasiou et al. 2004; Ueda et al. 2003).

Skeletonisation and narrow pedicles may injure IMA, precluding good results of surgery and Cunningham (1996) and Yacoub (1996) suggested that avoiding the

use of electrocautery near the IMA can prevent or minimize endothelial dysfunction. Although a wider pedicle may minimise the damage done to the endothelium, physiological studies to verify this are lacking (Matsumoto et al. 2006).

4.1.1 Hypothesis

The width of pedicles harvested by monopolar electrocautery influences calcium ionophore A23187 mediated relaxation of IMA harvested from patients undergoing CABG.

4.1.2 Aim

To determine the effect of pedicle width on calcium ionophore A23187 mediated relaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG.

4.1.3 Experimental approach

1) IMA pedicle width and IMA diameter will be determined post harvest.
2) Relaxation to calcium ionophore A23187 (endothelium-dependent vasodilatator) and sodium nitroprusside (endothelium-independent vasodilatator) will be determined in IMA from patients undergoing CABG.
3) Cardiovascular risk factors (age, sex, body mass index, current active smoking, diabetes mellitus status, diastolic blood pressure, total cholesterol) and current drug therapy will be recorded.

4.2 METHODS

4.2.1 Study population

Between April 2001 and January 2004, 264 patients undergoing elective CABG were prospectively recruited from the Western Infirmary Glasgow. At admission a careful clinical history was taken. Total cholesterol level was obtained from reviewing the medical record of each patient for the most recent result, as described by Raggi et al. (2004). The general characteristics of the study population are summarised in Table 4.1.

4.2.2 Surgical technique

At the Western Infirmary Glasgow, the IMA is harvested as a pedicle with its venae commitantes from the thoracic wall by a no-touch technique, leaving the vessels in their anatomic environment surrounded by internal thoracic fascia. The internal thoracic fascia is incised with conventional monopolar high frequency electrocautery along both sides of the IMA approximately 0.5 -1.0 cm away from the concomitant veins. The flap of fascia, muscle, and fat tissue containing the IMA with concomitant veins is dissected with electrocautery, working from its distal to proximal end. The major IMA branches are ligated with metal clips and the minor branches are electrocoagulated with electrocautery.

The discarded distal end (1 – 2 cm) of the IMA was carefully removed and placed in ice-cold Krebs/HEPES buffer-solution on ice, and immediately taken to the laboratory. Since the use of the vessels was at the discretion of the cardiothoracic surgeon, it was not possible to select patients in advance. There were, therefore, no specific exclusion criteria.

4.2.3 Laboratory methods

The width of the pedicle was measured perpendicular to the course of the IMA using a theatre ruler (Vicarey, Davidson & Company Limited, Glasgow, United Kingdom). The loose connective tissue was carefully removed under a light source using fixed position, through-the-lens surgical telescopes (3.5×; Designs for Vision, Ronkonkoma, New York, United States of America) and fine surgical grade instruments (Mercian Basic Micro Instrument Set BMS-1; Mercian Surgical Supply Co Ltd, Birmingham, United Kingdom) leaving the adventitia in situ. The vessel was straightened out by slightly stretching and pinning at either end before cutting 2 mm long rings of IMA with a Swann–Morton surgical steel disposable scalpel (LIG Supplies Ltd., Cambridge, United Kingdom). All rings were stored in Krebs-Hepes solution overnight at 4°C for study the next day. The next day the diameter of the IMA rings was recorded using a caliper ruler (Vicarey, Davidson & Company Limited, Glasgow, United Kingdom).

4.2.4 Vascular reactivity of IMA

2 mm long rings of IMA were studied in organ chambers according to methods described in Chapter 2. Vessels were constricted with phenylephrine (3 μmol/L) and relaxations to calcium ionophore A23187 (0.01 - 10 μmol/L) and sodium nitroprusside (0.01-10 μmmol/L) examined. Relaxation was expressed as a % of the constriction to phenylephrine.

4.2.5 Statistical Analysis

To investigate which risk factors were related to vasorelaxation multiple linear regression analysis was performed using data from the 264 CABG patients and

estimated effects, 95% confidence intervals (CI), and p values were tabulated. Multiple correlation coefficients were calculated as an overall measure of the relationship between risk factors and the dependent parameter - vasorelaxation to calcium ionophore A23187 (10 μmol/L). The multiple linear regression analysis included the following risk variables in the model: age in years; sex (0 = female, 1 = male); diastolic blood pressure (mmHg); tobacco smoking status (0 = non-smokers and ex-smokers of more than 30 days, 1 = current smoker); diabetes mellitus (0 = non-diabetes mellitus, 1 = on antidiabetic medication); body mass index (kg/m^2); blood levels of total cholesterol (mmol/L); statin therapy (0 = no therapy, 1 = on therapy) and ACE inhibitor / ARB therapy (0 = no therapy, 1 = on therapy). When risk factors were known to be interdependent, such as systolic and diastolic blood pressure, only one risk factor was included in the analysis. Note that quantitative variables, for example smoking status, were included as factors in the analysis. Linear relationships have been assumed between all continuous variables and outcomes. Normal distribution of data was examined by the Kolmogorov-Smirnov test. Coefficients and their confidence intervals indicate the estimated proportional increase in vasorelaxation to calcium ionophore A23187 (10 μmol/L) (the dependent parameter in the multivariate linear regression analysis) associated with a unit increase in the predictor. Where appropriate, Pearson correlation coefficients coefficients are displayed. Statistical analysis was performed using the statistical package MINITAB, version 13.1 (Minitab Inc, State College, Pennsylvania, United States of America). Results are shown as mean ± SEM (standard error of the mean) and $p < 0.05$ was considered statistically significant.

4.3 RESULTS

4.3.1 Patient characteristics

Demographics and clinical characteristics of the patients are shown in Table 4.1. The age range was 38 - 87 years. The high male to female ratio in Table 4.1 is a combined result of local referral patterns and insufficient vascular tissue left after CABG in women.

4.3.2 Vascular reactivity

For the rings used in the study, maximal calcium ionophore A23187 mediated relaxation (10 μmol/L) was 62.2 ± 1.1 % (mean ± SEM) (Figure 4.1) and maximal endothelium-independent relaxation to sodium nitroprusside (10 μmol/L) was 76.5 ± 1.2 % (mean ± SEM) (Figure 4.2).

Table 4.1 General demographics of the study population.

Variables	CABG
N	264
Age (y)	63 = 2
Gender (M / F)	155 / 56
Body Mass Index	28.0 ± 0.3
Blood pressure, mmHg	135.1 ± 1.2 / 87 ± 0.5
Total cholesterol, mmol/L	5.7 ± 0.7
Family history CAD (%)	43.6
Peripheral vascular disease (%)	12.2
Cerebrovascular disease (%)	6.1
Diabetes mellitus (%)	13.6
Currently smoke (%)	52.7
ACE inhibitor / ARB medication (%)	40.5
Statin medication (%)	68.5

Values shown are means ± SEM or %.

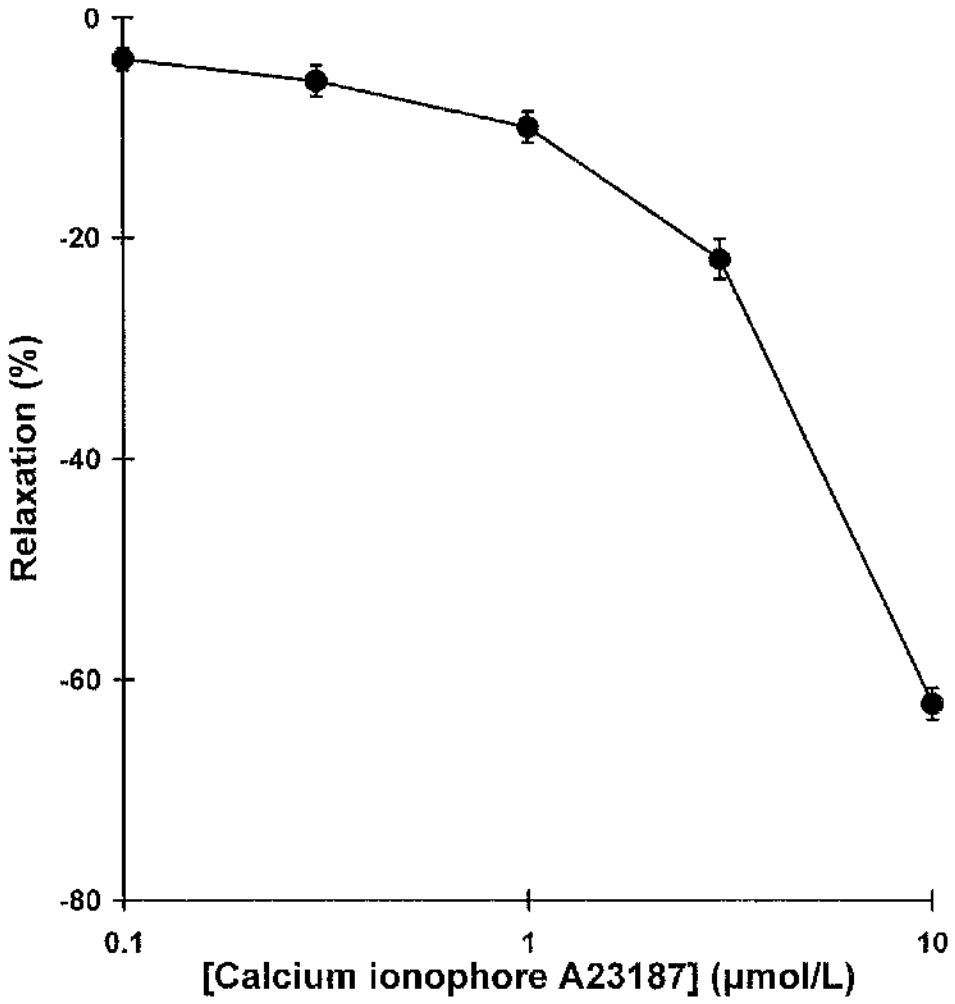

Figure 4.1 Vasorelaxation to calcium ionophore in rings of IMA from patients undergoing CABG (n = 264). The x-axis is on a logarithmic scale. Results are shown as mean ± SEM.

Maximal calcium ionophore A23187 mediated relaxation (10 µmol/L) was 62.2 ± 1.1 % (mean ± SEM).

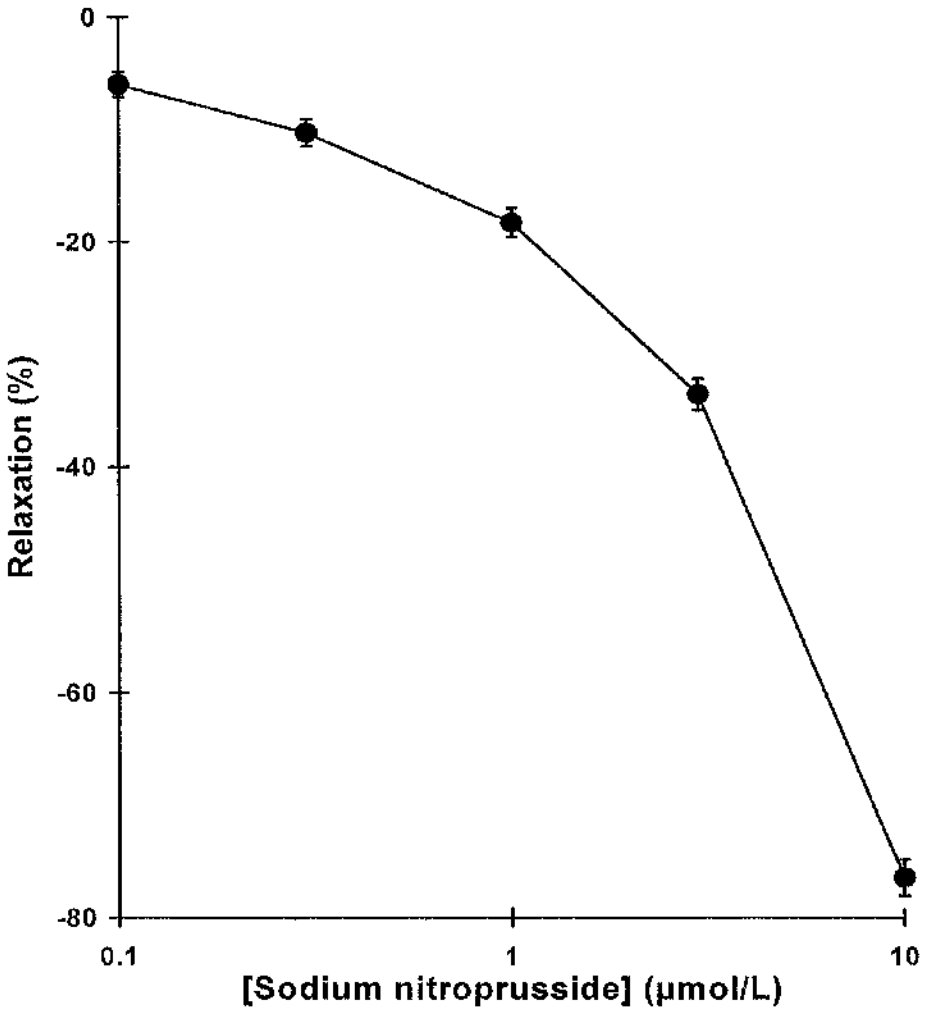

Figure 4.2 Vasorelaxation to sodium nitroprusside in rings of IMA from patients undergoing CABG (n = 264). The x-axis is on a logarithmic scale. Results are shown as mean ± SEM.

Maximal sodium nitroprusside mediated relaxation (10 µmol/L) was 76.5 ± 1.2 % (mean ± SEM).

4.3.3 Risk factor analysis

Multiple regression analysis using IMA pedicle width and cardiovascular risk factors that could be related to endothelial dysfunction was conducted on the CABG population studied (n = 264).

The analysis identified IMA pedicle width as a highly significant predictor of calcium ionophore A23187 mediated relaxation in the CABG patients (β coefficient, 0.79 per mm; $p < 0.001$). Total cholesterol level was also a significant determinant of calcium ionophore A23187 mediated relaxation in the CABG patients (β coefficient -2.90 per mmol/L; $p = 0.035$). There was no evidence that age, sex, body mass index, blood pressure, diabetes status, statin, and angiotensin-converting enzyme inhibitor therapy contributed to calcium ionophore A23187 mediated relaxation (Table 4.2). Tendencies to relationships between age and calcium ionophore A23187 mediated relaxation ($p = 0.07$) and statin therapy and calcium ionophore A23187 mediated relaxation ($p = 0.092$) were observed although these were not statistically significant (Table 4.2). Overall the model was able to account for 13.4 % of the variation in calcium ionophore A23187 mediated relaxation in the CABG patients (coefficient of determination) (Table 4.2).

Thus, a significant positive correlation between IMA pedicle width and a marker of endothelial function was observed (Table 4.2; Figure 4.3) and IMA pedicle width correlated directly to calcium ionophore A23187 mediated relaxation (IMA pedicle width versus calcium ionophore A23187 mediated relaxation: $p = < 0.001$) (Figure 4.3). Also, a negative correlation between total cholesterol and calcium ionophore A23187 mediated relaxation was observed (Table 4.2; Figure 4.4). However total cholesterol did not significantly correlate directly to calcium ionophore

A23187 mediated relaxation (total cholesterol versus calcium ionophore A23187 mediated relaxation: p = 0.077) (Figure 4.4).

There was no significant relationship between IMA pedicle width and endothelium-independent relaxation to sodium nitroprusside (10 μmol/L) (n = 264, p = 0.440) (Figure 4.5). There was no significant relationship between calcium ionophore A23187 mediated relaxation and IMA diameter (n = 264, p = 0.743) (Figure 4.6).

At follow up after a median of 32 months (range 19 - 51 months), none of these patients showed evidence of mediastinitis, deep sternal infection or sternal wound dehiscence.

Table 4.2 Analysis of variance p-values for the null hypothesis of no relationship between risk factors and calcium ionophore A23187 (10 µmol/L) mediated relaxation of IMA in CABG patients.

Variable	Calcium ionophore (n=264)	
	p	β coefficient (95% confidence intervals)
Age (10 years)	0.073	-0.17 (-0.36 – 0.01)
Sex (0=F, 1=M)	0.210	2.94 (-1.64 - 7.53)
Body Mass Index (kg/m²)	0.325	0.29 (-0.29 – 0.87)
IMA pedicle width (mm)	<0.001	0.79 (0.40 – 1.19)
Current Active Smoking (0=no, 1=yes)	0.851	-0.40 (-4.53 3.73)
Diabetes Mellitus (0=no, 1=yes)	0.657	1.38 (-04.71 – 7.48)
Diastolic Blood Pressure (10mmMg)	0.641	0.06 (-0.18 – 0.30)
Total Cholesterol (mmol/L)	0.035	-2.90 (-5.57 - -0.22)
Angiotensin Converting Enzyme Inhibitor (0=no, 1=yes)	0.342	2.01 (-2.12 – 6.13)
Statin (0=no, 1=yes)	0.092	3.87 (-0.62 – 8.73)

This analysis identified pedicle width (β coefficient 0.79 per mm, $p < 0.001$) and total cholesterol (β coefficient -2.90 per mmol/L; $p = 0.035$) as significant predictors of calcium ionophore A23187 mediated relaxation of IMA from CABG patients. There was no evidence that age, sex, body mass index, current active smoking, diabetes mellitus status, diastolic blood pressure, angiotensin-converting enzyme inhibitor therapy or statin therapy contributed to calcium ionophore A23187 mediated relaxation. Overall the model was only able to account for 13.4% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in calcium ionophore A23187 mediated relaxation with a unit increase in the predictor.

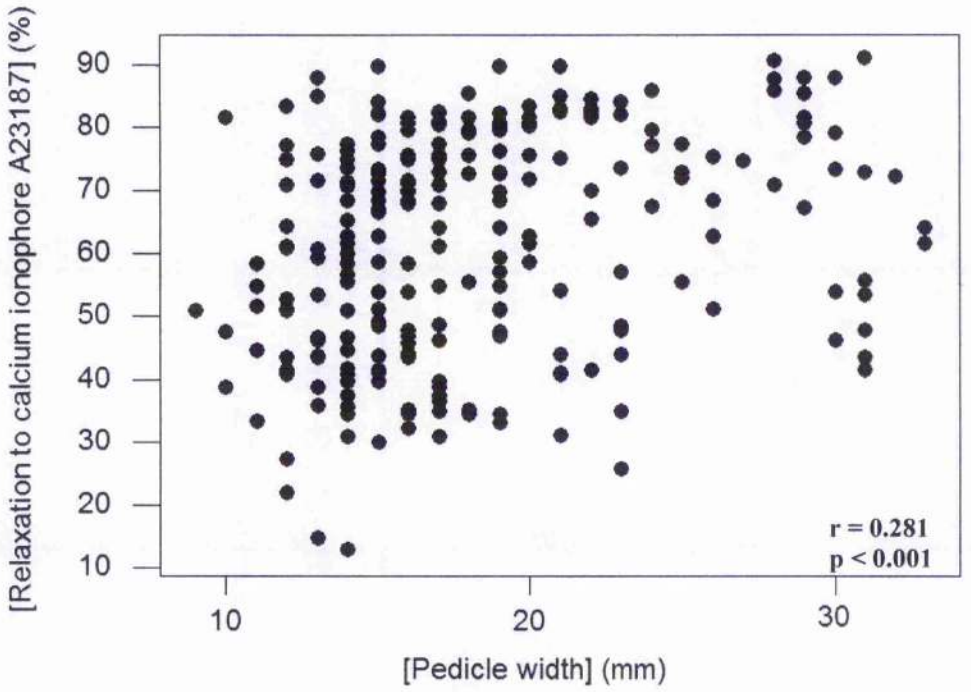

Figure 4.3 Scatter plots of IMA pedicle width (mm) and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA (n = 264).

Pearson correlation coefficient (r) and p-value are shown in the figure.

This scatter plot shows a significant correlation between pedicle width (mm) and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA ($p < 0.001$, $r = 0.281$).

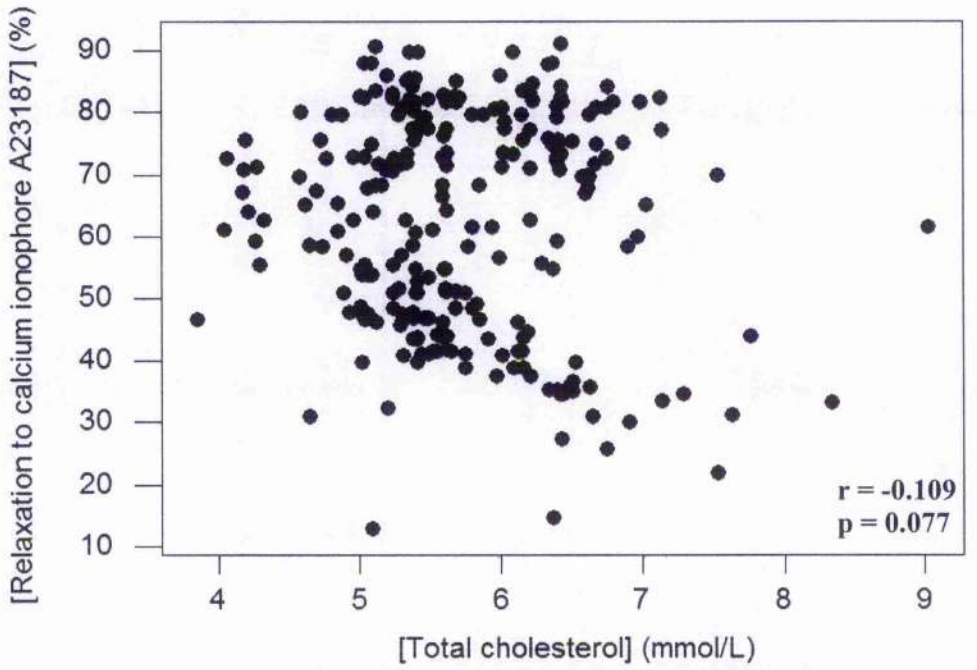

Figure 4.4 Scatter plots of total cholesterol concentration and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA (n = 264).

Pearson correlation coefficient (r) and p-value are shown in the figure.

This scatter plot shows no significant correlation between total-cholesterol and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA (p = 0.077, r = -0.109).

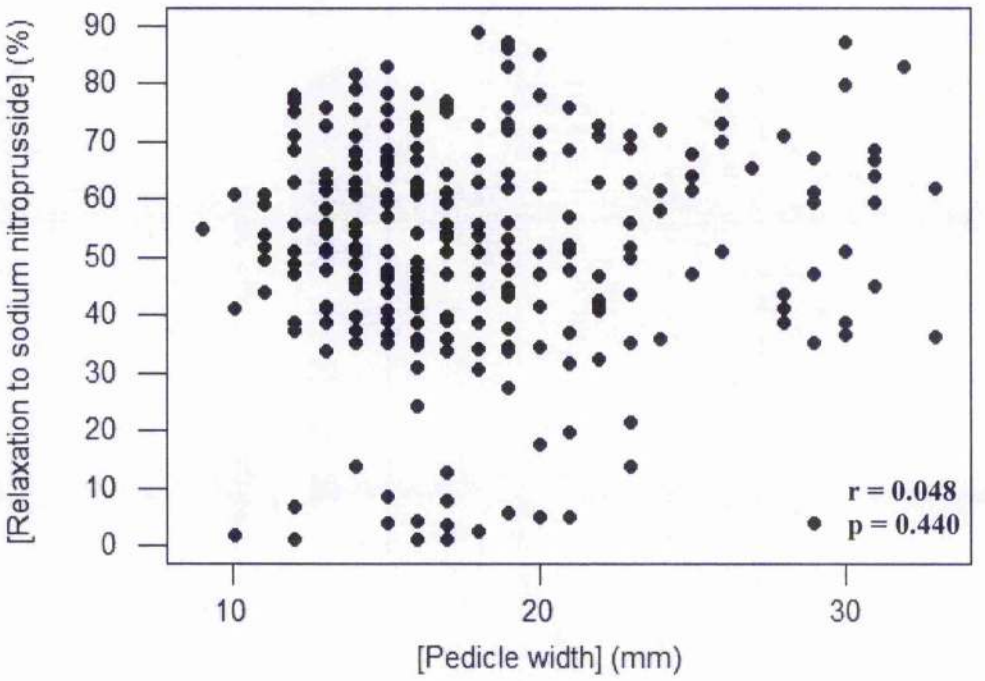

Figure 4.5 Scatter plots of IMA pedicle width (mm) and sodium nitroprusside (10 μmol/L) mediated relaxation of IMA (n = 264).

Pearson correlation coefficient (r) and p-value are shown in the figure.

This scatter plot shows no significant correlation between pedicle width and sodium nitroprusside (10 μmol/L) mediated relaxation of IMA (p = 0.077, r = -0.287).

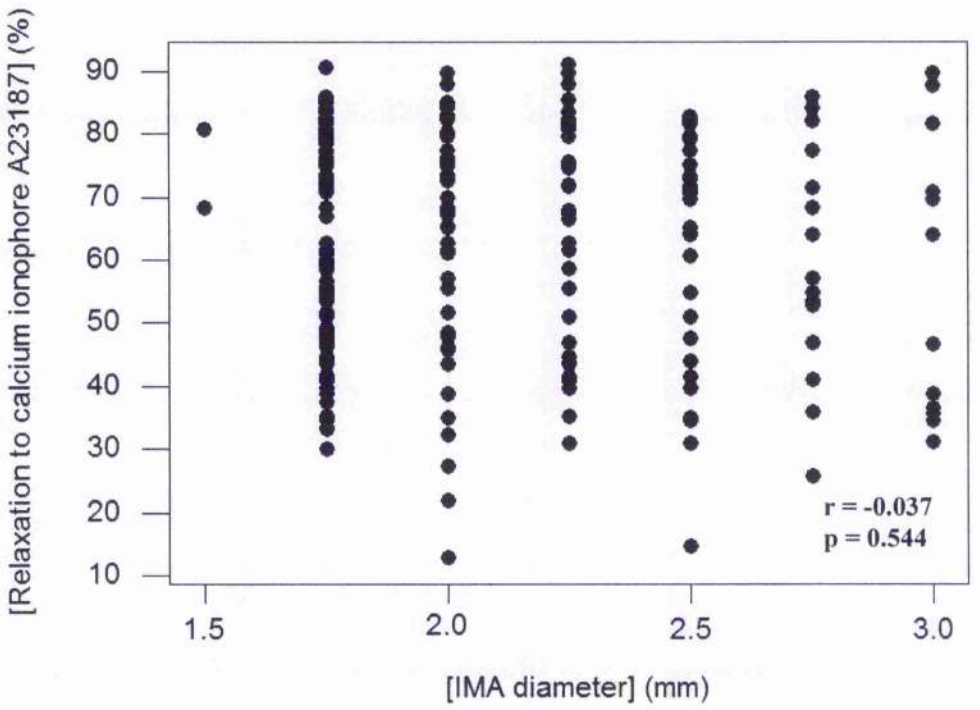

Figure 4.6 Scatter plots of IMA diameter (mm) and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA (n = 264).

Pearson correlation coefficient (r) and p-value are shown in the figure. This scatter plot shows no significant correlation between IMA diameter and calcium ionophore A23187 (10 μmol/L) mediated relaxation of IMA (p = 0.544, r = -0.037).

4.4 DISCUSSION

4.4.1 Pedicle width and endothelium-dependent function

This study has demonstrated the predominant role of IMA pedicle width in determining calcium ionophore A23187 mediated relaxation of IMA in patients undergoing CABG. This study also showed a significant positive correlation between IMA pedicle width and calcium ionophore A23187 mediated relaxation of IMA maintained across the whole range of IMA pedicle widths.

This is the first study investigating IMA endothelial function demonstrating that a wider pedicle harvested with monopolar electrocautery preserves NO-dependent endothelial function. NO synthesised by healthy intact endothelium has a plethora of roles in maintaining vessel patency (Simionescu 2007; Yetik-Anacak and Catravas 2006; Félétou and Vanhoutte 2006; Moncada and Higgs 2006; Herman and Moncada 2005). Dissection of the IMA with a narrower pedicle may theoretically induce mechanical and physical damage to the vessel wall and result in detrimental effects on the functional integrity of the IMA (Ueda et al. 2003; Cunningham 1996).

Electrocautery means heating through radiofrequency electric current; heat is generated by passing this current through the tissues (Morris and Malt 1994). With monopolar electrocautery the current is passed through a large volume of tissue from an "indifferent" electrode of comparatively large surface area which is in good electrical contact with a large area of the body and the current then passes through an active electrode of very small contact surface, which is under control of the surgeon (Morris and Malt 1994). A very low current density is therefore passed through most of the body, but at the point of contact between the electrode and the tissues the current density is very high, and therefore has a large heating effect (Patricelli et al. 2001; Morris and Malt 1994). Tissue temperatures in of up to 129.5 °C have been

measured 5 mm from monopolar electrode placement (Patricelli et al. 2001) and tissue charring and vaporisation have been associated with tissue temperatures exceeding 100 °C (Panescu et al. 1995).

Light and scanning electron microscopy have shown complete loss of endothelium with electrocautery in blood vessels associated with mural thrombus formation (Gaudino et al. 2000; Acedia et al. 1995; Yoshida et al. 1995; Sparmann et al. 1992; Noera et al. 1993; Waleczek et al. 1993; Lehtola et al. 1989) and 70% of early graft occlusions in CABG are caused by thrombi overlying areas of endothelial loss (Alrawi 2001). In an animal model, skeletonised IMA harvested using electrocautery, showed a higher incidence of thrombosis, intimal thickening, and medial injury that pedicled grafts (Daly et al. 1988). The demand for a safe distance from vital structures when using electrocautery has also been demonstrated (Waleczek et al. 1993; Sparmann 1992). These histological studies corroborate this study's functional findings.

Although the main focus of this study was into the effect of pedicle width on NO-dependent endothelial-function, there are other potential sources of NO that are maintained with IMA pedicles (Gao et al. 2005). A recent study described the release of a transferable relaxing factor from the perivascular adipose tissue surrounding the human IMA and suggested that its retention when harvesting the IMA may play an important role in the superior patency rate of the IMA (Gao et al. 2005).

To prove that IMA harvested with a wider pedicle ameliorates survival, will be difficult because of the low sensitivity of clinical endpoints and the good results that are available with the use of normal harvested IMAs (Rosamund et al. 2007; Cho et al. 2006; Eagle et al. 2004; Sergeant et al. 1998). The Western Infirmary Glasgow on behalf of the National Adult Cardiac Surgical Database prospectively follows up their

CABG patients and five year freedom-from-reintervention and mortality data will be available to further investigate the influence of pedicle width on freedom-from-reintervention and survival.

This study has demonstrated the predominant role of IMA pedicle width in determining calcium ionophore A23187 mediated relaxation of IMA harvested with monopolar electrocautery. This study proposes that by reducing vascular injury to the IMA, using the less traumatic surgical technique (Ueda et al. 2003; Cunningham 1996) of a wider pedicle, NO-dependent endothelial function is maintained and this will result in improved graft performance (Luscher et al. 1988).

4.4.2 Total cholesterol and endothelium-dependent function

In this study, total cholesterol level was also a significant determinant of calcium ionophore A23187 mediated relaxation and a negative correlation between total cholesterol and a calcium ionophore A23187 mediated relaxation was observed. However total cholesterol did not correlate directly to calcium ionophore A23187 mediated relaxation. A tendency to a relationship between statin therapy and calcium ionophore A23187 mediated relaxation was observed although these were not statistically significant. Lipid-lowering therapy with statins has been demonstrated to improve endothelial function (Fichtlscherer et al. 2006; Tsunekawa et al. 2001; Anderson et al. 1995; John et al. 2001; John et al. 1998; O'Driscoll et al. 1997b) and reduce the occurrence of major cardiovascular events (Larosa et al. 2005; Nissen et al. 2005; Cannon et al. 2004). Apart from lipid-lowering therapy (Jeremy et al. 2007), no intervention has hitherto proved clinically effective in preventing late graft failure (Datani et al. 2000; Campeau et al. 2000; The Post Coronary Artery Bypass Graft Trial Investigators 1997). This study adds support to lipid-lowering in CABG patients

(Campeau et al. 2000; The Post Coronary Artery Bypass Graft Trial Investigators 1997). A recent study demonstrated that simvastatin treatment significantly reduced endothelial CD40-sCD40L, a culprit link between local inflammation and the coagulation cascade, in both venous and arterial grafts, and speculated that this effect of statins, in addition to enhancement of eNOS (Lefer et al. 2001; Miller et al. 2001), may help prevent vein graft thrombosis and graft failure (Chello et al. 2006).

The focus of this study was into the effect of pedicle width on NO-dependent endothelial-function. This study was not intended to examine the relationship between endothelial dysfunction and CVD. Given the important prognostic significance of endothelial dysfunction (Endemann and Schiffrin 2004; Widlansky et al. 2003; Gokce et al. 2002; Heitzer et al. 2001; Al Suwaidi et al. 2000; Schachinger et al. 2000), further studies were undertaken in the subsequent chapters to address the relationship between endothelial function and cardiovascular risk factors.

Chapter 5

Comparison of vasorelaxation and superoxide production in LSV from patients undergoing CABG and patients undergoing surgery for removal of varicose veins:

1) Effect of calcium ionophore A23187, allopurinol, apocynin, and sodium nitroprusside on relaxation of LSV

2) Measurement of superoxide production in LSV

3) Measurement of circulating indicators of oxidative stress

5.1 INTRODUCTION

Chapter 1 outlined that endothelial dysfunction occurs in conjunction with CAD and it is observed both in the coronary and peripheral vasculature (Félétou and Vanhoutte 2006). Moreover, risk factors for CAD have been almost universally associated with a degree of endothelial dysfunction in humans (Landmesser et al. 2004). Increased production of ROS, in particular, superoxide and radicals derived from superoxide, has been associated with endothelial dysfunction in animal models of disease, and there is increasing evidence of a link between oxidative stress and endothelial dysfunction in humans (Touyz and Schiffrin 2004; Taniyama and Griendling 2003; Harrison and Griendling 2003; Hamilton et al. 2002; Alexander and Dzau 2000). However these studies have been limited by the absence of control vascular tissue from subjects without clinical manifestation of coronary artery disease (CAD). The accepted means of comparison in this instance had been to compare arteries to veins (Berry et al. 2000) or to stratify subjects according to risk factors or disease severity (Simic et al. 2006; Redon et al. 2003; Guzik et al. 2000a). However, comparative data on endothelial function, direct measures of superoxide in human vessels, and circulating indicators of oxidative stress are not available in patients with CAD nor in control subjects with no documented CVD.

5.1.1 Non-varicose LSV from patients undergoing surgery for removal of varicose veins: A reasonable control model?

Mashiah et al. (1991) found no changes in the intima or adventitia of non-varicosed segments of varicose LSV with a scanning electron microscope. The abnormalities that were found coincided with areas of varicose dilatation that were between areas that retained their normal configuration (Mashiah et al. 1991). Khan et

al. (2000) studied the tunica intima of varicose veins with light and electron microscopy and found that the endothelial lining was intact in varicose sections and was not significantly different from those seen in control vein samples. A recent review article by Somers and Knaapen (2006) called "The Histopathology of Varicose Vein Disease" emphasised that intimal thickening found in varicose veins can be ascribed to phlebosclerosis due to ageing (Milroy et al. 1989) and is therefore not attributable to changes of the vein wall as observed in varicose vein disease (Somers and Knaapen 2006). MacFarlane et al. (1985) documented that LSV segments below the knee do not become varicose even in patients with extensive below-knee varicosities. Therefore LSV segments, particularly below knee non-varicosed LSV segments, from patients who are undergoing surgery for removal of varicose veins, who have no conventional risk factors for CVD, no documented CVD and a normal electrocardiogram (ECG) are proposed as a reasonable control model system to compare to LSV from subjects undergoing CABG.

5.1.2 Hypothesis

Patients with severe CAD have endothelial dysfunction and increased levels of oxidative stress compared to healthy control subjects with no documented CVD.

5.1.3 Aims

1. To study endothelium-dependent and -independent relaxation of LSV in a group of patients with severe CAD compared to controls.
2. To study superoxide production levels in blood vessels in a group of patients with severe CAD compared to controls.

3. To study circulating indicators of oxidative stress in a group of patients with severe CAD compared to controls.

5.1.4 Experimental Approach

1. Relaxation to calcium ionophore A23187 (endothelium dependent vasodilatator), sodium nitroprusside (endothelium independent vasodilatator), allopurinol (inhibitor of xanthine oxidase) and apocynin (reduced NAD(P)H oxidase inhibitor) will be determined in LSV from patients undergoing CABG and age- and sex-matched control patients with no documented CVD who were undergoing surgery for removal of varicose veins.
2. To evaluate circulating oxidative stress indicators. The GSH/GSSG ratio and the TAC will be determined in blood from patients undergoing CABG and age- and sex-matched control patients with no documented CVD who were undergoing surgery for removal of varicose veins.
3. To measure superoxide production using lucigenin chemiluminescence and oxidative fluorescent microtopography with hydroethidine from patients undergoing CABG and age- and sex-matched control patients with no documented CVD who were undergoing surgery for removal of varicose veins.

5.2 METHODS

5.2.1 Study population

51 subjects undergoing elective CABG and 51 subjects undergoing elective varicose vein surgery were recruited as controls. All were outpatients recruited at the Cardiothoracic Unit at the Western Infirmary Glasgow or the Vascular Unit at

Gartnavel General Hospital. Patients undergoing CABG surgery had obstructive CAD demonstrated by coronary angiography (CAD subjects). The control subjects had no history of CVD (angina, coronary artery disease or peripheral vascular disease), no conventional risk factors for CVD and a normal ECG. These control subjects underwent general health screening test, including a physical examination and ECG and they were enrolled if they had neither a history nor clinical evidence of atherosclerosis. In addition, any control subject taking any pharmacological therapy was not included in the study. The study was approved by the local ethics committee and all subjects gave informed written consent. Below-knee, non-varicose LSV were collected at the time of surgery. In addition, blood samples were collected 5-10 days prior to CABG surgery or within 1 month of varicose vein surgery. Subjects were fasted overnight and rested supine for 30 min prior to donating blood.

5.2.2 Vascular reactivity of veins

3 mm long rings of undistended LSV were studied in organ chambers according to methods described in Chapter 2. Only non-varicosed portions of vein were studied. Vessels were constricted with phenylephrine (3 μmol/L) and relaxations to calcium ionophore A23187 (0.01 - 10 μmol/L), sodium nitroprusside (0.01 - 10 μmol/L), apocynin (0.01 - 0.3 mmol/L) or allopurinol (0.01 - 0.3 mmol/L) examined. Relaxation was expressed as a % of the constriction to phenylephrine.

5.2.3 Circulating indicators of oxidative stress

Blood samples were collected from control and CAD subjects for determination of TAC, GSH, GSSG, GSH/GSSG molar ratio and LDL-cholesterol according to methods described in Chapter 2.

TAC was measured in plasma using a commercially available kit (AOP-490; Oxis International Inc., Portland, Oregon, United States of America) based upon the reduction of Cu^{2+} to Cu^{+}.

The GSH/GSSG ratio was measured in whole blood using a commercially available kit (GSH/GSSG-412, Oxis International Inc., Portland, Oregon, United States of America). GSH reacts with Ellman's reagent to form a spectrophotometrically detectable product at 412 nm. In a separate reaction GSSG is reduced to GSH which is then determined in the same manner.

5.2.4 LDL-cholesterol

The Bayer® ADVIA 1650 chemistry system with standard reagents (Bayer Diagnostics, Newbury, Berkshire, United Kingdom) was used to determine measure total cholesterol, HDL-cholesterol and triglycerides in plasma. The plasma total cholesterol concentration was measured by cholesterol esterase and cholesterol oxidase conversion, plasma triglyceride concentration by lipoprotein lipase/glycerol kinase enzymatic determination and HDL cholesterol measured cholesterol by polyethylene glycol-linked cholesterol esterase and oxidase after serum incubation with sulphated cyclodextrin buffer. Measurement of the total cholesterol, HDL cholesterol and triglyceride levels permitted the estimation of LDL-cholesterol concentrations, based upon the Friedwald formula (Friedwald et al. 1972).

5.2.5 Superoxide production in blood vessels

All experiments to investigate superoxide production in LSV were performed by Dr CA Hamilton (Senior Lecturer in Pharmacology at University of Glasgow) and

Mrs EJ Jardine (Laboratory Technician at University of Glasgow). Superoxide levels were measured in LSV from CAD (n = 15) and age- and sex-matched control patients (n = 15). Superoxide was measured using lucigenin chemiluminescence as described by Berry et al. (2000). 3 × 5 mm lengths of vein were blotted, weighed and placed in 2ml of Krebs buffer at pH 7.4 ± 2 and maintained in atmospheric conditions at room temperature. Next, either Krebs buffer, allopurinol (0.1 mmol/L) or apocynin (0.1 mmol/L) were added and after 60 minutes incubation at room temperature, lucigenin (0.25 mmol/L) was added and counts were recorded every 10 seconds for 3 minutes in a liquid scintillation counter (Tricarb 2100TR, Hewlett Packard; Hercules, California, United States of America) switched to the out-of-coincidence mode. Absolute counts were quantified with a xanthine / xanthine oxidase calibration curve for superoxide generation and reported as nmol min^{-1} mg tissue. In all experiments, superoxide production was measured in paired samples with a matched control from the same vessel in every case.

For oxidative fluorescent microtopography with hydroethidine, frozen sections were prepared and incubated with the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL for 2 minutes) followed by hydroethidine (2 μmol/L for 20 minutes). The oxidative fluorescent dye hydroethidine was used to evaluate the in situ formation of superoxide as described by Mollnau et al. (2005), Hink et al. (2001) and Miller et al. (1998). Fluorescence was detected using a laser scanning confocal microscope (MRC 1024, Bio-Rad Laboratories; Hercules, California, United States of America) with an optical band pass filter of 470 ± 30 nm for DAPI (2-photon excitation 750 nm) and a band pass of 580 ± 16 nm for hydroethidine (single-photon excitation 488 nm). Vessels from CAD and control patients were analyzed in parallel under identical imaging parameters. Relative superoxide levels within vessel

segments were quantitated by determining relative fluorescence in the red (hydroethidine) and blue (DAPI) channels over a defined area, using Scion Image version 4.0 software (Scion Corporation Inc., Frederick, Maryland, United States of America) (Chamseddine and Miller 2003).

5.2.6 Statistical analysis

All comparisons between responses in CAD and control vessels, superoxide production and circulating indicators of oxidative stress were made in age and sex matched samples. Age matching was performed using subjects at most two years apart from each other (Goodman et al. 2000; Terán-Santos et al. 1999; Cassidy et al. 1999; Strom et al. 1998; Duggan et al. 1998; Jick et al. 1998). Normal distribution of data was examined by the Kolmogorov-Smirnov test. Where appropriate, unpaired Student t tests were used for the comparisons of relaxation at different concentrations to calcium ionophore, sodium nitroprusside, apocynin, and allopurinol between the 2 groups. Plots of mean vessel responses together with their SEM are shown, with asterisks indicating when there was significant evidence of a difference between CAD and control subjects' response curves ($p < 0.05$). Statistical analyses of vascular superoxide concentrations were undertaken by use of the unpaired Student t test. The Mann-Whitney U test was used for comparison of non-normally distributed data between the groups (total antioxidant capacity and reduced/oxidized glutathione ratio). Unless otherwise indicated, results are shown as mean ± SEM, including 95 % CIs where appropriate, and $p < 0.05$ was considered significant.

5.3 RESULTS

5.3.1 Subject Characteristics

Demographics characteristics of the 2 subject groups are shown in Table 5.1. Vessels and blood from all subjects was available for study of circulating indicators of oxidative stress. The average age of the CAD subjects was lower and the proportion of females higher than that of a typical CABG population (see chapters 3 and 4). This was due to subject selection to match with control subjects who underwent varicose vein surgery, who tended to be younger women. This may also account for the high prevalence of risk factors present in the CAD group of subjects.

Table 5.1 CAD and control patient demographics.

Variables	CAD	Controls
Number	51	51
Age, (y)	55 ± 11	55 ± 11
Gender, (M/F)	22 / 29	22 / 29
Body Mass Index	30 ± 6	27 ± 4 *
Systolic Blood Pressure, (mm Hg)	140 ± 14	120 ± 11 *
Diastolic Blood Pressure, (mm Hg)	83 ± 9	71 ± 10 *
Total cholesterol, (mmol/L)	4.7 ± 0.7	3.7 ± 0.7
LDL-cholesterol, (mmol/L)	2.9 ± 0.6	1.8 ± 0.4 *
Triglycerides, (mmol/L)	1.0 ± 0.6	1.4 ± 1.3 *
Diabetes mellitus, (%)	31	0
Smokers (%) (active / stopped / none)	35 / 35 / 30	53 / 20 / 27
New York Heart Association heart failure score (%) (I/II/III/IV)	4/40/48/8	n/a
Canadian Cardiovascular Society angina score (%) (I/II/III/IV)	10/29/49/12	n/a
ACE inhibitor / ARB medication (%)	47	0
Antiarrhythmic (Amiodarone or Digoxin) (%)	4	0
Aspirin (%)	75	0
Beta Blockers (%)	58	0
Calcium Channel Antagonists (%)	45	0
Clopidogrel (%)	6	0
Diuretic (%)	29	0
Nicorandil (%)	32	0
Nitrate (%)	20	0
Statin medication (%)	86	0

Results are shown as mean ± standard deviation.

*$p<0.001$ between age and sex matched CAD and control patients

5.3.2 Vascular reactivity

Endothelium-dependent relaxations to calcium ionophore A23187 were attenuated in vessels from CAD subjects compared to control subjects across the full concentration response curve (Figure 5.1) maximal relaxation (calcium ionophore A23187 10μmol/L) being 26 ± 2 % and 60 ± 1 % respectively in CAD and control subjects ($p < 0.001$ 95 %, CI 29 % to 39 %). In contrast, endothelium-independent relaxation to sodium nitroprusside was not attenuated in the CAD patients; in fact, at the highest dose (10 μmol/L), relaxation was greater in CAD than in control patients [77 ± 3 % and 64 ± 2 % respectively ($p < 0.01$ 95 %, CI 21 % to 29 %)] (Figure 5.2). At the highest concentrations examined both the xanthine oxidase inhibitor, allopurinol, and the NAD(P)H oxidase inhibitor, apocynin, caused greater relaxations in vessels from CAD subjects compared to control subjects (Figure 5.3; Figure 5.4). In the presence of allopurinol, 0.3 mmol/L, relaxations were 25 ± 2 % and 16 ± 1% ($p < 0.001$ 95 %, CI -14% to -4%), while in the presence of apocynin, 0.3 mmol/L, relaxations were 23 ± 2% and 10 ± 1% ($p < 0.001$ 95 %, CI -18 % to -8 %) in CAD and control tissues respectively.

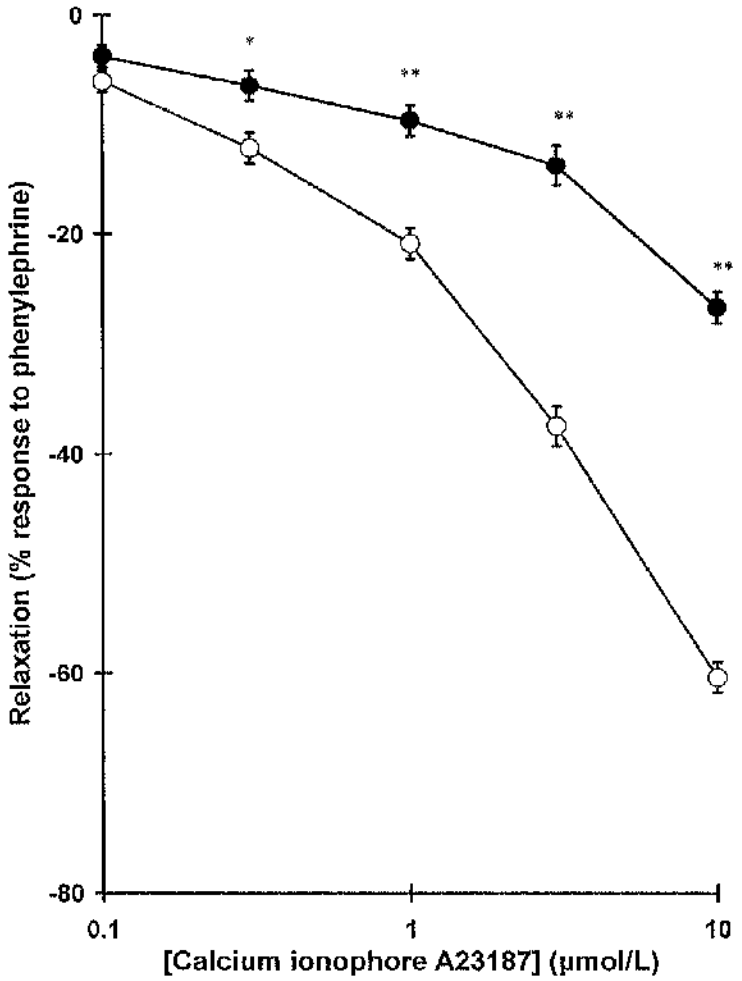

Figure 5.1 Vasorelaxation to calcium ionophore A23187 in rings of LSV from age and sex matched CAD (n = 51) and control (n = 51) patients. The x-axis is on a logarithmic scale. ○ Control ●CAD

Results are shown as mean ± SEM. Differences in relaxation between CAD and control patients were examined using unpaired t-tests.

* $p < 0.01$, ** $p < 0.001$.

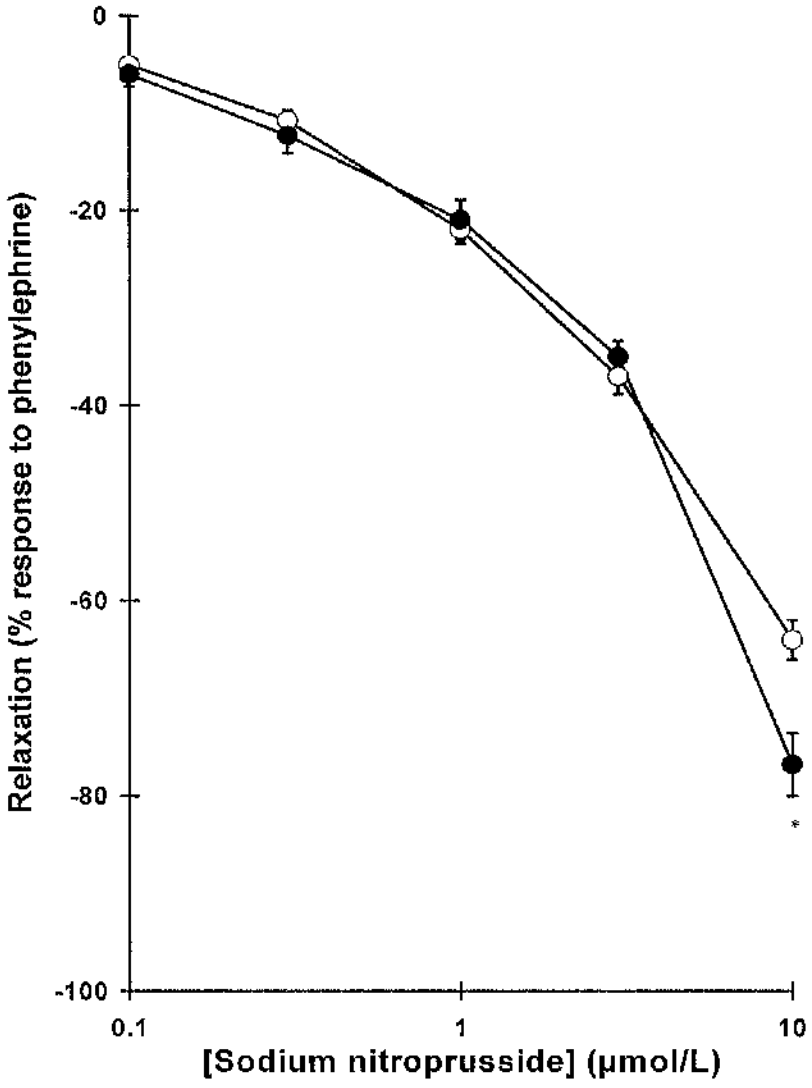

Figure 5.2 Vasorelaxation to sodium nitroprusside in rings of LSV from age and sex matched CAD (n = 51) and control (n = 51) patients. The x-axis is on a logarithmic scale. ○ Control ●CAD

Results are shown as mean ± SEM. Differences in relaxation between CAD and control patients were examined using unpaired t-tests.

* $p < 0.01$.

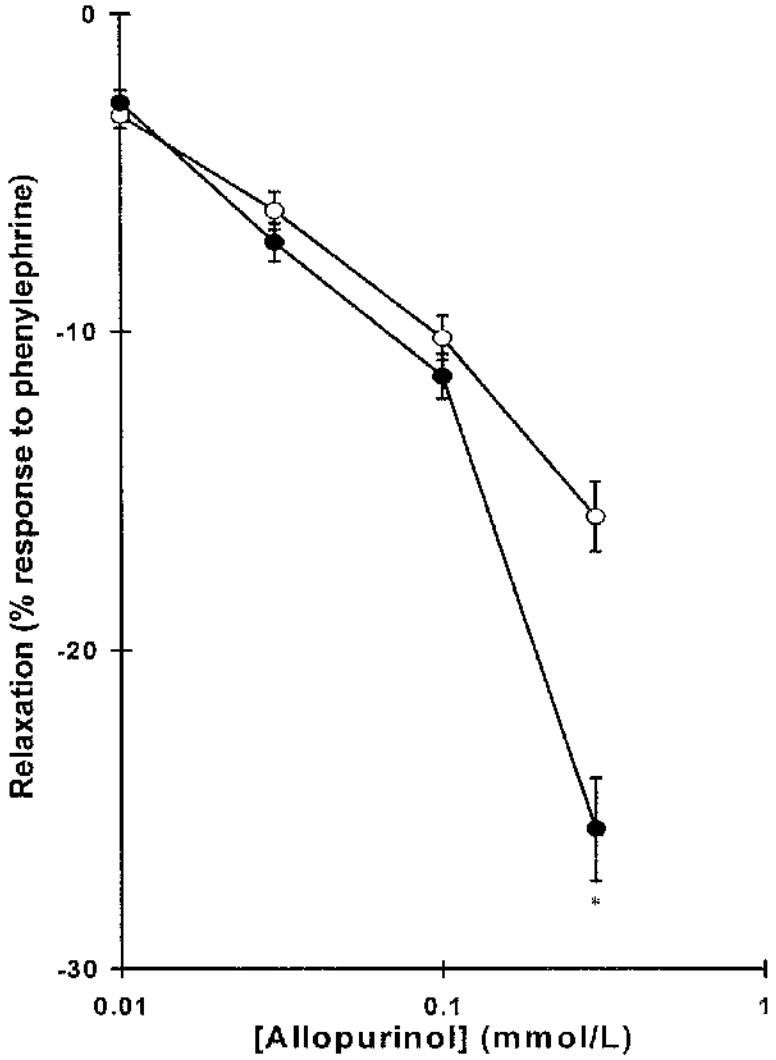

Figure 5.3 Vasorelaxation to allopurinol in rings of LSV from age and sex matched CAD (n = 51) and control (n = 51) patients. The x-axis is on a logarithmic scale. ○ Control ●CAD

Results are shown as mean ± SEM. Differences in relaxation between CAD and control patients were examined using unpaired t-tests.

* $p < 0.001$.

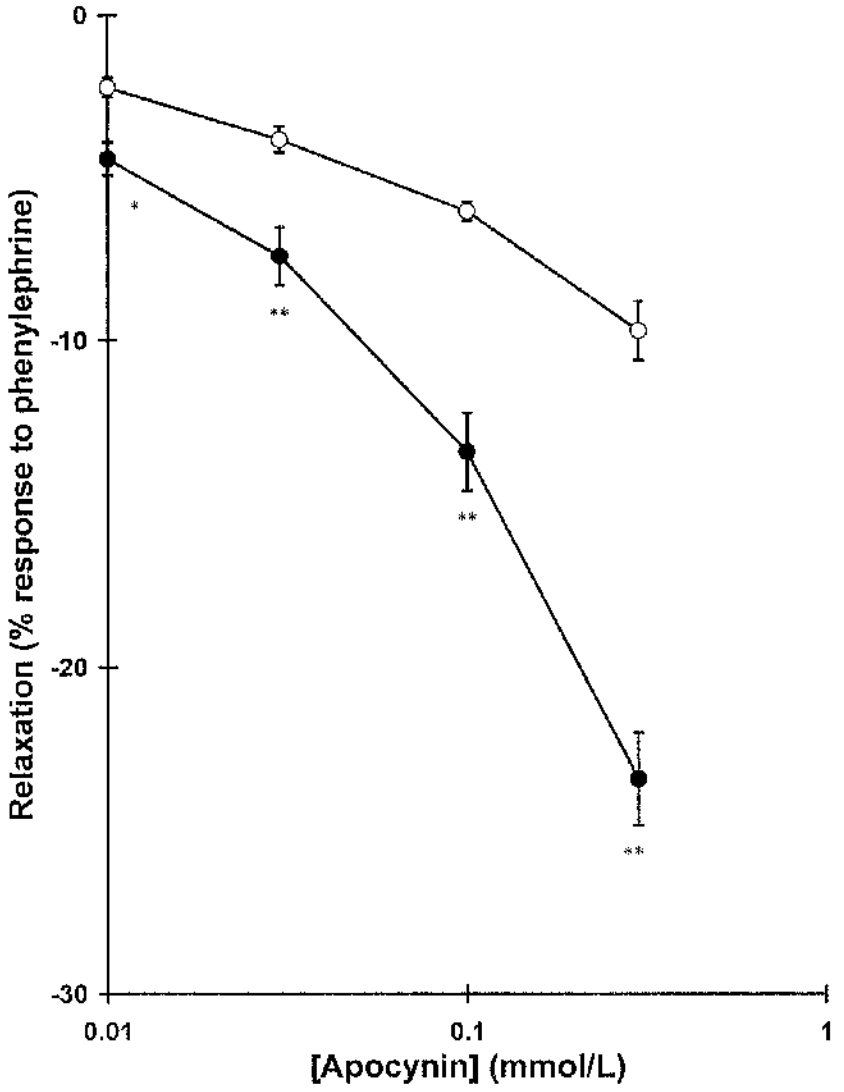

Figure 5.4 Vasorelaxation to apocynin in rings of LSV from age and sex matched CAD (n = 51) and control (n = 51) patients. The x-axis is on a logarithmic scale. ○ Control ●CAD

Results are shown as mean ± SEM. n=51 for all groups. Differences in relaxation between CAD and control patients were examined using unpaired t-tests. * $p < 0.01$, ** $p < 0.001$.

5.3.3 Direct measurement of vascular superoxide

Lucigenin chemiluminescence measurements of superoxide were significantly greater in blood vessels from CAD than from control patients (0.89 ± 0.10 versus 0.55 ± 0.06 nmol/mg/min; p = 0.008; 95% CI, 10.1 to 59.5 nmol/mg/min; Figure 5.5) when compared by unpaired Student t test. Apocynin (p = 0.021) and allopurinol (p = 0.038) reduced superoxide production significantly by 0.12 ± 0.05 nmol/mg/min (95% CI, 0.02 to 0.21 nmol/mg/min) and 0.22 ± 0.10 nmol/mg/min (95% CI, 0.14 to 0.43 nmol/mg/min) when compared with baseline values in patients with CAD (Figure 5.6). The higher levels of superoxide production in patients with CAD were confirmed using hydroethidine (Figure 5.8; Figure 5.9; Figure 5.11; Figure 5.12) and were observed throughout the vessel wall. In contrast to hydroethidine the intensity of DAPI staining was similar in vessel sections from CAD and control patients (Figure 5.7 and Figure 5.10).

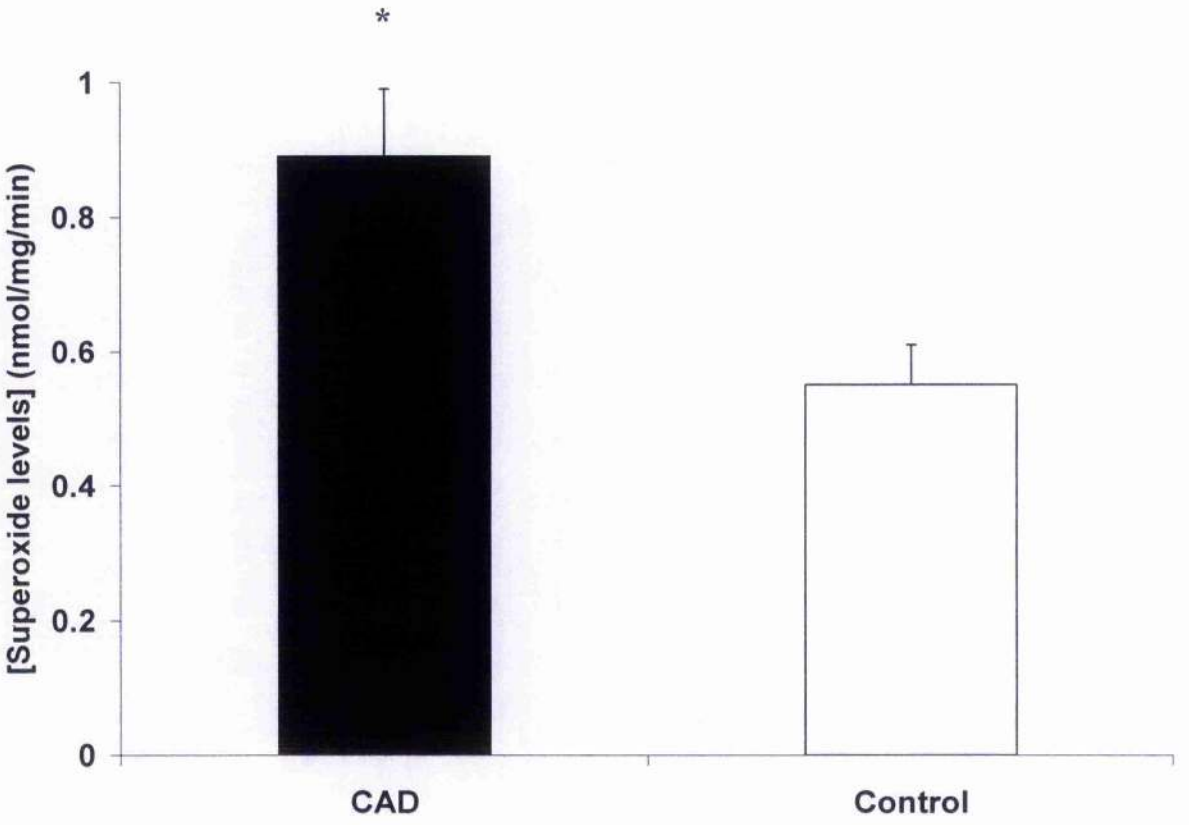

Figure 5.5 $^{\bullet}O_2^-$ levels in LSV from age- and sex-matched CAD (n = 15) and control (n = 15) patients. Samples were incubated with DAPI 0.5 μg / mL for 2 minutes followed by hydroethidine 2 μmol / L for 20 minutes. Superoxide levels measured by lucigenin chemiluminescence.

● CAD ○ Control

* $p < 0.01$ when superoxide production in vessels from CAD and control patients compared by unpaired Student t test.

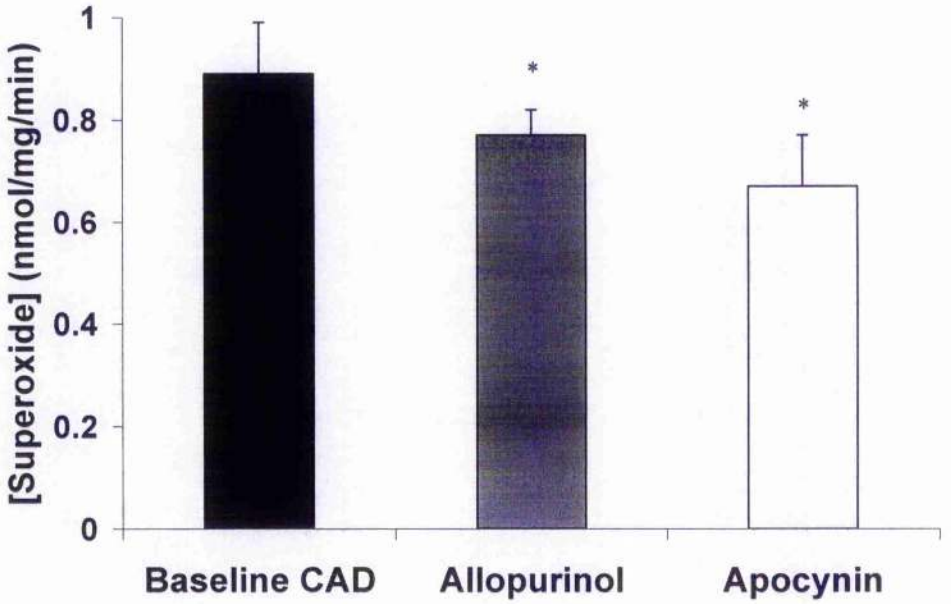

Figure 5.6 Superoxide levels in LSV from the age- and sex-matched CAD (n = 15) subgroup at baseline and after incubation (60 minutes) with allopurinol (0.1 mmol / L) and apocynin (0.1 mmol / L). Samples were incubated with DAPI 0.5 μg / mL for 2 minutes followed by hydroethidine 2 μmol / L for 20 minutes. Superoxide levels measured by lucigenin chemiluminescence.

● Baseline CAD ● Allopurinol ○ Apocynin

* $p < 0.05$ when superoxide production after incubation with allopurinol or apocynin is compared with baseline CAD values by unpaired Student t tests.

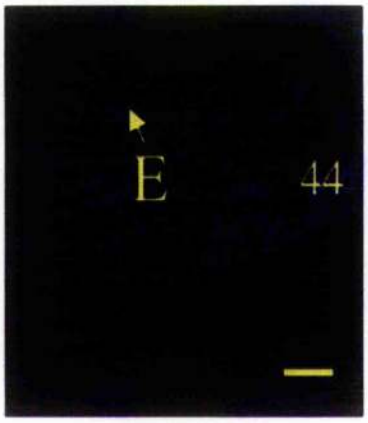

Figure 5.7

Figure 5.8

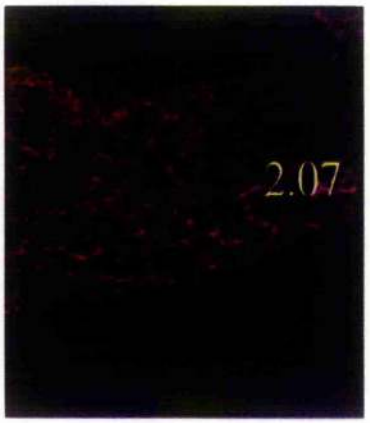

Figure 5.9

Figure 5.10

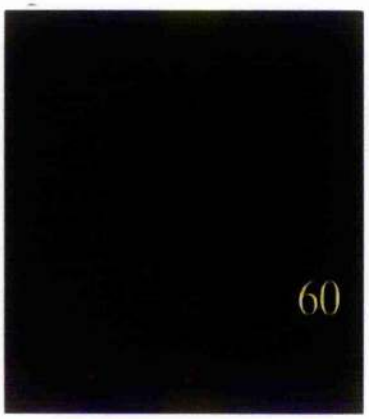

Figure 5.11

Figure 5.12

Figure 5.7 DAPI staining in a vein from a CAD patient; **Figure 5.8** Hydroethidine fluorescence in the same vein; **Figure 5.9** Merged images of Figure 5.7 and Figure 5.8; **Figure 5.10** DAPI staining in a vein from a control patient; **Figure 5.11** Hydroethidine fluorescence in the same vein; **Figure 5.12** Merged images of Figure 5.10 and Figure 5.11.

Bar in Figure 5.9 represents 100 μm. E indicates endothelium.

Numbers in the right bottom corner of the vessels in Figure 5.7, Figure 5.8, Figure 5.9, and Figure 5.10 represent average fluorescence on a scale of 0 to 256 over the area of the vessel whereas the numbers in Figure 5.9 and Figure 5.12 show the ratio of DAPI/hydroethidine fluorescence. The higher levels of superoxide production in patients with CAD are demonstrated using hydroethidine (Figure 5.8; Figure 5.9; Figure 5.11; Figure 5.12) and were observed throughout the vessel wall. In contrast to hydroethidine the intensity of DAPI staining was similar in vessel sections from CAD and control patients (Figure 5.7 and Figure 5.10).

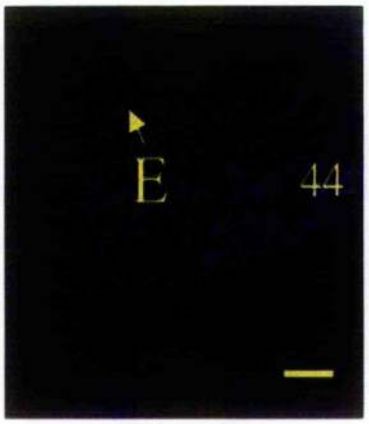

Figure 5.7

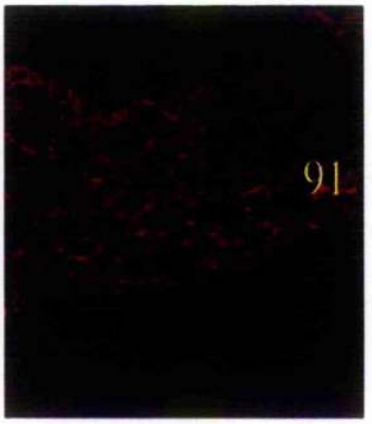

Figure 5.8

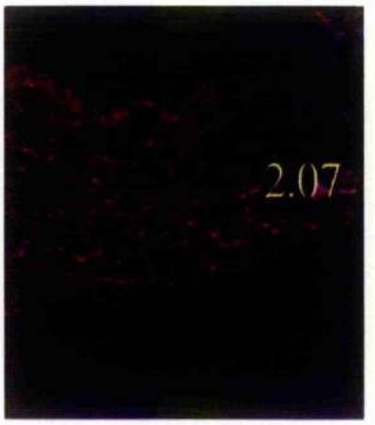

Figure 5.9

Figure 5.10

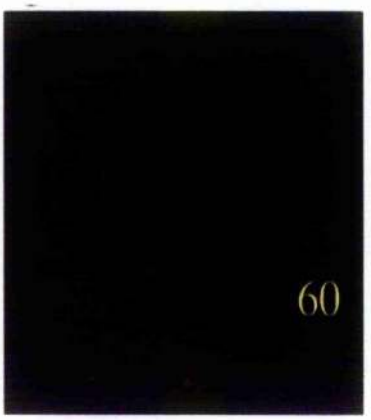

Figure 5.11

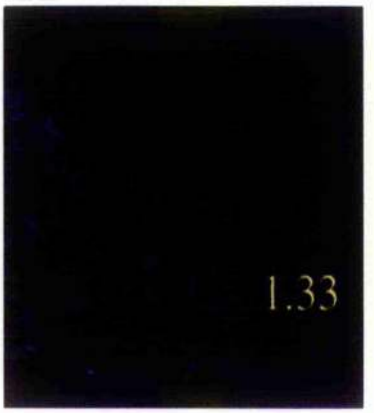

Figure 5.12

Figure 5.7 DAPI staining in a vein from a CAD patient; **Figure 5.8** Hydroethidine fluorescence in the same vein; **Figure 5.9** Merged images of Figure 5.7 and Figure 5.8; **Figure 5.10** DAPI staining in a vein from a control patient; **Figure 5.11** Hydroethidine fluorescence in the same vein; **Figure 5.12** Merged images of Figure 5.10 and Figure 5.11.

Bar in Figure 5.9 represents 100 μm. E indicates endothelium.

Numbers in the right bottom corner of the vessels in Figure 5.7, Figure 5.8, Figure 5.9, and Figure 5.10 represent average fluorescence on a scale of 0 to 256 over the area of the vessel whereas the numbers in Figure 5.9 and Figure 5.12 show the ratio of DAPI/hydroethidine fluorescence. The higher levels of superoxide production in patients with CAD are demonstrated using hydroethidine (Figure 5.8; Figure 5.9; Figure 5.11; Figure 5.12) and were observed throughout the vessel wall. In contrast to hydroethidine the intensity of DAPI staining was similar in vessel sections from CAD and control patients (Figure 5.7 and Figure 5.10).

5.3.4 Circulating indicators of oxidative stress

The levels of GSSG were significantly higher in blood from CAD compared with control patients (18 ± 3 versus 4 ± 1 μmol/L; P < 0.001) (Table 5.13 A), and the ratio of GSH/GSSG was lower (129 ± 31 versus 641 ± 115; p < 0.001) (Table 5.13 C). In contrast, levels of GSH did not differ between CAD and control patients (1127 ± 81 versus 1113 ± 49 μmol/L; p = 0.556) (Table 5.13 B). Consistent with these findings, the TAC was significantly reduced in plasma from the same group of CAD patients (1703 ± 57 versus 2012 ± 67 Cu^{2+}-reducing equivalents; p < 0.001) (Table 5.14).

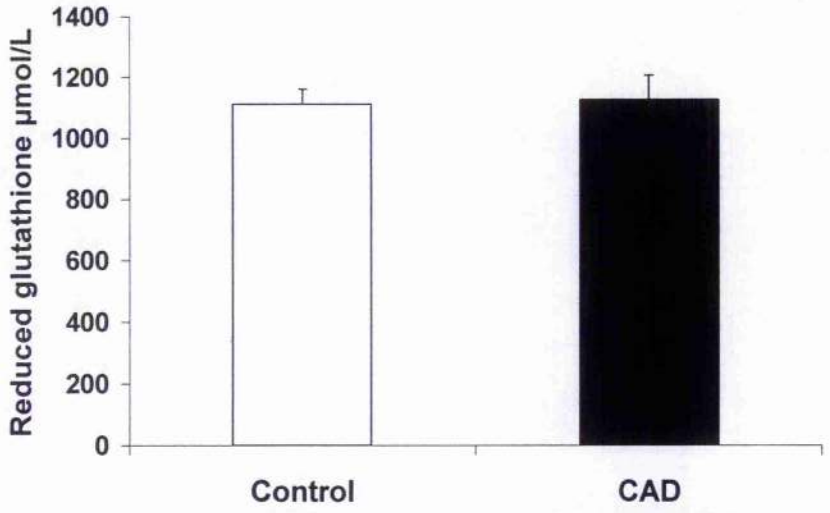

Figure 5.13 A Levels of reduced glutathione.

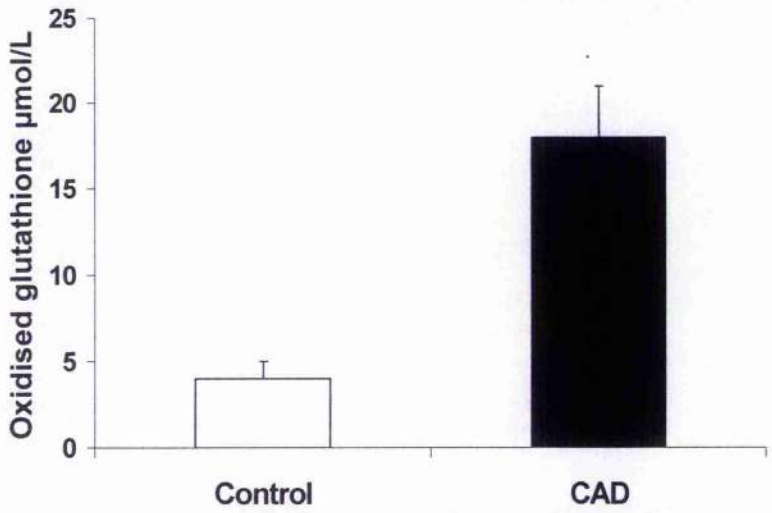

Figure 5.13 B Levels of oxidised glutathione.

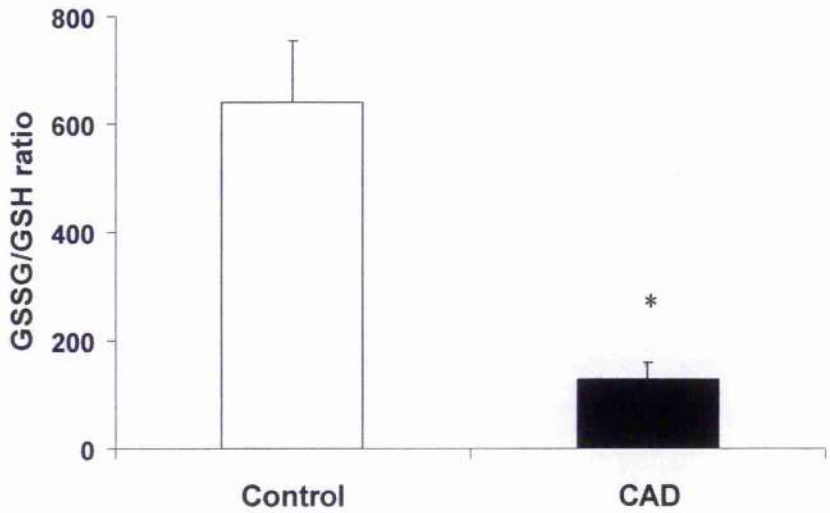

Figure 5.13 C Ratio of reduced to oxidised glutathione.

Figures 5.13 A-C Indicators of oxidative stress in whole blood from age and sex matched control and CAD subjects (n=51 for both patient groups).

□ Control patients

■ CAD patients

Results shown as mean ± SEM.

*$p<0.001$ when examined by unpaired t-tests.

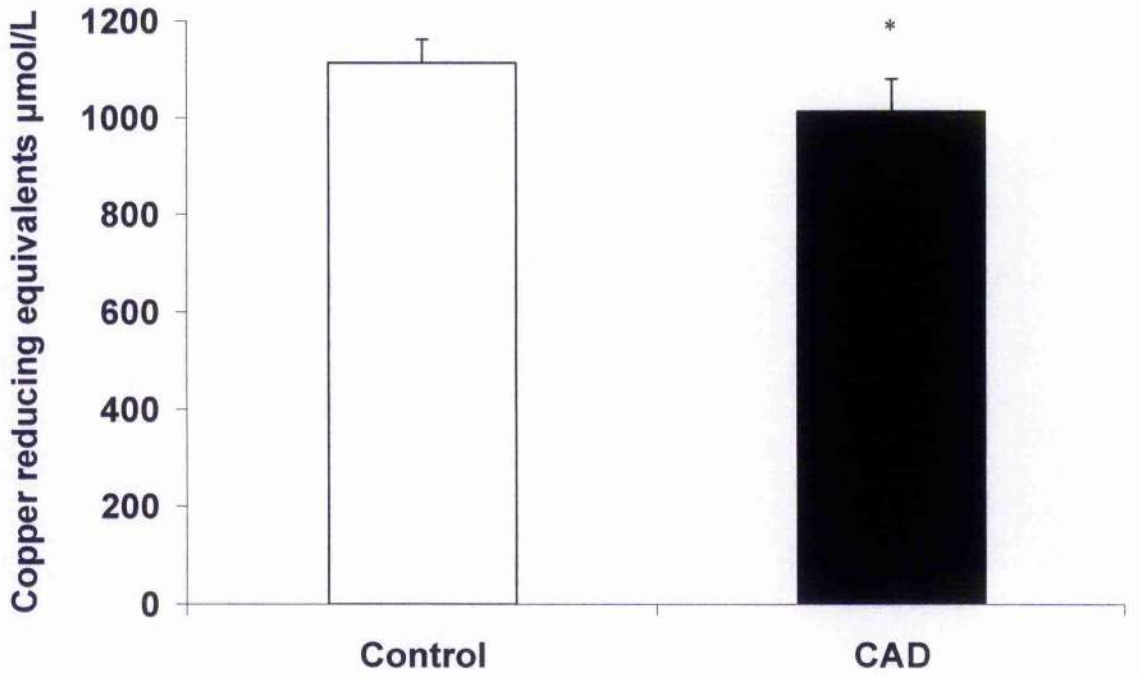

Figure 5.14 TAC measured as Cu^{2+} reducing equivalents in plasma from age and sex matched CAD and control patients (n=51 for both patient groups).

Control patients

CAD patients

Results shown as mean ± SEM.

*$p<0.001$ when examined by unpaired t-tests.

5.4 DISCUSSION

This study demonstrated contrasting levels of oxidative stress and endothelial function between patients with severe CAD and age- and sex-matched individuals with no documented CVD. This is the first study to assess endothelial function, superoxide production, and oxidative stress, not only in vessels used for revascularization in coronary artery bypass graft surgery, but also in the equivalent vessels from healthy control subjects.

The organ bath studies demonstrated that CAD subjects had significantly attenuated vasorelaxation to the NO-dependent endothelial vasodilator calcium ionophore A23187 that maximally stimulates endothelial NO synthase independent of any receptor-mediated pathway. In contrast, similar relaxations to the endothelial-independent NO donor sodium nitroprusside were observed when compared to the individuals with no documented CVD, suggesting a specific endothelial dysfunction in CAD subjects.

The studies performed do not allow an adequate explanation of the mechanism underlying endothelial dysfunction in CAD subjects. Endothelial dysfunction could be the result of either a diminished endothelial capacity to synthesize and release NO or an increased inactivation of NO after its synthesis. However, inhibition of superoxide production with allopurinol (xanthine oxidase inhibitor) and apocynin (NAD(P)H oxidase inhibitor) enhanced vasorelaxation in vessels from CAD subjects but had minimal effects in vessels from individuals with no documented CVD. This suggests that both xanthine oxidase and NAD(P)H oxidase contribute to excess superoxide production in CAD subjects. This excess superoxide may interact with NO reducing NO bioavailability and thus endothelial function.

Consistent with these observations, increased xanthine oxidase activity has been suggested in subjects with CAD (Harrison et al. 2003). In addition, allopurinol treatment has been reported to improve endothelial function in subjects with type 2 diabetes mellitus (Butler et al. 2000) and oxypurinol to improve forearm blood flow in hypercholesterolaemic subjects (Cardillo et al. 1997). No specific NAD(P)H oxidase inhibitors are licensed for use in man. However angiotensin II is known to upregulate NAD(P)H oxidase and increase superoxide production in blood vessels from animals and man (Touyz et al. 2005; Cruzado et al. 2005; Gao et al. 2005; Zuo et al. 2005; Erdos et al. 2005; Touyz et al. 2002; Berry et al. 2000; Rajagopalan et al. 1996a) and inhibition of the renin-angiotensin system by ACE inhibition and angiotensin receptor blockade has been shown to reduce superoxide production in blood vessels from CAD subjects (Berry et al. 2001).

This study is consistent with studies that demonstrated increased NAD(P)H oxidase activity is closely associated with the presence of cardiovascular risk factors and impaired endothelium-dependent vasodilatation in human arteries (Guzik et al. 2000a). In the latter study, diabetes mellitus and hypercholesterolaemia were independently associated with NAD(P)H-dependent superoxide production. In vessels from the animals with atherosclerotic disease up regulation of NAD(P)H oxidase in endothelial cells can occur (Zalba et al. 2001).

Of note, quantitative analyses of the contribution of NAD(P)H oxidase and xanthine oxidase to superoxide production are not possible from these experiments. Apocynin acts by blocking the assembly of the NAD(P)H oxidase complex (Ximenes et al. 2007; Barbieri et al. 2004). NOX4 has been shown to be the predominant homologue in vascular smooth muscle from conduit vessels (Lambeth 2004) NOX4 is constitutively active (Clempus and Griendling 2006; Cheng et al. 2006); thus,

apocynin may have low potency in the LSV and only inhibit a proportion of NAD(P)H oxidase activity.

Free radical generation and oxidative stress have been proposed as putative mechanisms of endothelial dysfunction. Healthy people are protected against free radicals by several defence mechanisms. Reduced GSH is the most important intracellular scavengers of free radicals (Kir et al. 2006). GSH serves as a reductant in oxidation reactions resulting in the formation of GSSG. Thereby decreased GSH levels and increased GSSG levels may reflect depletion of the antioxidant reserve (Halliwell and Gutteridge 1999). In particular, oxidative damage occurs when the delicate balance between pro- and antioxidants is alterated. In the body exist various antioxidant molecules to be used against free radicals injury. Among them, GSH and the enzyme and glutathione peroxidase are critical for maintaining the redox balance of the cell (Penckofer et al. 2002; Yu 1994). However, this balance may be destroyed by certain vascular features, such as by certain risk factors for atherosclerosis including hypertension, hyperlipidaemia, diabetes mellitus and cigarette smoking (Rojas et al. 2006; Wassmann et al. 2006; Yung et al. 2006; Maytin et al. 1999). Once oxidant stress is evoked, characteristic adverse pathological conditions, namely reduced vessel reactivity is established (Rojas et al. 2006; Wassmann et al. 2006; Yung et al. 2006).

The balance between pro-oxidants and antioxidants may depend on the oxidative stress evoked as well as the antioxidant defence system (Rojas et al. 2006; Wassmann et al. 2006; Yung et al. 2006). The simultaneous evaluation of the TAC together with GSH/GSSG ratio as undertaken here may represent the optimum approach for the evaluation of circulating indicators of oxidative stress in the field of CVD and may be useful when evaluating therapeutic interventions directed against

pathologic processes related to atherosclerosis. In the present study, CAD subjects showed not only enhanced GSSG concentration and a decrease in the GSH/GSSG ratio, but also significantly reduced TAC. This study is consistent with studies that revealed increased oxidative stress in hypertensive subjects (Simic et al. 2006; Redon et al. 2003).

The present findings demonstrate marked differences in vascular function and oxidative stress between the two discrete populations and defines for the first time endothelial function in conduit veins from a 'low risk' population. This study was not designed to examine in detail the relationship between oxidative stress and risk factors including pharmacological regimes but to compare levels of endothelial function and oxidative stress in a CAD population with those found in a 'normal' low risk population. The CAD and the varicose vein individuals with no documented CVD present two distinct populations. Each population is homogenous but they are discrete populations. There are extremely marked differences between the two groups; atherosclerotic disease is severe in the CAD subjects and is associated with many cardiovascular risk factors and atherosclerotic disease is minimal in the varicose vein individuals with no documented CVD who have no cardiovascular risk factors. Therefore detailed analysis of risk factors was not performed in this unsuitable group of subjects. In the next chapter detailed analysis of risk factors is performed in a homogenous group of 188 subjects undergoing CABG surgery.

Chapter 6

Relationship of LDL-cholesterol and circulating indicators of oxidative stress to calcium ionophore A23187, apocynin and allopurinol mediated relaxation of LSV obtained from CABG patients

6.1 INTRODUCTION

The work in chapter 5 demonstrated marked endothelium dysfunction and increased oxidative stress in CAD patients compared to varicose vein patients with no documented CVD matched for age and sex. This raised a number of questions:

1) What causes the deterioration in A23187 mediated relaxation of LSV from the CAD patients compared to the varicose vein patients with no documented CVD?

2) Is this marked deterioration in A23187 mediated relaxation of LSV due to increased oxidative stress?

3) What are the risk factors that contribute to this increased oxidative stress?

Circulating indicators of oxidative stress have been investigated in human patients with essential hypertension and in control subjects (Simic et al. 2006; Redon et al. 2003), but the relationship between these circulating indicators of oxidative stress and endothelial function was not examined. In addition, although the degree of endothelial function has been consistently linked to the number of risk factors present in patients with CAD, the relative importance of individual risk factors in determining levels of oxidative stress and endothelial function remains uncertain (Guzik et al. 2000; Huraux et al. 1999).

6.1.1 Hypothesis

Cardiovascular risk factors modify endothelial function.

6.1.2 Aim

To determine which study cardiovascular risk factors influence A23187 mediated relaxation of LSV in patients undergoing CABG.

6.1.3 Experimental Approach

1) Relaxation to calcium ionophore A23187 (endothelium dependent vasodilatator), sodium nitroprusside (endothelium independent vasodilatator), allopurinol (inhibitor of xanthine oxidase) and apocynin (reduced nicotinamide-adenine dinucleotide phosphate (NAD(P)H) oxidase inhibitor) will be determined in LSV from patients undergoing CABG.

2) To evaluate circulating oxidative stress indicators: the GSH/GSSG ratio and the TAC will be determined in blood from patients undergoing CABG and age- and sex-matched control patients with no documented CVD who were undergoing surgery for removal of varicose veins.

3) Cardiovascular risk factors (age, sex, body mass index, current active smoking, diabetes mellitus status, diastolic blood pressure, LDL-cholesterol) and current drug therapy will be recorded.

6.2 METHODS

6.2.1 Subjects

188 subjects undergoing elective CABG surgery were studied. A subset of 51 of these subjects were involved studied in chapter 5. All were outpatients recruited at the Cardiothoracic Unit at the Western Infirmary Glasgow. Patients undergoing CABG surgery had obstructive CAD demonstrated by coronary angiography. The study was approved by the local ethics committee and all patients gave informed written consent. Vein samples were collected at the time of surgery, stored in Krebs-HEPES buffer overnight and assayed the following day. Blood samples were collected 5-10 days prior to CABG surgery. Patients were fasted and rested supine for

30 min prior to donating blood. At the time of the blood sampling, all subjects gave a complete history which included cardiovascular risk factors such as smoking habits, hypertension, diabetes mellitus and dyslipidaemia. No patients were receiving antioxidant therapy and none had acute or chronic inflammatory diseases, immunological diseases and history or presence of neoplastic disease.

6.2.2 Vascular reactivity of veins

3 mm long rings of undistended LSV were studied in organ chambers, according to methods described in Chapter 2. Vessels were constricted with phenylephrine (3 μmol/L) and relaxations to calcium ionophore A23187 (0.01 - 10 μmol/L; endothelium dependent vasodilatator), sodium nitroprusside (0.01 - 10 μmol/L; endothelium independent vasodilatator), allopurinol (0.01 - 0.3 mmol/L; inhibitor of xanthine oxidase) or apocynin (0.01 - 0.3 mmol/L; NAD(P)H oxidase inhibitor) were examined. Relaxation was expressed as a percentage of the constriction to phenylephrine.

6.2.3 Circulating indicators of oxidative stress

Blood samples were collected from control and CAD subjects for determination of TAC, GSH, GSSG, GSH/GSSG molar ratio and cholesterol.

TAC was measured in plasma using a commercially available kit (AOP-490; Oxis International Inc.; Portland, Oregon, United States of America) based upon the reduction of Cu^{2+} to Cu^{+} as described in chapter 2.

The GSH/GSSG ratio was measured in whole blood using a commercially available kit (GSH/GSSG-412, Oxis International Inc.; Portland, Oregon, United States of America) as described in chapter 2. GSH reacts with Ellman's reagent to

form a spectrophotometrically detectable product at 412 mm. In a separate reaction GSSG is reduced to GSH which is then determined in the same manner.

6.2.4 LDL-cholesterol

The Bayer® ADVIA 1650 chemistry system with standard reagents (Bayer Diagnostics, Newbury, Berkshire, United Kingdom) was used to determine measure total cholesterol, HDL-cholesterol and triglycerides in plasma. The plasma total cholesterol concentration was measured by cholesterol esterase and cholesterol oxidase conversion, plasma triglyceride concentration by lipoprotein lipase/glycerol kinase enzymatic determination and HDL cholesterol measured cholesterol by polyethylene glycol-linked cholesterol esterase and oxidase after serum incubation with sulphated cyclodextrin buffer. Measurement of the total cholesterol, HDL cholesterol and triglyceride levels permitted the estimation of LDL-cholesterol concentrations, based upon the Friedwald formula (Friedwald et al. 1972).

6.2.4 Statistical analysis

To investigate which risk factors were related to vasorelaxation multiple linear regression analysis was performed using data from the 188 CAD patients and estimated effects, 95% confidence intervals (CI), and p values were tabulated. Multiple correlation coefficients were calculated as an overall measure of the relationship between risk factors and vasorelaxation. The varicose vein patients with no documented CVD had been selected in the previous study chapter to have contrasting clinical characteristics to the CAD patients and were therefore not included in the regression analysis. The multiple linear regression analysis included the following risk variables in the model: age in years; sex (0 = female, 1 = male);

diastolic blood pressure (mmHg); tobacco smoking status (0 = non-smokers and ex-smokers of more than 30 days, 1 = current smoker); diabetes mellitus (0 = non-diabetes mellitus, 1 = on antidiabetic medication); body mass index (kg/m^2); serum levels of LDL-cholesterol (mmol/L); statin therapy (0 = no therapy, 1 = on therapy) and ACE inhibitor / ARB therapy (0 = no therapy, 1 = on therapy). When risk factors were known to be interdependent, such as systolic and diastolic blood pressure or total cholesterol and LDL-cholesterol, only one risk factor was included in the analysis. Note that quantitative variables, for example smoking status, were included as factors in the analysis. Linear relationships have been assumed between all continuous variables and outcomes. Data not following normal distributions (TAC and GSH/GSSG ratio) were logarithmically transformed for this analysis. Coefficients and their confidence intervals for these 2 analyses are given in Tables 6.6 to 6.7 have been back transformed from the log scale, and they indicate the estimated proportional increase in the response associated with a unit increase in the predictor. The dependent parameter in the five multivariate linear regression analyses were vasorelaxation to 10 μmol/L calcium ionophore A23187, vasorelaxation to 0.3 mmol/L apocynin, vasorelaxation to 0.3 mmol/L allopurinol, GSH/GSSG ratio and TAC. Where appropriate, Pearson correlation coefficients and Spearman correlation coefficients are displayed. Statistical analysis was performed using the statistical package MINITAB, version 13.1 (Minitab Inc, State College, PA, United States of America). Results are shown as mean ± SEM and $p < 0.05$ was considered statistically significant.

6.3 RESULTS

6.3.1 Patient characteristics

Demographics and clinical characteristics of the patients are shown in Table 6.1. Vessels and blood from all subjects was available for study of circulating indicators of oxidative stress. This larger group of CAD patients showed a similar degree of oxidative stress and endothelial dysfunction to the sub-group matched to control patients (Table 6.2). The total CAD population contained a significantly higher proportion of males ($p < 0.001$) than the population selected for comparison with the patients undergoing varicose vein surgery in Chapter 5, but was comparable in all other respects.

6.3.2 Endothelium-dependent vasorelaxation to calcium ionophore A23187

The multivariate linear regression analysis identified LDL-cholesterol to be the only independent factor associated with A23187 mediated vasorelaxation to 10 μmol/L calcium ionophore A23187 in the CAD patients ($F=10.93$, $p=0.001$) (Table 6.3). There was an inverse relationship found between LDL-cholesterol and endothelium-dependent vasorelaxation to calcium ionophore A23187 ($r = -0.287$, $p = 0.001$) (Figure 6.1).

Age, sex, BMI, diastolic blood pressure, diabetic status, ACE inhibitor and statin therapy did not contribute to endothelium-dependent relaxation (Table 6.3). Overall the model was only able to account for 12.1% of the variation in relaxation (coefficient of determination) (Table 6.3). The Joint British Societies' Guidelines 2 recommend that LDL-cholesterol should be below 2.0mmol/L. (British Cardiac Society et al. 2005). The LDL-cholesterol is substantially elevated (mean 3.0 mmol/L

± SEM 0.1) in many of the CAD patients studied in this chapter despite 76% of them being on lipid lowering therapy with statins.

Table 6.1 General characteristics of the study population

	Total CAD
Number	188
Age (years)	58 ± 12
Sex M/F	135 / 53
Systolic Blood Pressure mmHg	139 ± 1.0
Diastolic Blood Pressure mmHg	83 ± 0.8
Total cholesterol (mmol/L)	4.8 ± 0.1
LDL-cholesterol (mmol/L)	3.0 ± 0.1
Triglycerides (mmol/L)	1.3 ± 0.1
Body Mass Index (kg/m^2)	29 ± 0.4
Diabetes Mellitus %	24
Smokers % (active / stopped / none)	38 / 38 / 24
NYHA heart failure score % (I / II / III / IV)	9 / 37 / 47 / 7
CCS angina score % (I/II/III/IV)	13 / 27 / 51 / 9
Prior MI % (no prior MI / 1 to 90 days from prior MI / > 90 days from prior MI)	56 / 10 / 34
ACE Inhibitors and/or ARBs %	46
Antiarrhythmic (Amiodarone or Digoxin) %	4
Aspirin %	80
Beta Blockers %	58
Calcium Channel Antagonists %	42
Clopidogrel %	10
Diuretic %	25
Nicorandil %	32
Nitrate %	30
Statins %	76

Results expressed as mean ± SEM.

Table 6.2 Comparison of vasorelaxation and circulating indicators of oxidative stress in full CAD group compared to CAD subgroup which was age and sex matched to the varicose vein group with no documented CVD in chapter 5.

	Total CAD Group	CAD subgroup	p
Number of patients	188	51	
% Relaxation calcium ionophore A23187 (10 μmol/L)	26.3 ± 1.0	26.2 ± 1.9	0.980
% Relaxation to sodium nitroprusside (10 μmol/L)	73.5 ± 1.8	76.8 ± 3.2	0.414
% Relaxation apocynin (0.3 mmol/L)	23.4 ± 1.2	23 ± 2.3	0.978
% Relaxation allopurinol (0.3 mmol/L)	25 ± 1.4	24 ± 1.8	0.816
TAC	1625 (1575;1677)	1662 (1575;1774)	0.521
GSH/GSSG ratio	91 (77;108)	83 (63;109)	0.587

Student's t-test was used to compare between the groups. TAC and GSH/GSSG were log transformed before Student's t-test was applied. Note the absence of any significant difference between the groups.

Data following normal distribution are expressed as mean ± SEM, otherwise geometric mean (95% CI of geometric mean) is given.

In this table it can been seen that the larger group of 188 CAD patients investigated this chapter showed a similar degree of oxidative stress and endothelial dysfunction to the 51 CAD patient sub-group matched to the varicose vein group with no documented CVD in chapter 5.

Table 6.3 Analysis of variance p-values for the null hypothesis of no relationship between risk factors and calcium ionophore A23187 mediated relaxation of LSV in CAD patients.

Variable	Calcium ionophore A23187 (n=134)	
	p	β coefficient (Confidence Intervals)
Age (10 years)	0.807	-0.26 (-2.4, 1.9)
Sex (0=F, 1=M)	0.876	-0.38 (-5.1, 4.4)
Body Mass Index (kg/m^2)	0.427	-0.19 (-0.66, 0.28)
Current Active Smoking (0=no, 1=yes)	0.789	0.68 (-4.3, 5.7)
Diabetes Mellitus (0=no, 1=yes)	0.436	-2.2 (-7.7, 3.3)
Diastolic Blood Pressure (10mmMg)	0.188	-1.3 (-3.3, 0.65)
LDL Cholesterol (mmol/L)	0.001	-4.7 (-7.6, -1.9)
ACE inhibitor / ARB (0=no, 1=yes)	0.401	-1.9 (-6.3, 2.5)
Statin (0=no, 1=yes)	0.937	-0.21 (-5.6, 5.1)

This analysis identified LDL cholesterol as a significant predictor of calcium ionophore A23187 mediated relaxation of LSV in the CAD patients (β coefficient, -4.7 per mmol/L; P=0.001). There was no evidence that age, sex, body mass index, current active smoking, diabetes mellitus status, diastolic blood pressure, ACE inhibitor / ARB therapy or statin therapy contributed to calcium ionophore A23187 mediated relaxation. Overall the model was only able to account for 12.1% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in calcium ionophore A23187 mediated relaxation with a unit increase in the predictor.

Table 6.4 Analysis of variance p-values for the null hypothesis of no relationship between risk factors and apocynin mediated relaxation of LSV in CAD patients.

Variable	Apocynin (n=142)	
	p	β coefficient (Confidence Intervals)
Age (10 years)	0.751	-0.43 (-3.2, 2.3)
Sex (0=F, 1=M)	0.461	2.3 (-3.8, 8.4)
Body Mass Index (kg/m^2)	0.234	0.35 (-0.24, 0.94)
Current Active Smoking (0=no, 1=yes)	0.357	-3 (-9.3, 3.3)
Diabetes Mellitus (0=no, 1=yes)	0.377	3.2 (-4.1, 11)
Diastolic Blood Pressure (10mmMg)	0.546	-0.77 (-3.3, 1.7)
LDL Cholesterol (mmol/L)	0.019	4.4 (0.64, 8.2)
ACE inhibitor / ARB (0=no, 1=yes)	0.433	2.2 (-3.3, 7.7)
Statin (0=no, 1=yes)	0.321	3.5 (-3.4, 10)

This analysis identified LDL cholesterol as a significant predictor of apocynin mediated relaxation of LSV in CAD patients (β coefficient, 4.4 per mmol/L; P=0.019). There was no evidence that age, sex, body mass index, current active smoking, diabetes mellitus status, diastolic blood pressure, ACE inhibitor / ARB therapy or statin therapy contributed to apocynin mediated relaxation. Overall the model was only able to account for 8.0% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in apocynin mediated relaxation with a unit increase in the predictor.

Table 6.5 **Analysis of variance p-values for the null hypothesis of no relationship between risk factors and allopurinol mediated relaxation of LSV in CAD patients.**

Variable	Allopurinol (n=142)	
	p	β coefficient (Confidence Intervals)
Age (10 years)	0.386	-0.94 (-3.1, 1.2)
Sex (0=F, 1=M)	0.190	3.2 (-1.7, 8.1)
Body Mass Index (kg/m^2)	0.186	-0.31 (-0.77, 0.15)
Current Active Smoking (0=no, 1=yes)	0.356	2.4 (-2.7, 7.5)
Diabetes Mellitus (0=no, 1=yes)	<0.0005	17 (11, 23)
Diastolic Blood Pressure (10mmMg)	0.947	-0.07 (-2, 1.9)
LDL Cholesterol (mmol/L)	0.326	-1.5 (-4.5, 1.5)
ACE inhibitor / ARB (0=no, 1=yes)	0.020	5.3 (0.55, 10)
Statin (0=no, 1=yes)	0.374	-2.5 (-8, 3)

This analysis identified diabetes mellitus (β coefficient, 17 per mmol/L; P<0.0005) and ACE inhibitor / ARB therapy (β coefficient, 5.3 per mmol/L; P=0.020) as significant predictors of allopurinol mediated relaxation of LSV in CAD patients. There was no evidence that age, sex, body mass index, current active smoking, diastolic blood pressure, LDL cholesterol or statin therapy contributed to allopurinol mediated relaxation. Overall the model was only able to account for 27.6% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in allopurinol mediated relaxation with a unit increase in the predictor.

Table 6.6 Analysis of variance p-values for the null hypothesis of no relationship between risk factors and GSH/GSSG ratio in CAD patients.

Variable	GSH/GSSG ratio (n= 108)	
	p	β coefficient (Confidence Intervals)
Age (10 years)	0.043	17.35 (0.12, 37.54)
Sex (0=F, 1=M)	0.576	10.52 (-22.68, 57.97)
Body Mass Index (kg/m²)	0.864	0.3 (-3.03, 3.74)
Current Active Smoking (0=no, 1=yes)	0.021	55.27 (6.5, 126.38)
Diabetes Mellitus (0=no, 1=yes)	0.437	17.35 (-22.64, 78.02)
Diastolic Blood Pressure (10mmMg)	0.005	24.61 (7.38, 44.6)
LDL Cholesterol (mmol/L)	<0.0005	-53.14 (-63.07, -40.54)
ACE inhibitor / ARB (0=no, 1=yes)	0.891	2.02 (-25.73, 40.15)
Statin (0=no, 1=yes)	0.871	-2.96 (-34.75, 44.32)

This analysis identified age (β coefficient, 17.35; P=0.043), current active smoking (β coefficient, 55.27; P=0.021), diastolic blood pressure (β coefficient, 24.61; P=0.005) and LDL cholesterol (β coefficient, -53.14; P<0.0005) as significant predictors of GSH/GSSG ratio in CAD patients. There was no evidence that sex, body mass index, diabetes mellitus or ACE inhibitor / ARB therapy contributed to GSH/GSSG ratio. Overall the model was only able to account for 36.0% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in GSH/GSSG ratio with a unit increase in the predictor.

Table 6.7 Analysis of variance p-values for the null hypothesis of no relationship between risk factors and TAC in CAD patients.

Variable	TAC (n = 142)	
	p	β coefficient (Confidence Intervals)
Age (10 years)	0.097	-2.37 (-5.23, 0.57)
Sex (0=F, 1=M)	0.447	-2.47 (-8.63, 4.11)
Body Mass Index (kg/m²)	0.039	-0.66 (-1.28, -0.03)
Current Active Smoking (0=no, 1=yes)	0.052	-6.57 (-12.65, -0.07)
Diabetes Mellitus (0=no, 1=yes)	0.142	-5.64 (-12.64, 1.93)
Diastolic Blood Pressure (10mmMg)	0.006	-3.73 (-6.43, -0.95)
LDL Cholesterol (mmol/L)	<0.0005	-12.28 (-15.68, -8.74)
ACE inhibitor / ARB (0=no, 1=yes)	0.174	4.19 (-1.82, 10.56)
Statin (0=no, 1=yes)	0.301	3.98 (-3.36, 11.87)

This analysis identified body mass index (β coefficient, -0.66; P=0.039), diastolic blood pressure (β coefficient, -3.73; P=0.006), and LDL cholesterol (β coefficient, -12.28; P<0.0005) as significant predictors of TAC in CAD patients. There was no evidence that age, sex, current active smoking, diabetes mellitus, ACE inhibitor / ARB therapy or statin therapy contributed to TAC. Overall the model was only able to account for 36.6% of the variation in relaxation (coefficient of determination).

The β coefficients indicate the estimated proportional increase in TAC with a unit increase in the predictor.

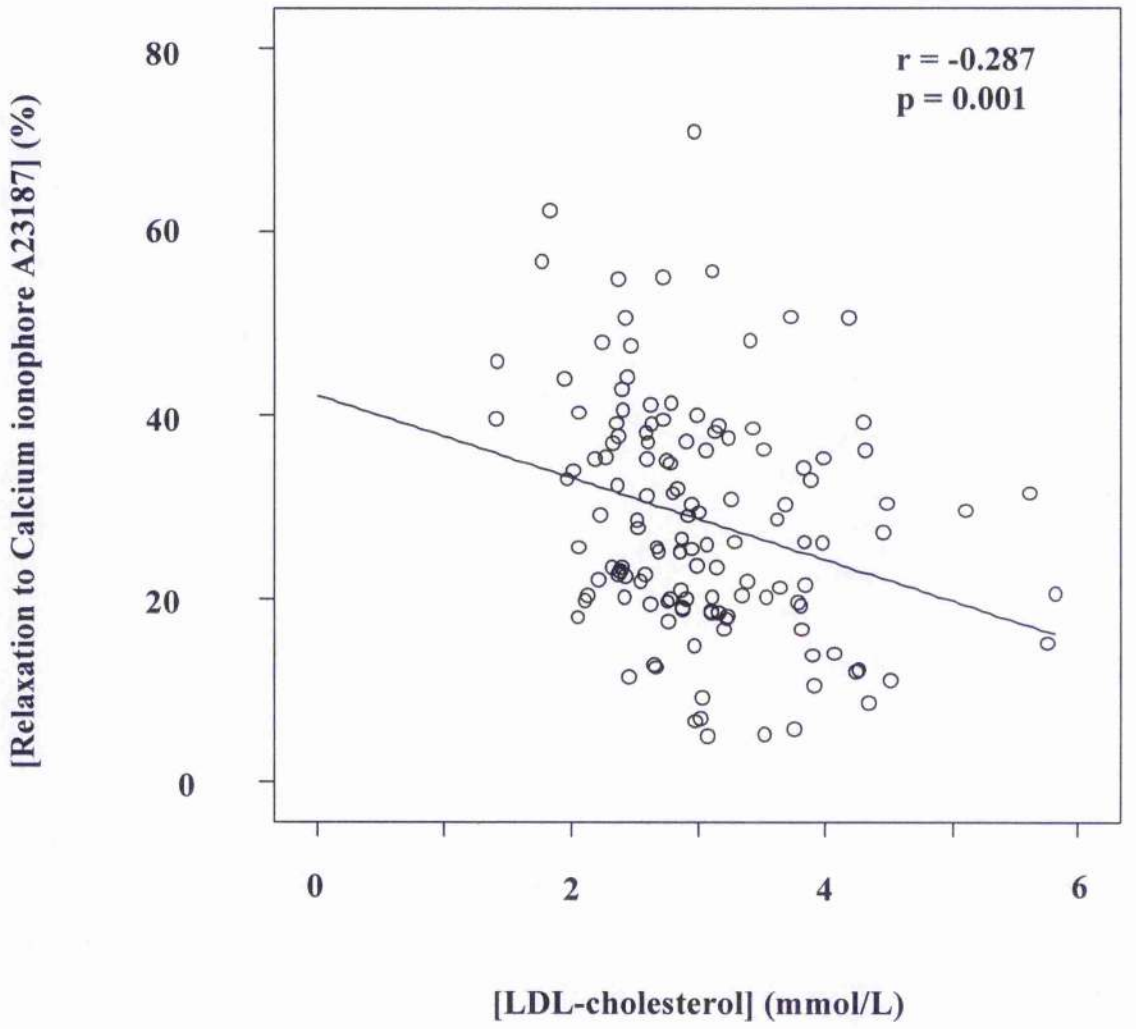

Figure 6.1 Scatter plots of LDL-cholesterol concentration and calcium ionophore A23187 mediated relaxation of LSV (n = 134). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows a significant correlation between LDL-cholesterol and calcium ionophore A23187 mediated relaxation of LSV (p = 0.001, r = -0.287)

6.3.3 Circulating indicators of oxidative stress

6.3.3.1 GSH / GSSG ratio

In a second model of multivariate linear regression analysis, in which log GSH/GSSG ratio represented the dependent parameter, LDL cholesterol again showed a strong association with log GSH/GSSG (Table 6.6). Log GSH/GSSG showed a highly significant independent inverse relationship with LDL-cholesterol ($r = -0.490$, $p < 0.0005$) (Figure 6.2). In addition the potential cardiovascular risk factors, age ($p = 0.043$), tobacco smoking ($p = 0.021$) and more strongly diastolic blood pressure ($p=0.005$) were related to log GSH/GSSG (Table 6.6). Overall the model was able to account for 36.0 % of the variation in relaxation (coefficient of determination) (Table 6.6).

6.3.3.2 TAC

In a third model of multivariate linear regression analysis, in which log TAC represented the dependent parameter, log TAC showed a strong association with LDL-cholesterol and showed a highly significant inverse relationship with LDL-cholesterol ($r = -0.514$, $p < 0.0005$) (Table 6.7; Figure 6.4). In addition the potential cardiovascular risk factors, body mass index ($p = 0.039$) and once again diastolic blood pressure ($p = 0.006$) represented risk factors for log TAC (Table 6.7; Figure 6.5). Tendencies to relationships between log TAC and age ($p = 0.097$) and log TAC and tobacco smoking ($p=0.052$) were observed although these were not statistically significant (Table 6.7). This model was able to account for 36.6 % of the variation in relaxation (coefficient of determination) (Table 6.7).

Although the multivariate analysis showed that both TAC and GSH/GSSG were strongly associated with diastolic blood pressure when direct correlations

between diastolic blood pressure and both log TAC and log GSH/GSSG ratio were performed neither log TAC nor log GSH/GSSG ratio showed a significant correlation with the potential cardiovascular risk factor diastolic blood pressure (Figure 6.6; Figure 6.7). Both circulating indicators of oxidative stress showed a strong association with diastolic blood pressure.

Linear regression analysis had shown LDL-cholesterol was the most important determinant followed by diastolic blood pressure for both circulating indicators of oxidative stress, however when direct correlations between the circulating indicators of oxidative stress were performed both log TAC and log GSH/GSSG ratio ($p = 0.163$) and TAC and GSH/GSSG ($p = 0.725$) failed to demonstrate a significant correlation (Figure 6.6; Figure 6.7).

Moreover although correlations between LDL-cholesterol and markers of endothelial function and oxidative stress were observed (Figure 6.1), the circulating indicators of oxidative stress did not correlate directly with endothelium-dependent relaxation to calcium ionophore A23187 (log TAC vs calcium ionophore A23187 $p=0.072$; log GSH/GSSG vs calcium ionophore A23187 $p=0.651$).

6.3.4 Endothelium-independent vasorelaxation to superoxide inhibitors

LDL cholesterol was also a significant predictor of relaxation to the NADPH oxidase inhibitor apocynin (β coefficient, –4.42 per mmol/L, $p=0.019$), although this was weaker than for other relationships (Table 6.4). No association between ACE inhibitor / ARB treatment and response to apocynin was demonstrated. No other factors included in the analysis had any effect on relaxation to apocynin.

In contrast to the other phenotypes examined, relaxation to the xanthine oxidase inhibitor allopurinol was not determined by LDL cholesterol but was very

significantly associated with the presence of diabetes mellitus (β coefficient, –17 with the presence of diabetes mellitus, $p < 0.0005$) (Table 6.5). Figure 6.8 demonstrates that in diabetic subjects, xanthine oxidase inhibition with allopurinol had a greater effect on the percentage of vasorelaxation than in nondiabetic subjects (35 ± 3 versus 21 ± 1 %; $p < 0.001$; 95 % CI, –20 % to –7 %). ACE inhibitor / ARB therapy was the only other variable that was related to the xanthine oxidase inhibitor allopurinol ($p = 0.020$) (Table 6.5).

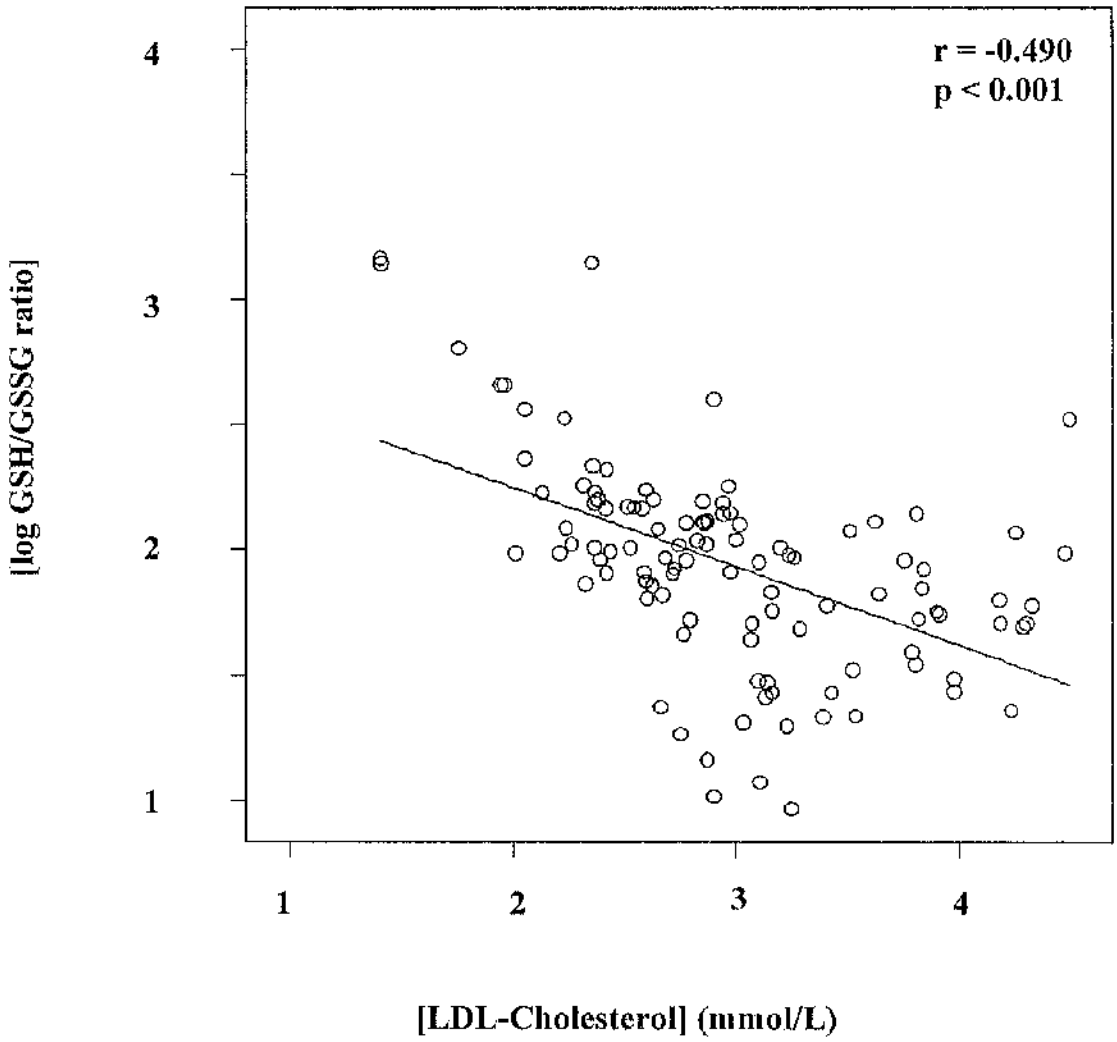

Figure 6.2 Scatter plots of LDL-cholesterol concentration and log GSH/GSSG ratio. (n = 108). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows a significant correlation between LDL-cholesterol and log GSH/GSSG ratio of LSV ($p < 0.001$, $r = -0.490$).

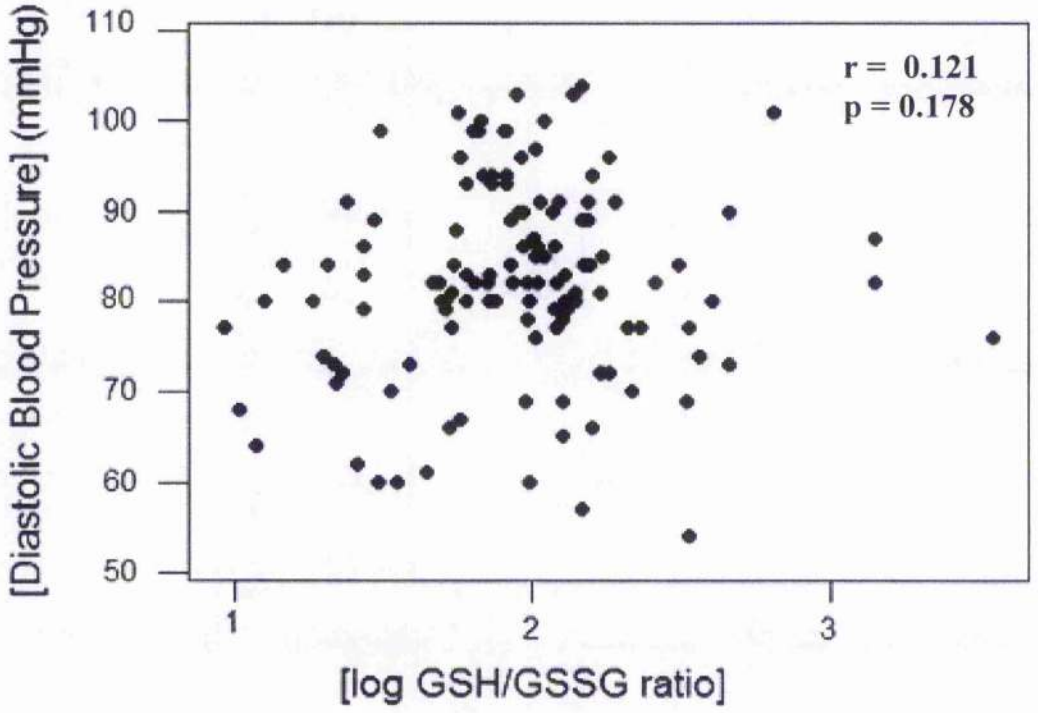

Figure 6.3 Scatter plots of log GSH/GSSH and diastolic blood pressure (mmHg) in the CAD patients (n = 108). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows no significant correlation between log GSH/GSSG ratio and diastolic blood pressure ($p = 0.178$, $r = 0.121$).

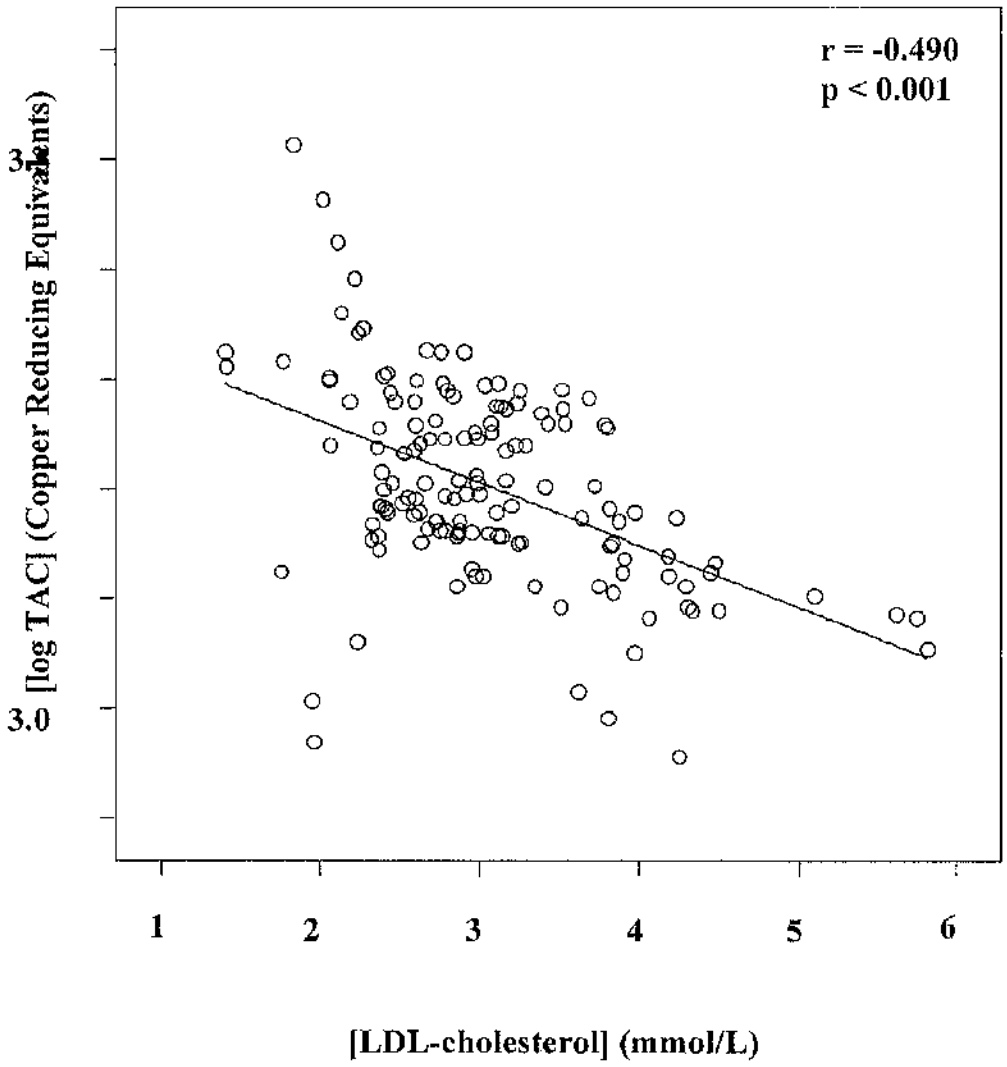

Figure 6.4 Scatter plots of LDL-cholesterol concentration and log TAC (Cu^{2+} reducing equivalents) (n = 142). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows a significant correlation between LDL-cholesterol concentration and log TAC ($p < 0.001$, $r = -0.490$).

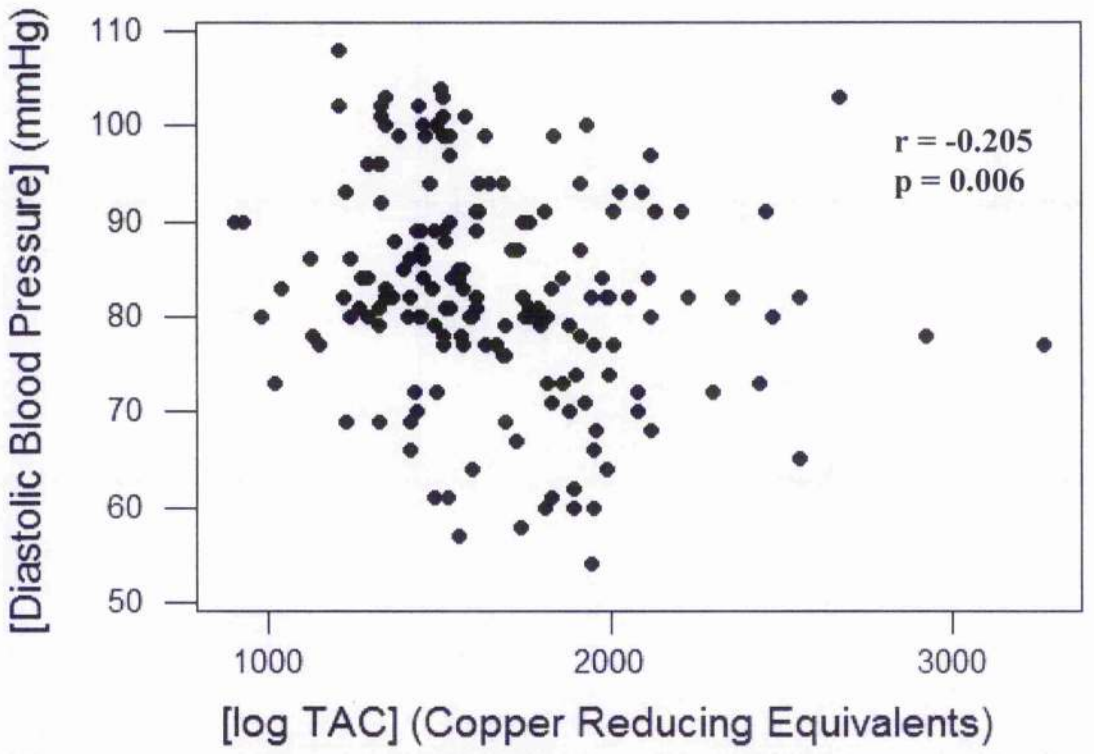

Figure 6.5 Scatter plots of TAC and diastolic blood pressure (mmHg) in the CAD patients (n = 142). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows a significant correlation between log TAC concentration and diastolic blood pressure (p = 0.006, r = -0.205).

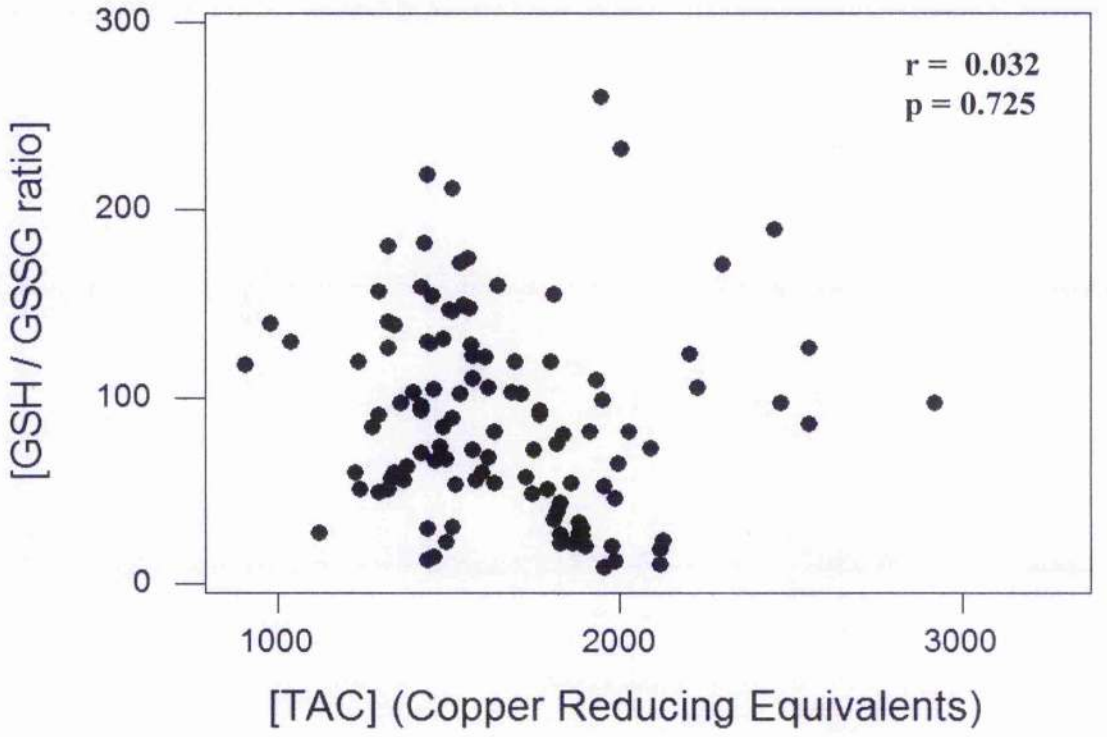

Figure 6.6 Scatter plots of TAC and GSH/GSSG ratio in the CAD patients (n = 108). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows no significant correlation between TAC and GSH/GSSG ratio (p = 0.725, r = 0.032).

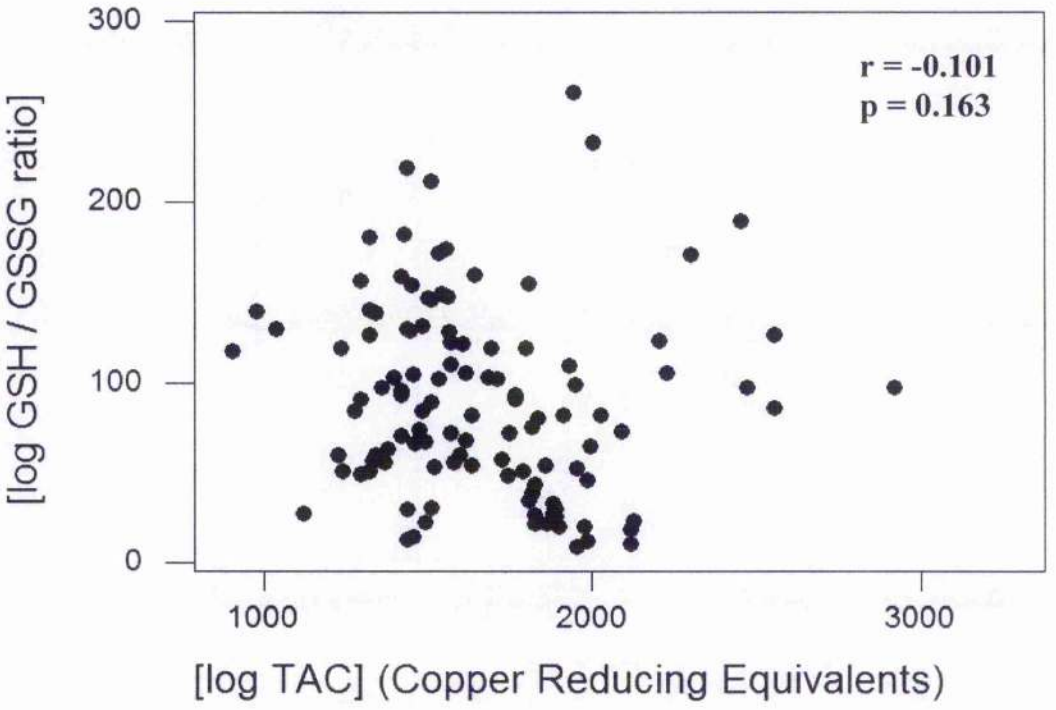

Figure 6.7 Scatter plots of log TAC and log GSH/GSSG ratio in the CAD patients. (n = 108). Pearson correlation coefficient and p value are shown in the figure. This scatter plot shows no significant correlation between log TAC and log GSH/GSSG ratio (p = 0.163, r = -0.101).

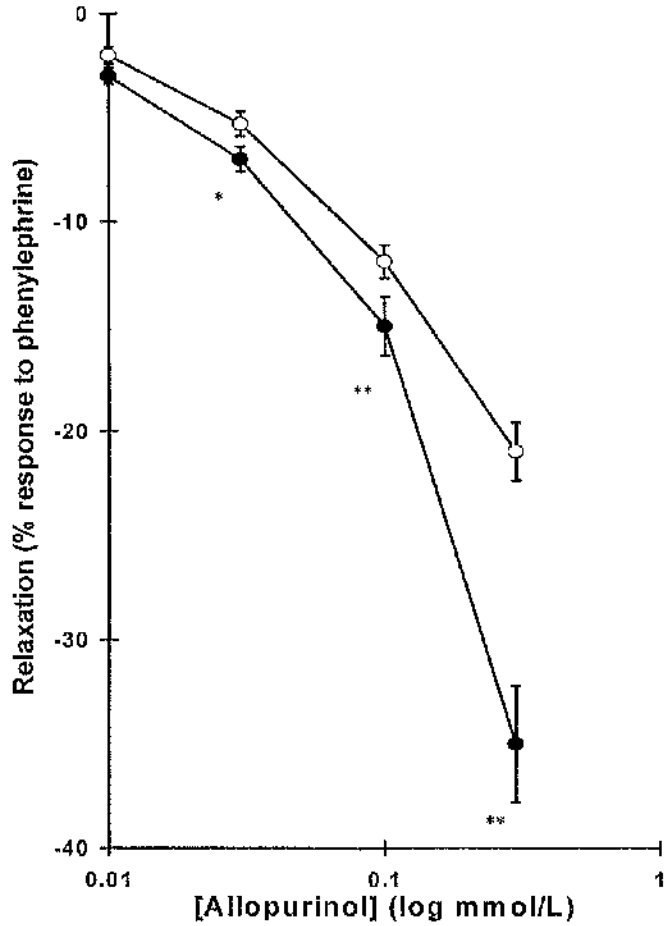

Figure 6.8 Vasorelaxation to allopurinol in rings of LSV from CAD patients with diabetes mellitus (n=45) and non-diabetic CAD patients (n=143). The x-axis is on a logarithmic scale. ○ Non-diabetic ● Diabetes mellitus Results are shown as mean ± SEM. Differences in relaxation between CAD and control patients were examined using unpaired t-tests.

* $p < 0.01$, ** $p < 0.001$.

In diabetic subjects, xanthine oxidase inhibition with allopurinol had a greater effect on the percentage of vasorelaxation than in nondiabetic subjects (35 ± 3 versus 21 ± 1 %; $p < 0.001$; 95 % CI, –20 % to - 7 %).

6.4 DISCUSSION

Data provided in this study showed that severity of LDL-cholesterol levels represents an independent factor for both endothelium-dependent vasorelaxation and circulating indicators of oxidative stress in patients with CAD. The 'oxidative modification hypothesis' of lipids is now supported by many lines of evidence, and it is well known that oxidized low density proteins (LDL) may contribute to the onset and progression of atherosclerotic lesions by numerous mechanisms, including its proinflammatory, immunogenic and cytotoxic actions (Steinberg and Witztum 2002). The role of lipoprotein oxidation in atherosclerosis is well established. A decreased resistance to oxidation of LDL (shortened lag phase) indicates an atherogenic nature of LDL (Holvoet et al. 2000; Ahotupa and Vasankari 1999) and is associated with an increased risk of atheromatosis (Regnström et al. 1992). Hypercholesterolemia has been shown to attenuate endothelial dependent vasorelaxation in numerous vascular beds including human coronary arteries (Quyyaimi et al. 1997) and forearm resistance vessels (Chowienczyk et al. 1992). In animal models of hypercholesterolaemia, attenuated endothelial function associated with increased superoxide production is observed (Müggе et al. 1994). Both native and oxidised LDL-cholesterol may cause uncoupling of eNOS and superoxide production in cultured endothelial cells (Vergani et al. 2000). In addition, components of oxLDL have been reported to stimulate superoxide production via NAD(P)H oxidase (Rueckschloss et al. 2001). Investigators demonstrated in a small series of hypercholesterolemic patients that lipid-lowering therapy reverses disturbed endothelium-dependent vasorelaxation in peripheral arteries and that endothelial dysfunction returns rapidly when hypercholesterolemia is restored (Stroes et al. 1995).

The Joint British Societies' guidelines on prevention of CVD in clinical practice recommend that the target for LDL-cholesterol should be under 2.0 mmol/L for patients with clinically established CVD and those considered at risk of developing the disease, including those with diabetes mellitus (British Cardiac Society et al. 2005). Only 4% (7/188) of CAD patients included in this study had a cholesterol in this recommended target range despite 76% of them being on statin therapy.

Although LDL-cholesterol showed significant association with both circulating indicators of oxidative stress and endothelial function there was no obvious relationship between either of the indicators of oxidative stress examined and endothelial function. Other factors including surgical preparation of vessels (Dashwood et al. 2007; Souza et al. 2006; Dashwood et al. 2005) and levels of oxygen radical degrading enzymes such as ecSOD and catalase within the vessel wall (Landmesser et al. 2004, Breaman et al. 2002) will have a major impact on endothelial function.

Both circulating indicators of oxidative stress also showed an association with blood pressure which was not observed for endothelial function. The reason for this is not clear. Guidelines produced by the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure recommend that the target for diastolic blood pressure should be under 75 mmHg for individuals. The guidelines also state that patients with a high cardiovascular risk profile can benefit from reducing blood pressure below the goal of diastolic blood pressure < 75 mmHg (Chobanian et al. 2003). Yet 80.3 % of these CAD patients have a diastolic blood pressure above this recommended target range despite 93.6 % of these CAD patients taking anti-hypertensive therapy.

LDL-cholesterol also influenced superoxide production via NAD(P)H oxidase as measured by relaxation to apocynin, although the significance of the association in this case was relatively low. NAD(P)H oxidase is the predominant source of superoxide in the human vasculature. Smoking, hypertension, hypercholesterolemia and diabetes mellitus have all been reported to upregulate this enzyme (Grindling et al. 2000) but showed no association in this study.

Tobacco smoking was only classified as yes or no; to control for smoking, total pack-years of smoking is a more accurate measure to incorporate all past smoking experience. One cigarette pack-year is equivalent to having smoked one pack or 20 cigarettes per day for an entire year. A dose related relationship between total amount (often expressed as "cigarette pack years") of cigarettes smoked and relaxation to apocynin would have been a better study and might have revealed an association with endothelial function and / or NAD(P)H oxidase derived superoxide.

Similarly as the CAD patients were on polypharmacy (Table 6.1) for diabetes mellitus and hypertension, this makes interpretation of the data in subjects with these disorders more difficult.

In contrast to the other analyses, LDL-cholesterol had no significant influence on superoxide production via xanthine oxidase as measured by relaxation to allopurinol, but activity of this enzyme was enhanced in diabetic subjects consistent with the positive effect of allopurinol on endothelial function in diabetics (Butler et al. 2000). Xanthine oxidase inhibition has also been suggested to have therapeutic potential in patients with heart failure (Berry and Hare 2004). ACE inhibitor therapy was the only other variable that was weakly associated with relaxation to the xanthine oxidase inhibitor allopurinol. There is evidence in the literature for interactions between xanthine oxidase, angiotensin converting enzyme and NAD(P)H oxidase

(Berry and Hare 2004; Usui et al. 1999). Allopurinol has been shown to prevent increases in ACE activity (Usui et al. 1999). Additionally, it has been stated that xanthine oxidase can generate superoxide via NAD(P)H oxidase activity and can produce NO via nitrate and nitrite reductase activities (Berry and Hare 2004).

There has been much interest recently in inhibition of vascular NAD(P)H oxidase as a means to reduce oxidative stress and progression of CVD (Cai et al. 2003, Jacobson et al. 2003). This data would indicate further research into the benefit of xanthine oxidase inhibition in diabetic subjects but most importantly this study highlights the role of LDL-cholesterol in modulating endothelial function and levels of oxidative stress and supports the mandatory use of cholesterol lowering drugs in patients with CAD. It was noted in the previous chapter that the CAD patients have higher cholesterol levels than the control patients. This may suggest that more vigorous treatment with cholesterol lowering therapy may be beneficial despite over three quarters of CAD patients already being on statin therapy.

Neither of the circulating indicators of oxidative stress were directly related to endothelial function but the simultaneous evaluation of the two circulating indicators of oxidative stress, TAC together and GSH/GSSG, may represent fundamental indicators for the evaluation of oxidative stress levels in the field of CVD and also constitute a potential tool to monitor therapeutic interventions and disease progression directed against pathologic processes related to atherosclerosis.

In conclusion, the most important finding in this study was the principal role of LDL-cholesterol in determining oxidative stress and endothelial dysfunction in CAD patients. This study supports vigorous intervention and therapy to reduce the severity of risk factors and oxidative stress both before and after CAD surgery.

Chapter 7

Discussion

Endothelial dysfunction is a common pathophysiological feature which develops in the evolution of CVD (Simonescu 2007). Strategies to maintain a healthy endothelium or to reverse endothelial dysfunction are crucial for the normal function of the cardiovascular system and the maintenance of cardiovascular health (Simonescu 2007). Endothelial dysfunction is observed both in the coronary and peripheral vasculature (Drexler 1997).

The main objective of this thesis was to study the effect of harvesting techniques and cardiovascular risk factors on endothelial function of human coronary artery bypass grafts.

The chapter 3 study found no significant difference in calcium ionophore A23187 mediated vasorelaxation or sodium nitroprusside mediated vasorelaxation in LSV harvested by traditional vein open harvesting and minimally invasive techniques. These results demonstrate no evidence that the increased manipulation of the LSV, consequent upon using the Mayo stripper, affected endothelial function any more than the traditional open harvest method. In order to eliminate the effect of surgical expertise, the study used 2 very experienced surgeons who were equally comfortable with either technique to carry out all of the vein harvesting. This study supports the notion that harvesting vein through multiple incisions using the Mayo vein stripper is quicker, results in better wound healing and has no more deleterious effect on endothelial function than the open technique.

This study has several noteworthy limitations that might influence interpretation of results. The number of patients, and therefore the number of LSV samples, is relatively small, and statistical power is thereby limited. Although a type II error may be present with the small sample size in the present study, the similarity in the two groups suggests a larger sample size would, at most, only detect a small

difference that, although interesting, may not be clinically relevant. Also, sampling only two rings from harvested veins may miss areas of injury at other sites within the vein. For long-term quality control, post-operative assessment of graft patency by angiography may give further insight into possible differences between traditional open and minimally invasive harvesting techniques.

Although the main focus of the chapter 3 study was into the effect of harvesting techniques on NO-dependent endothelial function, there are other considerations to the study that should be addressed. As the Mayo vein stripper requires dissection intimate to the adventitial layer to visualize the side branches, the adventitia and the surrounding tissue was removed. The adventitia is also removed during TOVH (Dashwood and Loesch 2007). This measure may play an important part in the subsequent processes involved in endothelial dysfunction and graft occlusion, because the adventitia does not merely provide structural support for the media, but contains the vasa vasorum, a microvascular network responsible for the exchange of gases and supply of nutrients to the vein wall (Dashwood et al. 2007). The preservation of the perivascular tissue around the LSV might be important as a natural external support (Souza et al. 2006), thereby protecting the vein against the effects of aortic shear stress (Vijayan et al. 2004; Tsui and Dashwood 2002), and has been associated with improved graft patency (Souza et al. 2006). It has been proposed that by reducing vascular injury using atraumatic surgical techniques, NO-dependent endothelial function is maintained and that this will result in improved graft performance (Dashwood et al. 2007; Dashwood et al. 2005; Tsui et al. 2002). Preservation of endothelial function of LSV grafts used in CABG surgery is an important determinant of patency up to 18 months postoperatively (Cook et al. 2004). It would be interesting to repeat this study incorporating the "Souza no-touch

technique" (Souza et al. 2006) to compare calcium ionophore A23187 mediated relaxation in LSV harvested with perivascular tissue by traditional open and minimally invasive techniques.

The aim of the chapter 4 was to determine the effect of IMA pedicle width on endothelium-dependent vasorelaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG. This study demonstrated the predominant role of IMA pedicle width in determining calcium ionophore A23187 mediated relaxation of IMA harvested by monopolar electrocautery from patients undergoing CABG. This study demonstrated that a wider IMA pedicle harvested with monopolar electrocautery better preserves the NO-dependent endothelial function of the IMA. Although the main focus of this study was into the effect of pedicle width on NO-dependent endothelial-function, there are other potential sources of NO that are maintained with IMA pedicles (Gao et al. 2005). A recent study described the release of a transferable relaxing factor from the perivascular adipose tissue surrounding the human IMA and suggested that its retention when harvesting the IMA may play an important role in the superior patency rate of the IMA (Gao et al. 2005). To prove that IMA harvested with a wider pedicle ameliorates survival, will be difficult because of the low sensitivity of clinical endpoints and the good results that are available with the use of normal harvested IMA (Rosamund et al. 2007; Cho et al. 2006; Eagle et al. 2004). Long-term follow-up including angiographic assessment of the patients may add valuable data regarding outcome with regard to this harvesting technique. The Western Infirmary Glasgow on behalf of the National Adult Cardiac Surgical Database prospectively follows up their CABG patients and five year freedom-from-reintervention and mortality data will be available to further investigate the influence of pedicle width on freedom-from-reintervention and survival.

Damage to segments of the LSV and IMA during harvesting as a graft has been described as inevitable (Dashwood and Loesch 2007; Gaudino et al. 2003) and a recent publication stated "...as basic research scientists, we are surprised by the degree of damage inflicted on the saphenous vein during conventional bypass surgery...It seems, when harvesting this vessel as a graft, many surgeons are in effect preparing a "tube" or "pipe" (common definitions of conduit) with no regard to its many vital vascular structures..." (Dashwood and Loesch 2007).

Maintaining structural and functional integrity of the endothelium of the graft is an important feature of any procurement technique (Dashwood and Loesch 2007; Dashwood et al. 2007; Dashwood et al. 2005; Ueda et al. 2003; Thatte and Khuri 2001). There are many factors that contribute to endothelial dysfunction in grafts for CABG (Hinokiyama et al. 2006; Barker et al. 1994; Lawrie et al. 1990; Angelini et al. 1989). Such factors may be categorized as unavoidable and avoidable. Unavoidable factors include the exposure of the vein to arterial pressure. However, some factors such as high-pressure distention, stripping the adventitia (Dashwood et al 2007; Souza et al. 2006) and the use of scissors to avoid electrocautery heat injury (Deja et al. 2005) are avoidable. Relatively simple technical changes aimed at preserving the integrity of endothelial function and preventing endothelial dysfunction may have considerable impact on graft patency (Dashwood and Loesch 2007; Hinokiyama et al. 2006; Ueda et al. 2003; Cunningham 1996).

The Chapter 5 studies demonstrated contrasting levels of oxidative stress and endothelial function between patients with severe CAD and age- and sex-matched individuals with no documented CVD. This is the first study to assess endothelial function, superoxide production, and oxidative stress, not only in vessels used for revascularisation in CABG surgery, but also in the equivalent vessels from healthy

control subjects. Moreover, the predominant role of LDL cholesterol in determining oxidative stress and endothelial dysfunction in CAD patients has been demonstrated

The studies showed severe depression of relaxation to calcium ionophore A23187 in veins from CAD patients despite normal responses to sodium nitroprusside, consistent with a specific defect in endothelium-dependent nitric oxide pathways. Superoxide levels, measured directly within the vessel wall using 2 methods, were elevated in blood vessels from CAD compared with control patients. In addition, in the organ bath studies, inhibition of xanthine oxidase and NADPH oxidase with allopurinol and apocynin, respectively, caused significantly greater relaxation in vessels from CAD compared with control patients. These findings indicate excess superoxide production as an important cause of the attenuation of endothelium-dependent relaxations in CAD patients and suggest that both xanthine oxidase and NADPH oxidase contribute to superoxide production in these patients. Consistent with these observations, increased NADPH oxidase and xanthine oxidase activity has been suggested in patients with CAD (Landmesser et al. 2007; Guzik et al. 2006; Simic et al. 2006; Redon et al. 2003; Spiekermann et al. 2003), and inhibitors of these enzymes have been shown to inhibit superoxide production in LSV from CAD patients (Berry et al. 2000). Of note, quantitative analyses of the contribution of nicotinamide-adenine dinucleotide phosphate oxidase and xanthine oxidase to superoxide production are not possible from these experiments. Apocynin acts by blocking the assembly of the NADPH oxidase complex (Bedard and Krause 2007). NOX4 has been shown to be the predominant homologue in vascular smooth muscle from conduit vessels (Lambeth et al. 2004). NOX4 is constitutively active (Bedard and Krause 2007); thus, apocynin may have low potency in the LSV and only inhibit a proportion of NAD(P)H oxidase activity.

The degree of endothelial dysfunction has been reported to be related to the number of risk factors present in CAD patients (Guzik et al. 2000; Huraux et al. 1999). However, Huraux et al. (1999) failed to show any relationship between superoxide levels and endothelial dysfunction in IMA from a group of 97 CAD patients, whereas Redon et al. (2003) reported that no relationship was observed between 24-hour mean blood pressure and reduced/oxidized glutathione ratio in hypertensive subjects.

The findings in chapter 6 show a significant relationship between LDL cholesterol levels and both endothelium-dependent vasorelaxation and markers of oxidative stress in patients with CAD. Hypercholesterolemia has been shown to attenuate endothelium-dependent vasorelaxation in numerous vascular beds, including human coronary arteries (Quyyumi et al. 1997) and forearm resistance vessels (Chowienczyk et al. 1992). In animal models of hypercholesterolemia, attenuated endothelial function associated with increased superoxide production is observed (Mugge et al. 1994). The studies performed in this thesis do not provide a mechanistic explanation for the relationship between LDL cholesterol and superoxide generation. However, LDL cholesterol and oxidized LDL cholesterol have been shown to affect the trafficking of eNOS to caveolae (Shaul 2002). Both native and oxidized LDL cholesterol may cause an uncoupling of eNOS, resulting in superoxide production in endothelial cells (Vergnani et al. 2000). In addition, components of oxidized LDL have been reported to stimulate superoxide production via NADPH oxidase (Rueckschloss et al. 2001). Lipid-lowering therapy with statins has been demonstrated to improve endothelial function (Fichtlscherer et al. 2006; John et al. 1998).

In the chapter 6, the relationship between LDL cholesterol and endothelial dysfunction and oxidative stress was maintained across the whole range of LDL

cholesterol concentrations. These results are consistent with those from recently published studies, which showed that intensive lipid-lowering therapy in patients with stable CAD reduced the occurrence of major cardiovascular events (Larosa et al. 2005; Nissen et al. 2005; Cannon et al. 2004) and reduced the progression of atherosclerosis (Nissen et al. 2004). Significantly fewer events have been observed in patients in whom LDL cholesterol levels were 2.0 mmol/L compared with those with a mean LDL cholesterol of 2.6 mmol/L (Larosa et al. 2005).

Although LDL cholesterol was a significant predictor of both markers of oxidative stress and endothelial function in our study, there was no obvious relationship between either of the circulating markers of oxidative stress examined and endothelial function. Other factors, including surgical preparation of vessels (Dashwood et al. 2007; Dashwood and Loesch. 2007; Hinokiyama et al. 2006; Souza et al. 2006; Dashwood et al. 2005) and levels of oxygen radical degrading enzymes, such as extracellular superoxide dismutase and catalase within the vessel wall, will have a major impact on endothelial function (Landmesser et al. 2004; Brennan et al. 2002). In addition, C-reactive protein has been reported to influence endothelial function (Clapp et al. 2005; Fichtlscherer et al. 2000). CRP renders oxidized LDL more susceptible to uptake by macrophages, induces the expression of vascular-cell adhesion molecules, stimulates the production of tissue factor, and impairs the production of nitric oxide (Verma et al. 2002; Torzewski et al. 2000; Cermak et al. 1993; Zouki et al. 1997). We cannot rule out a contribution of C-reactive protein to endothelial function in addition to that of LDL cholesterol. Interestingly, both the reduced/oxidized glutathione ratio and total antioxidant capacity showed an association with blood pressure that was not observed for endothelial function.

LDL cholesterol was also related to superoxide production via NADPH oxidase as measured by relaxation to apocynin, although at a lower level of significance. NADPH oxidase is the predominant source of superoxide in the human vasculature. (Miller et al. 2005; Griendling 2004; Touyz et al. 2004; Bengtsson et al. 2003; Cai et al. 2003) Smoking, hypertension, hypercholesterolemia, and diabetes have all been reported to upregulate this enzyme (Griendling 2004; Griendling et al. 2000). In contrast, LDL cholesterol had no influence on the production of superoxide from xanthine oxidase, but superoxide production via this enzyme was significantly enhanced in diabetic subjects, consistent with the positive effect of allopurinol on endothelial function in patients with diabetes (Butler et al. 2000). ACE inhibitor / ARB therapy was related to superoxide production via xanthine oxidase as measured by relaxation to allopurinol. A recent study demonstrated that angiotensin II–dependent stimulation of endothelial xanthine oxidase activity plays a major role for increased endothelium bound xanthine oxidase activity in patients with CAD (Landmesser et al. 2007). Xanthine oxidase inhibition has been suggested to have therapeutic potential in patients with heart failure (Berry and Hare 2004).

There has been much interest recently in the inhibition of vascular NADPH oxidase as a means to reduce oxidative stress and progression of CVD (Guzik and Harrison 2006; Miller et al. 2005; Calo et al. 2005). Our data indicate that there is a significant relationship between LDL cholesterol and vascular oxidative stress across the entire range of LDL cholesterol concentrations. This provides a mechanistic explanation in support of intensive LDL cholesterol-lowering therapy as suggested by recent clinical trials.

In conclusion, this thesis proposes that a multifactorial strategy aimed at prevention of endothelial dysfunction, should include improved surgical techniques

and the use of specific pharmacologic agents, including LDL cholesterol-lowering therapies, as a form of intervention. The successful application of this strategy, and the development of newer strategies, including the use of new pharmacological agents (Lorusso et al 2006), gene therapy (George et al. 2006; Baker et al. 2006) and the use of mechanical devices (Jeremy et al. 2007), may impact on long-term graft patency.

Chapter 8

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