Intimal Hyperplasia in Human Long Saphenous Vein: The Effects of Statins

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Thank you all.

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1. Pre-operative ischaemia of the long saphenous vein predisposes to intimal hyperplasia in bypass grafts through enhanced smooth muscle cell migration.

Ruiz, M Carmen ;Orr, DJ ;Teenan, RP; Wadsworth, RM.

British Journal of Surgery. 93(3):379, March 2006.

> Winner of the British Journal of Surgery Prize for the Best Scientific Paper.
>
> Oral Presentation at the Vascular Society of Great Britain & Ireland Annual General Meeting, Bournemouth, November 2005.

2. The influence of statin therapy on infrainguinal vein bypass graft patency.

Ruiz, M Carmen; Teenan, RP; Orr, DJ.

[Abstract] British Journal of Surgery. 93 Supplement S1:123, September 2006.

3. The influence of pre-operative ischaemia on the development of intimal hyperplasia in vein bypass grafts is mediated through enhanced smooth muscle cell migration.

Ruiz, M Carmen ;Orr, DJ ;Teenan, RP; Wadsworth, RM.

Poster Presentation at the 16th European Chapter Congress of the International Union of Angiology, Eurochap. October 2005.

Summary

The overall aims of the study were: to establish whether long saphenous veins from ischaemic limbs were more susceptible to developing intimal hyperplasia in organ culture compared with veins from non-ischaemic limbs; to investigate the effect of simvastatin on this process; to compare the proliferation and migration of vascular smooth muscle cells explanted from ischaemic and non-ischaemic veins and the effect of simvastatin on these processes; and to audit the outcomes of all infrainguinal vein bypass grafts performed at Glasgow Royal Infirmary between 1993-2003, correlating graft occlusion with statin use.

Veins were obtained from two patient groups: those undergoing lower limb amputation due to severe ischaemia- ischaemic veins; and those undergoing coronary artery bypass grafting using the long saphenous vein- non-ischaemic veins. These were placed in organ culture for 14 days in either culture medium with growth factors alone or culture medium with growth factors and simvastatin. At the end of this period, vein rings were fixed and processed for histological analysis and the area of Tunica Intima was calculated using Image Analysis software. Vascular smooth muscle cells were explanted from the vein rings and grown on for use in proliferation and migration assays. Cell proliferation was assayed using a ^{3}H labelled Thymidine incorporation technique. Cell migration was assayed using a modified Boyden chamber technique.

Veins from both ischaemic and non-ischaemic limbs had the same amount of vessel area as Tunica Intima in the fresh fixed state. Veins from both groups showed significant growth of the intimal layer following 14 days in culture. Veins from the ischaemic limbs developed significantly more intimal growth compared with the veins from non-ischaemic limbs. Simvastatin abolished the growth of intima in the veins from ischaemic limbs and reduced the growth of intima in veins from non-ischaemic limbs. The proliferation of vascular smooth muscle cells from both ischaemic and

non-ischaemic veins was inhibited in a dose-dependent manner by simvastatin. There was no difference in the mean 50% inhibitory concentration of simvastatin for the two groups of vein cells. Vascular smooth muscle cell migration in response to platelet-derived growth factor was enhanced in cells from the ischaemic veins compared with cells from the non-ischaemic veins. Simvastatin appeared to inhibit vascular smooth muscle cell migration.

Between 1993-2003, 207 infrainguinal vein bypass grafts were created in Glasgow Royal Infirmary. Data was available for 133 of these grafts. The graft patency in patients taking a statin at 1,3 and 5 years was 97%, 91% and 83% compared with 88%, 72% and 56% for patients not taking a statin. The median survival of grafts was 9.8 years for those taking a statin and 6.7 years for those not taking a statin. These differences approach statistical significance. Additionally, female gender was associated with increased risk of graft occlusion.

In conclusion, ischaemia predisposes the long saphenous vein to develop intimal hyperplasia in organ culture and simvastatin abolishes this process. This tendency to develop intimal hyperplasia may be partly explained by the finding that vascular smooth muscle cells from ischaemic veins have an accelerated migration response in cell culture compared with cells from non-ischaemic veins. Simvastatin inhibits vascular smooth muscle cell proliferation and migration *in vitro* and this can explain the effect of simvastatin in organ culture. These findings raise the possibility of using simvastatin to prevent intimal hyperplasia in infrainguinal vein grafts. The concentration of simvastatin used in these experiments, however, was much higher than can be attained with normal oral doses and, as such, may lead to the development of novel drug delivery techniques such as drug eluting sutures. Clinically, simvastatin has been associated with improved infrainguinal graft patency. How this relates to the *in vitro* findings here is not clear but is likely to involve more than the known anti-proliferative and anti-migratory effects of statins.

Chapter 1

INTRODUCTION

1.1 Atherosclerosis

1.1.1 General aspects of atherosclerosis

Atherosclerosis is a multifactorial, chronic, systemic disease process which causes significant morbidity and mortality worldwide. The disease is known to begin in childhood with early changes to major blood vessels having been identified during post-mortem examinations of young people. The process progresses gradually, however, and symptoms usually manifest clinically in middle age (1). The pathological lesion in atherosclerosis is the atherosclerotic plaque. These are lipid-rich areas within the vessel wall which are infiltrated by inflammatory monocytes and contain proliferating smooth muscle cells and fibroblasts as well as areas of collagen and lipid deposition. The presence of the atherosclerotic plaque reduces the luminal diameter of the affected vessel, thereby impairing the blood supply to the tissues supplied by that artery. Additionally, plaques are capped by a fibrocalcific layer which, if breached, exposes the flowing blood to highly thrombogenic material and leads to thrombosis of the vessel. This can either reduce the luminal diameter of the vessel further or lead to complete vessel thrombosis and infarction of distal tissues. Clinically, this manifests as myocardial ischaemia if the coronary vessels are affected, cerebral ischaemia if the cerebral vessels are affected and lower limb ischaemia if the circulation to the lower limbs is affected. Most research into the process of atherosclerosis is in relation to cardiac and cerebrovascular disease. However, the same process is the cause of lower limb ischaemia.

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1.1.2 Cellular Aspects of Atherosclerosis

Atherosclerosis is multifactorial. Processes which are known to be involved at a cellular level include endothelial dysfunction, infiltration of inflammatory cells, vascular smooth muscle cell (VSMC) proliferation and matrix and lipid deposition (2). Systemic risk factors for the development of atherosclerosis include hypertension, hyperlipidaemia, diabetes and smoking. These cause endothelial dysfunction which triggers a cascade of events resulting in the development of an atherosclerotic plaque. An established atherosclerotic plaque consists of an area of intimal thickening which is laden with lipid deposits both extracellularly and within foam cells, an intense inflammatory response with activated leukocytes and macrophages, and sheets of proliferating VSMCs. This is capped by a fibrocalcific plaque which may show signs of previous rupture and subsequent thrombus organisation.

1.1.2.1 Role of the Endothelium

The endothelium plays a major role in the development of atherosclerosis. Damaged endothelial cells express adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), monocyte chemoattractant molecules (MCP-1) and E-selectin. These are important as they allow adhesion and migration of monocytes into the intima of the vessel (3;4) which then sets up a cascade of events resulting in the development of an atherosclerotic plaque. Additionally, damaged endothelium causes a lowering of the bioavailability of Nitric Oxide (NO). This may be through decreased secretion of NO or through increased NO inactivation via reactive oxygen species. NO is known to be involved in repression of cell proliferation by inhibiting upregulation of p21 and causing cell cycle arrest through blockade of cyclin A mRNA (5). As such,

the relative lack of NO in damaged endothelium contributes to the inflammatory process and allows VSMC activation and proliferation into the intima of the vessel.

1.1.2.2 Role of Monocytes

Monocytes are a type of circulating white blood cell which, upon stimulation, migrate out of the circulation through the vascular endothelium and differentiate into an activated form within the tissues. Once monocytes have migrated out of the circulation into tissues they are often termed macrophages. Monocyte migration into the intima of affected arteries is one of the earliest steps in the development of an atherosclerotic plaque. Stimulated by the presence of circulating lipoproteins, especially oxidised low density lipoprotein (LDL) which has been shown to be chemotactic for monocytes in vitro (6), monocytes adhere to endothelium and migrate into the intima. Subsequent phagocytosis of lipoproteins initiates the differentiation from monocytes into foam cells (7). There is evidence of foam cell accumulation in early atherosclerotic lesions found in children and young adults (1). Foam cells, unlike monocytes, are fixed within the tissue and will not migrate out. They represent an activated form of monocyte and, as such, generate an inflammatory cascade (8), releasing cytokines which, in turn stimulate the recruitment of more monocytes as well as stimulating the migration and proliferation of VSMC from the medial layer (9). Furthermore, monocytes secrete extracellular proteinases including collagenases and matrix metalloproteinases (MMPs) which serve to weaken and thin the surrounding fibrocalcific plaque (10) which may predispose to plaque rupture and subsequent vessel thrombosis.

1.1.2.3 Role of Vascular Smooth Muscle Cells

Migration and proliferation of VSMC into the intima is a predominant feature of atherosclerotic plaques. The VSMC are stimulated by both the endothelium and monocytes to migrate and proliferate. Cytokines and growth factors such as platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF) and Angiotensin II released from damaged endothelium, platelets and monocytes all contribute to the migration and proliferation of VSMC (2;3;5;8-10). The resultant effect of this process, along with the accumulation of monocytes laden with lipid, is a thickening of the intima which leads to luminal narrowing in the blood vessel. Furthermore, the VSMC appear to change from a contractile phenotype to a synthetic phenotype during this process (11). They subsequently secrete extracellular matrix which propagates the inflammatory response and may also contribute to the formation of the fibrous plaque on the surface of the lesion.

1.1.3 Consequences of Atherosclerosis

Impaired blood flow to any organ due to arterial luminal narrowing causes ischaemia. The end effect of this process depends on the organ affected. For cardiac atherosclerosis, the result is angina or myocardial infarction. In the case of cerebrovascular ischaemia, the consequence may be stroke or multi-infarct dementia. When occlusive atherosclerosis affects the peripheral circulation, the most commonly affected site is the lower limb. This process is most often felt as pain in the calf muscles on walking (claudication). In some patients, however, the lack of oxygenated blood to the distal tissues is so severe that necrosis develops. This is known as critical limb ischaemia (CLI) which, without treatment, necessitates lower limb amputation. The major effective treatment for peripheral arterial disease is arterial bypass grafting. This is normally performed using the long saphenous vein

and is most commonly in the form of a bypass graft from the common femoral artery to the popliteal artery. These grafts can restore a normal blood supply to the limb. Unfortunately, these grafts are susceptible to a process of narrowing which can lead to graft occlusion and recurrence of the ischaemic state of the limb. The process which leads to vein graft narrowing and occlusion is known as intimal hyperplasia and is the major focus of this thesis.

1.2 Lower Limb Ischaemia

1.2.1 Prevalence and Natural History

Chronic lower limb ischaemia secondary to atherosclerosis is a common disease worldwide. Accurately measuring the prevalence of the condition is difficult as many people have asymptomatic disease detectable only by physical examination which reveals reduced or absent peripheral pulses and or low ankle brachial pressure index (ABPI) (12). Based on epidemiological studies, the prevalence of lower limb ischaemia in Europe and North America is estimated at 16% of the adult population or 27 million people (13). The majority of these people (60%) are asymptomatic. Within the symptomatic population, the prevalence of disease increases with age to >20% in the over 80 age group (14). The Fontaine classification (Table 1) is routinely used to categorise severity of limb ischaemia.

Stage I	Asymptomatic
Stage II	Intermittent claudication
Stage III	Rest pain
Stage IV	Ulceration and / or gangrene

Table 1: Fontaine classification of lower limb ischaemia.

The majority of patients with symptomatic lower limb ischaemia experience claudication. That is, they suffer calf or buttock pain on walking. The pain is relieved by rest and is reproducible at the same walking distance. Although other disease processes such as osteoarthritis can also cause leg pain on walking, intermittent claudication can be confirmed by measuring the ankle brachial

pressure index (ABPI) at rest and also after exercise. An ABPI of <0.95 at rest or following exercise confirms the presence of occlusive arterial disease affecting the lower limb and warrants further investigation and management. It is worth noting that certain disease states such as Diabetes Mellitus and Rheumatoid Arthritis can lead to calcification of vessels giving falsely elevated ABPI measurements. In these patients, a careful history and examination of the patient is of prime importance to reach the diagnosis. Intermittent claudication has a major impact on quality of life measures. Mobility scores are reduced, anxiety scores are increased and sleep, emotional behaviour and social interactions are affected (15). Additionally, between 3-8% of patients suffering from claudication (and 10% of claudicants over the age of 80 (12)) will experience disease progression to suffer pain at rest and / or gangrene with tissue ulceration. This progression of disease is classified as Fontaine Stages III and IV. These two stages of disease are termed critical limb ischaemia (CLI). There is no internationally agreed definition of critical limb ischaemia but one is that patients with persistent rest pain of over two weeks' duration requiring regular analgesia and with an ankle pressure of less than 30mmHg or patients with ulceration or gangrene of the feet, again with the same reduction of ankle blood pressure would be classed as having CLI. The term critical limb ischaemia implies that the circulation has deteriorated to such an extent that, if uncorrected, there will be significant tissue loss and gangrene usually necessitating major amputation. Critical limb ischaemia causes severe pain necessitating opiate use which alters mental functioning and reduces quality of life (15). Additionally, chronic opiate use in other patient groups such as cancer patients, leads to malnutrition through appetite loss (16) and the same can be expected in patients with opiate use for critical limb ischaemia. A recent study showed the median time to being seen in a hospital outpatient clinic was 25 days for patients with critical ischaemia and that patients had experienced symptoms for a median of 8 weeks prior to this (17), giving more than enough time for significant alteration of quality of life.

Development of critical limb ischaemia necessitates urgent hospital admission for surgical assessment. If the patient is found to be suitable for intervention, they will undergo revascularisation using either a bypass graft or endovascular techniques. If neither of these are possible, the only other alternative is amputation. In 1997, a successful infrainguinal bypass graft was estimated to cost £4320 per patient, whereas the estimated cost of primary amputation was £12730. A failed bypass graft which then led to amputation was put at £17066 per patient (18). Other studies have found the same trend in costs for each procedure (19-21). Critical limb ischaemia is, therefore, a significant problem, both in terms of morbidity and mortality to patients, and in cost to society. In addition, as the average age of our population increases, peripheral arterial disease will continue to be a significant burden on health care resources.

Additionally, up to 30% of patients who undergo successful revascularisation for CLI will require amputation within 2 years of infrainguinal grafting due to graft occlusion (22). Graft occlusion within this time is mostly due to development of stenosed areas within the bypass graft caused by areas of intimal hyperplasia. The process of intimal hyperplasia is incompletely understood but the principal abnormality seen is thickening of the intimal layer with cellular proliferation and matrix deposition. This results in narrowing of the graft lumen which, if not corrected, leads to graft thrombosis and occlusion. Development of intimal hyperplasia within a graft is the major limiting factor in the success of bypass grafting and therapies aimed at preventing these stenoses could, therefore, be expected to improve the longevity of such bypass grafts.

1.2.2 Medical Treatment of Lower Limb Ischaemia

Medical treatment of lower limb ischaemia is primarily aimed at altering or halting the progression of systemic atherosclerosis, the underlying cause of arterial disease. Patients with peripheral vascular disease presenting with intermittent claudication have a threefold increased risk of death from cardiovascular disease when compared with age matched controls (23). 28% of patients presenting to a clinician with intermittent claudication will die within 5 years from cardiovascular causes (24). Medical management of peripheral arterial disease is principally aimed at altering this risk and includes antiplatelet therapy, smoking cessation, hypertension management, reduction of cholesterol levels and detection and management of diabetes. It is important to emphasise that patients with intermittent claudication (i.e. Fontaine Stage I & II) do not usually undergo surgical intervention and the medical management of these patients is of paramount importance. This contrasts with those patients suffering from critical limb ischaemia (i.e. Fontaine Stage III & IV) who must undergo surgical intervention if the limb is to be salvaged. In the latter group, the main purpose of medical management is to prevent cardiac or cerebrovascular events. Medical treatments, currently, cannot replace surgical management of critical limb ischaemia.

The Antithrombotic Trialists' Collaboration showed that antiplatelet therapy reduced the incidence of any serious vascular event by 25% in patients with any manifestation of atherosclerotic disease (25). They also found a one third reduction in non fatal myocardial infarction, 25% reduction in non-fatal stroke and one sixth reduction in vascular mortality. Within the subgroup of patients with peripheral arterial disease, the reduction in serious vascular events was 23%. The most commonly used antiplatelet agent is aspirin but up to 20% of patients are intolerant of its gastro-intestinal side effects. Clopidogrel, a newer antiplatelet agent, is significantly more expensive than aspirin but has a better side effect profile. The Clopidogrel versus Aspirin in Patients at Risk of Ischaemic Events

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(CAPRIE) study found a 10% reduction in serious vascular events in patients with a history of myocardial infarction, stroke or peripheral arterial disease treated with clopidogrel compared with aspirin (26).

Smoking is the strongest risk factor for the development and the progression of peripheral arterial disease. Therapies to help people give up smoking have, however, poor efficacy rates. In total, only 10-15% of attempts to stop smoking are successful (27). Continued smoking in patients with peripheral arterial disease is associated with decreased survival and increased risk of developing critical limb ischaemia (28;29). Additionally, smokers have lower graft patency and survival rates after revascularisation when compared with non-smokers (30).

Antihypertensive therapy is associated with reduced risk of stroke, ischaemic heart disease and vascular death (31). However, lowering of blood pressure in patients with peripheral arterial disease may worsen symptoms. This is of particular concern with reference to β-blockers which may cause additional peripheral vasoconstriction and thus, ischaemia. Despite this, a recent Cochrane systematic review of the treatment of hypertension did not find any strong evidence that β-blockers should not be used in peripheral arterial disease (32). Angiotensin converting enzyme inhibitors (ACE inhibitors) have been shown to reduce cardiovascular morbidity and mortality in patients with cardiovascular disease. The Heart Outcomes Prevention Evaluation (HOPE) study randomised patients to ramipril 10mg or placebo. The treatment group showed a 22% reduction in myocardial infarction, stroke or cardiovascular death in patients with left ventricular dysfunction and hypertension (33). Additionally, there was a 25% reduction in cardiovascular morbidity and mortality in those patients with peripheral arterial disease (34). The results of the European trial on Reduction Of cardiac evens with Perindopril in patients with stable coronary Artery disease (EUROPA) study strengthened the case for use of ACE inhibitors in established cardiovascular disease. In this study, patients with chronic cardiovascular disease were randomised to perindopril

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8mg or placebo. After a mean follow up of 4.2 years, there was at 20% reduction in cardiovascular death, myocardial infarction or cardiac arrest in the treatment group (35).

Reduction in plasma cholesterol levels using statins (3-Hydroxy 3 methylglutaryl CoA reductase inhibitors) has been proven to reduce cardiovascular morbidity and mortality in patients with ischaemic heart disease and peripheral vascular disease (36-38). (See section 1.4.3). Three recent trials have shown various degrees of improvement in claudication in patients treated with statins (39-41). Aronow *et al* found an increase in mean walking time of 54 and 95 seconds before onset of claudication after treatment with simvastatin for 6 months and one year, respectively. However, the use of walking time before onset of claudication rather than walking distance makes this study difficult to interpret fully. Additionally, only 69 patients were enrolled in the study. Mohler *et al* randomised 354 patients and found an improvement in pain-free walking time in patients treated with 80mg atorvastatin compared with placebo but no difference in maximal walking time. Patients in the treatment group, however, did report an improvement in mobility scores assessed by the Low Level Physical Activity Recall (LOPAR) questionnaire but there was no difference in quality of life measures as assessed by the Short-Form 36 (SF36) questionnaire or walking function as assessed by the Walking Impairment Questionnaire (WIQ). Mondillo *et al* showed an improvement in pain-free walking distance and maximum walking distance of 90 and 126 metres respectively after 6 months treatment with simvastatin 40mg. The authors also found an increase in resting and post-exercise ABPI of 0.09 and 0.19 respectively in the treated group. In addition, sub-group analysis of the Scandinavian 4S study showed a reduction in the incidence of new-onset intermittent claudication in patients treated with simvastatin (42). Statins are the most commonly prescribed drugs for hypercholesterolaemia. Other cholesterol lowering agents include the fibrates which have proven beneficial effects especially with regard to lowering triglyceride levels and may be used with caution in combination with a statin (43). One of the fibrates, bezafibrate has also been shown to

reduce the severity of intermittent claudication in elderly men (44). Overall, the evidence suggests that cholesterol lowering treatment of patients with peripheral vascular disease does improve claudication to some extent.

Diabetes mellitus confers a more than 2-fold increase in the risk of developing peripheral arterial disease (45). The combination of peripheral arterial disease and diabetes puts patients at increased risk of cardiovascular events (46). Diabetic patients with intermittent claudication have an increased risk of disease progression to critical limb ischaemia (47). The multi-level nature of diabetic atherosclerotic disease means that revascularisation is less often possible and patients have a higher risk of amputation (48). Treatment of diabetes by maintaining tight glycaemic control reduces the risk of cardiovascular disease morbidity and mortality (49). For every 1% reduction in the glycosylated haemoglobin A1c level (HbA1c), a 21% reduction in all deaths related to diabetes has been shown. Additionally, there is a 14% reduction in risk of myocardial infarction and a 37% reduction in microvascular complications (50).

While these therapies are of proven benefit in reducing overall cardiovascular risk, none has been clearly shown to improve the symptoms of lower limb ischaemia, other than statins producing some mild improvement in intermittent claudication. Exercise programmes have been shown to increase the claudication distance (51) but this benefit is only seen in patients who follow supervised exercise programmes and the cost benefit is low (52). More recently, cilostazol (a phosphodieterase III inhibitor) has been introduced to the market and evidence suggests that claudication distances are increased on a par with exercise programmes (53;54). Overall, however, little can be done to treat the symptom of lower limb ischaemia medically and the majority of the treatment is aimed at halting systemic atherosclerosis progression and preventing secondary cardiac or cerebrovascular events.

For patients with critical limb ischaemia, the only chance of returning the limb to a pain-free state is through surgical intervention.

1.2.3 Surgical Treatment of Lower Limb Ischaemia

Once critical ischaemia develops, the only surgical treatment options are revascularisation or amputation. Revascularisation may be in the form of angioplasty with or without stent insertion or bypass grafting. Before a decision can be made between these two options, the anatomical level of disease must be considered. Occlusive arterial disease occurring in the aorto-iliac segment can be successfully treated by either approach. Open surgery to bypass the aorto-iliac arterial tree using synthetic grafts such as Dacron or Polytetrafluoroethylene (PTFE) has good long term success rates (55;56). More recently, endovascular techniques to angioplasty and / or stent the suprainguinal vessels has been established as less invasive than open surgery and, in suitable lesions, is safe and effective (57;58)

Treatment of occlusive disease below the level of the inguinal ligament is different to that of aorto-iliac disease. Here, angioplasty is recommended only for short (<3cm) focal arterial stenoses (Trans-Atlantic Inter-Society Consensus (TASC) type A lesions) (59) as long term results of infrainguinal angioplasty in critical limb ischaemia are not good. Angioplasty is, therefore, usually reserved for patients whose medical condition precludes surgical bypass (60). Bypass surgery involves surgically inserting a conduit, either synthetic or autogenous, from the patent artery proximal to the occlusion to a patent artery distal to the occlusion. The technique of using autogenous vein to bypass an occluded arterial segment in the lower limb was first performed in 1948 by Jean Kunlin. The same technique was then applied in cardiac surgery after cardiopulmonary bypass became possible in the 1960s. In infrainguinal disease, the benefits from a successful bypass operation are immense. The

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lower limb can be saved and the patient restored to an almost pain free situation. The physical and mental benefits of this are great and quality of life is improved (61;62). Some studies, however, report an increase in anxiety scores following infrainguinal bypass (63) and that patient perceived benefits are greater than objectively measured mobility scores (64).

Infrainguinal bypass grafting is usually only performed for critical limb ischaemia and less often for patients with claudication. In the latter group, the benefits of infrainguinal bypass surgery are less clear. Although claudicants can have a very definite improvement in symptoms in the short term following infrainguinal bypass grafting, it is generally accepted that the risk of developing critical ischaemia when the graft eventually occludes is greater than if no bypass surgery had been performed. As such, infrainguinal bypass grafting is usually only performed for critical limb ischaemia. In claudicants with disease above the inguinal ligament, however, the role of revascularisation is clearer. These patients can often be treated by endovascular methods to relieve the arterial stenosis or occlusion or, in the case of more severe aorto-iliac disease, by open surgery. These interventions have improved outcomes when compared to interventions for infrainguinal disease (65).

1.3 Bypass Grafting For Infrainguinal Disease

1.3.1 Conduit

The long saphenous vein is the most commonly used conduit for infrainguinal bypass surgery. It lies in an ideal anatomical location for use in lower limb bypass, running from the ankle to the groin making it long enough even for very distal grafting. The most common site of arterial occlusion in the lower limb is the superficial femoral artery in the thigh. Grafting to bypass this occlusion usually runs from the common femoral artery in the groin to the popliteal artery either above or below the knee joint level. Should the occlusion include the popliteal artery, the distal graft site can be extended to the tibial vessels. The vein may be used in 2 ways: either dissected out completely and its orientation reversed before anastomosis, or left *in situ* and valves destroyed by a valvulotome. *In situ* vein grafts were initially expected to be better than reversed vein grafts for several reasons. Firstly, the diameter match between the proximal and distal anastomoses is better, theoretically aiding the ease of the surgical procedure. Additionally, by not dissecting the vein out from all of its surrounding tissue, it was thought that the vein and nutrient vessels were preserved. However, the *in situ* technique requires valve disruption and this inevitably leads to endothelial damage. A few studies have compared reversed vein grafts with *in situ* vein grafts and no difference in graft patency or limb salvage has been shown. In some patients, the long saphenous vein is either unsuitable for grafting or has been removed previously. As a result of this problem synthetic alternatives Dacron and PTFE were introduced in the 1970s. These alternatives, however, have significantly poorer patency rates when compared to autogenous vein and are less resistant to infection (66;67).

1.3.2 Graft Failure

Graft failure is a result of graft occlusion and can be divided into early, medium and long term failure. Early graft failure (within the first 4 weeks of surgery) is due to technical problems with the graft. Failure of grafts after this time but within the first 12-18 months is usually due to the development of areas of graft stenosis secondary to intimal hyperplasia. Late graft failure after 2 years is most often due to progression of arterial disease either in the vessels proximal to the graft origin or in the distal vessels. Little can be done to avoid early or late graft failure but 80% of all graft failures occur between 1 and 18 months of surgery. As such, therapies aimed at preventing graft stenoses due to intimal hyperplasia can be expected to have a major impact in improving graft patency.

Patency rates of infrainguinal grafts depend on the distal anastomosis site and the graft material used (68;69). For above-knee popliteal artery bypass, the patency rate at 2 years is similar for autogenous vein and synthetic conduits at around 70-75%. For below-knee popliteal or tibial vessel bypass, the patency rates at 2 years are poorer - 70% for autogenous vein compared to 40% for synthetic grafts (67-69). If the graft does occlude, the conduit used is also of relevance. Occlusion of synthetic grafts more often leads to recurrence of critical ischaemia than autogenous vein graft occlusion (70). This is true regardless of the distal anastomosis site. One theory for this is that synthetic grafts are more likely to fail due to stenosis at the distal anastomosis which then leads to thrombosis and occlusion of the downstream vessels. In the case of vein grafts, however, the cause of occlusion is more often due to a mid-graft stenosis which does not then lead to distal thrombosis of vessels (67). Therefore, the long saphenous vein is the conduit of choice for infrainguinal revascularisation.

In attempts to improve the patency of synthetic grafts, surgeons have attempted to improve the anastomotic site where the synthetic graft is joined to the native artery. Cuffs or patches of autogenous vein have been interposed between the synthetic material and the artery in various ways. The aim of these techniques is to reduce shear stress at the anastomosis site which reduces endothelial damage thus reducing intimal hyperplasia at the anastomosis leading to improved patency rates (71;72).

1.3.3 Graft Surveillance to Detect Stenoses

Vein graft stenoses develop in up to one third of infrainguinal bypasses, the majority of these occurring within one year of surgery (73;74). Detection of a critical stenosis by graft surveillance using duplex ultrasound allows for either endovascular or surgical correction of the problem. Without repair, these grafts would occlude and thrombose, potentially threatening the viability of the limb. The technique of graft surveillance and stenosis correction improves intermediate term patency of vein grafts but has not been shown to affect eventual limb salvage (74). Beyond the first year after surgery, the rate of developing a new graft stenosis is around 3% per year. Lifelong surveillance of vein grafts has been proposed but is currently not done routinely in the UK.

1.3.4 Vein Graft Stenosis Development

Autogenous vein transplanted into the arterial circulation as a bypass graft undergoes arterialisation, a process thought to be due to a combination of factors including higher flow rates and changes in wall pressures. The result of arterialisation is a thickening of the intimal layer with sparing of the

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medial and adventitial layers (75). Some veins, however, develop a more aggressive response to arterialisation and areas of focal luminal narrowing result. Such graft stenoses are due to intimal hyperplasia. The development of these stenoses is incompletely understood but is the major limiting factor in the success of both infrainguinal bypass grafts and cardiac bypass grafts. Many factors are thought to be involved in the process of vein graft stenosis, including endothelial dysfunction, VSMC migration and proliferation, vessel remodelling and matrix deposition. Additionally, it has been hypothesised that pre-existing vein abnormalities such as areas of existing stenosis may contribute to an accelerated process post-operatively (76). Other systemic factors which have been postulated as influencing vein graft stenosis rate are systemic factors such as diabetes, renal failure, homocysteine levels, fibrinogen levels and lipoprotein-(a) levels (77).

1.3.4.1 Cellular Processes Involved in Vein Graft Stenosis

The term intimal hyperplasia is used to describe the abnormal arterial response to injury such as balloon angioplasty and is also the term applied to stenotic lesions which develop in vein grafts. The process of intimal hyperplasia is similar to the atherosclerotic process: the principal abnormality seen is thickening of the intimal layer with cellular proliferation and matrix deposition (75;78). Lipid deposition and foam cell accumulation resembling atherosclerotic plaque has also been reported in stenotic coronary vein grafts (79;80). Additionally, remodelling of the medial and adventitial layers are seen with angiogenesis in these layers. The VSMC, endothelial cells and monocytes all play a significant role in intimal hyperplasia as discussed below.

The result of intimal hyperplasia in an autogenous vein bypass graft is significant intimal thickening, hypercellularity and marked matrix deposition. This results in luminal narrowing (81). Once this

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narrowing reaches a critical level, a vein graft stenosis will result in thrombosis and graft occlusion. The consequences of an occluded bypass graft depend on the degree of concomitant arterial disease but often results in a return of the critical ischaemia, necessitating amputation (70).

1.3.4.2 Role of Endothelium

Damage to the endothelium of autogenous vein graft is an inevitable consequence of the surgical procedure. Additionally, in reversed vein grafts, the shear forces associated with reversed flow direction and acute pressurisation by arterialisation of the vein are important (82). For *in situ* vein grafts, the disruption of valves by use of a valvulotome causes endothelial denudation. Furthermore, relative ischaemia of the vein wall by disturbance of the nutrient vessels (vasa vasorum) during dissection causes disturbance of the endothelial layer. Together, both stress and relative ischaemia cause a decrease in the availability of endothelial factors especially nitric oxide but also prostacyclin and adenosine (83). Loss of these factors promotes cell adhesion, cell migration and thrombosis in the vein by expressing adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), monocyte chemoattractant molecules (MCP-1) and E-selectin (3;4). A biological relationship exists between replicating endothelial cells and smooth muscle cells. Replicating endothelial cells release factors that stimulate smooth muscle cell proliferation whereas confluent endothelial cells inhibit smooth muscle cell proliferation and migration. It can thus be expected that the disruption of the endothelial layer contributes to initiation of and propagates the process of intimal hyperplasia.

1.3.4.3 Role of Vascular Smooth Muscle Cells

Vascular smooth muscle cells derived principally from the vein medial layer proliferate and migrate into the intimal layer (81). In doing so, they change from a contractile to a synthetic phenotype. Control of this phenotypic modification is poorly understood. Stenotic vein grafts show the highest level of cell proliferation in the tunica media where the majority of cells are VSMC. In the tunica intima, although the cell type is predominantly VSMC, the level of cellular proliferation is lower than that of the media and only one third of the proliferating cell type are VSMC (81). This suggests that VSMC proliferate and dedifferentiate whilst still within the tunica media and then migrate into the tunica intima where they are more involved with secretion of extracellular products. Stimuli for this response of VSMC include platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), basic fibroblast growth factor (bFGF), plasminogen activators and cell-surface receptors.

1.3.4.4 Role of Monocytes

Within the intimal layer of stenotic vein grafts, monocytes account for the minority of cell types but represent one third of replicating cells (81). The exact role of monocytes in stenotic vein lesions is poorly understood. It is noted, however, that some vein grafts develop areas of intima laden with lipid rich proliferating foam cells similar to arterial atherosclerotic plaques (79). It is expected that these proliferating cells act in a similar fashion to those found in arterial atherosclerosis and secrete inflammatory cytokines which further propagate vascular smooth muscle cell proliferation and migration. However, a recent study showed an association between the number of foam cells in a vein graft stenosis and the degree of VSMC apoptosis (80). The authors suggest that the result of

this VSMC apoptosis could weaken the intimal plaque and predispose to plaque rupture similar to that seen in arterial atherosclerotic lesions causing graft thrombosis.

1.3.4.5 Role of Extracellular Matrix

An event of prime importance in the migration and dedifferentiation of vascular smooth muscle cells is the secretion of extracellular matrix metalloproteinases (MMPs). These degrade the surrounding extracellular matrix, allow VSMC migration and promote VSMC proliferation. Vein graft stenoses are associated with increased levels of MMP-2 activation and MMP-9 secretion (84). Vascular smooth muscle cells, endothelial cells and monocytes have been shown to express these proteases. Regulation of these proteases is complex. Cytokines such as interleukin-1 alpha and growth factors (PDGF-BB) act to increase expression of MMP-9 in vein graft stenoses (85).

1.3.4.6 Role of Pre-existing Disease

It has long been suspected that pre-existing vein abnormalities such as areas of hypertrophy, sclerosis or calcification influence the outcome of vein bypass grafting (86;87). Other studies, however disagree with these findings (78;88-90). Recently, another study showed an association between hyperhomocysteinaemia, pre-existing vein hypertrophy and subsequent vein graft failure (77). Diabetes has also been implicated as a factor which is more likely to lead to vein graft failure through insulin stimulated VSMC proliferation (91;92).

1.3.4.7 Role of Pre-Operative Tissue Ischaemia

One factor, which has not been investigated with respect to vein graft intimal hyperplasia, is the effect of pre-operative ischaemia. In critical limb ischaemia, there is profound tissue hypoxia which is often present for many months before revascularisation. The effect of ischaemia on the skin and subcutaneous tissue of the affected limb is clearly visible with areas of ulceration and / or gangrene. The nociceptive response to the profound ischaemia is equally prominent with patients requiring 24-hour opioid analgesia. Furthermore, small arterioles from distal muscle biopsies from amputated limbs show structural changes as well as functional changes including impairment of pressure dependent myogenic tone (93). It is possible, therefore, that profound tissue ischaemia also affects the long saphenous vein adversely. There may, therefore, be structural, functional or cellular changes within the vein which could influence the response of the long saphenous vein when used in bypass grafting.

This thesis aims to investigate the role of pre-operative ischaemia on the long saphenous vein and its propensity to develop intimal hyperplasia.

1.4. Statins

1.4.1 Pharmacology of Statins

The statins are a group of drugs, developed for their cholesterol lowering properties. They exert their effect by inhibiting the intracellular enzyme 3-Hydroxy-3 methylglutaryl CoA (HMG-CoA) reductase, thus inhibiting the formation of mevalonate, a precursor in the intracellular synthesis of cholesterol. This decrease in available intracellular cholesterol stimulates the production of membrane LDL receptors, which pull cholesterol from the plasma, thereby reducing plasma cholesterol levels. The main site of action is in the liver.
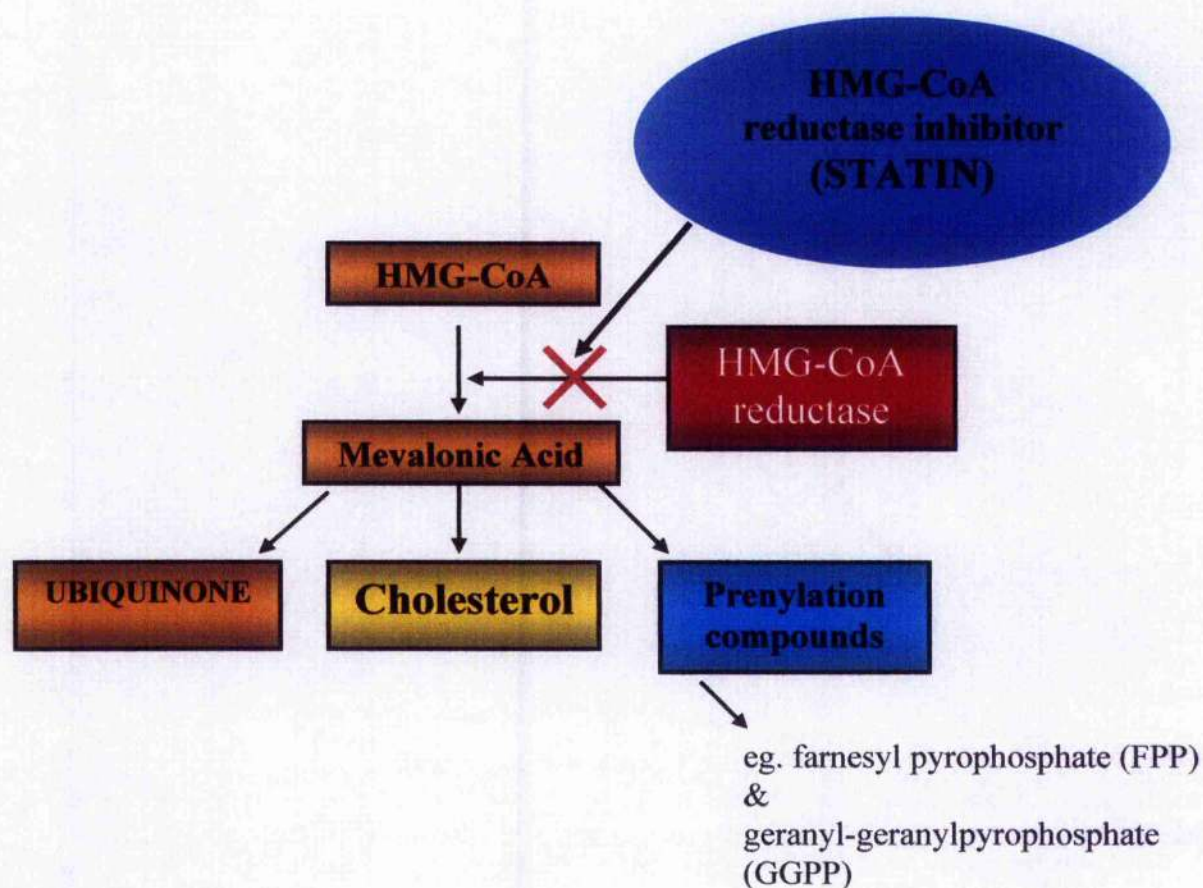
Figure 1: The mevalonic acid production pathway, the point of action of statins.

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First generation statins simvastatin, lovastatin and pravastatin are derived from fungal metabolites. Lovastatin is produced by the fungus *Aspergillus terreus*, simvastatin is obtained by replacing the 2-methylbutyryl side chain with a 2,2-dimethylbutyryl group. Simvastatin and lovastatin are hydrophobic compounds whereas pravastatin is hydrophilic. Simvastatin is absorbed orally and activated by hydrolysation to its active form, simvastatin acid *in vivo*. Only 5% of orally administered simvastatin reaches the circulation in its active form, the majority being metabolised by first pass metabolism in the liver, its site of maximal action. Both simvastatin and lovastatin are metabolised by the cytochrome P450 enzyme system and care must be taken not to administer these drugs in patients who are also taking drugs which inhibit the cytochrome P450 system. When this occurs, the bioavailability of simvastatin and lovastatin can increase dramatically, possibly leading to significant side effects (94;95). Pravastatin, however, is not metabolised by the cytochrome P450 pathway. The second generation statins atorvastatin, fluvastatin and cerivastatin are entirely synthetic compounds. They are all hydrophobic and metabolised principally by the cytochrome P450 system. Metabolites of both cerivastatin and atorvastatin also have HMG-CoA reductase inhibitive effects. Cerivastatin is an extremely potent HMG CoA reductase inhibitor, having equivalent cellular effects at concentrations 100-150 fold lower than the other statins. Cerivastatin, however, has been removed from the drug formulary due to a raised incidence of adverse effects, principally myotoxicity (94).

1.4.2 Side Effects of Statins

Overall, statins are well tolerated. The most common side effects are myalgia and raised transaminase levels. Less commonly, myopathy, myositis and rhabdomyolysis can occur, although these are usually related to enzyme deficiencies or concurrent therapy with cytochrome P450

inhibitors. The exact mechanism of these skeletal muscle effects are poorly understood but since mevalonate is also a precursor of products essential in the process of cell replication and glycoprotein synthesis it is possible that the cell membrane is disrupted, threatening the viability of the cell.

1.4.3 Cardiovascular Effects of Statins

Beneficial cardiovascular effects have been shown to result from statin therapy. This is true for both secondary and primary prevention. The major studies showing a beneficial outcome in secondary prevention are the Scandinavian Simvastatin Survival (4S) study, the Cholesterol and Recurrent Events (CARE) study and the Heart Protection Study (HPS) (36-38).

The 4S study looked at patients with proven ischaemic heart disease and raised cholesterol levels. 4444 patients were randomised to either 40mg simvastatin or placebo and followed up over 5 years. In the treatment group, total cholesterol and LDL cholesterol were reduced by 25% and 35% respectively. Also, major cardiac events (coronary death, myocardial infarction or resuscitated cardiac arrest) were reduced in the treatment group (relative risk (RR) 0.66), there was also a reduction in coronary death (RR 0.58) as well as total mortality (RR 0.7).

The CARE study was similar, involving patients with known ischaemic heart disease but with cholesterol levels within the "normal range" (5.4 +/- 0.4 mM). Patients were randomised to pravastatin 40mg or placebo. Over 4000 patients were randomised and followed up for a median of 5 years. The treatment group had reduced total and LDL-cholesterol levels of 20% and 28% respectively. The outcome measures of coronary death or non-fatal myocardial infarction were

reduced by 24% in the treatment group. Also, the need for coronary revascularisation was reduced by 26% for coronary bypass and 23% for coronary angioplasty.

The Heart Protection Study enrolled over 20000 patients with a high risk of atherosclerotic disease, that is, prior history of coronary disease, non-coronary disease (cerebrovascular or peripheral vascular) or diabetes. Patients were randomised to simvastatin 40mg or placebo regardless of baseline cholesterol levels. The treatment group showed a 24% reduction in all cause cardiovascular events, with a decreased rate of all cause mortality, principally due to an 18% reduction in cardiac mortality. There was also a significant reduction in vascular mortality as well as a 9% reduction in revascularisation procedures (coronary and non-coronary).

Taken together, these three studies demonstrate overwhelming benefit with statin therapy for patients with existing cardiovascular disease across all baseline levels of total and LDL-cholesterol.

Statins have also been shown to have beneficial cardiovascular effects in primary prevention, that is, in patients without a history of cardiovascular disease. Both the West of Scotland Coronary Prevention Study (WOSCOPS) and Air Force / Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS) addressed this issue (96;97).

In WOSCOPS, 6595 men with raised cholesterol levels (mean 7.03mM +/- 0.57) but no prior history of cardiovascular disease, were randomly assigned to pravastatin 40mg or placebo. There was a reduction in total and LDL cholesterol of 20% and 25% in the treatment group as well as a 31% reduction in all coronary events, 32% reduction in cardiovascular mortality and a 37% reduction in revascularisation procedures.

In the AFCAPS/TexCAPS study, 5608 men and 997 women with no history of cardiovascular disease were randomised to receive either lovastatin 20-40mg or placebo. Mean total cholesterol at the beginning of the study was 5.7mM. After a mean follow up of 5.2 years, there was a reduction in serum cholesterol levels (mean 3.96mM), as well as a reduction in first major coronary events (RR 0.63) in the treatment group. Additionally, there was a reduction in coronary revascularisation procedures (RR 0.67).

1.4.4 Anticipated Effects of Statins on Vein Grafts

Two recent publications have shown improved infrainguinal graft patency in patients being treated with statins (98;99). Both papers are retrospective reviews of patients undergoing infrainguinal bypass surgery. Henke *et al* investigated the drug history of 293 patients undergoing 338 infrainguinal bypass grafts. The authors found that the majority of patients had co-existing cardiovascular disease (70% with hypertension, 52% with diabetes, 51% had ischaemic heart disease and 37% with hyperlipidaemia) but, despite this, prescription of cardioprotective medications was low. Only 56% of patients were prescribed a statin and 54% were prescribed an Angiotensin Converting Enzyme Inhibitor (ACE Inhibitor) despite national guidelines which recommend the use of these drugs in all patients with systemic atherosclerosis. The majority of patients (94%) were prescribed an antiplatelet agent or warfarin. At a mean follow up of 17 months, the authors found a positive correlation with graft patency and statin use (odds ratio (OR), 3.7) as well as a correlation with decreased amputation rate and statin use (OR, 0.34).

These findings are supported by the findings of Abbruzzese *et al* who analysed outcomes of 189 infrainguinal bypass grafts on 172 patients with specific reference to statin prescription. Statin

prescription was associated with increased secondary patency rate (97% +/-2% vs 87% +/- 4%) and the risk of graft failure was 3.2 fold higher in the untreated group. Neither of these papers sought to explain the mechanism of action of statins. However, both papers noted no significant difference in serum total cholesterol levels at the time of surgery between both groups (data only available in 47% of patients in the study by Abruzzesse *et al*), suggesting the effect is independent of cholesterol lowering. Possible mechanisms by which statins affect infrainguinal graft patency include endothelial effects, antiproliferative effects, extracellular matrix effects, anti-oxidant effects, anti-inflammatory effects and anti-thrombotic effects.

1.4.5 Pleiotropic Effects of Statins

Not all of the effects of statins can be directly attributed to lowering of serum cholesterol levels (100;101). They have been shown to inhibit vascular smooth muscle cell proliferation as well as migration (102), activate endothelial NO release (103;104), act as an antioxidant (105;106), reduce secretion of inflammatory cytokines (107;108) and act as an antithrombotic agent (101;109;110). The combination of these effects is thought to be the means by which statins stabilise atherosclerotic plaques, contributing to their beneficial cardiovascular effects. Additionally, these cellular mechanisms are central to the development of intimal hyperplasia as discussed previously. As such, it could be anticipated that statins may also play a role in the prevention or attenuation of vein graft intimal hyperplasia. Other possible actions include interference with angiogenesis (111) and immune-modulation (112).

1.4.5.1 Endothelial Effects

Endothelial dysfunction serves as a surrogate marker for cardiovascular disease (113). A measure of endothelial function can be made by assessing the vasoconstricting and vasodilating mechanisms of vessels. The most important mediator of vascular vasodilatation is nitric oxide (NO) and the most potent vasoconstrictors are endothelin-1 (ET-1) and angiotensin. By affecting the metabolism of these vasoactive mediators, statins enhance endothelial-dependent vasodilatation. Statins reduce the production of ET-1 from endothelial cells by down-regulating the production of pre-pro ET-1 mRNA (114). Additionally, statins cause an increase in the local generation of NO from endothelial cells by increasing the half life of endothelial NO synthase (eNOS) (104;115).

Statin therapy has been shown to improve endothelial function in diabetic men (116). Patients had baseline measurements of brachial artery dilatation in response to ischaemia induced reactive hyperaemia. They were then were given oral cerivastatin for 3 days following which the test was repeated. There was a significant increase in the amount of brachial artery dilatation after only three days treatment but no further increase after continuation of cerivastatin treatment for three months. The effects seen at three days were in the absence of any change in serum cholesterol level, although a reduction in cholesterol was seen at three months. Another study investigated healthy males with normal cholesterol levels in a similar manner (117). This study found that oral atorvastatin caused an increase in forearm blood flow after four days. However, serum cholesterol levels were found to have fallen within two days of commencing oral atorvastatin. Cessation of atorvastatin treatment caused forearm blood flow measurements to return to normal after only one day, whereas cholesterol levels took twelve days to return to pre-treatment values.

1.4.5.2 Antiproliferative Effects

The major antiproliferative effect of statins on VSMC is related to reduction in intracellular mevalonate production (102;118). This substrate is a precursor of farnesyl-pyrophosphate (FPP) and geranyl-geranyl-pyrophosphate (GGPP). Replacement of these compounds reverses the antiproliferative effects of statins. FPP and GGPP are isoprenoid compounds necessary for the post-translational prenylation of Ras, Rho and Rac. Without this post-translational modification, these small GTP-binding proteins cannot localise to the cell membrane (their point of action) and they cannot trigger other intracellular mechanisms required for proliferation and migration (119). By inhibiting cell proliferation and migration, statins can be expected to inhibit the development of intimal hyperplasia in vein grafts as these are two of the most important events within this process.

1.4.5.3 Extracellular Matrix Effects

Matrix metalloproteinases (MMPs) are secreted by VSMC and monocytes in the development of vein graft intimal hyperplasia (102;120). MMP-9 secretion allows extracellular matrix degradation which potentiates VSMC migration and proliferation. By inhibiting MMP secretion, VSMC proliferation is inhibited. Statins have been shown to reduce secretion of MMP 9 in organ culture, causing a reduction in intimal hyperplasia (102;121).

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1.4.5.4 Anti-oxidant Effects

By reducing the bioavailability of nitric oxide (NO), free radicals and reactive oxygen species contribute to endothelial dysfunction. Also, this reduction in the bioavailability of NO contributes to the increase in expression of chemoattractant molecules by endothelial cells which allow monocyte adhesion and migration into the intimal layer as well as promoting VSMC proliferation. Furthermore, oxidation of LDL-cholesterol plays a pivotal role in the pathogenesis of atherosclerosis.

NAD(P)H oxidase is expressed at increased levels in balloon injured arteries and in atherosclerotic vessels (122;123). It has also been identified within dedifferentiated VSMC in neointimal lesions of aortocoronary vein grafts (124). The result of increased levels of NAD(P)H oxidase is an increase in free radical formation and NO inactivation. Statins have been shown to interfere with the function of NAD(P)H oxidase (125) creating antioxidant effects. Additionally, these antioxidant properties of statins would be expected to reduce oxidised-LDL levels and enhance endothelial NO bioavailability. Together, these would contribute to attenuating intimal hyperplasia in vein grafts.

1.4.5.5 Anti-inflammatory Effects

Statins have been shown to reduce the production of pro-inflammatory cytokines including Interleukin-1 (IL-1), tumour necrosis factor alpha (TNFα), Interleukin-6 (IL-6) and cyclo-oxygenase-2 (COX-2) from endothelial cells, VSMCs and leukocytes (107;108). These effects may result in reduced adhesion molecule expression such as ICAM-1, VCAM-1 and reduced leukocyte activation. Together these effects would be expected to attenuate the process of vein graft intimal hyperplasia.

1.4.5.6 Anti-thrombotic Effects

By inhibiting platelet aggregation and improving platelet function through increased NO bioavailability and thromboxane A2 inhibition (119), statins exert an anti-thrombotic effect. Furthermore, tissue factor (TF) expression by monocytes, endothelial cells and VSMC has been shown to be reduced by statins, further contributing to their anti-thrombotic role (109;126). These combined anti-thrombotic effects could be expected to reduce the risk of vein graft thrombosis and contribute to the beneficial effects of statins.

1.5. Overall Summary & Aims of the Project

Intimal hyperplasia closely resembles the process of atherosclerosis, with VSMC migration and proliferation, intimal thickening and extracellular matrix deposition. Intimal hyperplasia leads to significant stenoses in one third of infrainguinal vein grafts within the first year of surgery and is the major limiting factor in the success of bypass grafting using the long saphenous vein. Patients undergoing lower limb bypass surgery have profound tissue ischaemia affecting the skin, subcutaneous tissue, small arterioles and nociceptors. Ischaemia may also adversely affect the long saphenous vein before it is used as a bypass graft.

Statins (3-Hydroxy 3-methylglutaryl CoA reductase inhibitors) have well established cardioprotective effects related to cholesterol lowering. They have also been shown to have many other (pleiotropic) effects including improved endothelial function and inhibition of VSMC proliferation and migration. More recently, statin therapy has been associated with improved patency rates of infrainguinal bypasses.

Project Aims:

1. Compare the susceptibility of human saphenous vein to develop intimal hyperplasia in organ culture between two groups: Ischaemic and Non-Ischaemic Veins, and to investigate the effect of simvastatin on this process.

2. To determine the effects of simvastatin on vascular smooth muscle cell proliferation on cells explanted from the two groups of veins: Ischaemic and Non-Ischaemic.

3. To determine the effects of simvastatin on vascular smooth muscle cell migration on cells explanted from the two groups of veins: Ischaemic and Non-Ischaemic.

4. Perform a retrospective analysis of all infrainguinal bypass grafts performed at Glasgow Royal Infirmary between 1993 and 2003 and to correlate outcome with statin therapy.

Chapter 2

MATERIALS AND METHODS

2.1. Ethical Approval

Ethical approval was sought and obtained from the North Glasgow Hospitals NHS Trust Research Ethics Committee. Authorisation was obtained to approach patients to obtain consent for removing a portion of long saphenous vein for use in this study. The patient groups to be included were those undergoing lower limb amputation and those undergoing coronary artery bypass grafting. Informed consent was obtained from all patients involved in the study.

2.2 Patient Groups and Tissue Collection

2.2.1 Patients Undergoing Amputation

Patients were identified as being suitable for inclusion in the study by Consultant Vascular Surgeons at Glasgow Royal Infirmary and Gartnavel General Hospital. All patients were scheduled to undergo lower limb amputation due to ischaemia from chronic peripheral vascular disease. Patients were deemed suitable for inclusion if they met the criteria in Table 2 below. Consent was obtained to remove the long saphenous vein from the amputated lower limb after amputation had been performed.

	Inclusion Criteria	Exclusion Criteria
Age	All ages	
Sex	Both genders	
Aetiology	Ischaemia Combined ischaemia / infection	Infection alone Absence of ischaemia
Amputation Type	Primary Above or Below Knee Amputation	Minor Amputation Revision of Amputation
Mental State		Unable to give informed consent

Table 2: Inclusion & exclusion criteria for enrolling patients undergoing amputation.

2.2.2 Patients Undergoing Coronary Artery Bypass Grafting

All patients in this group were due to undergo aorto-coronary bypass grafting in Glasgow Royal Infirmary. Patients were identified as being suitable for inclusion in the study by the primary investigator. Patients were deemed suitable for inclusion if they met the criteria detailed in Table 3 below. Consent was obtained for the primary investigator to be given any leftover portion of long saphenous vein not required for the bypass grafting.

	Inclusion Criteria	Exclusion Criteria
Peripheral Circulation	Palpable pedal pulses	ABPI <=0.85 Previous Vascular Surgery
Gender	Both	
Age	All	
Mental State		Unable to give informed consent

Table 3: Inclusion & exclusion criteria for enrolling patients undergoing coronary artery bypass grafting.

2.2.3 Collection of Tissue from Ischaemic Limbs

After amputation, the lower limb was placed on a sterile drape on a theatre trolley. This was taken to a side room off theatre where the long saphenous vein was identified and dissected out along with its surrounding subcutaneous tissue. This was placed in sterile culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution) and transported back to the laboratory at the University of Strathclyde at 4°C.

2.2.4 Collection of Tissue from Non-Ischaemic Limbs

After coronary artery bypass grafting was completed, any leftover long saphenous vein was placed in sterile saline solution at 4°C. The primary investigator was telephoned by theatre staff to collect the vein. The vein was transferred to sterile culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution) and transported back to the laboratory at the University of Strathclyde.

2.3. Organ Culture

2.3.1 Preparation of and Maintaining Vein Rings in Organ Culture

The long saphenous vein was dissected out from its surrounding tissue in a sterile culture hood (Class 2) in the laboratory. Following this, the vein was cut into 2-4mm rings using a sterile scalpel. Each ring was numbered sequentially. Rings 1,4,7,10, 13 and 16 were fixed immediately in 10% formal saline. Rings 2,5,8,11,14 and 17 were placed in wells of a 6 well culture plate (Corning) along with culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution) containing 15% Foetal Calf Serum and simvastatin at 5µM. Rings 3,6,9,12,15 and 18 were placed in wells of a 6 well culture plate along with culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution) containing 15% Foetal Calf Serum and equal volume of vehicle (Sodium Hydroxide/ Ethanol solution) used to prepare the simvastatin. Plates were incubated in a culture incubator at 37°C in the presence of 20% O_2 and 5% CO_2. Media, along with drug or vehicle, was replaced every 48 hours for a total of 14 days. At this point, vein rings were washed in phosphate buffered saline (PBS) then transferred to 10% formal saline for fixing.

In situations where there was insufficient length of vein to obtain 18 rings, fewer were used.

2.3.2 Fixing and Processing of Vein Rings

Rings were placed in a solution of 10% formal saline for 24 hours. These were then transferred to individual tissue cassettes for placement through the tissue processor for preparation for wax embedding. Tissue was passed through a cycle of: 70% ethanol; 90% ethanol; Histo-clear /ethanol 50:50 mix; Histo-clear x2; and wax x2. Following this, tissue was embedded in wax blocks in the correct orientation for cutting in the microtome (Leica). Sections were cut at 4μm and floated on a paraffin section mounting bath (Electrothermal) before being mounted on glass slides. Slides were treated in the oven at 60°C for 35 minutes then allowed to cool before proceeding to staining.

2.3.3 Staining of Vein Rings

Slides were stained with Haematoxylin and Eosin using an autostainer (Varistain 24-4, Thermo Shandon). The cycle of staining was as follows: Histo-clear 1 min x3; absolute ethanol 1 min x2; distilled water 1 min; Haematoxylin 6 min; distilled water 1 min; acid alcohol (0.5%) 1 min; distilled water 1min; Scott's tap water substitute 2 min; distilled water 1 min; Eosin 3% 5 min; absolute ethanol 1 min x3; Histo-clear 1 min x4. Following staining, tissue was sealed onto the microscope slide by placement of a cover slip using DPX as a mounting solution.

2.3.4 Analysis of Vein Rings

Vein sections were photographed using a Cool SNAP-Pro Color digital camera which was attached to an inverted light microscope. Images were taken with the vein ring under 2x magnification. At this magnification, the whole of the vein ring section was included in the image. These images were then analysed using Image-Pro Express V 4.0. This package has a facility to calculate absolute areas of a 2-dimensional image by tracing around the area concerned. For each image, calibration was performed by photographing a micrometer at the same magnification (x2) and using the calibration facility of the package which calibrated the programme by counting the number of pixels which corresponded with one micrometer. Measurements taken were: area of lumen; area to outer edge of tunica intima; area to outer edge of tunica media; and area to outer edge of tunica adventitia. In order to calculate the area of intima, the area of the lumen was subtracted from the area the outer edge of tunica intima. In order to calculate the area of media, the area to the outer edge of tunica intima was subtracted from the area to the outer edge of tunica media. For each vein ring, 3 measures were made and the mean of this used in the analysis. The area of intima and area of media were expressed as a percentage of the total area to the outer edge of the tunica media. These different areas are shown diagrammatically in Figure 2. The amount of tunica adventitia varied greatly between veins since it was dependent on the amount of dissection that had been performed.
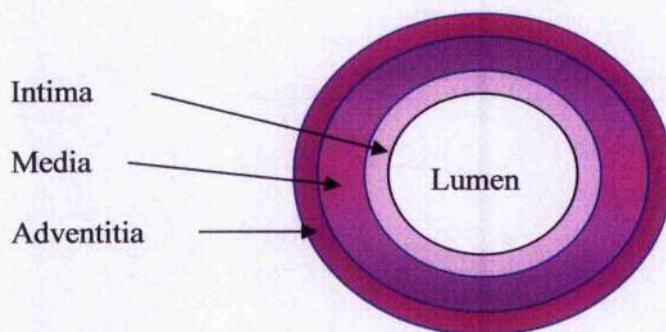
Figure 2: Areas measured on vein rings for analysis of intimal growth.

2.3.5 Statistical Analysis

The unpaired t test was used to analyse comparisons between the ischaemic and non-ischaemic veins. A p value of <0.05 was considered significant. To analyse differences within each group (i.e. ischaemic veins with ischaemic veins and non-ischaemic veins with non-ischaemic veins), one way ANOVA using randomised blocks was used. A p value of < 0.05 was considered significant. All of the above analyses were performed using Graph Pad Prism version 4.03 for Windows.

2.4 Cell Culture

2.4.1 Explanting Vascular Smooth Muscle Cells

It was noted that vascular smooth muscle cells (VSMC) spontaneously explanted from the vein rings in organ culture during the 14 day culture period. This event was expected as it had been observed in similar projects in the laboratory using pig coronary artery rings. When vascular smooth muscle cell explantation occurred, the cells were maintained after vein ring removal at day 14. These cells were grown to confluency then transferred to culture flasks (Nunc) for further culture. No VSMC were seen to explant from vein rings in the simvastatin treated group.

2.4.2 Maintenance of Vascular Smooth Muscle Cells in Cell Culture

Vascular smooth muscle cells were maintained in a culture incubator at $37^{\circ}C$ in the presence of 20% O_2 and 5% CO_2 in culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution) containing 15% Foetal Calf Serum (FCS). Culture medium and FCS was replenished every 48 hours. When cells reached confluency in the well of the 6 well plate, they were detached using Accutase ^{TM} (Sigma Aldrich) and transferred to culture flasks. Cells were grown on in the same fashion and used in cell culture experiments between Passages 3 and 5.

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2.4.3 Cell Detachment and Flask Transfer Technique

In order to detach vascular smooth muscle cells from the culture flasks, culture medium was aspirated from the flask, Accutase ^{TM} (2-5ml depending on flask size) was added to the flask which was then returned to the cell incubator for 10-20 minutes. Cell detachment was confirmed visually before neutralising the Accutase ^{TM} using culture medium (50:50 mix of Waymouth's Medium and F12 Ham with 1% Penicillin/Streptomycin solution). The resulting cell suspension was transferred to a centrifuge tube and spun at 10000 rpm for 5mins. Supernatant was aspirated off before re-suspending the cell pellet in culture medium. This cell suspension was then either transferred in whole to a new flask or split between 2 or more flasks or plates for use in cell culture experiments.

2.5. Proliferation Assay

2.5.1 Preparation of Vascular Smooth Muscle Cells

Vascular smooth muscle cells were seeded onto the wells of a 24 well culture plate in the presence of culture medium with 15% FCS. Once cells had reached 60-70% confluency on visual inspection, the culture media was removed and replaced with culture media containing 0.1% FCS (quiescing media). Each experiment was performed in triplicate.

2.5.2 Stimulation of Vascular Smooth Muscle Cells

After a 24 hour period of incubation with quiescing medium, VSMC were stimulated by the addition of 15% FCS (150μl per ml of media). Some wells were left unstimulated to provide background counts. At the same time point, simvastatin was added to the wells in varying concentrations as well as mevalonate.

2.5.3 Addition of Radiolabelled Thymidine

A solution of ^{3}H labelled Thymidine (Amersham Biosciences) was prepared by addition of 25μl of stock to 975μl of culture medium. This contained a total activity of 925kBq. 10μl of this solution was added to each well of the 24 well plate at 18 hours after addition of FCS. This timing was to correspond with the S phase of DNA replication in the cell cycle (Figure 3).

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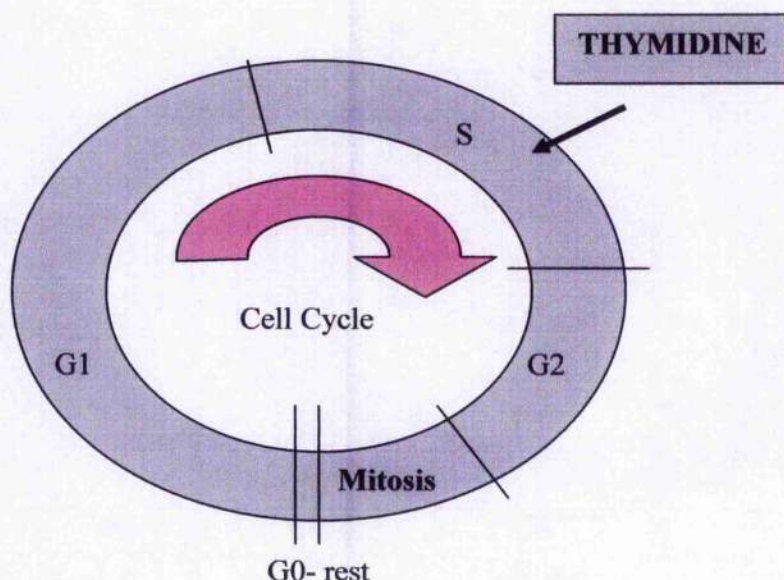
Figure 3: The cell cycle. Thymidine is added to the assay at 18 hours to coincide with DNA replication in the S phase.

2.5.4 Assay Termination

At 24 hours following stimulation with FCS, the assay was terminated. Firstly, plates were visualised to confirm VSMC remained attached to the wells. Following this, media was aspirated from each well and cells were washed in phosphate buffered saline at 4°C. Cell membranes were then permeablised using 10% trichloroacetic acid (TCA) solution washes. Up to six 15 minute washes were required before adequate permeabilisation was visualised at high magnification. Once permeabilisation had been confirmed visually, remaining TCA was aspirated off before addition of 250μl of Lauryl sulphate / Sodium Hydroxide to lyse cells. This was left overnight at 4°C.

2.5.5 Scintillation Counting

Beta vials were loaded with 2ml of Liquid scintillation counting fluid, Emulsifier-Safe [TM] (Canberra-Packard) before addition of the fluid from the wells of the 24 well plate. These were sealed and numbered before being placed in counting racks. Disintegrations per Minute (DPM) for each sample were counted using a Wallac 1409 DSA Liquid Scintillation counter.

2.5.6 Statistical Analysis

Data, in the form of DPM, were normalised to controls and expressed as a percentage inhibition of growth. These data were compared within groups with one way ANOVA using randomised blocks. Also, growth inhibition curves were constructed and the 50% inhibitory concentration (IC50) was calculated for each experiment using the Hill equation. These were compared using the unpaired t-test. A p value of <0.05 was considered significant. All of the above analyses were performed using Graph Pad Prism version 4.03 for Windows.

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2.6. Migration Assay

2.6.1 Preparation of Transwell [TM] Inserts

The upper surfaces of Transwell [TM] Inserts with a pore size of 6μm were coated with a layer of 1% Collagen solution. This was allowed to dry overnight at 37°C. The inserts were maintained at this temperature in a 24 well plate until use.

2.6.2 Preparation and Seeding of Vascular Smooth Muscle Cells

Vascular smooth muscle cells were grown in a T75 culture flask. Once confluency was reached, cells were quiesced for 24 hours in medium containing 0.1% FCS (quiescing medium). Following this, cells were lifted from the plate using Accutase [TM]. The cell pellet was resuspended in 1ml of quiescing medium and cell density was counted using a slide haemocytometer and light microscopy at x100 magnification. The initial cell suspension was then diluted to a density of $5x10^6$ cells per ml and 100μl of this diluted cell suspension was seeded onto the upper surface of the Transwell [TM] filter (a final cell density of $5x10^5$ cells per filter). The lower chamber was charged with plain quiescing media with or without PDGF (20ng/ml) and / or simvastatin 5μM. Plates and filters were returned to the cell culture incubator for 4 hours. Each experiment was performed in duplicate.

2.6.3 Termination of Migration Assay and Staining of Filters

At the end of the 4 hour stimulation period, Transwell ^{TM} inserts were placed in phosphate buffered saline and the remaining non-migrated cells were rubbed from the upper surface of the filter by a cotton bud. Following this, inserts were placed though the sequence of 3 Diff-Quik ^{TM} solutions for 3 minutes each to fix then stain the cells that had migrated onto the lower surface of the filter. Inserts were rinsed in distilled water before being allowed to dry overnight.

2.6.4 Cell Counting

Dried filters were cut away from the plastic housing of the insert and mounted on glass microscope slides using DPX. Cells were counted at high power (x400) for six different fields and the mean of this used. Counting was performed blind and verified by a second counter.

2.6.5 Statistical Analysis

The unpaired t test was used to compare the migration between VSMC from ischaemic and non-ischaemic veins. A p value of <0.05 was considered significant. To analyse differences within each group (i.e. ischaemic veins with ischaemic veins and non-ischaemic veins with non-ischaemic veins), one way ANOVA with randomised blocks was used. A p value of < 0.05 was considered significant. All of the above analyses were performed using Graph Pad Prism version 4.03 for Windows.

2.7. Audit of Infrainguinal Grafts 1993-2003

2.7.1 Database Design

In order to collect information regarding patients at Glasgow Royal Infirmary who had undergone infrainguinal bypass grafting between 1993 and 2003, a database was created using Microsoft Access 2000. Data collected included patient details such as: date of birth, gender, pre-operative medications; smoking status and past medical history. For the operation: date of operation; which leg was operated on; Ankle-Brachial pulse index (ABPI) pre-operatively; graft inflow; graft outflow; and type of vein graft (*in situ* or reversed) used were recorded. Information on subsequent surveillance scans included: proximal anastomosis; flow through graft; and distal anastomosis. These were recorded as satisfactory, unsatisfactory or unknown. Any areas of concern (stenosis, fistula, aneurysm) were noted. Any secondary operations performed on the graft were recorded. Outcome measures recorded were: graft patency; major amputation; smoking status; medication status and death.

2.7.2 Identifying Patients to be Included in the Database

Patients to be included in this database were identified by use of the Surveillance Programme records held at Glasgow Royal Infirmary Vascular Laboratory. Since 1991, all patients who have undergone infrainguinal bypass grafting have been entered prospectively into the graft surveillance programme at the hospital. The programme involves ultrasound scanning at 1 month, 3 months, 6 months and twelve months postoperatively. All scans were performed by one of two trained vascular technologists. Further scans are performed if there are concerns about the graft such as poor flow or

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areas of narrowing. Only patients who underwent infrainguinal bypass grafting using the long saphenous vein were included. Furthermore, patients undergoing grafting for aneurysmal disease or trauma were excluded from the database. Patients who died within 30 days of surgery were also excluded.

2.7.3 Data Collection

Surveillance Programme records were reviewed to identify which patients had undergone graft surveillance between 1993 and 2003. Once these patients had been identified, their case notes were requested through the hospital records system. These were then used to collate further patient data as well as outcome data. As regards drug history, drugs were deemed positive if recorded as being used pre-operatively or during hospital stay. Post-operative drug use was recorded based on clinic letter or further hospital admissions. Smoking history was deemed positive if the pre-operative clerking sheet or post-operative notes indicated so. Graft patency was judged on clinical findings either at outpatient clinic visit or surveillance scan. Amputation was recorded only if a major amputation had been required but not for digital or forefoot amputation as this is not a reliable measure of graft function. Date of death and cause was recorded where known. Some case notes had been destroyed or were incomplete. In the case of destroyed notes, the patient was excluded from further analysis. For incomplete notes, data that could be obtained was recorded.

2.7.4 Statistical Analysis

Descriptive statistics were used to describe the general characteristics of the group studied, quoting means and percentages where appropriate and quoting medians and ranges for non-parametric data.

The outcomes of graft occlusion and major amputation were analysed using a univariate model with regard to the operative characteristics of the graft (i.e. proximal anastomosis site, distal anastomosis site and orientation of long saphenous vein), patient characteristics (diabetes, gender, smoking status) and medication use (statin, antiplatelet and warfarin) using Fishers exact test quoting relative risks and 95% confidence intervals.

Kaplan-Meier survival curves were constructed to display the time to graft occlusion with respect to patient factors and medications. The Logrank test was used to compare curves. The same method was used to create Kaplan-Meier survival curves for the time to major amputation, again with respect to patient factors and medications. Deaths and patients lost to follow up were treated as censored events at the time of the event. All of the above analyses were performed using Graph Pad Prism version 4.03 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com.

To investigate graft occlusion further, a multivariate analysis was performed using logistic regression with forward conditional analysis. Variables were included in the model if $p<0.05$ and excluded if $p>0.10$. This analysis was performed using SPSS for Windows v13.0.

2.8. Drugs, Reagents and Consumables Used

2.8.1 List of Drugs and Reagents Used by Supplier

Amersham Biosciences, Buckinghamshire, UK.	[methyl-^{3}H]Thymidine
McQuilkin & Co., East Kilbride, UK.	Diff-Quik ®
Canberra Packard Ltd., Pangbourne, UK.	Emulsifier-Safe ^{TM}
Cambrex Bioscience Wokingham Ltd., Berkshire, UK.	Penicillin / Streptomycin
Fisher Scientific, Loughborough, UK.	Histo-clear
Inverclyde Biologicals, Bellshill, UK.	Phosphate buffered saline
Invitrogen Ltd.,Paisley, UK.	F12 (Ham) + L – Glutamine Waymouth Medium 752/1 + L – Glutamine
Merk Biosciences Ltd, Nottingham, UK.	Simvastatin
Sigma – Aldrich Co Ltd., Poole, UK.	Accutase ^{TM} Collagen Type I (from rat tail) Ethanol absolute Foetal Calf Serum Magnesium Sulphate Mevalonic Acid Platelet Derived Growth Factor AB Sodium Bicarbonate Sodium Dodecyl Sulphate Trichloroacetic Acid
Surgipath Europe Ltd., Peterborough, UK.	Haematoxylin Gill III
VWR International, Poole, UK.	Acetic acid DPX mountant for microscopy Eosin yellowish Hydrochloric acid Sodium hydroxide

Table 4: List of drugs and chemicals used by supplier.

2.8.2 Formulae of Solutions Used

Lauryl Sulphate/ Sodium Hydroxide

0.1g Sodium Dodecyl Sulphate

0.6g Sodium Hydroxide

50ml distilled H_2O

Scott's Tap Water Substitute

20g Sodium Bicarbonate

3.5g Magnesium Sulphate

1000ml distilled H_2O

0.5 % Acid Alcohol

70ml Ethanol absolute

30ml distilled H_2O

0.5ml concentrated Hydrochloric Acid

2.8.3 Plasticware Used

VWR International, Poole, UK.	Corning Costar 6 well plate
	Corning Costar 24 well plate
	Costar Cell Culture Inserts, Transwell [TM]
	Polycarbonate Membranes, 6µm pore
	Nunc Cell Culture Flasks, Easy Flask [TM]

Table 5: List of plasticware used by supplier.

Chapter 3

RESULTS

3.1 Patients Involved in the Experimental Study

3.1.1 Introduction

The principal aim of this study was to determine whether profound tissue ischaemia in a lower limb affected the way in which the long saphenous vein would respond to organ culture. Long saphenous vein taken from patients undergoing amputation due to severe ischaemia was to constitute one of the groups. It was essential that non-ischaemic veins were taken from a comparable patient group. Obtaining long saphenous vein from patients undergoing varicose vein surgery would certainly have provided large amounts of non-ischaemic tissue but the patient characteristics would have been very much different when compared to patients undergoing amputation. It was decided that the most comparable group of patients would be those undergoing aorto-coronary bypass grafting for ischaemic heart disease.

3.1.2 Summary of Patients Involved in the Study

A total of twenty patients were consented and entered into the study. A total of 11 ischaemic veins (IV) and 9 non-ischaemic veins (NIV) were obtained. A portion of long saphenous vein was obtained from each patient. Table 6 lists the order in which these vein specimens were obtained as well as the age and sex of each patient.

PATIENT NUMBER	Age	Sex	ISCHAEMIC OR NON-ISCHAEMIC LIMB
2	72	M	Ischaemic
3	86	F	Ischaemic
5	45	F	Ischaemic
6	62	M	Ischaemic
8	60	M	Ischaemic
9	77	M	Ischaemic
10	75	F	Ischaemic
11	65	F	Non-Ischaemic
12	68	F	Non-Ischaemic
13	76	M	Non-Ischaemic
14	76	M	Non-Ischaemic
15	74	M	Non-Ischaemic
16	78	M	Non-Ischaemic
18	81	F	Ischaemic
19	65	M	Ischaemic
20	47	M	Non-Ischaemic
21	68	M	Ischaemic
22	66	M	Non-Ischaemic
23	55	M	Ischaemic
24	50	M	Non-Ischaemic

Table 6: Summary of characteristics of patients involved in the study in order of obtaining vein portion.

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3.1.3 Comparison of Patients Involved in the Study

It was expected that the two groups of patients would be of comparable age and sex distribution. Other factors such as diabetes, total number of medications in use and current smoking history were thought to possibly be less comparable. Table 7 shows a comparison of these factors.

Characteristic	Ischaemic (n=11)	Non-Ischaemic (n=9)
M: F	7:4	7:2
Median Age (range)	68 (45-86)	68 (47-78)
Smokers	4	2
Diabetic	6	2
Median number of medications (range)	5 (2-12)	5 (2-10)
Statin prescription	4*	8*

*Table 7. Comparison of characteristics of the two patient groups involved in the study. Statistics: Mann-Whitney test to compare age and total number of medications, p=1.00 and p=0.90, respectively; Fishers exact test used to compare sex, p=0.64; prevalence of smoking, p=0.64; and prevalence of diabetes, p=0.20. * Fishers exact test to compare statin prescription, p=0.03 .*

It can be seen that the two groups are well matched with regard to age, smoking history, diabetes and total number of medications in use. The only significant difference between the two groups other than the presence of profound tissue ischaemia is statin prescription. Patients undergoing coronary artery bypass grafting are significantly more likely to be prescribed a statin than those patients undergoing lower limb amputation for ischaemia.

3.2 Organ Culture

3.2.1 Introduction

Vein rings maintained in organ culture develop thickening of the intimal layer which is similar, in many ways, to the pathological changes seen in vein graft stenoses. In both cases, the intimal layers contain an abundance of VSMCs of a secretory phenotype and the composition of the extracellular matrix is very similar (127). Intimal thickening in the organ culture model is maximal at 14 days and does not change after this time (127). In addition, the majority of proliferating cells are VSMC that have migrated into the intima from the vessel media (128) and use of this organ culture technique as a model of intimal hyperplasia has been validated as a useful model of vein graft stenosis (92). Previous authors have demonstrated intimal thickening in vein rings maintained in organ culture from patients undergoing coronary artery bypass grafting and that simvastatin reduced the amount of intimal thickening in a dose-dependent manner, and by 75% at a concentration of 5µM (102). The technique of vein ring organ culture was chosen in this thesis to compare the effects of organ culture on vein rings from two groups of patients: those undergoing lower limb amputation; and those undergoing coronary artery bypass grafting (CABG). The veins from the patients undergoing lower limb amputation have been exposed to severely ischaemic conditions prior to use in this model (Ischaemic veins), whereas the veins from the CABG group have not (Non-Ischaemic veins). It may be that the Ischaemic veins are more susceptible to the development of intimal hyperplasia in organ culture compared with Non-Ischaemic veins due to the adverse effects of prolonged tissue ischaemia and that simvastatin may have an effect on this process of intimal hyperplasia in organ culture.

3.2.2 General Observations of the Effect of Organ Culture

From published data, it was expected that organ culture for 14 days would result in intimal growth in the human long saphenous vein from non-ischaemic limbs. The response of veins from ischaemic limbs was not known. Figures 4, 5 and 6 are photographs from vein rings and have been chosen to give a representative view of the differences between the groups and the changes seen after organ culture.

In freshly fixed vein (Figure 4a) the three layers of the vein wall (Tunica Intima, Tunica Media and Tunica Adventitia) can be easily identified. The very thin intimal layer is shown in more detail in Figure 4b and contains few cells, mainly myointimal cells and endothelial cells.

Following culture for 14 days in culture medium alone, the intimal layer is much thicker and contains many more cells, often appearing in groups (Figure 5). These cells appear to be smooth muscle cells. Between these cellular areas, there are areas of increased extracellular matrix which has been deposited.

Figure 6 is a photograph of a vein ring following 14 days of culture in culture medium plus Simvastatin 5µM. It can be seen that the development of intimal hyperplasia in this specimen is less than that in Figure 5. There is still an increase in the thickness and cellularity of the intimal layer although it is not as pronounced as in the absence of simvastatin. There is also an increase in extracellular matrix, but again, not as pronounced as in Figure 5.

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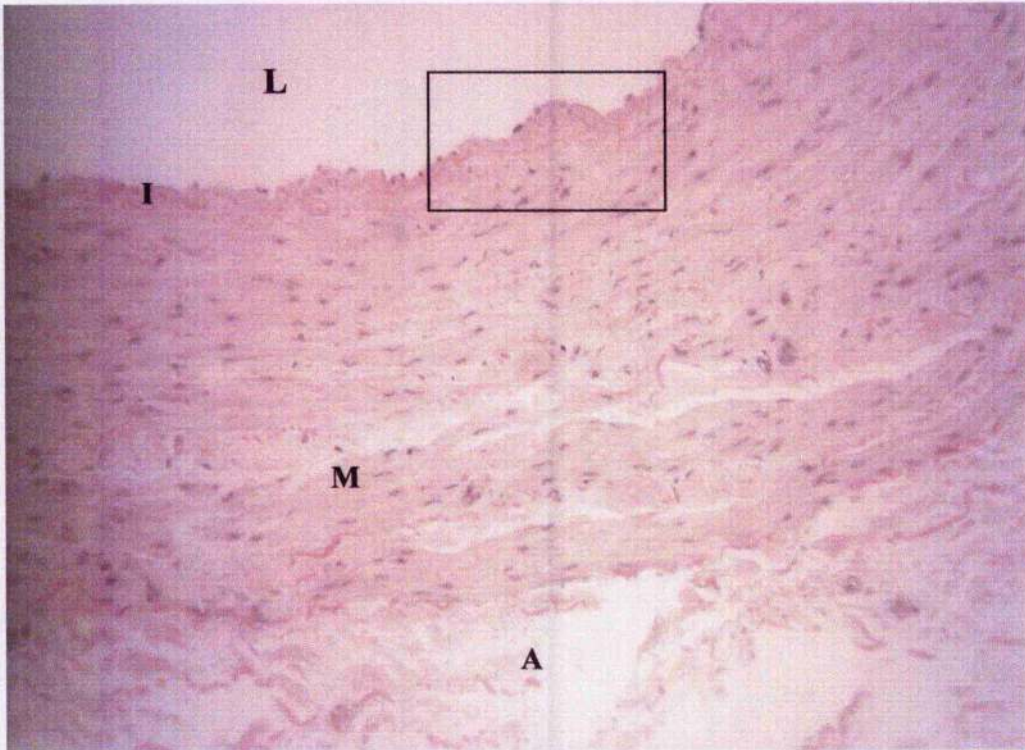
Figure 4a: Section of fresh fixed vein at x100 magnification. L=Lumen; I= Tunica Intima; M= Tunica Media; A= Tunica Adventitia. Area enclosed in box is shown at x400 magnification in Figure 4b below. Staining is with Haematoxylin and Eosin.
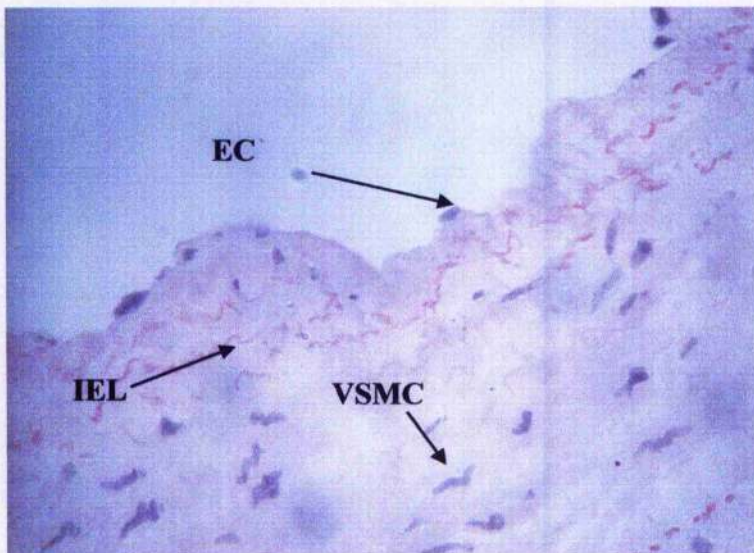
Figure 4b: Detail from Figure 4a at x400 magnification. IEL=Internal elastic lamina, EC=endothelial cell, VSMC=vascular smooth muscle cell.
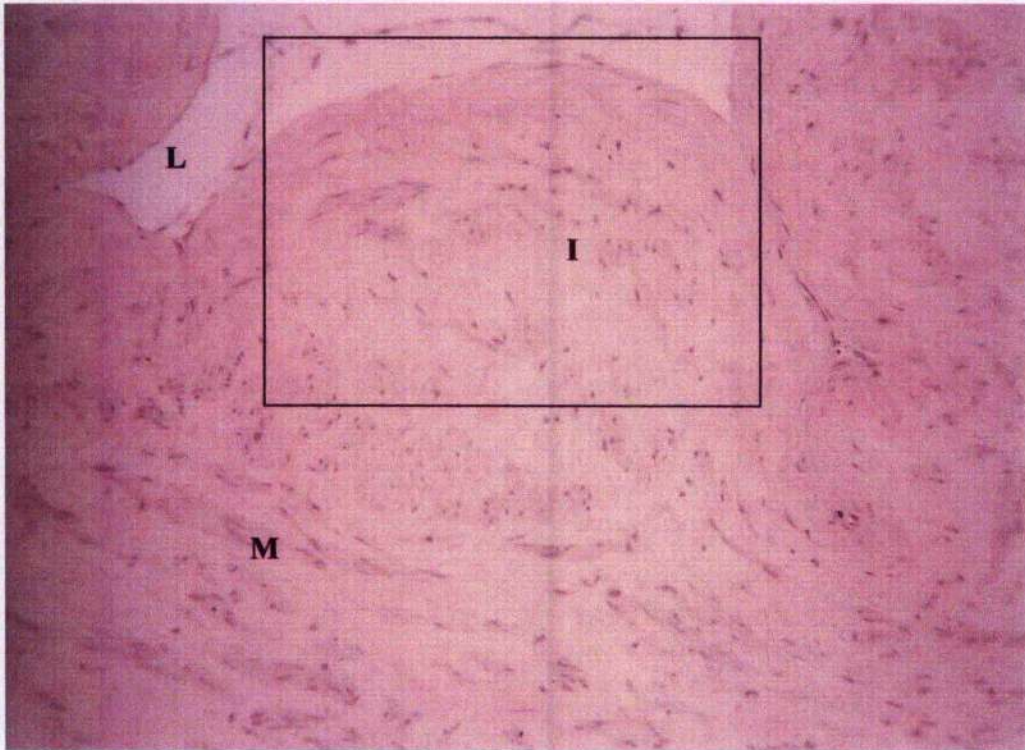
Figure 5a: Section of vein after culture for 14 days in culture medium alone at x100 magnification. L=Lumen; I= Tunica Intima; M= Tunica Media. Area enclosed in box is shown at x200 magnification in Figure 5b below. Staining is with Haematoxylin and Eosin.
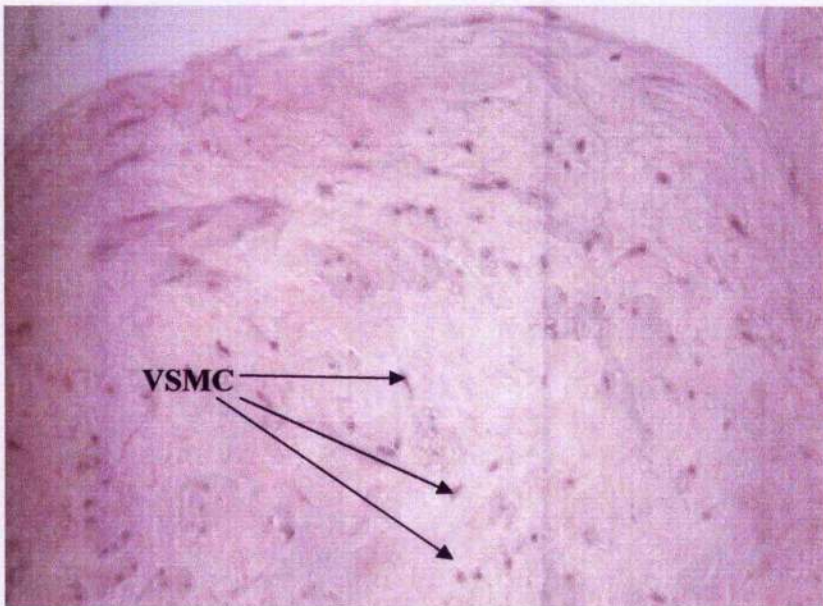
Figure 5b: Detail from Figure 5a at x200 magnification showing intimal hyperplasia with numerous vascular smooth muscle cells (VSMC) within extracellular matrix.
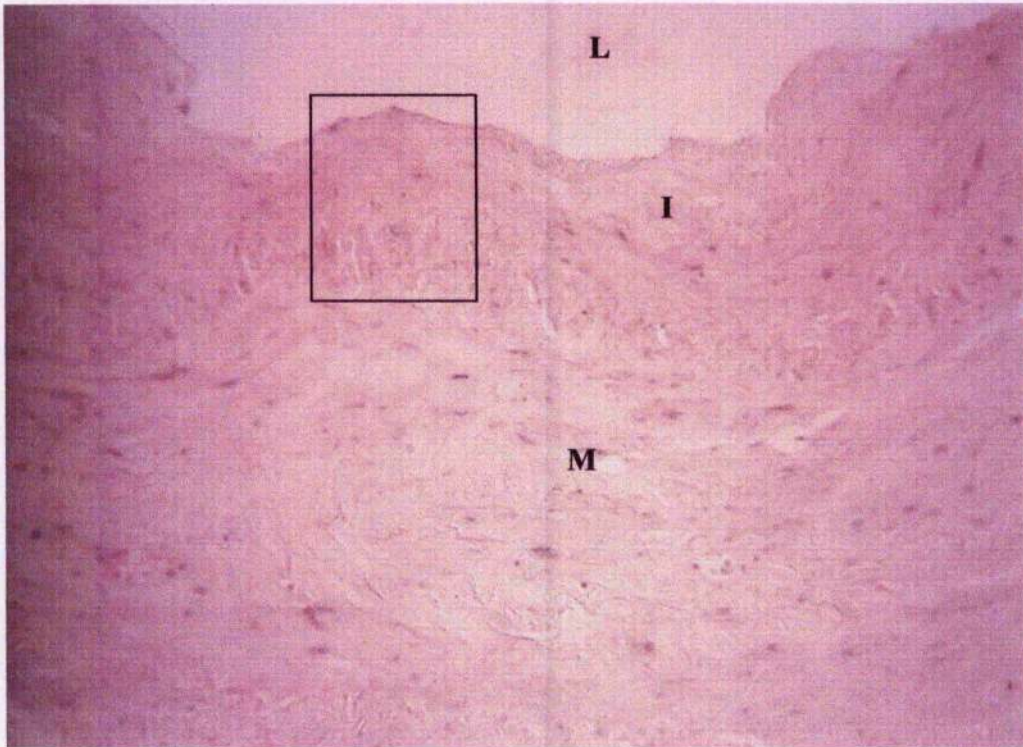
Figure 6a: Section of vein after culture for 14 days in culture medium with 5μM simvastatin at x100 magnification. L=Lumen; I= Tunica Intima; M= Tunica Media. Area enclosed in box is shown at x400 magnification in Figure 6b below. Staining is with Haematoxylin and Eosin.
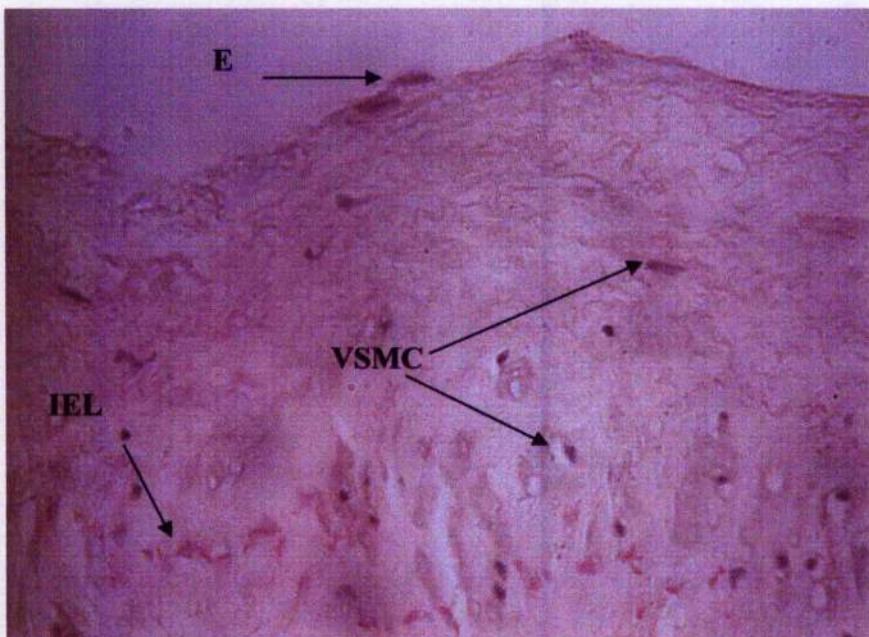
Figure 6b: Detail from Figure 3a at x400 magnification. IEL=Internal elastic lamina, E=endothelial cell, VSMC=vascular smooth muscle cell.

3.2.3 Summary of Organ Culture Data from Ischaemic Veins

Organ culture was not completed in veins from patients number 2 and 5. In both cases, the vein rings showed evidence of bacterial infection within the first 72 hours of culture, suggesting that contamination occurred at the time of dissection. Of the remaining 9 veins, organ culture was continued for the full 14 days as described in Section 2.3. Therefore, $n=9$ for ischaemic vein rings in the following results.

3.2.4 Observed Effects of Organ Culture on Ischaemic Veins

Vein rings from ischaemic limbs which had been maintained in organ culture for 14 days showed growth of the intimal layer when compared with the fresh fixed vein rings. The presence of simvastatin at 5μM for the duration of organ culture appeared to abolish this process. One-way ANOVA using randomised blocks was used to compare intimal areas between the 3 groups: fresh fixed, culture alone and culture with simvastatin. There was a significant difference in the percentage area of intima between fresh fixed and culture alone groups and between culture alone and culture with simvastatin. There was no difference between the fresh fixed and culture with simvastatin groups. Figure 7 is a graphic representation of the differences in intimal areas.

3.2.5 Summary of Organ Culture Data from Non-Ischaemic Veins

Organ culture was completed all veins except vein number 16 from the non-ischaemic limbs. Therefore, $n=8$ for non-ischaemic vein rings in the following results.

3.2.6 Observed Effects of Organ Culture on Non-Ischaemic Veins

Vein rings from non-ischaemic limbs, which had been maintained in organ culture for 14 days, showed growth of the intimal layer when compared with the fresh fixed vein rings. The presence of simvastatin at 5μM for the duration of organ culture suppressed this process. One-way ANOVA using randomised blocks was used to compare intimal areas between the 3 groups: fresh fixed, culture alone and culture with simvastatin. As in the comparisons for ischaemic veins, there was a significant difference between fresh fixed and culture alone groups and culture alone and culture with simvastatin. There was also a significant difference between fresh fixed and culture with simvastatin in the non-ischaemic veins. This suggests that the vein rings were less responsive to the presence of simvastatin in the organ culture than the veins from ischaemic limbs. Figure 8 is a graphic representation of the differences in intimal areas.
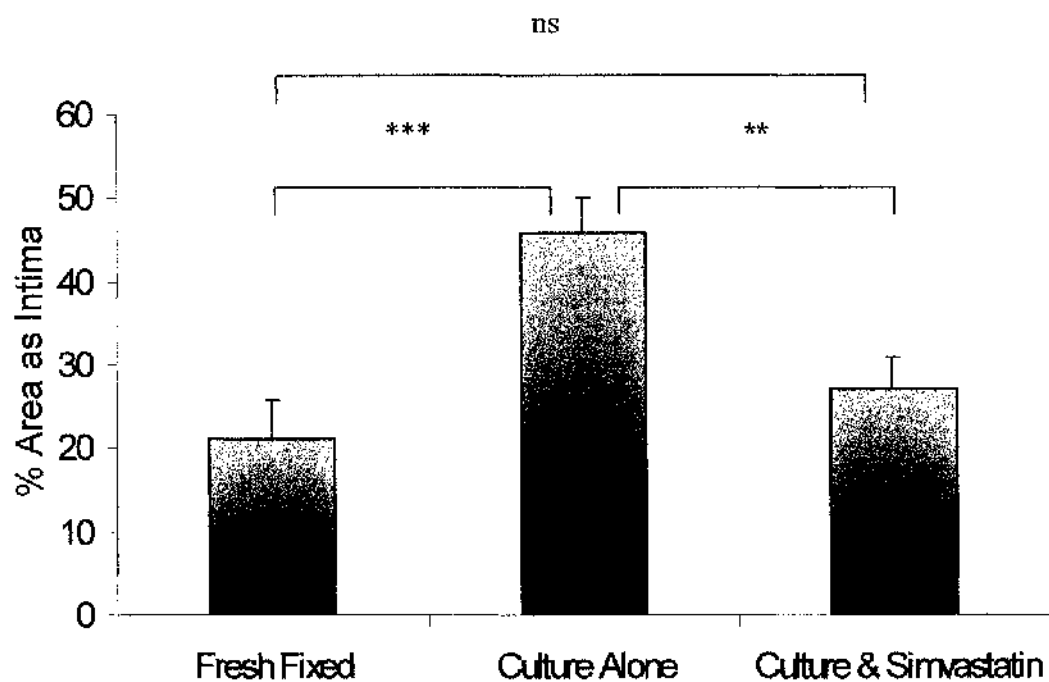
*Figure 7: Comparison of percentage areas as intima between ischaemic veins. ***p<0.001, ** p<0.01 (n=9). One-way ANOVA.*
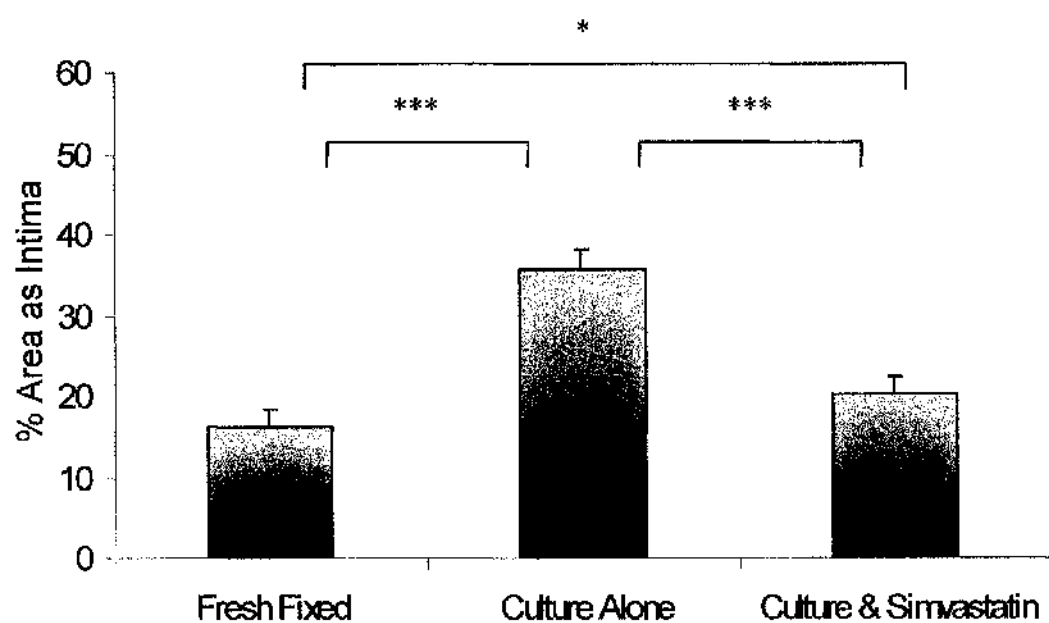
*Figure 8: Comparison of percentage areas as intima between non-ischaemic veins. *** p<0.001, *p<0.05 (n=8). One-way ANOVA.*

3.2.7 Comparison of Ischaemic and Non-Ischaemic Vein Organ Culture

Ischaemic vein rings developed a greater amount of intima compared with Non-Ischaemic vein rings (45.8% from 22.3% vs 35.8% from 16.2%) in culture alone. In the fresh fixed and culture with simvastatin vein rings, there was no difference between the amount of intima between the two groups. This is shown in Figure 9.
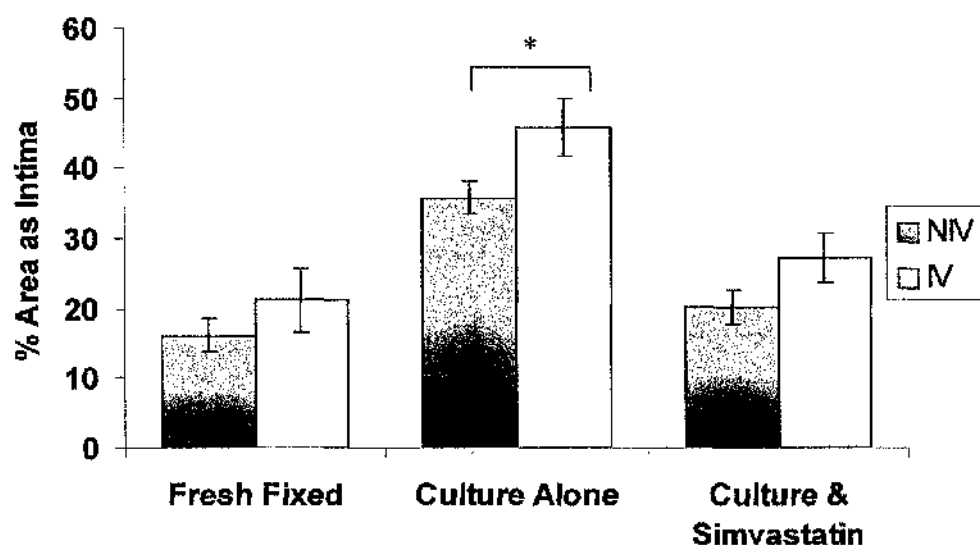
*Figure 9: Comparison of percentage areas as intima between Ischaemic Veins (n=9) (IV) and Non-Ischaemic Veins (n=8) (NIV). * p=0.04. Unpaired t-test.*

3.2.8 Changes in the Absolute Area of Tunica Intima and Tunica Media

The absolute area of tunica intima and tunica media was calculated for each vein ring. Table 8 shows the mean absolute intimal and medial areas for Non-Ischaemic vein rings and Table 9 shows this for Ischaemic vein rings.

	Mean intimal area, μm^2 (SEM)	Mean medial area, μm^2 (SEM)
Fresh Fixed	0.495 (0.095)	2.649 (0.380)
Culture with Simvastatin	0.674 (0.142)	2.538 (0.373)
Culture Alone	0.874 (0.157)	1.796 (0.334)

Table 8: Intimal & Medial areas for Non Ischaemic vein rings. n=8

	Mean intimal area, μm^2 (SEM)	Mean medial area, μm^2 (SEM)
Fresh Fixed	0.523 (0.197)	1.558 (0.435)
Culture with Simvastatin	0.674 (0.202)	1.530 (0.366)
Culture Alone	1.045 (0.332)	1.056 (0.331)

Table 9: Intimal & Medial areas for Ischaemic vein rings. n=9

The mean absolute intimal area increased in both groups from the fresh fixed group to the culture with simvastatin group and also from the fresh fixed group to the culture alone group. There was a corresponding overall decrease in the area of tunica media. On analysing this data using one-way ANOVA with repeated measures, the difference between the fresh fixed intimal area and intimal area after culture alone was significant in both groups (Table 10). This confirms that there is a change in the intima area whether expressed as percentage of the total vessel wall or as an absolute value. However, the difference in absolute intimal area between fresh fixed rings and rings cultured with simvastatin was not statistically significant. Partially, this is a reflection of the variability of intimal area from vein to vein which can also be seen in the high standard errors.

The mean absolute medial areas show a decrease from the fresh fixed group to the culture with simvastatin group and also from the fresh fixed group to the culture alone group. This change in area of media mirrors the changes seen in the tunica intima of the vein rings and is likely to be due to migration of the smooth muscle cells from the media into the intima. However, the change in medial area is statistically significant only for the non-ischaemic vein rings between the fresh fixed and culture alone groups and between the culture alone and culture with simvastatin groups (Table 11). This may reflect the variability of medial area from vein to vein which can also be seen in the high standard errors.

	Comparison of Intima Areas	p
Non-Ischaemic Veins	Fresh fixed vs Culture Alone	<0.05
	Fresh fixed vs Culture with Simvastatin	>0.05
	Culture Alone vs Culture with Simvastatin	>0.05
Ischaemic Veins	Fresh fixed vs Culture Alone	<0.05
	Fresh fixed vs Culture with Simvastatin	>0.05
	Culture Alone vs Culture with Simvastatin	>0.05

Table 10: Statistical comparison of absolute tunica intima areas for Non-Ischaemic and Ischaemic Veins. One-way ANOVA with repeated measures.

	Comparison of Media Areas	p
Non-Ischaemic Veins	Fresh fixed vs Culture Alone	<0.01
	Fresh fixed vs Culture with Simvastatin	>0.05
	Culture Alone vs Culture with Simvastatin	<0.01
Ischaemic Veins	Fresh fixed vs Culture Alone	>0.05
	Fresh fixed vs Culture with Simvastatin	>0.05
	Culture Alone vs Culture with Simvastatin	>0.05

Table 11: Statistical comparison of absolute tunica media areas for Non-Ischaemic and Ischaemic Veins. One-way ANOVA with repeated measures.

3.2.9 Discussion of Organ Culture Findings

In organ culture for 14 days, vein rings from Ischaemic and Non-Ischaemic limbs develop significant growth of the intimal layer. This response was expected in the veins from Non-Ischaemic limbs as it had been demonstrated previously (92;102). However, direct comparison of this data with that from these authors is not possible as both used intimal thickness rather than intimal area. The problem with using intimal thickness is that it cannot distinguish between a vessel which has remodelled in culture and a vessel which has developed intimal growth as both will appear to have a thicker tunica intima. Additionally, Porter *et al* did not quote baseline intimal thickness (that is, before organ culture (fresh fixed). Instead, the authors use veins cultured in culture medium with growth factors alone as their control measure of intimal growth compared with organ culture in the presence of variable concentrations of drug. Huang *et al* also use intimal thickness but compare this with baseline pre-culture thickness. The data presented in this thesis is, therefore, novel in two ways. Firstly, it gives a more accurate reflection of actual changes in the vessel wall composition by measuring absolute intimal and medial areas in order to calculate percentage contribution to the vessel wall. Additionally, this is the first study showing data on veins from Ischaemic limbs. It was observed that the amount of intimal growth was significantly greater in the Ischaemic vein rings when compared with the Non-Ischaemic vein rings. This finding suggests that the vein response has been pre-programmed by the conditions prior to organ culture, that is, tissue ischaemia.

The addition of simvastatin at a concentration of $5\mu M$ to the organ culture reduced the intimal growth in both Ischaemic and Non-Ischaemic vein rings. This effect was expected in the Non-Ischaemic vein rings but had not previously been demonstrated in vein rings from Ischaemic limbs. The effect of simvastatin may be related to both its antiproliferative and anti-migratory effects on VSMC. From my data, the Ischaemic vein rings appear to show a greater response to the presence of

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simvastatin than the Non-Ischaemic vein rings. A possible explanation is that there is enhanced VSMC proliferation and migration in the Ischaemic Vein rings, thereby making the cells more susceptible to the effect of the statin. This effect may suggest a therapeutic target for simvastatin in patients undergoing both infrainguinal bypass grafting for lower limb ischaemia as well as patients undergoing coronary artery bypass grafting to prevent graft stenosis. It is important, however to also point out that the numbers achieved in these organ culture experiments were small and the apparent differences between the two groups may be due to a Type I statistical error.

The concentration of simvastatin chosen for these organ culture experiments, 5µM, was based on my observations in experiments on cell proliferation (see Section 3.3). This concentration also compares with work by previous authors (102;109). How this concentration in organ culture compares with expected tissue levels of simvastatin during oral administration is not clear. Oral doses of simvastatin vary from 10mg to 80mg per day. Studies on the pharmacokinetics of simvastatin show that it is readily absorbed orally but is extensively metabolised by first pass hepatic metabolism. As such, plasma concentrations of simvastatin are in the region of 5-20nM (129;130). Furthermore, 95% of circulating simvastatin is protein-bound and more than three quarters of this is in the inactive form (129;131). Based on these facts, it can be seen that the concentration used in my organ culture experiments is much greater than levels that could be expected in patients treated with normal doses of simvastatin. However, the concentration chosen for these experiments is in line with the concentrations used in previous studies demonstrating antiproliferative effects of statins (102;132). How this fits with the observations of Henke *et al* and Abbruzzese *et al* who both associated improved graft patency with statin therapy, is not clear. The patients in both of these studies received oral statins in normal doses. This could not have delivered sufficiently high enough local concentrations to the vein grafts in order to exert a direct antiproliferative effect. It is possible, however, that the improved graft patency seen by these authors

was not due to a direct inhibition of VSMC proliferation. Instead, the effects may have been mediated by other factors, such as increased bioavailability of nitric oxide (NO) which has been shown to repress cell proliferation by inhibiting p21 upregulation. Certainly, normal oral doses of statins have been shown to improve endothelial dependent vasodilation measured by forearm blood flow. Since simvastatin has been shown in the present study to have antiproliferative effects on VSMC explanted from ischaemic long saphenous veins (see section 3.3), it would not be unreasonable to attempt to use this effect in order to prevent graft stenosis. Possible ways to do this include use of local high dose delivery systems. This could be in the form of a drug eluting suture which would release the drug over a prolonged period of time. Another possibility would be to bathe the vein in a statin solution just prior to inserting it as a graft.

The only differences between the two groups of veins obtained in this study were presence or absence of prolonged tissue ischaemia before culture and statin prescription (see Section 3.1.3). The effect of prolonged tissue ischaemia has not previously been investigated. It is likely, however, that the tissue, and therefore cellular ischaemia activates stress pathways in the vein cells. Possible pathways include the mitogen activated protein kinase (MAP Kinase) p38 pathway. This pathway stimulates cell proliferation responses and has been shown to cause vascular hypertrophy in patients with pulmonary vascular hypoxia leading to pulmonary hypertension (133). Another pathway that may be involved is the MAP Kinase p42/p44 pathway which leads to cellular proliferation and migration (134). Further work investigating the role of these MAP Kinase pathways would clarify their role. Another question is what would be the response of vein rings cultured under hypoxic conditions.

It is possible, that the prior medication of the patient with statin influenced the outcome of the experiments. This is unlikely for a few reasons. Firstly, based on known pharmacokinetics of statins,

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the plasma concentrations are very low and most of the drug is protein bound. Additionally, all vein rings were washed in blank media before culture which would remove any surplus drug and the frequent replenishment of culture medium would likely have facilitated the removal of any residual statin. These arguments, however, do not account for any cellular-level changes which may have occurred as a result of statin therapy before vein harvest.

Organ culture was completed in nine out of eleven ischaemic veins. Of these veins, three came from patients with prior treatment with a statin (vein numbers 8, 9 and 19). One of the veins in which organ culture was not completed came from a patient taking a statin. Unfortunately, these numbers are too low to perform any statistical subgroup analysis, but on reviewing the data on intimal areas, there is no apparent trend in the responses of these veins compared to veins from patients who did not receive a statin pre-operatively. Of course, it cannot be said with certainty that prior treatment with a statin could influence the experimental results. Unfortunately, however, in light of the overwhelming evidence that patients with atherosclerotic disease should be prescribed a statin, there is little feasibility of performing a study to investigate this point.

3.3 Vascular Smooth Muscle Cell Proliferation

3.3.1 Introduction

Vascular smooth muscle cells (VSMCs) in the walls of arteries and veins are normally in a relatively quiescent state. Activation of VSMC leading to proliferation and migration are key steps in the development of intimal hyperplasia and atherosclerosis.

3.3.2 Proliferation of Cells Explanted from Ischaemic and Non-Ischaemic Vein Rings

VSMCs were explanted from vein rings and used in cell proliferation studies (see Section 2.5). Each experiment was performed in triplicate between Passage 3 and 5. (Ischaemic Veins, n=6; Non-Ischaemic Veins, n=6).

3.3.3 Statistical Analysis of Proliferation Data from Cells Explanted from Ischaemic and Non-Ischaemic Vein Rings

Simvastatin caused a concentration-dependent inhibition of VSMC proliferation as measured by incorporation of ^{3}H-thymidine. This was reversed by the addition of mevalonate 100μM. Data is presented (Figures 10 & 11) as absolute values, and is shown normalised to controls (Figure 12) as a percentage reduction in proliferation. The mean 50% inhibitory concentration (IC_{50}) for VSMC explanted from Ischaemic Vein rings was 1.16μM (+/- Standard error of mean (SEM), 0.46μM). The mean IC_{50} for VSMC explanted from Non-Ischaemic Vein rings was 1.22μM (+/- SEM, 0.33μM). The IC_{50}s were calculated using the Hill equation and were not statistically significantly different when compared using the unpaired t test.
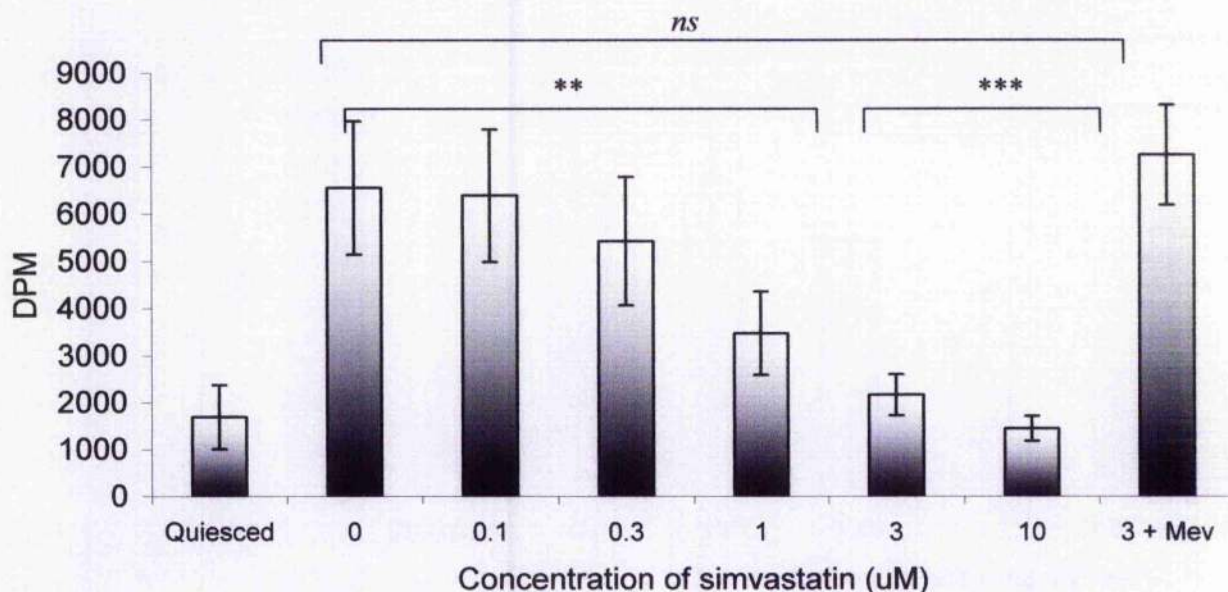
Figure 10: Effect of Simvastatin on 3H-Thymidine incorporation of VSMCs from Ischaemic Vein Rings. Quiesced= unstimulated cells, Mev= Mevalonate 100μM. One way ANOVA with randomised blocks, **p<0.01, ***p<0.001. n=6
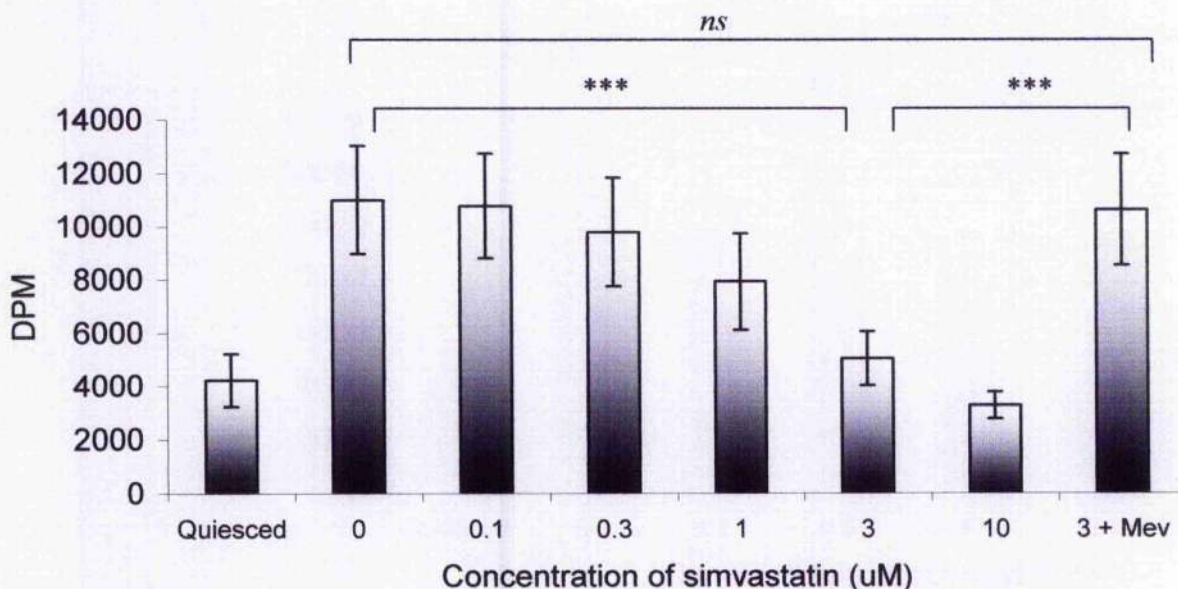
Figure 11: Effect of Simvastatin on 3H-Thymidine incorporation of VSMCs from Non-Ischaemic Vein Rings. Quiesced= unstimulated cells, Mev= Mevalonate 100μM. One way ANOVA with randomised blocks, ***p<0.001. n=6
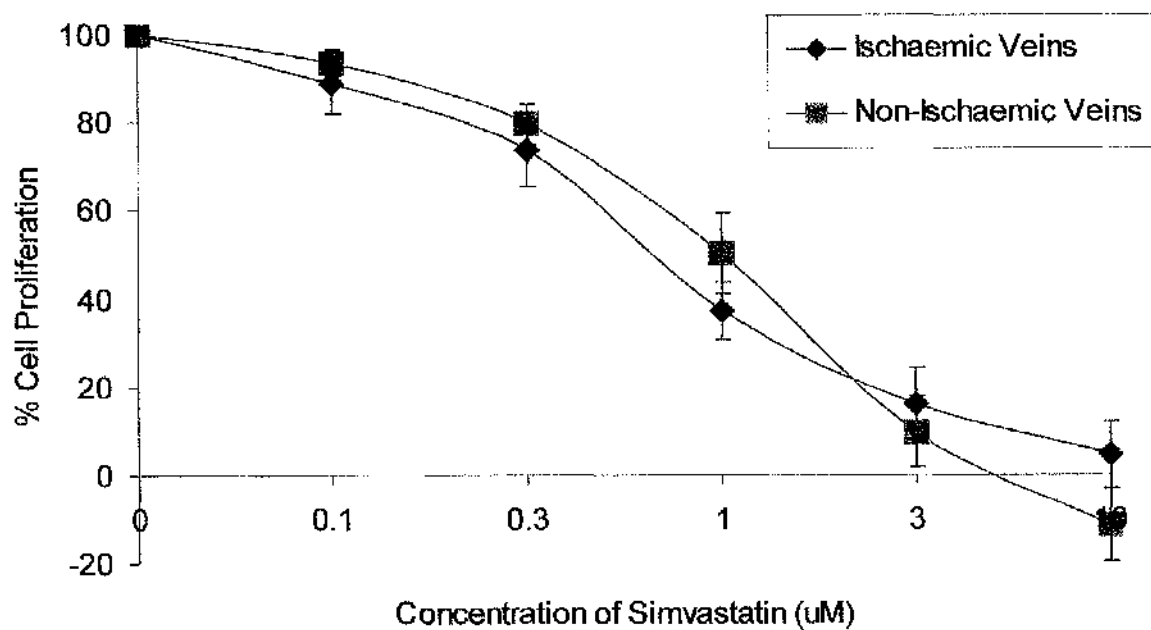
Figure 12: Effect of Simvastatin on VSMC proliferation from Ischaemic and Non-Ischaemic Vein Rings. IC$_{50}$ 1.16µM (+/- SEM 0.46µM) and 1.22µM (+/- SEM 0.33µM) for Ischaemic and Non-Ischaemic rings respectively (p=ns). Unpaired t test.

3.3.4 Discussion of Cell Proliferation Results

Vascular smooth muscle cell proliferation is stimulated by activation of the mitogen-activated protein kinase (MAP Kinase) pathways. The MAP Kinase pathways are usually activated in response to growth factor stimulation and include the extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2) as well as the stress-activated protein kinases (SAPK or p38 MAPK). These pathways are activated by various signals from second messengers and result in cell proliferation or differentiation (135). Statins have been shown to inhibit VSMC proliferation *in vitro* both in cell culture and organ culture models (102;136). However, the response of VSMCs to statins appears to be dependent on factors such as animal of origin and also tissue of origin. For example, Corsini et al found that cultured rat aorta VSMCs had a similar IC_{50} for simvastatin and fluvastatin at 2.2 and 2.8µM, respectively. However, the authors found that proliferation of human femoral artery VSMCs is inhibited at lower concentrations of these two statins (137). Furthermore, another rat model showed that VSMCs explanted from post-angiopasty neointimal lesions are more susceptible to statin-induced apoptosis than VSMCs explanted from the rat thoracic aorta (138).

Given these observed differences it may also be expected that VSMCs from Ischaemic and Non-Ischaemic veins could respond differently to simvastatin. The principal aim of my experiments was to delineate any such difference. My data from the organ culture of vein rings (see section 3.2.6 and 3.2.7) has suggested a difference in the proliferative response to culture and the inhibition of this response to the presence of simvastatin between Ischaemic and Non-Ischaemic veins. The Ischaemic vein rings were more susceptible to the presence of simvastatin and this could possibly have been due to an enhanced antiproliferative effect of simvastatin on the VSMCs. My cell culture experiments, however, have not demonstrated any difference in inhibition of VSMC proliferation between cells from Ischaemic and Non-Ischaemic veins. This may suggest that there is an additional

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effect of the surrounding vein wall matrix on the VSMC proliferation response which is lost once the VSMCs are freed from this surrounding environment.

With regard to the possible mechanism of VSMC proliferation inhibition, addition of mevalonic acid to the assays completely reversed the effect of simvastatin. This is evidence that the mechanism of inhibition is through reduction of intracellular mevalonic acid, a precursor of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These are lipid attachments necessary for the membrane localisation of G proteins including, ras, rho and rac. Although these experiments were not designed to establish the cellular mechanism of inhibition of VSMC proliferation by simvastatin, it is likely that its action is by this mechanism.

3.4 Vascular Smooth Muscle Cell Migration

3.4.1 Introduction

Migration of vascular smooth muscle cells (VSMCs) from the tunica media into the tunica intima is an important step in the development of intimal hyperplasia. Given that veins from ischaemic limbs showed a greater degree of intimal hyperplasia in organ culture compared with veins from non-ischaemic limbs, it was hypothesised that this may be due, in part, to a greater degree of VSMC migration in veins from the ischaemic limbs.

3.4.2 Migration of Cells Explanted from Ischaemic Vein Rings

VSMCs were explanted from vein rings in organ culture and used in cell migration studies (see Section 2.6). Each experiment was performed in duplicate. For the data presented here, n=5.

3.4.3 Statistical Analysis of Migration Data from Cells Explanted from Ischaemic Vein Rings

In the absence of simvastatin, PDGF 20ng/ml caused extensive migration of VSMC compared with quiesced cells. Addition of mevalonate to the PDGF did not significantly alter the amount of VSMC migration. In the presence of simvastatin, PDGF still caused significant VSMC migration compared with quiesced cells. Addition of mevalonate did not alter the degree of VSMC migration. VSMC migration in the presence of PDGF appeared to be reduced but not significantly by the addition of simvastatin. These results are displayed in Figure 13.
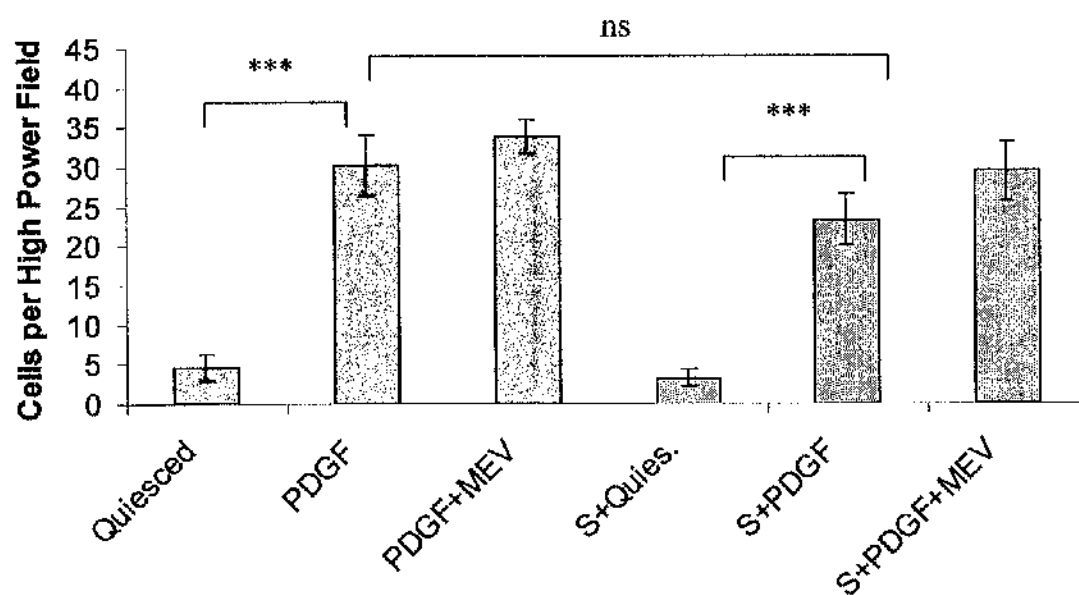
Figure 13: VSMC migration from cells explanted from Ischaemic Veins. PDGF= Platelet derived growth factor, MEV= mevalonate, S= Simvastatin, Quiesc .= Quiesced,.***p<0.001. One way ANOVA with randomised blocks. n=5

3.4.4 Migration of Cells Explanted from Non-Ischaemic Vein Rings

VSMCs were explanted from vein rings in organ culture and used in cell migration studies (see Section 2.6) at passage 3 or 4. Each experiment was performed in duplicate. For the data presented here, n=8.

3.4.5 Statistical Analysis of Migration Data from Cells Explanted from Non-Ischaemic Vein Rings

In the absence of simvastatin, PDGF 20ng/ml caused significant migration of VSMC compared with quiesced cells. Addition of mevalonate to the PDGF did not significantly alter the amount of VSMC migration. In the presence of simvastatin, PDGF still caused significant VSMC migration compared with quiesced cells. Addition of mevalonate did not alter the degree of VSMC migration. Again, VSMC migration in the presence of PDGF appeared to be reduced but not significantly by the addition of simvastatin (Figure 14).
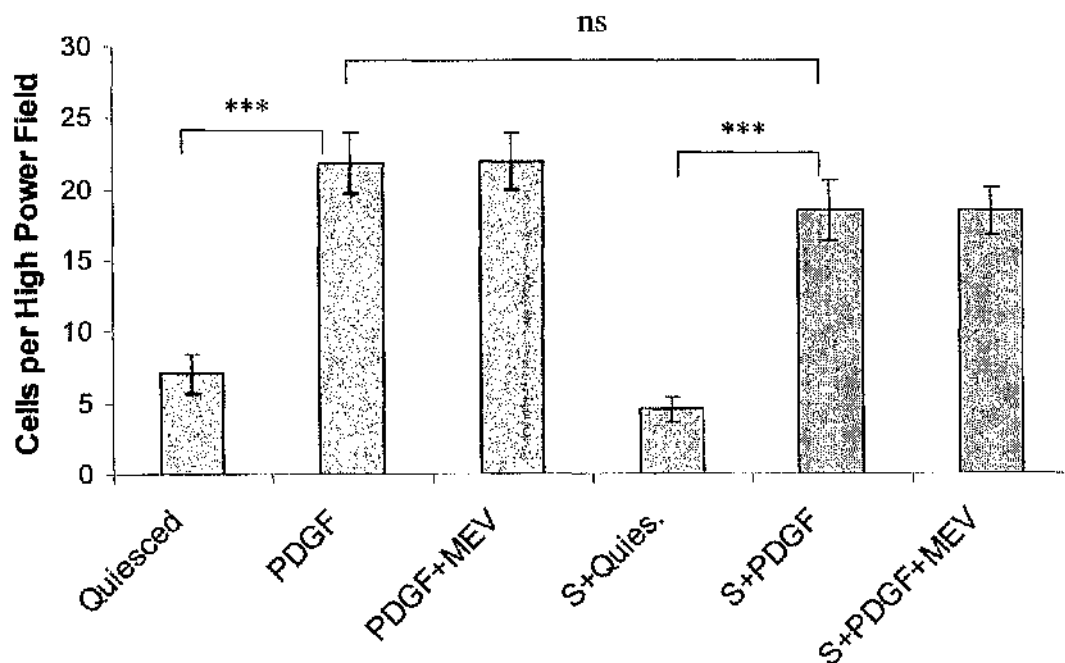
Figure 14: VSMC migration from cells explanted from Non-Ischaemic Veins. PDGF= Platelet derived growth factor, MEV= mevalonate, S= Simvastatin, Quiesc .= Quiesced. *** p<0.001. One way ANOVA with randomised blocks. n=8

3.4.6 Comparison of Migration Between Ischaemic and Non-Ischaemic Vein Cells

The basal level of VSMC migration in quiesced cells was not significantly different in cells from the Ischaemic and Non-Ischaemic groups. However, PDGF stimulated migration of VSMCs from the Ischaemic veins more strongly when compared with the response of VSMC from Non-Ischaemic Veins. This remained true in the presence of mevalonate (Figure 15).
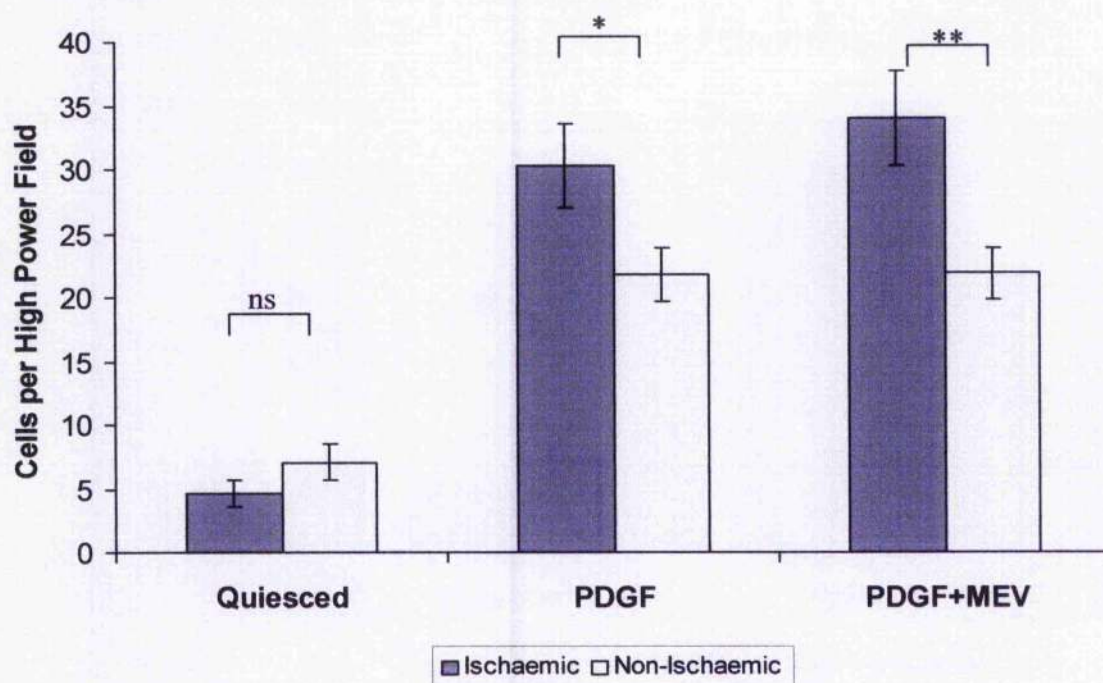
Figure 15. Comparison of VSMC migration between cells from Ischaemic and Non-Ischaemic Veins. PDGF= Platelet derived growth factor, MEV = mevalonate. *p=0.04, **p=0.009 . Unpaired t-test. Ischaemic veins, n=5, Non-Ischaemic veins, n=8.

3.4.7 Discussion of Cell Migration Results

From this data, VSMC explanted from Ischaemic vein rings demonstrate a greater degree of migration when stimulated with PDGF compared with cells explanted from Non-Ischaemic vein rings. No significant alteration in the migration response was elucidated by the addition of simvastatin at a concentration of 5μM.

The process of cell migration involves three key stages. Firstly, an external stimulus causes polarisation of the cell and the extension of a cell protrusion in the direction of movement. Following this, the protrusion adheres to the substance on which the cell is migrating. Finally, contraction of the cell in the direction of migration along with release of adhesions at the rear of the cell, allows the cell to move forward (145). The cellular mechanisms involved in this process are complex. An early key step in cell polarisation is the activation of cell division cycle 42 (Cdc42). Inhibition of Cdc42 causes cells to lose the ability to migrate directionally in response to a concentration gradient. Assembly and polymerisation of actin filaments in the cell leading edge is controlled by the small GTP-binding proteins, Rho and Rac (139;140).

The increased rate of migration, demonstrated by VSMCs from the Ischaemic vein rings when stimulated by PDGF, could be explained by changes in membrane PDGF receptor expression. PDGF receptor expression (α and β) is known to be altered in response to autocrine factors such as transforming growth factor – β (TGF-β) (141). Another possibility is a change in the intracellular signalling responses such as upregulation of the MAP Kinase p38 system. This system lies downstream of the GTP-binding proteins Cdc42 and Rac. It may be that profound tissue hypoxia leads to activation of MAP Kinase p38, with subsequently amplified responses to a migratory

stimulus. Hypoxia has previously been shown to stimulate the MAP Kinase p38 pathway in pulmonary fibroblasts and cardiac myocytes (142;143).

Mevalonate is a precursor, not only of intracellular cholesterol synthesis, but also of isoprenylation compounds farnesyl pyrophosphate (FPP) and geranyl geranyl prophosphate (GGPP). These compounds are necessary for the membrane localisation of small GTP-binding proteins including Ras, Rac and Rho. By blocking the membrane translocalisation of these proteins, statins could be expected to inhibit the effects of these proteins, including VSMC migration.

In my data, there was some inhibition of migration of smooth muscle cells by simvastatin. This effect approached but did not reach statistical significance. It is possible that this reflects a Type II error as the numbers are low (n=5 for cells explanted from ischaemic vein rings and n=8 for those explanted from the non-ischaemic vein rings.) in both groups. Further experiments would be needed to clarify this. Another possibility is that the concentration of simvastatin used was too low. However, previous studies have used similar concentrations of simvastatin and found an inhibition of cell migration (102;144). This raises the possibility that the stimulus for migration, PDGF was used in too high a concentration (20ng/ml).

3.5 Audit of Infrainguinal Graft Outcome 1993-2003

3.5.1 Introduction

The outcome of infrainguinal bypass grafting is most commonly measured by graft patency and limb salvage rates. Section 1.3 describes factors which are known to influence vein graft patency. This section of the thesis aims to analyse the graft patency rates and limb salvage rates for infrainguinal vein bypasses performed at Glasgow Royal Infirmary between 1993 and 2003. These outcomes are correlated with operative characteristics (graft inflow, outflow and conduit), patient characteristics (gender, diabetes and smoking) and medication use (statin, antiplatelets and warfarin).

Patients were identified to be included in the study from graft surveillance records at Glasgow Royal Infirmary. Since 1991, all infrainguinal bypass grafts performed at the hospital have undergone ultrasound surveillance for the first year after surgery. The case notes for all patients who underwent graft surveillance between Jan 1993 and Jan 2003 at Glasgow Royal Infirmary were reviewed. Previous studies have shown that one third of infrainguinal bypass grafts develop an area of significant stenosis in the first year after surgery (73;74). Surveillance of grafts using ultrasound can detect these stenoses and allow for repair before the graft occludes. This technique of surveillance and repair has been shown to improve the medium term patency of infrainguinal grafts but not eventual limb salvage rates (74).

3.5.2 Basic Analysis of Infrainguinal Grafts 1993-2003

Between January 1993 and January 2003, 207 infrainguinal grafts in 194 patients underwent surveillance. Of these, the case notes had been destroyed in 46 grafts and at the end of my research time frame, the case notes for a further 28 grafts could not be obtained. In total, I have reviewed the case notes for 133 of the 207 grafts (64%). With regard to the case notes which have been destroyed or were not available, some information was obtained from the surveillance database, allowing the following results to be collated. Figure 16 shows a breakdown of the number of grafts per year and the proportion of case notes that were destroyed or not seen. Table 12 shows some of the basic characteristics of the patients who underwent bypass grafting.
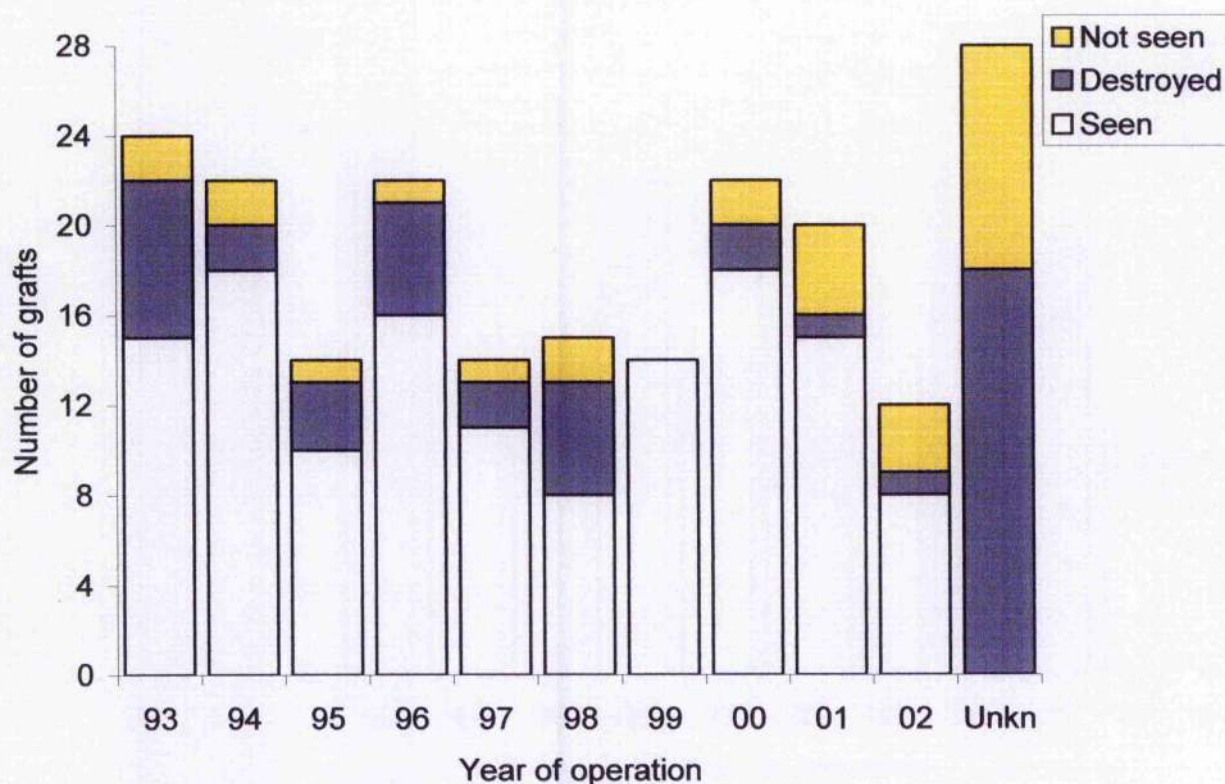
Figure 16: Number of infrainguinal grafts per year. Total number of grafts = 207.

	All Grafts	Grafts- notes seen
Number of grafts	207	133
Number of patients	194	122
Median Age at operation	67	66
Age Range	38-91	42-86
Males: Females	123:71	83:50
Side: Left:Right:Unknown	103:97:7	68:64:1

Table 12: Basic characteristics of the patients that underwent infrainguinal bypass grafting.

3.5.3 Concurrent Medical Conditions

Patients with peripheral vascular disease have a high incidence of concurrent medical conditions, mainly those associated with atherosclerosis. Of the 122 patients whose notes were reviewed, 115 had information regarding concurrent illnesses. The median number of concurrent illnesses was 2 (range 0-5). The commonest condition recorded was hypertension (46 patients), followed by diabetes (36 patients), ischaemic heart disease (30 patients), cerebrovascular disease (18 patients) and myocardial infarction (12 patients). Nineteen patients had also previously undergone major vascular surgery. These details along with the other conditions are shown in Table 13. It is interesting to note that very few patients were labelled with a diagnosis of hypercholesterolaemia at the time of bypass grafting. This is likely to be due to the fact that serum cholesterol levels were not routinely measured pre-operatively in these patients in the past. Furthermore, the evidence regarding the effect of statins in patients with "normal" cholesterol levels but a history of cardiovascular disease has only recently been established.

Condition	Number of Patients Affected (%)
Hypertension	46 (40%)
Diabetes	36 (31%)
Ischaemic Heart Disease / Angina	30 (26%)
Previous Vascular Surgery	19 (17%)
Cerebrovascular disease	18 (16%)
Previous Myocardial Infarction	12 (10%)
Chronic Obstructive Pulmonary Disease (COPD) /Asthma	13 (11%)
Congestive Cardiac Failure	6 (5%)
Atrial Fibrillation	5 (4%)
Hypercholesterolaemia	4 (3%)
Coronary Bypass Grafting	3 (2.6%)
Chronic Renal Failure	2 (1.7%)
Venous Ulceration / Thrombosis	3 (2.6%)
Epilepsy	2 (1.7%)
Alcoholism	2 (1.7%)
Polycythaemia	2 (1.7%)
Prostate Cancer	1 (0.8%)
Rheumatoid Arthritis	1 (0.8%)
Cognitive Impairment	1 (0.8%)
Myeloproliferative Disorder	1 (0.8%)
No concurrent illness recorded	**12 (10%)**

Table 13: Concurrent medical conditions of patients undergoing infrainguinal bypass grafting.

3.5.4 Types of Infrainguinal Grafts

Information regarding the proximal anastomosis site, distal anastomosis site and material used for the graft was extrapolated from a combination of the case notes and the surveillance scans. The proximal anastomosis site was the Common Femoral Artery (CFA) in 146 grafts, a previous Aortic Bifurcation Graft (ABG) in 15 grafts and from the External Iliac Artery (EIA) in 7 grafts. The site of distal anastomosis was at the above knee Popliteal Artery in 59 grafts, the below knee Popliteal Artery in 47 grafts, the tibial vessels in 55 grafts and unknown in 46 grafts. The conduit for all these grafts was the long saphenous vein (LSV). The LSV was reversed in 68 grafts and used *in situ* (that is, not reversed) in 69 grafts. This information was not recorded 70 grafts.

3.5.5 Graft Outcome and Operative Characteristics.

Of the grafts for which the case notes were reviewed (n=133), the mean follow up was 1093 days (median 843, range 12-4065). Of these 133 grafts, 33 (24.8%) occluded during the follow up period, at a mean time of 977 days (median 509, range 63-4065). Overall graft patency at 1, 3 and 5 years was 90%, 80% and 63%. This is shown in a survival curve in Figure 17.

Of the 33 grafts which occluded, 15 patients subsequently required amputation (45%), 18 did not (55%). Time to amputation was at a mean of 745 days (median 307, range 91-2428). One additional patient required amputation despite a functioning graft.
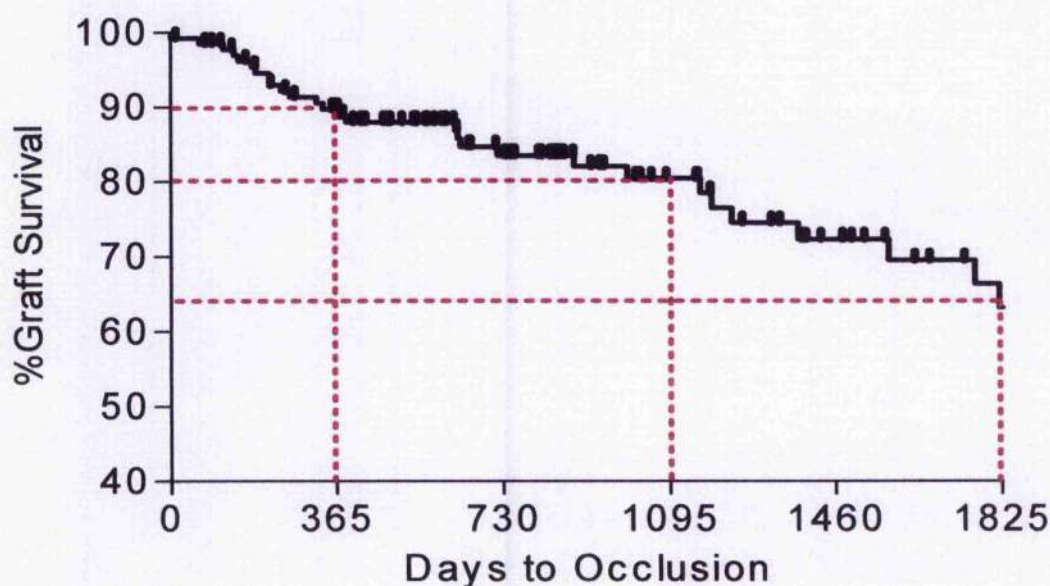
Figure 17: Overall graft patency for infrainguinal grafts 1993-2003. Broken red lines indicate 1, 3 and 5 year patency rates of 90%, 80% and 63%, respectively.

Graft occlusion was associated with below knee anastomosis at the popliteal artery or the tibial vessels compared with above knee anastomosis (p=0.0007, RR=3.8 (1.6-9.2) Fisher's exact test) and with *in situ* grafting compared with reversed vein grafting (p=0.025, RR=2.1 (1.1-4.0) Fisher's exact test). It is worth noting, however, that in the majority of distal grafts (i.e. to below knee popliteal artery or tibial vessels), the LSV was used *in situ*. The reason for this is that *in situ* grafting allows better matching of the diameter of the anastomoses. The proximal anastomosis site did not influence graft occlusion (p=0.77, RR= 0.89 (0.4-2.0) Fisher's exact test). These data are shown in Table 14.

The relative risk of amputation of the lower limb was not significantly affected by proximal or distal anastomosis site or reversal or *in situ* placement of the vein graft. However, the numbers of patients in this group was small and it is likely that the current data is insufficient to make this conclusion. These data are shown in Table 15.

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		Graft Occluded	Graft Working	p	RR (95%CI)
Proximal Anastomosis	CFA	27	82	0.77	0.89 (0.4-2.0)
	ABG/EIA	5	13		
Distal Anastomosis	Below knee	27	47	0.0007	3.80 (1.6-9.2)
	Above knee	5	47		
Long Saphenous Vein	In situ	23	42	0.025	2.1 (1.1-4.0)
	Reversed	10	49		

Table 14: Graft occlusion and its association with anastomosis site and vein use. Statistical analysis using Fishers exact test. CFA= Common Femoral Artery, ABG/EIA= Aortic Bifurcation Graft / External Iliac Artery.

		Not Amputated	Amputated	p	RR (95%CI)
Proximal Anastomosis	CFA	15	12	1.0	0.93 (0.42-2.0)
	ABG/EIA	3	2		
Distal Anastomosis	Below knee	14	14	0.11	0.5 (0.3-0.7)
	Above knee	4	0		
Long Saphenous Vein	In situ	13	10	1.0	1.13 (0.5-2.3)
	Reversed	5	5		

Table 15: Amputation and its association with anastomosis site and vein use. Statistical analysis using Fishers exact test. CFA= Common Femoral Artery, ABG/EIA= Aortic Bifurcation Graft / External Iliac Artery.

3.5.6 Graft Occlusion and Patient Characteristics

Patient factors that may influence graft occlusion include gender, diabetes and smoking status. Univariate analysis of graft occlusion and patient factors was performed using Fishers exact test. Female gender showed a trend approaching, but not reaching, statistical significance as a factor which was associated with increased graft occlusion (Relative Risk (RR) 1.7 (95% CI, 0.95-3.0), p=0.098). Diabetes and smoking were not associated with graft occlusion. This data is shown in Table 16 below.

		Graft Occluded	Graft Working	p	RR (95%CI)
Gender	Female	17	33	0.098	1.7 (0.95-3.0)
	Male	16	64		
Diabetes	YES	8	29	0.51	0.77 (0.38-1.5)
	NO	25	68		
Smoker pre or post-op	YES	57	18	0.29	1.14 (0.9-1.5)
	NO	28	14		

Table 16: Graft occlusion and its association with patient factors: gender, diabetes and smoking. Statistical analysis using Fishers exact test.

3.5.7 Graft Occlusion and Medication Use

Medication use may influence graft occlusion. The use of antiplatelet agents, warfarin and statins were recorded as either present or absent at the time of surgery and also at the final follow up. Data for the final follow up period was less often available in the case notes.

Univariate analysis of graft occlusion and medication use was performed using Fishers exact test. Warfarin prescription was found to be associated with an increased risk of graft occlusion. This is likely to be due to the fact that warfarin is only used in patients with a very high risk of occlusion, such as history of previous graft thrombosis, poor blood flow through the graft and extensive distal disease. Antiplatelet and statin use did not appear to influence graft occlusion. These data and their analyses by Fisher's exact test are shown in Tables 17, 18, and 19 below.

		Graft Occluded	Graft Working	p	RR (95%CI)
Warfarin pre-op	NO	29	94	0.10	0.39 (0.2-0.86)
	YES	3	2		
Warfarin post-op	NO	23	78	0.03	0.46 (0.25-0.8)
	YES	8	8		
Warfarin pre or post-op	NO	22	77	0.02	0.44 (0.25-0.8)
	YES	9	9		

Table 17: Warfarin use and graft occlusion. Statistical analysis using Fisher's exact test.

		Graft Occluded	Graft Working	p	RR (95%CI)
Antiplatelet pre-op	YES	14	53	0.31	0.70 (0.4-1.3)
	NO	18	42		
Antiplatelet post-op	YES	20	66	0.24	0.68 (0.4-1.3
	NO	11	21		
Antiplatelet pre or post-op	YES	23	74	0.58	0.58 (0.4-1.7)
	NO	6	14		

Table 18: Antiplatelet use and graft occlusion. Statistical analysis using Fisher's exact test.

		Graft Occluded	Graft Working	p	RR (95%CI)
Statin pre-op	YES	2	12	0.52	0.54 (0.14-2.0)
	NO	30	83		
Statin post-op	YES	5	24	0.22	0.55 (0.23-1.3)
	NO	25	55		
Statin pre or post-op	YES	5	24	0.22	0.56 (0.24-1.3)
	NO	24	54		

Table 19: Statin use and graft occlusion. Statistical analysis using Fisher's exact test.

3.5.8 Analysis of Graft Occlusion by Survival Curves

The influence of patient characteristics (gender, diabetes and smoking) as well as medication use (statins, antiplatelets and warfarin) on time to graft occlusion were calculated and compared using the Logrank test. Kaplan-Meier survival curves were generated and are shown below in figures 18-23. Only statin use showed a trend approaching, but not reaching, statistical significance as a factor which was associated with decreased graft occlusion.
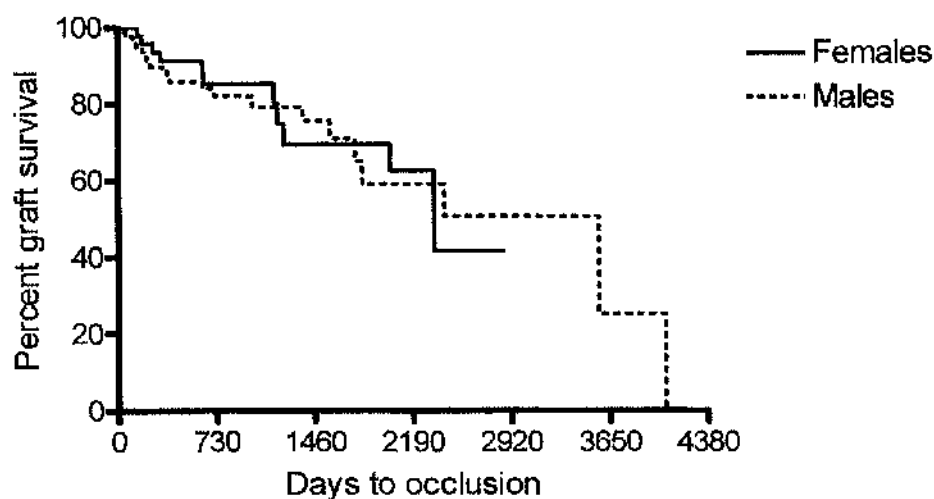
Figure 18: Survival curves for graft occlusion and gender using Logrank test, p=0.84.
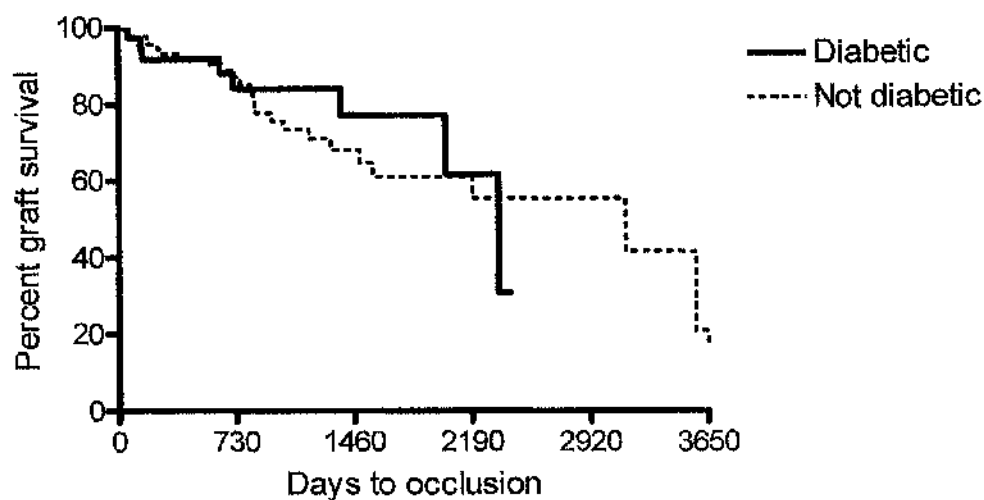
Figure 19: Survival curves for graft occlusion and diabetes using Logrank test, p=0.77.
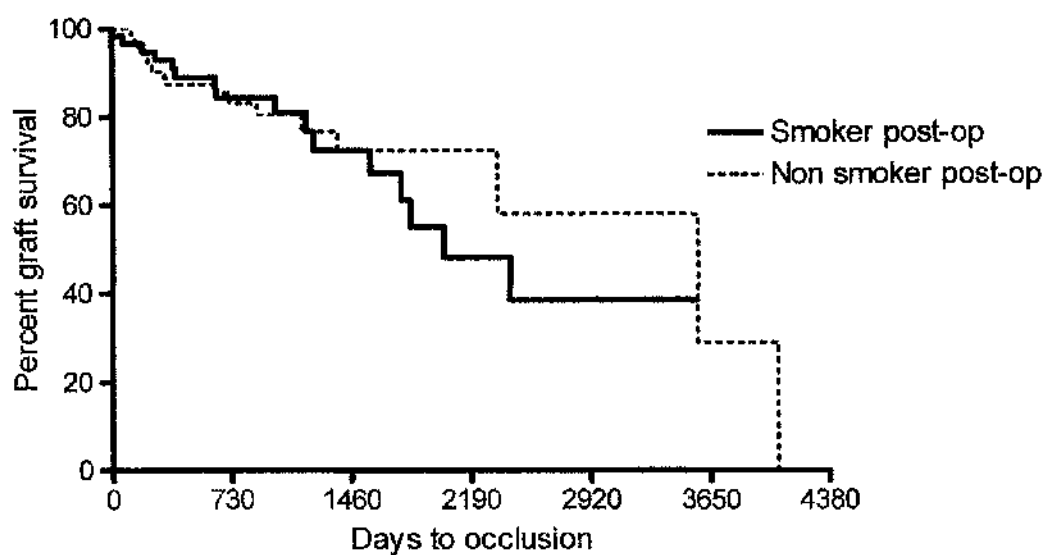
Figure 20: Survival curves for graft occlusion and smoking history using Logrank test, p=0.55.
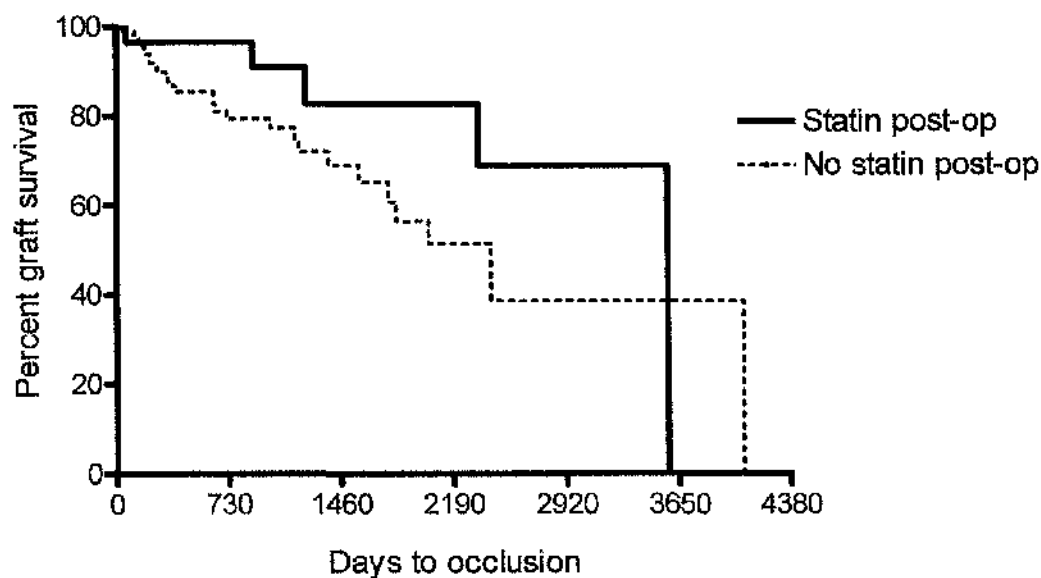
Figure 21: Survival curves for graft occlusion and statin use post-operatively using Logrank test, p=0.08 .

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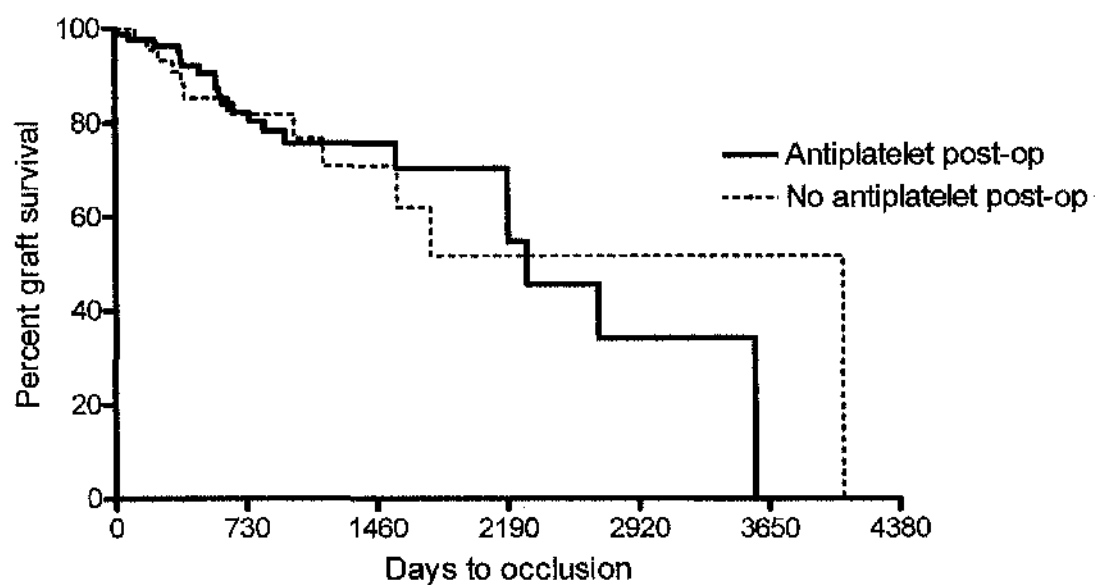
Figure 22: Survival curves for graft occlusion and antiplatelet use post-operatively using Logrank test, p=0.99.
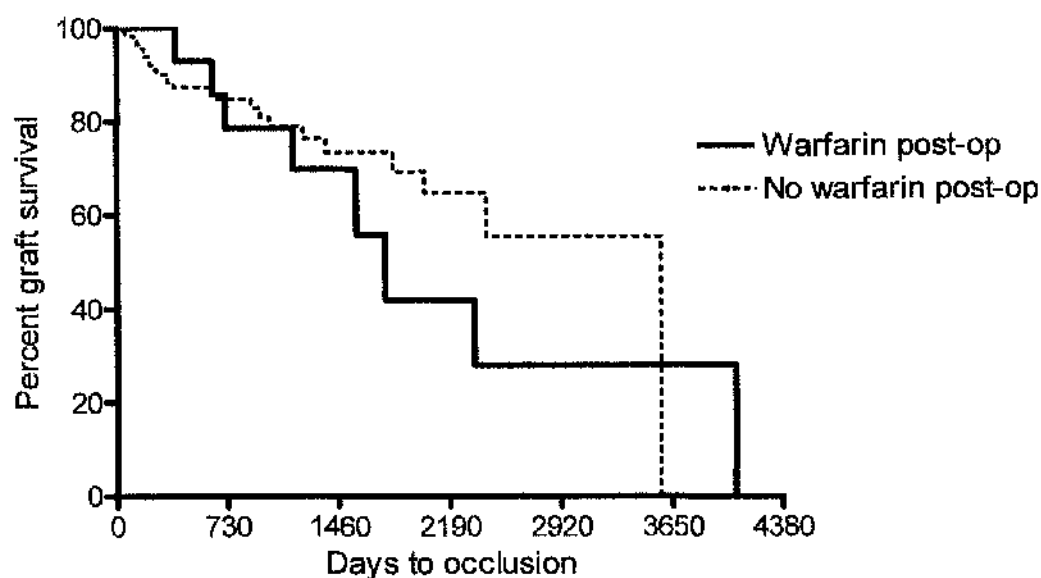
Figure 23: Survival curves for graft occlusion and warfarin use post-operatively using Logrank test, p=0.48 .

3.5.9 Analysis of Amputation by Survival Curves

The influence of patient characteristics (gender, diabetes and smoking) as well as medication use (statins, antiplatelets and warfarin) on time to amputation were calculated and compared using the Logrank test. Kaplan-Meier survival curves were generated and are shown below in figures 24-29. So few amputations were performed on the study group that only trends of possible differences may be seen in this data. It is notable, however, that no patient prescribed a statin post-operatively subsequently underwent amputation.
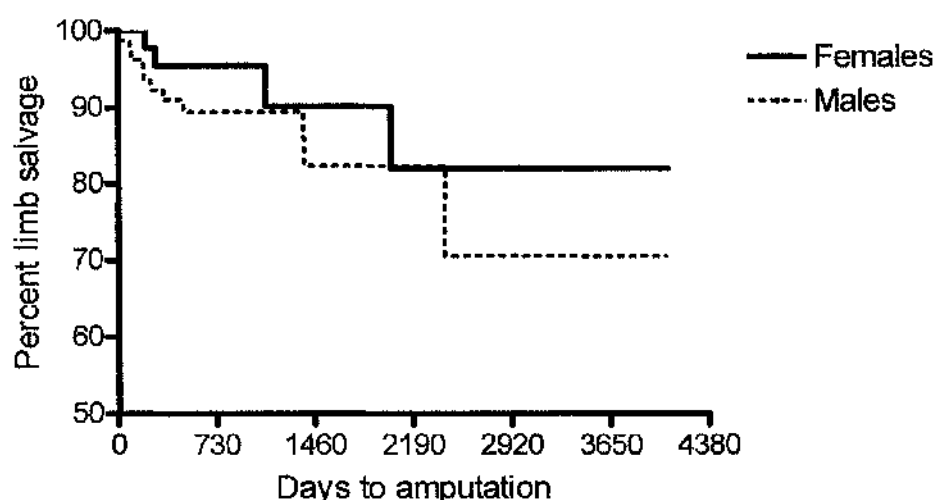
Figure 24: Survival curves for amputation and gender compared using Logrank test, p=0.33 .
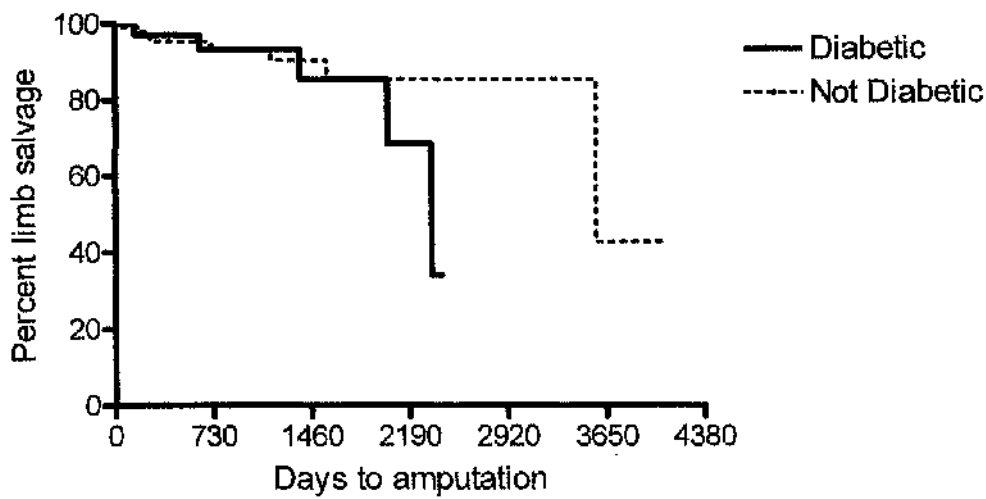
Figure 25: Survival curves for amputation and diabetes compared using Logrank test, p=0.31 .
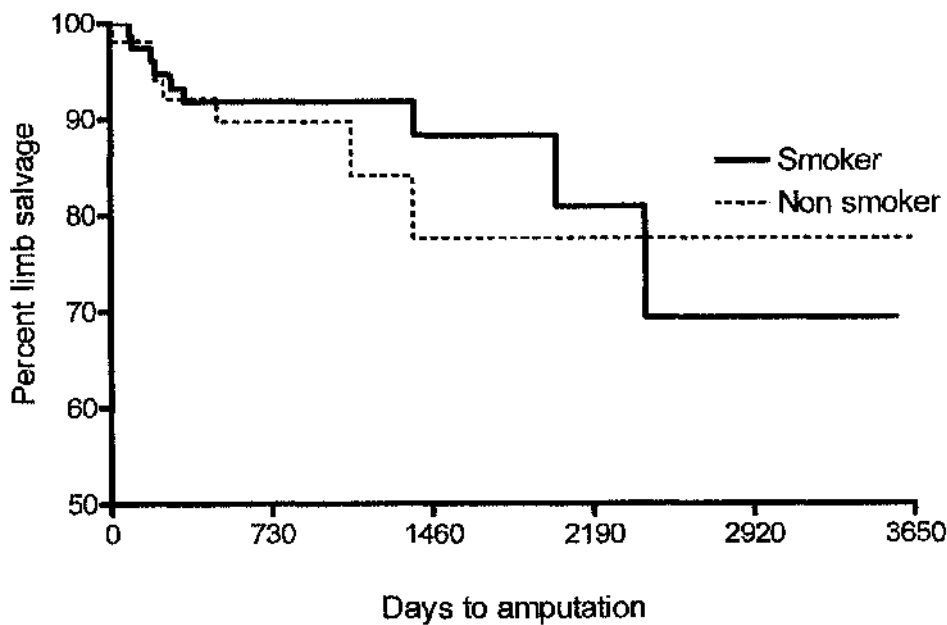
Figure 26: Survival curves for amputation and smoking post-operatively compared using Logrank test, p=0.66 .
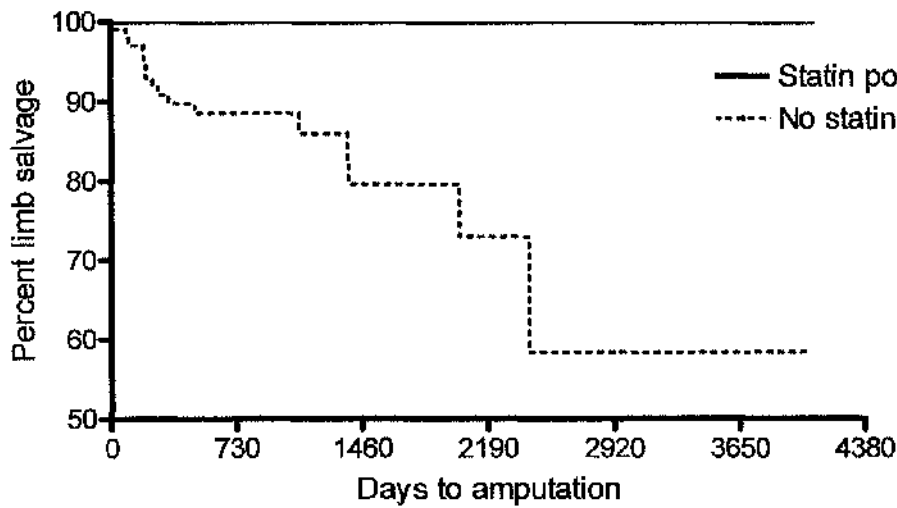
Figure 27: Survival curves for amputation and statin use post-operatively compared using Logrank test, p=0.001 .
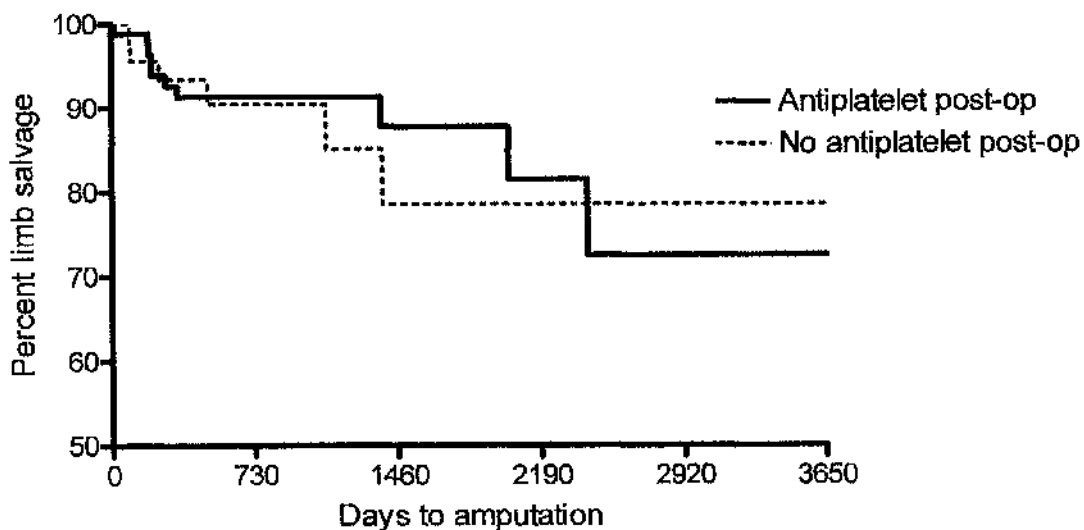
Figure 28: Survival curves for amputation and antiplatelet use post-operatively compared using Logrank test, p=0.72.

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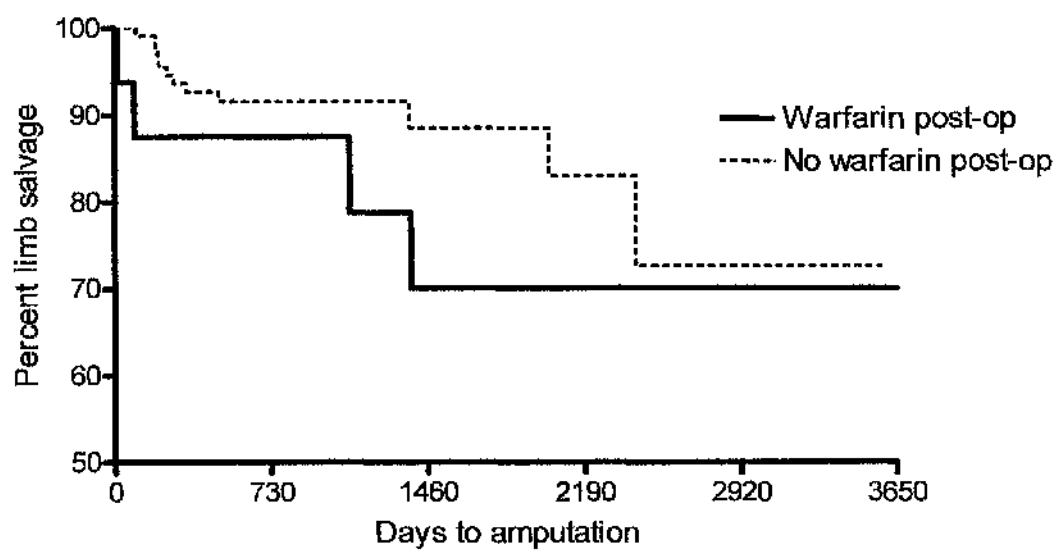
*Figure 29: Survival curves for amputation and warfarin use post-operatively using Logrank test,
p=0.25.*

3.5.10 Multivariate Analysis of Graft Occlusion

The effect of patient characteristics (gender, age, diabetes and smoking) as well as medication use (statin, antiplatelet and warfarin) on graft occlusion were analysed in a multivariate model to investigate which of the covariates influenced graft occlusion. These factors were analysed in a forward logistic regression model. Female gender and warfarin prescription were independent predictors of graft occlusion. This is shown in Table 20 below.

	B	S.E	Wald	df	Sig	Exp(B)	95% CI for Exp(B))	
							Lower	Upper
Age	-0.02	0.024	0.677	1	0.410	0.980	0.934	1.028
Female	-1.134	0.482	5.541	1	0.019	0.322	0.125	0.827
Antiplatelet	-0.394	0.534	0.545	1	0.46	0.674	0.237	1.919
Warfarin	-1.658	0.639	6.742	1	0.009	0.190	0.054	0.666
Statin	0.866	0.602	2.070	1	0.150	2.379	0.731	7.744
Smoker	-0.235	0.477	0.242	1	0.623	0.791	0.311	2.014
Diabetic	0.419	0.502	0.695	1	0.405	1.520	0.568	4.070

Table 20: Multivariate analysis of graft occlusion using logistic regression model. B=logistic regression coefficient, S.E = standard error of B; Wald = Wald statistic; df = degrees of freedom; Sig = statistical significance; Exp(B)= Hazard Ratio.

3.5.11 Secondary Operations

Of the 133 grafts for which the case notes were reviewed, 33 underwent secondary operations at a median time of 230 days (range 3-2592) from the initial operation. Of these, 9 grafts underwent 2 secondary operations and one graft underwent 3 secondary operations, giving a total of 44 secondary procedures. The majority (75%) of these secondary procedures were elective procedures, the remainder were emergency cases.

Twenty six people required secondary operations to repair a problem with the graft. Five of these people required 2 secondary procedures, giving a total of 31 secondary procedures on the grafts. The proximal anastomosis was revised in 17 grafts, 6 underwent revision of the mid-segment of the graft, 3 had the distal anastomosis revised, and 4 required emergency thrombectomy. Only one case of graft angioplasty was recorded formally in the case notes but it is likely that more patients underwent this procedure without a record being made in the case notes. Of these 31 secondary procedures, 26 (84%) had a recorded abnormality on the surveillance scan which prompted re-operation. One graft had a recognised problem which was not corrected and subsequently developed thrombosis of the graft requiring emergency thrombectomy. Two grafts did not appear to have any initial surveillance scans after the primary operation. These grafts required secondary operations at 462 and 236 days. How the problem with these grafts was identified is not clear but will likely have been following clinical examination.

Not all of the secondary operations were to revise a problem with the original graft. Three grafts were re-done with PTFE after the vein graft had occluded, one patient required an axillo-bifemoral graft, three patients required ilio-femoral grafting and one patient required a femero-femero

crossover graft for proximal disease. Four further patients underwent extension of the graft distally for progression of distal disease.

Additionally, 19 grafts had a problem identified at scanning which was not acted upon. The fate of these grafts was as follows: 11 continued to function normally until the final observation, 7 occluded but did not require amputation and 1 occluded requiring amputation. It is worth noting, however, that a further 21 grafts had a problem identified at scanning but these notes have been destroyed. Another 4 grafts had recorded problems but the notes have still to be seen. As such, information regarding possible secondary operations for these grafts are missing.

3.5.12 Discussion of Audit Findings

Within the time frame of this research project and with difficulties in obtaining case records, I was unable to review the case notes for all 207 grafts. Of the case records that were reviewed, many were incomplete or contained sparse amounts of information. This was especially true with regard to drug prescription and smoking status. As such, the results presented here may only suggest trends of observations.

Within the ten-year period of this audit, 207 infrainguinal vein grafts were created for occlusive arterial disease. The number of grafts performed per year did not change significantly over this time period. There have been recent reports that the number of infrainguinal grafts performed per year in the UK is falling. This is thought to be due to more widespread use of angioplasty. Our findings do not follow this trend, however. Reasons for this include the fact that infrainguinal grafting, in our unit, has almost always been reserved for patients with critical limb ischaemia which is not usually amenable to angioplasty. The reduction in infrainguinal grafting reported by other groups is likely to represent a reduction in the number of grafts performed for claudication rather than a reduction in the number performed for critical limb ischaemia.

Patients undergoing infrainguinal grafting had a high incidence of concurrent medical conditions. This was expected in this population group since patients with peripheral vascular disease have an increased risk of cardiovascular morbidity and mortality. Only 3% of the patients had a diagnosis of hypercholesterolaemia at the time of surgery, however. This is likely to be due to the fact that few patients underwent cholesterol measurements at that time. The importance of hypercholesterolaemia in atherosclerosis has been recognised only relatively recently. This may also explain why few patients were prescribed a statin either at the time of infrainguinal bypass or post operatively.

On univariate analysis, graft occlusion was associated with distal anastomosis (below the knee joint level) and *in situ* use of the long saphenous vein. Previous studies have also showed an association between distal grafting and graft occlusion but the association with *in situ* grafting is not a recognised factor. If the data are examined further, it is clear that the majority of distal grafts had *in situ* use of the long saphenous vein. This is to ensure that the anastomoses are better matched with respect to diameter of the vessels. It is extremely likely that this accounts for the apparent difference in patency rates between *in situ* and reversed grafts.

The expected effect of gender on infrainguinal graft patency rates is unclear. Initial studies suggested that female gender was a risk factor for graft occlusion but not all subsequent studies have supported this (145-148). Another feature is that females tend to come to infrainguinal bypass surgery at an older age than men, perhaps due to the protective effects of oestrogen. This difference in age at the time of surgery may influence graft patency rate (149). In the current study, univariate analysis of graft occlusion with respect to gender showed no statistically significant difference between males and females. However, the multivariate model did identify female gender as an independent predictor of graft occlusion. Furthermore, although there was a statistically significant difference in the age at operation between males and females (median age (range) of females = 70.5yrs (38-91), median age (range) of males = 66.5yrs (42-86), p=0.0029, Mann Whitney test) in the current study, age was not an independent predictor of graft occlusion in the multivariate model. One possible reason for decreased graft patency in females is that they have smaller blood vessels – both arteries and veins. This causes technical difficulty at the time of operative intervention but also means that subsequent graft occlusion is an increased risk due to decreased diameter of run-off vessels and decreased anastomosis diameter (150).

The role of diabetes with regard to the risk of graft occlusion is equally unclear. It is accepted that diabetic patients have poorer overall survival following surgical intervention mainly due to increased cardiac risk. This is reflected in their higher postoperative mortality and 5 year mortality compared with non-diabetics (150;151). Furthermore, some studies suggest that diabetic patients have an increased risk of graft occlusion but this is not a consistent finding (145;152;153). Univariate and multivariate analysis of the current study shows no significant difference in graft patency rates between diabetics and non-diabetics.

Smoking is a major risk factor with regard to the development and progression of peripheral arterial disease (154). Continued smoking following infrainguinal bypass grafting is generally accepted as being detrimental to graft patency rates (155;156). A recent meta-analysis of the effect of smoking on graft patency showed a 3 fold increased risk of graft occlusion (157). The current study, however, shows no association between smoking and graft occlusion. This probably reflects the retrospective nature of this study as patients often claim to have stopped smoking at both pre and post-operative clinic visits.

In investigating the relationship between drug prescription and graft outcome, statin use was weakly associated with improved graft patency and warfarin prescription was associated with increased graft occlusion. The findings with regard to warfarin prescription are very likely to be directly due to patients being prescribed warfarin as a last resort to try to maintain graft patency. Unfortunately, this information was not recorded in the case notes to allow this to be said with certainty. With regard to the association of statins with improved graft patency, this is in keeping with two recent studies showing similar effects (98;99). Henke *et al* investigated 338 infrainguinal grafts (both autogenous vein and synthetic grafts) and found an association between improved graft patency and lower amputation rate in patients taking statins. Abbruzzese *et al* investigated 189 autogenous vein grafts

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followed up for two years and found statin therapy to be associated with improved secondary graft patency. Neither of these two papers has sought to explain the mechanism of action of statins in improving graft patency. However, total cholesterol levels were noted to be no different between the groups studied at the time of surgery. This suggests that the action of statins was independent of the cholesterol lowering effects.

The Scottish Intercollegiate Guidelines Network (SIGN) guidelines for the management of patients with peripheral arterial disease were revised in October 2006. In light of recent evidence supporting the use of statins for patients with hypercholesterolaemia and a prior history of cardiovascular disease, statin therapy is now recommended for all patients with peripheral arterial disease and a cholesterol of >3.5mmol/l. The guidelines also recognise that statins may confer possible symptom improvement for patients with claudication and state this as a further reason for treating patients with peripheral arterial disease with a statin as first line therapy. No assessment has been made of recent studies which suggest that statin therapy may improve infrainguinal graft patency, however.

Given that there were relatively few amputations performed on the study population, it is difficult to associate this with drug prescription and smoking. Despite this, it is interesting that no patients who were recorded to be taking a statin required amputation. Further work would be required to clarify this apparent association.

The majority of secondary operations were to repair a specific problem with the graft whilst the remainder were to correct either the inflow or outflow of the graft, or to replace the graft with a synthetic conduit. Of the secondary operations on the grafts, only one angioplasty was recorded successfully in the case notes. From my experience working in the unit, it is extremely likely that

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other patients also underwent angioplasty but that no record appeared in the notes. Of the other procedures on the grafts, the majority (84%) were prompted by abnormal scans.

However, some grafts had abnormal scans which were not acted upon. Why this was the case is not clear. It is possible that some grafts were felt to have clinically insignificant scan abnormalities and were deliberately left without a secondary operation. It is also possible that some grafts were identified as requiring a secondary procedure but occluded before revision could be undertaken. Finally, it is also possible that the patient was felt to be medically unfit to undergo revisional surgery as this often requires prolonged general anaesthesia. The majority of grafts in which an abnormality was identified but no surgery was performed and then occluded, did not require an amputation. This is an important finding and may indicate that a conscious clinical decision was made not to operate on the failing graft.

Chapter 4

GENERAL DISCUSSION

4. General Discussion

The major aim of the experimental study was to establish whether prolonged hypoxia in the long saphenous vein predisposed the vein to develop intimal hyperplasia. In order to answer this question, the patients involved in the study had to be chosen carefully. It was important that the two patient groups were closely matched with regard to age, sex distribution, smoking history and other illnesses. The only patients from whom an ischaemic long saphenous vein could be obtained reliably were those undergoing major lower limb amputation. For the corresponding, non-ischaemic group, there would have been an abundance of long saphenous vein available from patients undergoing varicose vein surgery, however, these patients would have been much younger and would not have had similar medical histories to the amputees. As such, the patient group that was chosen to provide non-ischaemic vein tissue were those patients undergoing coronary artery bypass grafting. These patients were well matched with regard to age, sex, smoking history, diabetes and number of medications prescribed. The one factor which was seen to be different between the patient groups was statin prescription. The patients undergoing lower limb amputation were significantly less likely to be taking a statin compared with the patients undergoing coronary artery bypass grafting. This is an important clinical finding which may reflect the relative lack of understanding of the systemic nature of peripheral arterial disease amongst medical practitioners.

Patients with peripheral arterial disease have a three-fold increased risk of cardiovascular mortality compared with age-matched controls. Statins are principally cholesterol-lowering drugs. They have been proven to reduce the cardiovascular event rate in patients with established cardiovascular disease as well as in patients who only have risk factors for cardiovascular disease, even at cholesterol levels which would be classed as normal. Current clinical guidelines call for the treatment of patients with risk factors for atherosclerotic disease with a statin, even at normal

cholesterol levels. The new Scottish Intercollegiate Guidelines Network document for the treatment of patients with peripheral arterial disease also recommends treatment with a statin for patients with peripheral arterial disease and a cholesterol of >3.5mmol/l.

Organ culture of long saphenous vein leading to intimal hyperplasia has been validated as a suitable model of intimal hyperplasia. Although the numbers studied in this thesis were low, veins from the ischaemic limbs appeared to develop significantly more intimal growth compared with veins from the non-ischaemic limbs. This suggests that the ischaemic conditions prior to organ culture affected the vein in such a way as to predispose it to developing intimal hyperplasia. Possible mechanisms include activation of the mitogen activated protein kinase (MAP Kinase) p38 stress pathway. Activation of this pathway has been shown to cause vascular hypertrophy in patients with pulmonary vascular hypoxia leading to pulmonary hypertension. Another possible pathway which may be involved is the MAP Kinase p42/p44 pathway which leads to cellular proliferation and migration. Further work to assess the activity of these pathways in tissue from ischaemic and non-ischaemic veins would ascertain whether or not these pathways are upregulated in ischaemic vein tissue.

Addition of simvastatin to the organ culture model abolished the development of intimal hyperplasia in veins from ischaemic limbs. A similar effect was seen in the veins from the non-ischaemic limbs although the effect of simvastatin was less pronounced. The mechanism of action of simvastatin to inhibit the development of intimal hyperplasia is likely to be due to a combined inhibition of vascular smooth muscle cell (VSMC) proliferation and migration. This conclusion is based on the previous work of other authors as well as the findings in this thesis. Why there is a difference in the response of veins from ischaemic and non-ischaemic limbs is not clear. The statins may be inhibiting a pathway that is upregulated in the ischaemic veins. For example, the ischaemic veins

may have accelerated levels of VSMC migration and proliferation which makes the tissue more susceptible to the effects of simvastatin. This effect may indicate a therapeutic target for simvastatin to prevent vein graft stenosis in patients undergoing both infrainguinal grafting for peripheral arterial disease and patients undergoing coronary artery bypass grafting.

Vascular smooth muscle cells which were explanted from the vein rings were grown on in cell culture and used in proliferation and migration assays. In the cell proliferation assay, simvastatin was seen to dose-dependently reduce the amount of VSMC proliferation. The preceding organ culture experiments had shown that the ischaemic tissue was more susceptible to the effects of simvastatin than the non-ischaemic tissue. There was no difference, however, in the mean 50% inhibitory concentrations of simvastatin for the VSMCs from both the ischaemic and non-ischaemic veins. This indicates that VSMCs explanted from both groups have an equivalent degree of susceptibility to the effects of simvastatin. Despite this, it is possible that the VSMCs from the ischaemic vein rings could be proliferating at a higher rate compared with those from the non-ischaemic veins. Further work which would clarify this would be to look at the VSMC proliferation rate between the two groups using growth curves. Additionally, it is worth considering the potentially different response of vascular smooth muscle cells once removed from the extracellular matrix of the vein ring. Techniques to study cell proliferation within whole vein rings such as using confocal microscopy could be used to further clarify this issue.

Addition of mevalonic acid to the cell proliferation experiments abolished the effect of simvastatin. This is evidence that the effect of the drug is via inhibition of intracellular production of mevalonic acid. Mevalonic acid is also a precursor of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP). These are lipid attachments necessary for the post translational modification of G proteins including ras, rho and rac. Which of these pathways are being inhibited by simvastatin

could be investigated by the use of either FPP or GGPP instead of mevalonic acid in the cell culture experiments. Additionally, Western Blotting techniques could be used to look for reduced membrane or cytoplasmic localisation of these proteins in the presence of simvastatin to further extrapolate its effects.

Migration of vascular smooth muscle cells was investigated using a modified Boyden chamber technique and showed that VSMC migration was enhanced in cells from the ischaemic vein rings compared with cells from the non-ischaemic vein rings. A possible mechanism that may explain this accelerated migration is the up-regulation of the MAP Kinase p38 system. This system has been shown to be up-regulated in response to hypoxia in pulmonary fibroblasts and cardiac myocytes, and could equally be up-regulated in VSMCs from ischaemic vein tissue. The MAP Kinase p38 system is an intracellular signalling pathway which lies downstream of many processes including the activation of cell division cycle 42 (Cdc 42) and rac. These are two important systems which control cell migration and which are known to be stimulated by platelet derived growth factor (PDGF). Another possibility is that VSMCs from the ischaemic vein rings have an increased expression of membrane PDGF receptors. It is known that PDGF cell membrane receptor expression is altered in response to autocrine factors such as transforming growth factor beta (TGF-β) and this may explain the enhanced migratory response of the VSMC from the ischaemic vein group.

Simvastatin reduced the migration of vascular smooth muscle cells in these experiments although not to a statistically significant degree. It is likely that this represents a type II statistical error, as the numbers used in these experiments were small. Additionally, other authors have demonstrated an inhibition of VSMC migration in response to PDGF by simvastatin. The mechanism of action of simvastatin to inhibit VSMC migration is likely to be through the inhibition of membrane localisation of the G proteins, rho and rac. These control assembly and polymerisation of actin

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filaments in the leading edge of migrating cells. Both rho and rac require post-translational modification by the attachment of geranylgeranyl pyrophosphate (GGPP) in order that they may be functional in the cell membrane. GGPP is produced intracellularly by the mevalonate pathway which is directly inhibited by simvastatin.

Overall, the organ culture and cell culture experiments have demonstrated some important facts. Vein tissue from ischaemic limbs appears to be more susceptible to the development of intimal hyperplasia in organ culture compared with vein tissue from non-ischaemic limbs. This may be due to enhanced VSMC migration in cells from ischaemic vein rings compared with those from non-ischaemic vein rings. Furthermore, these findings may explain the relatively high rate of development of vein graft stenoses in patients undergoing infrainguinal bypass grafting for critical limb ischaemia. Additionally, simvastatin has been shown to inhibit the process of intimal hyperplasia in organ culture, this effect is likely to be due to a combined inhibition of VSMC migration and proliferation. This finding raises the possibility of using simvastatin therapeutically to prevent vein graft stenosis. The concentration of simvastatin used in these organ culture experiments was much higher than the serum levels of simvastatin attained at normal oral dosing, however. In order to attain the levels of simvastatin used in organ culture *in vivo*, novel delivery mechanisms of simvastatin could be employed. This could include the development of simvastatin-eluting sutures or bathing the vein in a simvastatin solution before anastomosing it as a bypass graft.

In auditing all infrainguinal bypass grafts performed in Glasgow Royal Infirmary between 1993-2003, a number of important observations were made. Firstly, the number of vein grafts performed each year during the observation period did not change. This reflects the fact that, in Glasgow Royal Infirmary, infrainguinal grafting is reserved for patients with critical limb ischaemia and the number of patients presenting with this condition each year has not changed. With an ageing population and

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a rise in the prevalence of diabetes, however, it is likely that more and more patients will present each year with critical limb ischaemia requiring surgery. It is therefore essential that these patients receive the best possible treatment and strategies aimed at prolonging graft patency are addressed.

Patients undergoing infrainguinal bypass grafting have a high rate of concurrent medical conditions. However, as in the amputees who were involved in the experimental study, few of these patients were prescribed a statin. This reflects the historic nature of the audit but it is imperative that this information is used in a positive manner and that all patients who undergo infrainguinal bypass grafting in future are assessed as to their cardiovascular risk and are placed on statin therapy accordingly. There should be very few patients who undergo bypass grafting that do not necessitate statin therapy.

Statin therapy was associated with improved graft patency and lower amputation rate in our data. This supports the findings of two recent studies which showed improved infrainguinal graft patency and limb salvage in patients taking statins. Neither study suggested a mechanism for the protective effect of simvastatin on infrainguinal vein grafts but it is likely to include reduced formation of intimal hyperplasia. This action is likely to be mediated through more than simple antiproliferative and anti-migratory effects that have been demonstrated in this study at a cellular and organ culture level. Indeed, as previously mentioned, the effects observed in the cell and organ culture experiments in this project were seen at much higher concentrations than can be achieved by normal oral administration of statins.

Within the multivariate model, female gender was associated with graft occlusion. This finding has been reported by previous studies but the reason for poorer graft outcome in females is unclear. Some studies have shown that females tend to undergo infrainguinal grafting at an older age than

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males, perhaps due to later presentation of disease. In this study, age was not an independent factor with poorer graft outcome despite females being 4 years older than males at the time of operation. The reason for poorer graft patency rates among females is not clear but technical reasons including reduced vessel diameter are likely. Unfortunately, this detail was not available in this study but future studies should, ideally, include a measure of arterial and venous diameters.

In conclusion, long saphenous veins exposed to prolonged hypoxia develop an enhanced potential for the development of intimal hyperplasia in culture. This is likely to be mediated through various cell mechanisms including the stress response MAP Kinase p38 pathway. Simvastatin inhibits this process of intimal hyperplasia in organ culture and its effect is likely to be mediated through inhibition of cell proliferation and migration via a number of mechanisms but all principally related to the inhibition of farnesylisation and geranylgeranlyation of essential G proteins including ras, rho and rac. *In vivo*, statins have been associated with improved graft patency as well as improved limb salvage in patients undergoing infrainguinal bypass grafting. This observation is likely to be due to a combination of factors but another therapeutic potential for the use of statins would be in local delivery to a vein graft in order to inhibit the formation of intimal hyperplasia.

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