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Cellular Aspects of Intimal Hyperplasia Formation

A thesis submitted to the

FACULTY OF MEDICINE, GLASGOW UNIVERSITY

For the degree of

M.D.

By

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ABSTRACT

Introduction.

12,000 infrainguinal bypass grafts are performed annually in the UK. Despite improvements in surgical technique, outcomes remain suboptimal: 20% of above knee grafts require intervention to maintain patency by 3 years. Only antiplatelet agents have been demonstrated thus far to improve graft survival. 80% of graft failure is as a result of intimal hyperplasia, an inflammatory process characterised by the proliferation and migration of vascular smooth muscle cells.

Toll Like Receptors (TLR), part of the innate immune system, have been implicated in atherosclerosis formation but not investigated in a model of infrainguinal graft failure.

When a vein is used as a conduit for infrainguinal bypass graft it has been exposed to ischaemic and hypoxic conditions: preliminary data has demonstrated that ischaemic vascular smooth muscle cell explants are hyperproliferative.

Phospholipase C γ (PLC γ) is a signalling pathway with potential links to innate immune pathways and pathways induced by hypoxia and ischaemia.

Methods:

Human vein tissue was obtained from patients undergoing amputation and coronary artery bypass surgery and used for immunohistochemistry and to obtain vascular smooth muscle cells by explant method.

Immunohistochemistry was used to determine the presence of TLR4 and PLC γ in human vein tissue.

Specific TLR Ligands were used to determine the functional response of TLR’s in vascular smooth muscle cells as measured by Interleukin 8 ELISA.

Radiolabelled Thymidine incorporation was used to measure proliferation of vascular smooth muscle cells in response to TLR4 activation, hypoxia and PLC γ inhibition.
Results:

TLR4 was demonstrated to be present in human vein tissue, and functionally active in human vascular smooth muscle cells. Furthermore stimulation with the specific ligand of TLR4 caused enhanced proliferation of vascular smooth muscle cells.

Hypoxia (5% and 10% Oxygen) significantly enhanced proliferative responses of vascular smooth muscle cells.

PLC γ was demonstrated to be present in human vein tissue, and inhibition, using U73122 in vascular smooth muscle cells reduced proliferation.

Conclusion:

TLR activation and hypoxia appear to enhance the proliferative responses of human vascular smooth muscle cells, a key cellular pathway of intimal hyperplasia formation and infrainguinal graft failure. Inhibition of PLC γ reduces proliferative responses. Further research is required to confirm that PLC γ is a key common pathway mediating enhances of proliferation caused by TLR activation and hypoxia.
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<tr>
<td>ABPI</td>
<td>Ankle:Brachial Pressure Index</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine Diphosphatase</td>
</tr>
<tr>
<td>APES</td>
<td>Aminopropylethoxysilane</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3-Diaminobenzidine</td>
</tr>
<tr>
<td>DAG</td>
<td>Diaglycerol</td>
</tr>
<tr>
<td>DPM</td>
<td>Disintegrations per Minute</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal Growth Factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial Nitric Oxide Synthase</td>
</tr>
<tr>
<td>ERK ½</td>
<td>Extra-Cellular signal related kinase</td>
</tr>
<tr>
<td>FCS</td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine Diphosphatase</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine Triphosphatase</td>
</tr>
<tr>
<td>HIF</td>
<td>Hypoxia Inducible Factor</td>
</tr>
<tr>
<td>HMG</td>
<td>3-Hydroxy-3 MethylGlutaryl</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human Umbilical Vein Endothelial Cell</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Intra-Cellular Adhesion Molecule-1</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin-Like Growth Factor-1</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>InsP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>JNK</td>
<td>cJun-nTerminal Kinase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low Density Lipoprotein</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Macrophage-CSF</td>
<td>Macrophage Colony Stimulating Factor</td>
</tr>
<tr>
<td>MAP-kinase</td>
<td>Mitogen Associated Protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte Chemoattractant Protein-1</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>NF-κβ</td>
<td>Nuclear Factor-κβ</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric Oxide</td>
</tr>
<tr>
<td>nTrKR</td>
<td>Non-Tyrosine Kinase Receptor</td>
</tr>
<tr>
<td>Pam₃CSK₄</td>
<td>Pam₃Cys-Ser-(Lys)₄·3HCL</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated Molecular Patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>PDGF</td>
<td>Platelet Derived Growth Factor</td>
</tr>
<tr>
<td>PI3-k</td>
<td>Phosphoinositol-3-kinase</td>
</tr>
<tr>
<td>PLCγ</td>
<td>Phospholipase Cγ</td>
</tr>
<tr>
<td>PolyI:C</td>
<td>Polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>PtdInsP</td>
<td>Phosphatidylinositol (4,5)-biphosphonate</td>
</tr>
<tr>
<td>TASC</td>
<td>Trans-Atlantic Society Consensus</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
</tr>
<tr>
<td>TRIS</td>
<td>Tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>TrKR</td>
<td>Tyrosine Kinase Receptor</td>
</tr>
<tr>
<td>U73122</td>
<td>1-6-(((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl]1H-pyrrole-2,5-dione</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular Cell Adhesion Molecule-1</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular Smooth Muscle Cell</td>
</tr>
</tbody>
</table>
Author’s Declaration

I declare that the work described in this thesis has been carried out by myself unless otherwise cited or acknowledged. It is entirely of my own composition and has not, in whole or in part, been submitted for any other degree.

Andrew J Jackson

July 2010
**Publications from Thesis**


Vascular Cell Responsiveness to Toll Like Receptors in Carotid Atheroma Erridge C, Burdess A, Jackson AJ et al European
CHAPTER 1
INTRODUCTION
1.1 Atherosclerosis.

1.1.1 General Aspects of Atherosclerosis.

Atherosclerosis is an inflammatory process characterised by the accumulation of lipids and fibrous elements in large and medium sized arteries.\textsuperscript{1} Its presence can lead to reduction in blood flow and subsequent ischaemia of the organ supplied.

Autopsy studies have shown the earliest lesions, fatty streaks, to be present in infants consisting of intimal foam cells and macrophages. These progress to intermediate lesions (composed of foam cells, macrophages and smooth muscle cells which have migrated from the medial layer to intimal)\textsuperscript{2} and advanced plaques.\textsuperscript{3} Advanced plaques are characterised by a dense fibrous plaque of connective tissue and smooth muscle cells with a necrotic, lipid rich core.\textsuperscript{4} Advanced plaque also contains large numbers of macrophages, T cells, and smooth muscle cells.\textsuperscript{1}

From the advanced plaque a complicated plaque develops with a thin cap, containing ulcers, erosions or cracks; this providing the site for platelet adherence aggregation and thrombosis.\textsuperscript{4}

Arterial remodelling is important in delaying the narrowing of the arterial lumen in plaque evolution.\textsuperscript{5} During the early phase of atherosclerosis plaque formation the vessel increases its diameter thereby maintaining normal flow in the vessel, however when the plaque occupies greater than 40% of the cross sectional area, dilatation can no longer compensate and the plaque intrudes, potentially limiting blood flow.\textsuperscript{6}

1.1.2 Pathophysiology of Atherosclerosis.
Atherosclerosis is multifactorial, inflammatory disease with each lesion representing a different stage of a chronic inflammatory process.\textsuperscript{7} Atherosclerosis formation can be initiated by endothelial dysfunction, following endothelial injury.\textsuperscript{8} Possible causes of endothelial cell dysfunction include elevated and modified Low Density Lipoproteins (LDL),\textsuperscript{9} free radicals from smoking, hypertension and diabetes. Other purported causes include elevated plasma homocysteine levels\textsuperscript{10} and infectious agents such as herpes virus and chlamydia pneumoniae.\textsuperscript{11} It has also been noted that specific sites of arteries such as bifurcations, where flow is not laminar are more susceptible to atherosclerosis.\textsuperscript{12} It has been demonstrated that changes in flow can alter the expression of genes that have pronuclear regions that respond to shear stress such as intracellular adhesion molecule 1(ICAM-1)\textsuperscript{13} potentiating atherosclerosis formation.

\subsection*{1.1.2.1 Endothelial cell dysfunction.}

Endothelial dysfunction is characterised by an imbalance of relaxing and contracting factors, procoagulant and anticoagulant substances and proinflammatory and anti-inflammatory mediators.\textsuperscript{14} In response to endothelial injury, endothelial cells initiate a protective response, altering the normal homeostatic mechanisms of the endothelium. Injury leads to increased adhesiveness of the endothelium with respect to leucocytes and platelets with an associated increased permeability. The endothelium shifts to have a procoagulant state, releasing vasoactive molecules, cytokines and growth factors. Nitric Oxide (NO) production and bioavailability is also reduced, reducing endothelium derived relaxation.\textsuperscript{15}

NO production and bioavailability is reduced as endothelial dysfunction effects endothelial Nitric Oxide Synthase (eNOS) production of NO. eNOS also becomes dysfunctional, producing superoxide rather than NO.\textsuperscript{16} Superoxide and its metabolites hydrogen peroxidase and peroxinitrite serve to further damage endothelial cells, and accelerate degradation of existing NO.\textsuperscript{17}
Aside from endothelium derived relaxation, NO is responsible for inhibiting Vascular Cell Adhesion Molecule 1 (VCAM-1) gene expression as well as repressing cell proliferation by causing cell cycle arrest via inhibition of p21 upregulation. NO also prevents platelet aggregation, has been demonstrated to reduce vascular smooth muscle cell proliferation and inhibit oxidation of LDL.

Increased adhesiveness of the endothelium to leucocytes occurs via expression of ICAM-1 and VCAM-1. Monocyte Chemoattractant Protein 1 (MCP-1) is also secreted, a chemokine to leucocytes. ICAM-1, VCAM-1 and MCP-1 expression is increased in endothelial cell dysfunction and allow adherence of leucocytes and entry by diapedesis at cell junctions.

1.1.2.2 Role of Leucocyte.

Circulating leucocytes migrate from the bloodstream through the dysfunctional endothelium to the intima as described where they contribute to atherosclerotic lesion formation both by lipid accumulation and release of inflammatory mediators enhancing other pathways of atherosclerosis formation.

Leucocytes in the intimal layer undergo morphological changes to form macrophages and foam cells. This occurs as leucocytes increase expression of scavenger receptors such as SRA (Scavenger Receptor Class-A) and CD36 (Cluster of Differentiation 36), allowing them to internalise modified lipoproteins under the control of Macrophage-CSF. Macrophage-CSF deficient mice show a marked reduction in macrophage accumulation in atherosclerotic plaques. Foam cells become fixed within the plaque and activate an inflammatory cascade which stimulates further leucocyte recruitment and monocyte replication as well as vascular smooth muscle cell proliferation and migration.
1.1.2.3 Role of Vascular Smooth Muscle Cells.

Vascular smooth muscle cells (VSMC) are present in the wall of normal arteries and contain the contractile proteins actin and myosin. In the normal state they display a contractile phenotype. Under the influence of proinflammatory cytokines they alter from a contractile to secretory phenotype, migrate to the intima and produce extracellular matrix\textsuperscript{26} where they become a predominant feature of atherosclerotic plaques. Factors released as a consequence of endothelial dysfunction such as Platelet-Derived Growth Factor (PDGF), Insulin-Like Growth Factor (IGF-1) and Epidermal Growth Factor (EGF) have all been demonstrated to promote this process.\textsuperscript{7}

The role of VSMC within the intima is still unclear. They almost certainly contribute to the development of atherosclerotic plaque by secretion of pro-inflammatory mediators in addition to production of extracellular matrix.\textsuperscript{27} However they are also thought to play a role in maintaining plaque stability by creating a firm fibrous cap. At the ‘shoulder’ areas of atherosclerotic plaques the caps are thin, and most prone to rupture. VSMC apoptosis can be seen in these areas in association with thinning of the cap.\textsuperscript{28} VSMC’s contribute to processes which lead to progression of the lesion and ischaemia of supplied organs, however they may be protective against plaque rupture and subsequent infarction of the organ.

1.1.3 Innate Immunity in Atherosclerosis.

Innate immunity is based upon detection of pathogen-associated molecular patterns (PAMPs) which, when activated initiate an inflammatory response.\textsuperscript{29} Macrophages express receptors which recognise PAMPs including Scavenger receptors and Toll-Like Receptors (TLR). Engagement of scavenger receptors leads to degradation of PAMP whereas engagement of TLRs causes activation of signalling pathways which encode genes for a wide array of inflammatory responses involved in atherosclerosis formation. Vascular Cells themselves have been shown to
express TLR’s and TLR expression has been demonstrated in atherosclerotic plaques\textsuperscript{30} which when activated via Nuclear Factor kβ (NF-kβ) and Mitogen Activated Protein (MAP) kinase pathways contribute further to the inflammatory response\textsuperscript{31,32} as well as expression of leucocyte adhesion molecules, eNOS and interleukin(IL)-1. This upsets the normal homeostasis of the blood vessel.\textsuperscript{33}

This has led to speculation that PAMP’s could contribute to the formation of atherosclerosis, with vascular TLR’s the purported mechanism by which bacterial degradation products such as lipopolysaccharide (LPS) and heat shock proteins (HSP) induce the inflammatory process.

1.1.4 The Clinical Consequence of Atherosclerosis.

The outcome of atherosclerosis is dependent upon the organ supplied by the affected vessel, the degree of flow limitation and the timeframe over which it occurs. Limitation of flow in the coronary vessels causes cardiac ischaemia and angina pectoris. If this occurs acutely, myocardial infarction can result.

In the lower limb, atherosclerosis causes a spectrum of disease from intermittent claudication to critical limb ischaemia as will be discussed.
1.2 Peripheral Vascular Disease.

1.2.1 General Aspects of Peripheral Vascular Disease.

Peripheral vascular disease encompasses a range of syndromes caused by arterial disease outside the coronary circulation. This includes cerebral and visceral arteries as well as those supplying limbs. Atherosclerosis is the commonest disease process causing peripheral vascular disease. This work focuses on peripheral vascular disease of the lower limb, where it can cause a spectrum of symptoms, from mild, non-lifestyle limiting intermittent claudication (pain in the legs on walking, relieved by rest) to severe: rest pain and gangrene. The Fontaine Classification allows the range of symptoms to be categorised as follows: 34

<table>
<thead>
<tr>
<th>Fontaine I</th>
<th>Asymptomatic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fontaine IIa</td>
<td>Intermittent Claudication with pain free walking &gt; 200m</td>
</tr>
<tr>
<td>Fontaine IIb</td>
<td>Intermittent Claudication with pain free walking &lt; 200m</td>
</tr>
<tr>
<td>Fontaine III</td>
<td>Rest Pain</td>
</tr>
<tr>
<td>Fontaine IV</td>
<td>Ulceration, Gangrene or Necrosis</td>
</tr>
</tbody>
</table>

Table 1.1: Fontaine Classification of Peripheral Vascular Disease.

Class III and IV correlate with critical limb ischaemia, which is defined as “limb pain that occurs at rest or impending limb loss that is caused by severe compromise of blood flow to the affected extremity.” 35

1.2.2 Epidemiology of Peripheral Vascular Disease.
Between 3 and 5% of the population have asymptomatic peripheral vascular disease. Prevalence rises to 20% in those over 70.\textsuperscript{36} Using Ankle: Brachial Pressure Index (ABPI) of < 0.9 as a marker of asymptomatic peripheral vascular disease, disease was detected in 29% of patients aged over 70 or those age 50 to 69 with a hard risk factor for peripheral vascular disease in the USA.\textsuperscript{37}

Symptomatic Peripheral Vascular Disease in the form of intermittent claudication has been reported to range from 1 to 10% depending upon age group studied and population. The Edinburgh Artery Study took 1592 individuals and determined the prevalence of intermittent claudication in this population to be 5%.\textsuperscript{38} Prevalence of intermittent claudication of 1% has been reported in those aged 55 to 60 and 6% in those aged over 80.\textsuperscript{35}

1.2.3 Natural History of Peripheral Vascular Disease.

The majority of Fontaine stage I and II disease does not progress to critical limb ischaemia, however patients with peripheral vascular disease are at increased risk of cardiovascular events due to concomitant coronary and cerebrovascular disease.\textsuperscript{39} There is a 2 – 4 times increased risk of coronary artery disease compared to age matched cohorts\textsuperscript{40} and angiography of patients with peripheral vascular disease has shown significant single vessel coronary disease in 60 to 80% of patients.\textsuperscript{41}

As a consequence, patients with peripheral vascular disease have a 20% increased risk of MI compared to age matched controls and a 2 to 6 times increased risk of death due to cardiac events.\textsuperscript{42}

1.2.4 Prognosis of Limb in Peripheral Vascular Disease.
The majority of Fontaine I/II disease remains stable. Deterioration is most likely in the first year of diagnosis. 7 to 9% deteriorate in this period, compared to 2 to 3% per year in the following period. A major amputation is required rarely, with 1 to 3% of patients presenting with intermittent claudication requiring amputation within 5 years. Those who progress to Fontaine III/IV disease have a particularly poor prognosis. It is estimated that within one year of diagnosis and primary treatment only 25% of patients disease will have resolved. 25% will have died, 30% will have had a major amputation and 20% will have ongoing Fontaine III/IV disease. (Estimates from 2007 TASC Guidelines)\textsuperscript{43} To put these figures in context, 5 year survival from regional breast cancer is 85%,\textsuperscript{44} indicating the poor prognosis that patients who progress to Fontaine III/IV disease have.

1.2.5 Management of Peripheral Vascular Disease.

Specific management of peripheral vascular disease is dependent upon the stage of the disease according to the Fontaine classification, however for all stages of the disease the same principles of management apply:

- Address Concomitant Risk of Death from other Cardiovascular Causes (Fontaine I to IV)
- Address Symptoms of Intermittent Claudication (Fontaine II)
- Address Critical Limb Ischaemia (Fontaine III/IV)

Surgical Intervention is only normally considered in class III/IV disease.\textsuperscript{45}

1.2.5.1 Management of Cardiovascular Risk in Peripheral Vascular Disease.

Peripheral vascular disease is a reflection of generalized atherosclerosis. Patients have significantly increased risk of cardiovascular events. Management requires optimization of the
medical management to reduce risk of cardiovascular events. At present this is poorly managed\textsuperscript{46} though publication of TASC and AHA/ACC guidelines should go a long way to rectify this.

1.2.5.1.1  \textit{Lifestyle Modification in Peripheral Vascular Disease.}

Cessation of smoking is the single most important factor in determining the outcome of patients with peripheral vascular disease, reducing the risk of death, cardiovascular events and amputation.\textsuperscript{47} Stopping smoking can also symptomatically improve peripheral vascular disease.\textsuperscript{48} Unfortunately success rates with smoking cessation remain poor with many patients failing and resorting to smoking after a short period of abstinence.\textsuperscript{49}

1.2.5.1.2  \textit{Antiplatelet Therapy.}

Patients with peripheral vascular disease have a high risk of cardiovascular events therefore lifelong antiplatelet treatment is recommended. The Antiplatelet trialist collaboration demonstrated a 23\% relative risk reduction in serious vascular events in 9706 patients with peripheral vascular disease treated with aspirin 75 to 150mg.\textsuperscript{50}

If patients are intolerant of aspirin, clopidogrel is a suitable alternative. The CAPRIE trial demonstrated an annual rate of serious cardiovascular events of 4.9\% in the aspirin group versus 3.7\% in the clopidogrel group, conferring a relative risk reduction of 24\%.\textsuperscript{51} Clopidogrel treatment is significantly more costly compared to aspirin therapy, so its use is only indicated in those who are intolerant of aspirin at present.

1.2.5.1.3  \textit{Antihypertensive Treatment.}
ACE inhibitors have been shown to reduce cardiovascular morbidity and mortality in patients with manifestations of cardiovascular disease. The HOPE study demonstrated a 22% reduction in myocardial infarction, stroke or death in patients treated with an ACE inhibitor with a 25% reduction in cardiovascular morbidity and mortality in the subgroup of 4051 patients with peripheral vascular disease.\textsuperscript{52}

The EUROPA study also demonstrated improved outcome in patients treated with ACE inhibitors, with a 20% reduction in cardiovascular death at 4 years in those treated with perindopril versus placebo.\textsuperscript{53}

It is recommended that ACE inhibitors are considered in patients presenting with peripheral vascular disease to reduce the risk of cardiovascular events.

It was a long held belief that non cardioselective beta-blockers could worsen intermittent claudication in patients with peripheral vascular disease, however a Cochrane Review and meta-analysis of randomised control trials of antihypertensive agents in peripheral vascular disease documented the safety of beta blockers in all but those with critical limb ischaemia.\textsuperscript{54}

1.2.5.1.4 \textit{Cholesterol Reduction.}

Statin use confers significant benefit in patients with cardiovascular disease when used for both secondary and primary prevention.

The 4S study demonstrated a relative risk reduction of 0.66 for major cardiac events in those treated with simvastatin in patients with ischaemic heart disease.\textsuperscript{55} The 4S Study also subclassified patients with peripheral vascular disease and demonstrated the risk of new or
worsening intermittent claudication to be 0.6 in patients treated with simvastatin compared to placebo.

The Heart Protection Study demonstrated that lowering cholesterol and LDL by 25% with a statin reduced the morbidity and mortality in patients with peripheral vascular disease by 21% irrespective of the initial absolute cholesterol measurement.56

The CARE study randomized patients with normal cholesterol levels to a statin or placebo and demonstrated a reduction in coronary death and myocardial infarction by 24% in the treatment group.57

Statins have also been demonstrated as effective in primary prevention in patients with raised cholesterol and no history of cardiovascular illness. Both the West of Scotland Coronary Prevention Study (WOSCOPS)58 and Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS)59 demonstrated a reduction in coronary events in patients with elevated cholesterol treated with statins.

Other evidence supporting the control of cholesterol in peripheral vascular disease comes from the program on the surgical control of hyperlipidaemias, which randomized patients with ileal bypass surgery to receive cholesterol lowering treatment or not. The risk of an abnormal ABPI was 0.6 and the risk of intermittent claudication or critical limb ischaemia was 0.7 when compared to the control group at 5 years.60

1.2.5.1.5 Management of Concomitant Diabetes Mellitus.
Diabetes and peripheral vascular disease often co-exist. Diabetics have a two-fold increased risk of developing peripheral vascular disease and an increased risk of disease progression to Fontaine III/IV disease. All diabetic patients with peripheral vascular disease should undergo a regime to ensure strict glycaemic control, with the aim of achieving a glycosylated haemoglobin of < 7%. A 1% reduction in glycosylated haemoglobin correlates with a 21% reduction in all-cause mortality. All secondary prevention measures as outlined above should be instigated.

With respect to the progression of peripheral vascular disease neither the Diabetes Control Complication Trial (1441 patients with type I diabetes) nor the UK Prospective Diabetes Study (3867 patients with type II diabetes) demonstrated a reduced risk of the development of peripheral vascular disease or amputation with improved glycaemic control.

1.2.5.2 Management of Fontaine I/II Disease.

Supervised exercise programmes have been demonstrated to improve walking distances. A meta-analysis of all trials demonstrated an increased walking distance of 120% and walking times of 180%. The Cochrane review of only randomized trials demonstrated improved walking distances of 150%. For exercise therapy to achieve these results it is recommended that participants have sessions of 30 to 60 minutes three times weekly for 3 months. Supervised exercise programmes are not freely available in the UK as yet, though patients should be advised that no harm will come from ‘walking through’ the pain of claudication.

A heel raise has been a long purported, anecdotal approach to improve walking distance in patients with SFA occlusion, however little objective data is available on this topic. A medline search on the topic showed no papers of any kind from 1950 to 2008.
Cilostazol, a phosphodiesterase inhibitor has been demonstrated to improve walking distance and pain free walking distance in patients with intermittent claudication. A meta-analysis of 6 randomised trials demonstrated a net improvement in walking distance of 50 to 70 metres.\textsuperscript{69} It is not known if this effect is due to cilostazol’s antiplatelet effect or vasodilatory effect.\textsuperscript{70,71}

Pentoxifylline has also been examined in various trials with respect to improving walking in patients with intermittent claudication. Early studies were promising, though meta-analysis of the studies have showed small improvements in walking distances at best.\textsuperscript{72} Pentoxifylline is still being investigated as to its potential to improve intermittent claudication.\textsuperscript{73}

Patients are only considered for revascularisation if they have lifestyle limiting symptoms and lesions in which intervention carries low risk and high probability of success.

In general patients with infrainguinal disease are not considered for surgical intervention for Fontaine II disease, though through advances in endovascular techniques TASC A infrainguinal lesions can be treated with angioplasty with growing success. For disease above the inguinal ligament, both open surgery and endovascular techniques produce better results than in infrainguinal revascularisation\textsuperscript{74} and is more often considered for the management of Fontaine II disease.

\textit{1.2.5.3 Management of Fontaine III/IV Disease.}

In the presence of Fontaine III/IV disease, in addition to optimal medical management, revascularisation is indicated if amputation is to be avoided. Revascularisation can be performed either through endovascular techniques such as angioplasty with or without stent deployment or surgical techniques such as bypass grafting using synthetic or autologous grafts.
The favoured intervention is determined by the anatomical level of disease. It is classified as follows:75

1. Localised Aorto-iliac disease: disease localized to the aorta and iliac arteries, in contrast to other atherosclerotic disease there is a 1:1 male to female ratio, often associated with a hypoplastic aorta.
2. Diffuse Aorto-Iliac disease:
   a. Confined to above the inguinal ligament
   b. Disease affecting both above and below the inguinal ligament
3. Disease below the inguinal ligament: normally affects the superficial femoral artery at the site of Hunters Canal.

Diabetic patients have a predilection for developing disease of the smaller vessels, the tibial or peroneal vessels.76

1.2.5.3.1 Management of Aorto-Iliac Disease.

The Transatlantic Intersociety Consensus (TASC) group have classified lesions to determine whether best dealt with by endovascular or surgical techniques, stratifying by length and morphology of lesions. In general short, focal lesions are suitable for endovascular intervention and longer, more complex lesions better dealt with surgically.77

<table>
<thead>
<tr>
<th>TASC A</th>
<th>Unilateral or Bilateral Stenosis of Common Iliac Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Short &lt;3cm Stenosis of External Iliac Artery.</td>
</tr>
<tr>
<td>TASC B</td>
<td>Short ≤3cm stenosis of infrarenal Aorta</td>
</tr>
<tr>
<td></td>
<td>Unilateral Common Iliac Artery Occlusion</td>
</tr>
<tr>
<td><strong>TASC C</strong></td>
<td>Single or Multiple Stenosis of EIA 3 to 10cm not extending to Common Femoral Artery.</td>
</tr>
<tr>
<td>------------</td>
<td>-----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td></td>
<td>Bilateral Common Iliac Artery Occlusion</td>
</tr>
<tr>
<td></td>
<td>Bilateral TASC B External Iliac Artery Lesions</td>
</tr>
<tr>
<td></td>
<td>Unilateral External Iliac Artery Lesion extending to Common Femoral Artery</td>
</tr>
<tr>
<td></td>
<td>Heavily Calcified TASC B Lesions</td>
</tr>
<tr>
<td><strong>TASC D</strong></td>
<td>Infra-Renal Aorto-iliac occlusion</td>
</tr>
<tr>
<td></td>
<td>Diffuse Aorto-iliac disease requiring treatment.</td>
</tr>
<tr>
<td></td>
<td>Diffuse multiple stenoses of unilateral Common Iliac, External Iliac and Common Femoral Arteries</td>
</tr>
<tr>
<td></td>
<td>Bilateral External Iliac Artery Occlusions</td>
</tr>
<tr>
<td></td>
<td>Unilateral Occlusion of Common Iliac and External Iliac Arteries.</td>
</tr>
<tr>
<td></td>
<td>Iliac Lesions in patients requiring open Abdominal Aortic Aneurysm repair or other lesions not amenable to Endovascular treatment.</td>
</tr>
</tbody>
</table>

Table 1.2 TASC Classification of Aorto-Iliac Disease

Endovascular treatment is preferred for type A and B lesions, and surgery for type D lesions. In type C lesions surgery is preferred if the patient has suitable health to undergo the procedure. With advances in stent and deployment technology, the boundary at which surgical intervention is required will no doubt further shift.

1.2.5.3.1.1 Endovascular Management of Aorto-Iliac Disease.
Angioplasty with or without stent deployment is most commonly used to treat iliac territory disease where a retrograde puncture of the common femoral artery is used (on either the contralateral side or ipsilateral). The axillary and brachial arteries can also be used if necessary. This technique has been established as safe and effective and less invasive than surgical management. Primary patency of 74% at 8 years has been reported with iliac bare metal stents. 78

1.2.5.3.1.2 Surgical Management of Aorto-iliac Disease.

Aorto-iliac bypass surgery is performed using synthetic grafts and these have also been shown to have good long term success rates. When compared with angioplasty there is a slightly higher complication rate but improved long term patency, and is therefore preferred in younger patients. 10 year patency when placed for Fontaine III/IV disease is as high as 80% and 87% when inserted for surgical management of Fontaine II disease. 79

1.2.5.3.2 Management of Infrainguinal Disease.

Infrainguinal disease has also been classified by TASC to optimize management. The classification is as follows:

<table>
<thead>
<tr>
<th>TASC A</th>
<th>Single Stenosis ≤ 10cm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Single Occlusion ≤ 5cm</td>
</tr>
<tr>
<td>TASC B</td>
<td>Multiple Lesions, each ≤ 5cm</td>
</tr>
<tr>
<td></td>
<td>Single Stenosis or occlusion ≤ 15cm, not involving infrageniculate popliteal artery.</td>
</tr>
<tr>
<td></td>
<td>Single or Multiple lesions in the absence of continuous tibial vessels to improve inflow for a distal bypass</td>
</tr>
</tbody>
</table>
Heavily Calcified occlusion ≤ 5cm
Single popliteal stenosis

TASC C
Multiple Stenosis or occlusions totaling > 15cm with or without calcification
Recurrent lesions that require intervention after two endovascular therapies.

TASC D
Chronic total occlusions of Common Femoral Artery or Superficial Femoral Artery > 20cm in length
Chronic total occlusion of popliteal artery and proximal trifurcation vessels

Table 1.3 TASC Classification of Infrainguinal Disease

Like in aorto-iliac disease, endovascular treatment is recommended for TASC A and B lesions where possible, with surgical intervention for TASC C and D lesions

1.2.5.3.2.1 Endovascular Management of Infrainguinal Disease.

Angioplasty for infrainguinal disease has poorer outcomes than when compared with iliac disease. One year patency has been reported to be as high as 77% when used to treat TASC A stenosis, however by 5 years the patency is only 55%. Stenting of lesions has demonstrated higher patency at 1 year for TASC A and B lesions when compared to angioplasty alone. These results include patients with Fontaine II disease, where the patency rates were more favourable, compared to interventions for critical limb ischaemia.

The BASIL trial compared balloon angioplasty to surgery for the management of critical limb ischaemia. 452 patients with Fontaine III/IV disease were randomized to surgery or angioplasty of their lesions. At 2 years there was little difference in amputation-free survival of the groups; however angioplasty had a significantly higher failure rate than surgery, with 27% of angioplasty having failed clinically and the patient requiring limb salvage surgery.
1.2.5.3.2.2 Surgical Management of Intrainguinal Disease.

Surgical intervention is still the treatment of choice for complex intrainguinal lesions causing critical limb ischaemia. Usually this will be in the form of a bypass graft either using autologous long saphenous vein graft, or prosthetic graft made of Dacron or PTFE. Human umbilical vein is also used.

A femoral popliteal bypass graft involves taking the conduit of choice, either autologous or synthetic and using it to bypass the narrowing in the vessel from a point above the occlusion to a point below. This is normally from the femoral artery to the popliteal artery, though the anterior and posterior tibial vessels as well as peroneal artery can also be used. Critical to the success of the graft is to have sufficient inflow (from the femoral artery) as well as sufficient outflow (patency of the distal artery) to allow blood to flow through the graft beyond the occlusion.

The material used as the conduit has been researched extensively. The current preference is to use autologous long saphenous vein. 5 year patency when autogenous vein is used is 76% compared to 52% using PTFE in above knee femoral-popliteal bypass grafts.83,84 In below-knee grafts, results using PTFE are poorer with 5 year patency of 39%85 compared to 70% with autologous saphenous vein.86,87

The consequences of failure of synthetic grafts also appear to be more severe than those of autogenous saphenous vein, with failure more likely to result in critical limb ischaemia.88

For these reasons, vein is the conduit of choice for infrainguinal revascularisation procedures, however despite refinements in technique, the graft failure rate still remains unsatisfactory with 30% becoming stenotic and requiring reintervention by 2 years.
1.3 Infrainguinal Graft Failure.

Infrainguinal graft failure can be classified as either short, medium or long term failure. Short term failure occurs within 4 weeks of surgery and is due to technical problems with the graft. Medium term graft failure occurs outwith this time frame and is due to intimal hyperplasia of the graft causing graft stenosis. Long term graft failure is due to progression of the arterial disease either proximal to the origin of the graft or in the distal vessels. 80% of graft failure is medium term graft failure, occurring between 1 and 18 months.

1.3.1 Intimal Hyperplasia.

Intimal hyperplasia describes the abnormal response of a vessel to injury, with intimal layer thickening, vascular smooth muscle cell (VSMC) proliferation and matrix deposition. The process is initiated by endothelial damage, with subsequent endothelial cell dysfunction causing an inflammatory response which drives the recruitment of leucocytes, proliferation and migration of VSMC’s and extracellular matrix deposition.

1.3.1.1 Role of the Endothelium.

Endothelial cells are critical to vascular integrity. They secrete NO, (formed via eNOS) and prostacyclins on a continuous basis. They also form a barrier, protecting the media from circulating growth factors. NO inhibits platelet activation and aggregation as well as inhibiting release of VCAM-1 and maintaining VSMC in a quiescent state.

Endothelial damage removes this protective mechanism and allows exposure of the subendothelial matrix. The expression of ICAM-1 and MCP-1 is also increased by damaged endothelium, promoting leucocyte migration and adhesion.
1.3.1.2 Role of Platelets.

Subendothelial matrix exposure causes tissue factor release\textsuperscript{92} with platelet activation and thrombus formation. Thrombus formation promotes further platelet activation and stimulates VSMC proliferation and migration via release of Thromboxane A2 and PDGF.\textsuperscript{93-95} P-selectin is also secreted, which binds to P selectin glycoprotein ligand 1 on leucocytes\textsuperscript{96}, welcoming a further, inflammatory component to the process.

1.3.1.3 Role of Leucocytes.

Activated leucocytes migrate to the endothelium, where they represent one third of the replicating cells.\textsuperscript{97} Their role is unclear, however they release Matrix Metalloproteinase (MMP)-9 and IGF-1,\textsuperscript{98} both of which promote VSMC proliferation and differentiation.

In some lesions of intimal hyperplasia, leucocytes behave as they do in an atherosclerotic plaque, consuming lipids to become foam cells, though this behaviour is not consistent.\textsuperscript{99} Increased numbers of atherosclerotic-like foam cells are associated with VSMC apoptosis, which has been linked to plaque rupture in both atherosclerotic and intimal hyperplasia lesions.\textsuperscript{100}

1.3.1.4 Role of Vascular Smooth Muscle Cells.

VSMC represent the largest cellular element in neointimal hyperplasia.\textsuperscript{101} In conjunction with the above response VSMC’s change from a quiescent contractile state to a synthetic motile state,\textsuperscript{94} facilitating migration to the intima. Motile cells secrete MMP 2 and 9, which degrade the existing extracellular matrix allowing further migration,\textsuperscript{97} as well as collagen and elastin deposition, increasing lesion density.\textsuperscript{101}
There is also evidence to suggest circulating VSMC-like precursor cells contribute to neointimal hyperplasia, migrating to the lesion and transforming into cells with VSMC characteristics. Peripheral mononuclear cells can differentiate into VSMC-like cells in culture and patients with coronary artery disease have significantly increased number of peripheral mononuclear cells capable of transforming to VSMC-like cells.\textsuperscript{102}

Neointimal hyperplasia formation progresses until the lumen reduces to a point where graft flow falls, symptomatic deterioration occurs and reintervention is necessary.

1.3.2 Cell Signalling Pathways in Intimal Hyperplasia.

Knowledge of the cell signalling pathways involved in neointimal hyperplasia has significantly increased, and is important in the search for new treatments.

1.3.2.1 MAP Kinases.

MAP kinases are a family of serine-threonine protein kinases. They are involved in the regulation of cell activation, proliferation and migration, and have been demonstrated to be central to VSMC activation.\textsuperscript{103} Those linked to neointimal hyperplasia are p38, cJun-nTerminal kinase (JNK) and extra-cellular signal related kinase (ERK 1/2). In general, p38 and JNK activation is in response to cell stress, such as hypoxia, heat or oxidative stress. ERK 1/2 activation is associated with response to mitogenic stimuli, such as PDGF.\textsuperscript{104} However there is clearly some overlap between the pathways: p38, JNK and ERK 1/2 all been shown to participate in PDGF stimulated migration\textsuperscript{105,106} and p38 pathways have also been associated with thrombin stimulation.\textsuperscript{107} In addition ERK 1/2 signalling has been shown as crucial to both MMP 2 and 9 production.\textsuperscript{108}
1.3.2.2 Small Guanine Triphosphatases (GTPase)s.

Small GTPase expression is increased following vein grafting\textsuperscript{109} and those implicated in neointimal hyperplasia are rho and ras and rac.\textsuperscript{110} The small GTPase proteins act as molecular switches, cycling between active GTP-bound and inactive GDP-bound forms, translating upstream signals to downstream effects.\textsuperscript{111} GTPases are linked with VSMC proliferation, migration and phenotype modulation.

Ras is associated with enhanced VSMC proliferation, with subsequent effects upon MAP kinase signalling\textsuperscript{112} Rho signalling influences the alteration of VSMC phenotype to the synthetic, motile state, as well as regulation of MMP-9.\textsuperscript{113}

Rac activation has been associated with a variety of VSMC effects including proliferation and migration and actin cytoskeleton reorganization.\textsuperscript{114,115} Rac1 is the small GTPase most closely associated with these effects, though recently Rac2 (normally expressed only in haematopoietic cells) has been demonstrated to be present in VSMC and respond to inflammatory cytokines and that overexpression can enhance VSMC proliferation and migration.\textsuperscript{116}

1.3.2.3 Other Signalling Pathways Implicated in Intimal Hyperplasia Formation.

Protein kinase C is a member of the serine/threonine protein kinase family. It is linked to activation of MAP kinase pathways in VSMCs\textsuperscript{117} and subsequent inhibition of VSMC proliferation and migration.
Phospholipase C-γ (PLC-γ) is an intracellular protein which participates in cell signalling pathways. PDGF stimulation leads to increased PLC-γ levels in VSMCs and phenotypic modulation.\textsuperscript{118} The role of PLC-γ will be fully discussed later.

![Diagram](image)

Figure 1.1: Cellular Aspects of Intimal Hyperplasia Formation: Endothelial dysfunction leads to loss of the normally protective NO secretion. A cascade of events subsequently occurs with platelet activation and leucocyte migration. These activated cells release proinflammatory mediators such as Thromboxane A2, PDGF and MMP-9, promoting the migration and proliferation of VSMC.
1.4 Improving Graft Survival

Graft survival remains suboptimal; therefore the search for interventions and treatments to improve outcomes in infrainguinal bypass grafts is important. This section will review treatments investigated to improve graft survival as well as potential new therapies. It is important to note that 80% of graft failure is due to intimal hyperplasia formation, making attenuating intimal hyperplasia an attractive pharmacological target, as will be further discussed.

1.4.1 Graft Surveillance Programmes.

As 80% of graft failure occurs within one to 18 months, graft surveillance during this period using duplex ultrasound can be used to detect areas of restenosis to facilitate reintervention before occlusion and more devastating clinical consequences occur.

Graft surveillance programmes have been shown to improve intermediate graft patency, but not overall limb salvage rates. To add controversy the recently reported vein graft surveillance randomized trial (VGST) of 594 patients demonstrated no improvement in patency rates at 18 months (79% vs. 80%) nor in limb salvage, with an amputation rate of 7% in both groups. Graft surveillance is not routinely practiced in the UK.

1.4.2 Pharmacological Interventions to Improve Infrainguinal Graft Patency.

Pharmacological interventions that improve graft survival are limited with few treatments demonstrating improved outcomes. The only recommendation in the latest TASC guidelines is that all patients are on an antiplatelet agent following infrainguinal bypass surgery – the only intervention with significant evidence supporting its use.
1.4.2.1 Antiplatelet Treatments.

Antiplatelet agents are well established in treatment of patients with peripheral vascular disease where use significantly reduces the risk of cardiovascular events.\textsuperscript{120} Aspirin is the agent most commonly used, though the efficacy of dipyridamole, ticlopidine and to a lesser extent clopidogrel have been assessed in improving graft patency. Aspirin inhibits platelets via irreversible inhibition of the prostaglandin H-synthase C (COX) enzyme\textsuperscript{121}, part of the arachidonic acid pathway that forms thromboxane A\textsubscript{2}. Dipyridamole inhibits cAMP phosphodiesterase enzyme, affecting the NO/cGMP signalling pathway\textsuperscript{122}, part of the platelet activation pathway. Ticlopidine and clopidogrel both irreversibly inhibit ADP-dependent pathways of platelet aggregation mainly via P2\textsubscript{Y12} G-protein coupled receptor which initiates platelet aggregation and amplifies the response to thromboxane A\textsubscript{2} and thrombin.\textsuperscript{123}

Platelet inhibition prevents thrombus formation within the graft and while this is important in preventing graft failure there are downstream effects potentially preventing neointimal hyperplasia formation. Activated platelets within a thrombus secrete PDGF, Thromboxane A\textsubscript{2} and P-selectin which as well as facilitating thrombin formation are associated with driving pathways of neointimal hyperplasia formation. (See figure 1.2)
Figure 1.2 How antiplatelet agents could reduce intimal hyperplasia: Aspirin, dipyridamole and clopidogrel all act via separate pathways to prevent platelet activation. Inhibition of platelet activation prevents formation of thrombin and PDGF which are strong VSMC mitogens as well as p-Selectin, which is chemoattractant for leucocytes. In addition, thromboxane A2 production is inhibited by aspirin. Thromboxane A2 is also a VSMC mitogen.

Current recommendations are that patients undergoing revascularisation procedures should be on an antiplatelet agent. This is based upon data from the antiplatelet trialists collaboration, which analysed 46 randomised trials of antiplatelet therapy versus control. 14 randomised trials analysed peripheral graft patency. Aspirin in dose range of 75mg to 1500mg per day was studied, along with dipyridamole (225mg to 450mg). Overall a 43% reduction in occlusion rates in patients treated with an antiplatelet agent was demonstrated. There was no significant difference between antiplatelet agents or combinations thereof, nor was there a difference between aspirin at high dose (500 – 1500mg) or medium dose (75mg to 325mg). No significant increase in bleeding risk...
was found. Various graft types were included in the analysis and no trend towards improvement in autologous or synthetic grafts was reported. A further meta-analysis of 32 studies compared the use of aspirin and dipyridamole, aspirin alone and placebo. Aspirin was studied in dose from 50mg to 990mg and dipyridamole 225mg to 450mg. The use of aspirin and dipyridamole (the objective in 11 of the 32 papers analysed) led to a reduction in occlusion rates, with an odds ratio of 0.69 when compared to placebo. The benefit of aspirin alone could not be determined, however only 2 eligible papers were included. Another meta-analysis of 5 papers demonstrated the relative risk of occlusion of patients on aspirin with or without other antiplatelet therapy to be 0.78. The dose range of aspirin studied was 325mg to 990mg with dipyridamole 225 to 300mg. A Cochrane review of 15 randomised trials confirmed that antiplatelet therapy (either aspirin alone or in combination with dipyridamole) reduced the risk of graft occlusion (Odds ratio 0.6). The benefit of antiplatelet therapy was greater in patients with prosthetic grafts.

Clopidogrel has superseded ticlopidine as the thienopyridine of choice, due to its superior side effect profile coupled with CAPRIE study data demonstrating efficacy similar to aspirin in improving cardiovascular risk. Clopidogrel can experimentally reduce neointimal hyperplasia in small animal models however its efficacy in preventing graft occlusion is unverified. Its use as an alternative to aspirin in those whom are aspirin intolerant is based upon evidence from a single RCT of 243 patients undergoing autologous femoral-popliteal or femoral-tibial bypass grafts, where two year patency was 82% in patients taking clopidogrel precursor ticlopidine 250mg bd compared with 63% in the placebo group at 2 years. Patency rates in the placebo group of this trial were inferior to high volume institutions, potentially limiting widespread implication.

More recently, the CASPAR trial has been provisionally reported, investigating the effect of clopidogrel (75mg) and aspirin (75 – 100mg) compared to placebo and aspirin in below knee bypass surgery. This demonstrated that clopidogrel and aspirin significantly improved outcomes in prosthetic grafts, with a hazard ratio of 0.63. There was no reduction in graft occlusion in patients with venous grafts. No significant increase in bleeding events was reported. While this
trial demonstrates promise that dual antiplatelet therapy may be beneficial in preventing failure of prosthetic grafts, larger confirmatory studies will be required in both above knee and below knee grafts.

The evidence pertaining to antiplatelet therapy in prevention of vein graft failure remains incomplete. While it is clear that all patients undergoing infrainguinal bypass surgery should be on an antiplatelet agent to reduce the risk of cardiovascular events as well as potentially improve graft survival, there is no consensus as to which antiplatelet agent is superior or if combination antiplatelet therapy further improves outcomes. It is also unclear if different patient subgroups benefit more from antiplatelet therapy. Some trials have demonstrated an improved patency in prosthetic grafts with antiplatelet therapy compared to autologous grafts, with the CASPAR trial raising the possibility that combined antiplatelet therapy may confer further advantages in this subgroup.

1.4.2.2 3-Hydroxy-3 methylglutaryl (HMG) CoA Reductase Inhibitors (statins).

Statins have significant beneficial cardiovascular effects and have been shown to reduce intimal hyperplasia via inhibition of smooth muscle cell proliferation and migration. Two clinical studies have demonstrated that patients taking statins have improved vein graft survival. One retrospective study of 172 patients demonstrated higher primary and secondary patency rates at 2 years in patients undergoing autologous infrainguinal bypass grafting on statin therapy (94% vs. 83% primary and 97% vs. 87% secondary). 65% of patients studied underwent below-knee bypass grafts. Although retrospective, the groups in this study were well matched however no data on compliance or duration of therapy was available and a variety of statin types were studies (64% simvastatin, 30% atorvastatin). Another retrospective study analysed 293 patients undergoing 338 infrainguinal bypass procedures where statin use was associated with an improved odds ratio for improved graft patency of 3.7, with median follow-up of 17 months. Again the patients included were on a variety of statin type, and duration of therapy was unclear. The study included 218 autologous vein grafts and 120 prosthetic or composite grafts.
There is evidence to suggest that the positive effects of statins is due not only to lipid-lowering but to pleiotropic (non-lipid lowering) effects. Pleiotropic effects are both anti-inflammatory and antiproliferative. Statins have been shown to inhibit VSMC proliferation and migration, activate endothelial NO release, reduce cytokine secretion and act as an antithrombotic agent.

The pleiotropic action is via the mevalonate pathway and its effect upon small GTPases, as a downstream effect of mevalonate inhibition on isoprenoid intermediates. Statins reduce rho and ras levels in VSMCs, in conjunction with reducing proliferation and migration. VSMC MMP-9 secretion is also reduced, and enhanced endothelial eNOS production via Rho kinase pathways has been demonstrated.

It is clear that statins could reduce neointimal hyperplasia via a number of synergistic mechanisms (see figure 1.3). While this data is suggestive that statins may potentially improve graft patency, it is far from definitive. Two retrospective studies are not enough to answer this question. A prospective randomised trial will not be possible as it is clear that all patients with peripheral vascular disease should be on statin therapy to reduce the risk of cardiovascular events. Further improvements in both graft survival and cardiovascular events may be demonstrated by gathering data comparing statin type and statin dosage. High dose statin pretreatment (atorvastatin 80mg) prior to coronary intervention for non-ST elevation MI reduces peri-procedural MI from 15% to 5%. This effect is independent of cholesterol level reduction. While not directly relevant to graft survival, similar trials with statins should be conducted in patients undergoing infrainguinal bypass surgery, with primary endpoints being mortality, cardiovascular events and graft survival.
Figure 1.3 The influence of statins and the mevalonate pathway on intimal hyperplasia:
Inhibition of the mevalonate pathway has been demonstrated to have a number of downstream effects, including increasing endogenous NO production, reducing the release of tissue factor, prevention of thrombus formation and inhibition of MMP-9. All of these potentially contribute to intimal hyperplasia formation.

1.4.2.3 Gene Therapy.

Gene therapy may play a significant role in the management of intimal hyperplasia. One large randomized control trial has been performed to date: PREVENT-III. PREVENT-III investigated edifoligide, a molecular therapy of oligodeoxynucleotides acting as a competitive inhibitor of the transcription factor E2F, which has been demonstrated to play a pivotal role in VSMC proliferation. Edifoligide could be delivered safely and effectively using an ex-vivo incubation method. While preliminary studies showed promise, PREVENT-III concluded no benefit in preventing reintervention for graft failure.142
The technology facilitating gene therapy has advanced, and further improvements will follow. Potential alternative molecular targets are also under investigation. Adenovirus-mediated gene transfer of N-terminal deletion mutant of the MCP-1 gene to block MCP has been shown to attenuate intimal hyperplasia in a canine model, as has reduction of NF-κβ in a rabbit model. Promise has also been demonstrated in human VSMC’s, where gene silencing of MMP-2 and MMP-9 has been shown to limit migration.

1.4.2.4 *Anticoagulant Treatments.*

Anticoagulant therapies have been investigated in improving graft survival. The coumarin derivative warfarin has been investigated most extensively, either alone or in combination with aspirin. The WAVE trial was designed to determine if oral anticoagulation and antiplatelet therapy was more effective than antiplatelet therapy alone in preventing secondary cardiovascular events. It showed that oral anticoagulation and antiplatelet therapy was no more effective in preventing such events than aspirin alone, with an additive risk of life threatening bleeding in those with additional anticoagulation. Unfortunately graft occlusion was not a primary endpoint of this study, however the group did publish a meta-analysis of 27 trials with a primary end-point of graft failure. 8 trials were included.

Meta-analysis of four trials of oral anticoagulation versus placebo demonstrated significantly lower incidence of graft occlusion (odds ratio 0.63) at the expense of increased major bleeding risk (6.5% vs. 0.3%). Anticoagulation targets were INR of or Quicktest 15% to 30% 1.5 to 2.5. Such a comparison is now irrelevant as it is clear all patients with peripheral vascular disease should be on an antiplatelet agent. Of the two trials comparing oral anticoagulation to antiplatelet agents, a reduction in graft occlusion was seen in one small randomized control trial of 91 patients (26.6% vs. 50.8%). All patients were undergoing autologous infrainguinal bypass grafts and oral anticoagulation (quicktest 25% to 35%) was compared to 325mg of aspirin. Follow-up
was 24 months and compliance rates were not reported. In the larger Dutch BOA trial (2690 patients),\textsuperscript{147} oral anticoagulation (target INR 3 to 4.5) with aspirin 80mg was compared to aspirin alone. No overall difference in graft occlusion was seen, with a two fold increase in major bleeding events. In subgroup analysis by graft material aspirin prevented more prosthetic graft occlusions (number needed to treat 17) and warfarin prevented more autologous graft occlusions (number needed to treat 15). Follow-up was for 24 months, but the anticoagulation arm suffered from poor compliance, with 14% of patients discontinuing therapy and only 50% of patient years being within the therapeutic target. This raised the possibility that oral anticoagulation is effective in maintaining the patency of autologous grafts only, and that no overall difference was seen due to the preferential effects of anticoagulation on autologous grafts and antiplatelet agents on prosthetic grafts. Combining these papers demonstrated no significant reduction overall in graft failure in patients assigned to warfarin. Two further trials have been conducted comparing oral anticoagulation and aspirin to aspirin alone. Sarac et al\textsuperscript{148} investigated the benefit of this approach in patients at high risk of graft failure. High risk in this study was defined as marginal autologous vein, poor run-off or previous failed graft. 56 patients (69% undergoing autologous femoral-tibial grafts) were randomized to post-operative heparin (aPTT target 1.5) followed by oral anticoagulation (target INR 2 to 3) and aspirin 325mg or aspirin alone. Graft occlusion was 22% in the combined anticoagulant and antiplatelets therapy compared to 41% in the antiplatelet group. Complete compliance was reported by the authors, though the percentage of patients achieving target INRs was not. Whilst encouraging, this study was limited in size and the reported haematoma formation was 35% in the combined treatment group compared with 3.7%, in control.

A larger randomized trial of 831 patients undergoing infrainguinal bypass grafting of all types was also performed. Oral anticoagulation with warfarin (commenced when the patient was tolerating oral intake) with a target INR of 1.5 to 2.8 and aspirin 325mg was compared with aspirin alone. No difference in survival of autologous vein grafts or 8mm prosthetic grafts was seen but in the subgroup of 212 patients with 6mm prosthetic grafts, graft failure was reduced by combining anticoagulant and antiplatelet therapy (28.6% vs. 42.1%). The bleeding risk reported was 8.4% compared to 3.6%, however 40% of patients assigned to anticoagulation discontinued
therapy and one third of patients had subtherapeutic INR levels, potentially masking any benefit of warfarin treatment.149

Taken together, these studies indicate that combined anticoagulant and antiplatelet therapy may have a role in the management of autologous grafts and appears to confer benefit in grafts at high risk of failure and small prosthetic grafts. The WAVE trial demonstrates that it adds no benefit in modification of cardiovascular risk and increases the risk of bleeding events.26 therefore anticoagulant and antiplatelet therapy should be avoided except in patients with grafts at high risk of failure. This is at the discretion of the individual clinician at present, as in the current TASC guidelines.

Future research should be aimed at better stratifying the risk associated with different graft types to formalize the definition of a high risk graft. Subsequently a randomized trial of anticoagulant and antiplatelet therapy compared to antiplatelet therapy alone could be conducted in high risk groups where the combination may potentially improve outcomes.

1.4.2.5 Angiotensin Converting Enzyme (ACE) Inhibitors.

Like antiplatelet agents and stains, ACE inhibitors have been demonstrated to reduce cardiovascular events in patients with peripheral vascular disease. Use of ACE inhibitors conferred a relative risk reduction of 25% in cardiovascular morbidity and mortality in the subgroup of 4051 patients with peripheral vascular disease in the HOPE study.52 Hypertension has been demonstrated to be a significant risk factor for graft occlusion in prosthetic grafts, with a hazard ratio of 3.1.150

In infrainguinal bypass surgery, a retrospective analysis of 293 patients has been performed taking into account the effect of ACE-inhibitors.150 While Kaplan-Meier survival curves demonstrated that ACE-inhibitors improved overall survival, no effect on graft patency was
demonstrated. No specific type of ACE-inhibitor was studied, and a variety of graft types were included in the analysis. To date only one group have demonstrated that ACE-inhibitors maintain prosthetic graft patency, in renal access surgery.\textsuperscript{151} This trial failed to control for differences in blood pressure between the two groups so it cannot be determined if the effect is due to better modulation of hypertension or a direct effect of the ACE-inhibitor. All patients with peripheral vascular disease should have appropriate management of blood pressure with an ACE-inhibitor if possible. While hypertension appears to be a risk factor for graft occlusion, ACE-inhibitor treatment has not been demonstrated to improve graft survival, however given the potential survival benefit its use should be encouraged.

\textbf{1.4.2.6 Prostaglandin Analogues.}

Prostaglandins have a vasodilatory effect, however also have been shown to have antiplatelet activity, improve endothelial cell function,\textsuperscript{152, 153} and improve short term haemodynamics in grafts.\textsuperscript{154} Based upon such findings, prostaglandin use has been investigated in graft survival. While initial results in small patient groups appeared promising, subsequent larger trials have demonstrated no effect on graft patency at 12 months when three days of perioperative iloprost was given to patients undergoing femoro-distal bypass grafts (82\% autologous, 18\% prosthetic).\textsuperscript{155} The most recent clinical trial investigated more prolonged use of lipo-ecraprost (60 ug/day) for 8 weeks following distal revascularizations for Fontaine III/IV disease. Enrolling 322 patients, no difference in graft patency or amputation was demonstrated at 180 days, with a high incidence of minor adverse events (2650 vs. 947). The trialists discouraged further research in this area.\textsuperscript{156}

\textbf{1.4.2.7 Other Antiplatelet Agents.}

Cilostazol has been approved for the symptomatic treatment of intermittent claudication and improvements in walking distance have been recorded through its use.\textsuperscript{70} Cilostazol is a
phosphodiesterase III inhibitor which reversibly inhibits platelet function, though it is thought that symptomatic improvements may relate to its vasodilatory effect.

Cilostazol has been demonstrated to reduce restenosis following coronary angioplasty, with some small pilot studies suggesting it to be as efficacious as aspirin. When used in combination with aspirin and clopidogrel the results in restenosis following angioplasty are even more encouraging, with the CREST trial demonstrating a 36% reduction in restenosis in those taking cilostazol. In the peripheral vasculature, cilostazol and aspirin has been shown to be superior to ticlopidine and aspirin therapy in preventing restenosis following angioplasty at 12, 14 and 36 months (87% vs. 65%; 82% vs. 57% and 73% vs. 48%). This was in a small randomised control trial of 127 patients, mostly with Fontaine II disease, though patients with Fontaine III/IV were included. Whilst groups were well matched, only 30% of patients enrolled were on statin therapy, which is in contrast to the number of patients taking statins with peripheral vascular disease in the USA and Europe. One paper attempted to compare cilostazol to ticlopidine in infrainguinal bypass grafts. Enrolling 198 patients, there was no difference in patency between the groups. Comparison to ticlopidine however is unfortunately flawed as the only available data for graft survival is from a solitary paper comparing ticlopidine to placebo. Despite this, future research into the role of cilostazol should be performed, initially with a comparison of aspirin and cilostazol to aspirin alone.

1.4.2.8 Work at experimental stage with promise.

1.4.2.8.1 Peroxisome proliferator-activated receptor agonists γ (Thiazolidinediones).

Thiazolidinediones are used in the management of type II diabetes. Recent research has shown reduction of intimal hyperplasia formation in diabetic animal models which may be independent of glucose levels. VSMC proliferation and migration has also been demonstrated to be reduced by thiazolidinedione treatment, giving promise to a potential therapeutic use in infrainguinal bypass surgery.
Translation of these results from animal studies into human trials may be delayed due to debate thiazolidinedione safety, with a potential association with myocardial infarction and heart failure.\textsuperscript{164} Though the issue has yet to be resolved\textsuperscript{165} investigation in a patient cohort at high risk of cardiovascular events would not be ethical at this point.

1.4.2.8.2 \textit{Nitric Oxide: exogenous administration and enhancing endogenous production.}

NO is critical to vascular integrity and protective against neointimal hyperplasia formation. As recently reviewed by Ahanchi\textsuperscript{166} NO precursors or NO donor compounds can be administered systemically or applied locally. Systemic use of a NO precursor has produced reductions in intimal hyperplasia animal models but not humans.\textsuperscript{167} Systemic therapy with NO donor compounds has demonstrated efficacy in animal models\textsuperscript{168} and has been successful in reducing luminal diameter in one human study following angioplasty,\textsuperscript{169} however a second human study did not correlate these findings, and a high incidence of side effects was reported, limiting clinical use.\textsuperscript{170} Local application to areas of injury has produced promising results in animal studies and would circumvent these problems.\textsuperscript{171}

As well as exogenous addition of NO, agents enhancing endogenous production are of interest. Celiprolol has been demonstrated to reduce neointimal hyperplasia in rabbits, with the proposed mechanism via enhanced NO function.\textsuperscript{172}

NO therapy appears to hold promise, with success in both human and animal models. Side effects of donor compounds limit use, however improving endogenous NO production or topical application may improve outcomes.
**1.4.2.8.3 MAP Kinase Inhibitors.**

MAP Kinase signalling pathways have a significant role in neointimal hyperplasia formation, therefore inhibition makes an attractive therapeutic target.

Systemic administration of both ERK \( \frac{1}{2} \)\(^{173} \) and p38\(^{174} \) inhibitors has been shown to reduce intimal hyperplasia in small animal models. Systemic use in humans is unrealistic as the ubiquity of MAP kinases is such that side effects would be unpredictable. Topical application or pre-incubation of transplanted vein circumvents this and success has been achieved in animal models where antiproliferative and anti-inflammatory effects have been recorded in the VSMCs of vein grafts.\(^{175} \)

**1.4.2.8.4 Small GTPase Inhibitors.**

Small GTPase signalling pathways are implicated in neointimal hyperplasia and local ras inhibitor delivery reduces restenosis rates by inhibiting early VSMC proliferation in an arterial porcine model.\(^{176} \)

The effect of statins on VSMCs has been consistently associated with Rho signalling pathways. Rho kinase inhibitors been demonstrate to reduce intimal hyperplasia in an arterial model\(^{177} \) and a vein graft model, where long term suppression of rho kinase was shown to reduce intimal thickening.\(^{178} \)
1.5 Toll Like Receptors.

1.5.1 General Aspects of Toll Like Receptors.

Toll Like receptors were first identified in monocytes as part of the immune response. They are pattern recognition receptors which recruit intracellular signalling mechanisms in response to appropriate binding, ultimately activating NF-κβ. There are ten human TLR’s, all of which serve to initiate inflammatory signalling in response to detection of pathogen associated molecular patterns (PAMPs). Each TLR recognizes different PAMPs, although no ligand for TLR10 has been indentified yet. TLR2 (in conjunction with TLR6 or TLR1) recognizes di-acyl or tri-acyl bacterial lipopeptides, TLR3 recognises double stranded RNA and TLR 4 recognises bacterial LPS. Ligand binding results in receptor dimerisation and recruitment of intracellular signalling (Myeloid differentiation primary response gene 88 (MyD88) and NF-κβ) and resultant expression of inflammatory mediators such as IL-8 and TNF-alpha.

1.5.2 Toll Like Receptors in Atherosclerosis.

A growing body of evidence associates TLR’s with atherosclerotic processes. TLR expression has been demonstrated in both atherosclerotic plaques and healthy arterial endothelial cells. Bjorkbacka et al demonstrated that MyD88 knockout mice (a TLR dependent signalling pathway) had significant reductions in atherosclerotic plaque size and presence of systemic proinflammatory cytokines.

Evidence for the role of TLR’s in atherosclerosis formation is also available on a population scale, examining polymorphisms of the genes which encode for TLR’s. Studies have demonstrated TLR’s to be associated with ischaemic stroke, myocardial infarction and
interestingly enhanced benefit of statin therapy. TLR2 has also been demonstrated to be more prevalent in patients with restenosis following cardiac angioplasty, linking it to the formation of neointimal hyperplasia in arterial models.

1.5.3 Toll Like Receptors in Vascular Smooth Muscle Cells.

TLR’s have been associated with VSMC. Increased levels of TLR4 signalling has been shown to promote a proinflammatory phenotype in vascular smooth muscle cells. TLR 3 has been associated with vascular smooth muscle cell proliferation, and TLR 4 has also been demonstrated to act via MAP kinase signalling pathways when stimulated by endotoxin. Both TLR 2 and 4 have been demonstrated to cause proliferation of venous VSMC when stimulated by heat shock protein 60, demonstrating the potentially important role of TLR activation in intimal hyperplasia formation.

1.5.4 Potential Role of Toll Like Receptors in Intimal Hyperplasia.

No previous studies have investigated the role of Toll Like Receptors in vein graft intimal hyperplasia in an infrainguinal graft model. TLR2 activation has been demonstrated to induce intimal hyperplasia following injury. Endotoxin has been shown to upregulate TLR4 expression as well as increase intimal hyperplasia formation in response to balloon injury in rabbits. This data lends argument as to the potential role of TLR’s in formation of intimal hyperplasia in a venous infrainguinal bypass graft. This project will determine TLR expression in vein tissue as well as functional activity.
1.6 Ischaemia and Hypoxia in Intimal Hyperplasia.

1.6.1 The Influence of Ischaemia on Infrainguinal Bypass Grafts.

The influence of ischaemia upon intimal hyperplasia formation has not been extensively considered. When the saphenous vein is dissected for use as a conduit, the nutrient supply from the vasa vasorum becomes disrupted, contributing to ischaemia and hypoxia of the vascular cells in the vein. When vein is utilised for infrainguinal bypass procedures, the ipsilateral saphenous vein is used. The vein has been exposed to the same ischaemic conditions that necessitated the bypass, conditions which may alter the cellular responses when it is used as a conduit and potentiate intimal hyperplasia formation. Non-Ischaemic saphenous vein when used for coronary artery bypass grafting (CABG) procedures appears much more durable, with only 9.8% of CABG’s requiring reintervention by 3 years.\textsuperscript{195} Whilst direct comparison between these procedures cannot be made, a potential influence upon this superior patency is the lack of ischaemia prior to use as a conduit. Indeed pilot data from this laboratory has demonstrated that vascular smooth muscle cells from ischaemic veins have enhanced proliferative and migratory responses when compared to VSMC from non-ischaemic veins.\textsuperscript{196}

1.6.2 Hypoxia and Vascular Smooth Muscle Cells.

The influence of hypoxia, a key component of ischaemia, has been investigated extensively in the pulmonary vasculature in models of pulmonary hypertension,\textsuperscript{197} where hypoxia has been shown to promote arterial human vascular smooth muscle cell proliferation. Increases in proliferation were maximal at 5% oxygen in these studies, though enhancement was also seen at 1%.\textsuperscript{198,199} In another study using pulmonary artery VSMC’s, proliferation was seen to increase two-fold at 3% oxygen.\textsuperscript{200}
The mechanisms of this proliferation have been identified. Hypoxic endothelial cells release VSMC mitogens and hypoxia itself enhances the proliferative effects of growth factors on VSMC. Cell signalling via RhoA pathways has also been implicated in the mitogenic response. Chronic hypoxia in pulmonary artery (similar to what would be seen in saphenous vein prior to use as a conduit) also significantly effects calcium handling in VSMC.

Hypoxia can also induce inflammatory processes which have been subsequently linked to intimal hyperplasia formation. IL-6 and IL-8 secretion is markedly upregulated in human VSMC exposed to hypoxia. Whilst IL-8 is primarily a chemoattractant (which can further contribute to inflammatory responses of intimal hyperplasia) it has also been demonstrated to be directly mitogenic for VSMC.

Cell signalling pathways associated with hypoxic proliferation in vascular smooth muscle cells included activation of the protein kinase C pathway as well as of G Protein coupled receptors, with subsequent activation of ERK. Other pathways of interest include hypoxia inducible factor (HIF)-1α. Stimulation of this pathway appears to promote proliferation in human vascular smooth muscle cells obtained from the pulmonary artery.

Most literature focuses upon hypoxia in a model of pulmonary hypertension. There is very little data available on the response of human saphenous VSMC to hypoxia. One paper included saphenous VSMC in proliferation studies and although proliferation was increased at 5% oxygen, VSMC were donated from a single patient.

1.6.3 Hypoxia in Atherosclerosis and Intimal Hyperplasia.

Hypoxia has been implicated in the formation of atherosclerosis, with systemic hypoxia enhancing the development of atherosclerosis and arterial hyperoxia inhibiting. Hypoxia has
also been linked to intimal hyperplasia formation where experimental hypoxia, by occlusion and formation of thrombus within the vasa vasorum causes accelerated intimal hyperplasia in porcine and murine models.\textsuperscript{213, 214}

It is likely that if these pathways are activated in a pulmonary artery model of hypoxia, similar pathways may be activated in the saphenous vein when exposed to ischaemia and hypoxia as part of the critically ischaemic limb and dissection for use as a conduit.
1.7 Phospholipase C Gamma (PLCγ)

1.7.1 General Review of PLCγ.

Phosphoinositide-specific phospholipase c (PI-PLC) causes hydrolysis of phosphatidylinositol(4,5)-biphosphate to inositol 1,4,5 trisphosphate and diacylglycerol in response to extracellular signalling. Fourteen mammalian families of PI-PLC exist: PLCβ(β1 to β4), PLCγ (γ1 & γ2), PLCδ(δ1 to δ4) PLCε. PLCζ and PLCη. Each is characterised by its organisational domain and signalling pathways regulating them. PLCγ is the best studied of the PI-PLC isoforms. Of the two PLCγ families, PLCγ1 is almost ubiquitously expressed, whereas PLCγ2 is expressed mainly in cells of haematopoietic origin.

PLCγ is an intracellular pathway linked to the Calcium (Ca+) signalling cascade. Activation of PLCγ by various receptors facilitates intracellular calcium release which has been demonstrated to be associated with a wide array of short and long term cellular responses, from rapid changes in contraction and secretion, actin reorganization as well as alteration of gene transcription, cell-cycle progression and apoptosis. PLCγ expression has also been shown to be upregulated during immune cell differentiation and in the nuclei of regenerating hepatocytes. Homozygous disruption of PLCγ1 gene causes a lethal embryonic phenotype, where growth is attenuated in all organs of the embryo including vasculogenesis, indicating the ubiquity of PLCγ expression.

1.7.2 Activation Pathways of PLCγ.

Growth factors with intrinsic tyrosine kinase receptor affinity (such as PDGF and EGF) can activate PLCγ. Once bound and activated, the tyrosine kinase receptors dimerise to recruit both PI3-kinase and PLCγ. Phosphorylation of tyrosine kinase residues in the PLCγ molecule,
whilst bound to the receptor (catalysed by kinases intrinsic to the receptor) causes PLCγ activation.\textsuperscript{223} Once activated, the PLCγ dissociates from the receptor and binds to PIP\textsubscript{3} (formed from PI3-kinase) which anchors it to the cell membrane.\textsuperscript{224}

PLCγ activation can also occur via non-tyrosine kinase receptors, whereby stimulation and formation of PI3-kinase leads to cell membrane anchoring and activation of PLCγ.\textsuperscript{223}

PLCγ activation causes intracellular calcium release. This occurs via formation of inositol 1,4,5-triphosphate (InsP\textsubscript{3}) and diacylglycerol (DAG). InsP\textsubscript{3} is generated by the hydrolysis of phosphatidylinositol (4,5)-bisphosphate PtdInsP\textsubscript{2} by activated PLCγ, thereby initiating intracellular Ca\textsuperscript{2+}-release and the subsequent functional effects.\textsuperscript{225} Figure 1.4 illustrates the key elements of PLCγ signalling pathways.

Figure 1.4 Intracellular Pathways of PLC-γ Activation: TrKR: Tyrosine Kinase Receptor, NTrKR: Non-Tyrosine Kinase Receptor, PI3-K: Phosphoinositol-3-kinase, PIP-3:
1.7.3 Links to Other Cell Signalling Pathways.

PLCγ activation and the products of its signalling pathway have also been demonstrated to interact with other signalling pathways, which may be responsible for the wide range of functional effects attributed to PLCγ.

PLCγ has been demonstrated to be activated by small G-protein beta subunits, with further evidence demonstrating that Rac GTPases are responsible for activation of PLCγ. PLCγ activation has been shown to downregulate rac expression in mast cells, giving rise to the possibility that PLCγ and rac exist as a negative feedback loop, however diaglycerol has also been implicated in negative regulation of rac and it may be downstream effects of PLCγ which ultimately affect this negative feedback.

Protein Kinase pathways associated with PLCγ are also of interest: protein kinase pathways have been noted to enhance proliferation of gastric and prostatic cell lines via rac downstream signalling pathways in response to hypoxia. Potentially then there is overlap between signalling pathways recruited in response to hypoxia and those pathways involved in PLCγ signalling.

PLCγ activation also results in formation of diaglycerol (DAG); dependent formation of DAG regulates increased ERK/12 MAP kinase formation via protein kinase C activation in vascular smooth muscle cells.
1.7.4 Functional Associations of PLCγ in Vascular Smooth Muscle Cells and Links to Neointimal Hyperplasia.

PLCγ has been associated with modulation of vascular smooth muscle cells from a quiescent to contractile phenotype, as seen in lesions of atherosclerosis and intimal hyperplasia.\textsuperscript{118} PLCγ activation has also been associated with activation of human vein smooth muscle cells in response to Hydrogen Peroxide in a model of ischaemia-reperfusion by facilitating intracellular calcium release as well as VSMC shape change.\textsuperscript{230} Most importantly inhibition of PLCγ has been demonstrated to significantly reduce proliferation in VSMC of rat pulmonary artery origin, indicating a potentially significant role in intimal hyperplasia formation.\textsuperscript{231}

PLCγ signalling pathways implicate that it should play a key role in intimal hyperplasia formation. InsP3 is rapidly formed in vascular smooth muscle cells in response to PDGF-AB, a potent VSMC growth factor and linked to enhanced VSMC proliferation.\textsuperscript{232}

1.7.5 Links of PLCγ to Toll Like Receptor Signalling Pathways.

Although TLR signalling mechanisms are usually associated with serine/threonine kinases, PLCγ signalling can be demonstrated in response to innate immune mechanisms. In peritoneal macrophages, Lipopolysaccharide (a TLR4 ligand) activates PLCγ signalling pathways.\textsuperscript{233} A confirmatory paper demonstrated PLCγ activation in response to both TLR2 and TLR4 Ligands in macrophages.\textsuperscript{234} These potential pathways of activation have not been studied in vascular smooth muscle cells.
1.7.6 Links of PLCγ to Pathways of Hypoxia.

While PLCγ activation is closely associated with tyrosine kinase receptor by growth factors, cell stressors have also been demonstrate to induce PLCγ activation, potentially via pathways not associated with tyrosine kinase. Oxidative stress can be demonstrated to increase PLCγ activation,\(^\text{235}\) and hypoxia of rat myocytes alters PLCγ localisation,\(^\text{236}\) indicating activation by pathways not limited to growth factors alone.

Also, as described in section 1.6, hypoxia alters calcium handling within vascular smooth muscle cells, as well as activation of PI-3K signalling pathways. Both of these processes are intrinsically linked to PLCγ signalling pathways, therefore it is possible that inhibition of PLCγ may not only reduce VSMC functional effects linked to intimal hyperplasia formation, but may preferentially do so under hypoxic conditions.

1.7.7 PLCγ: The rationale of investigation.

PLCγ gamma activation can be demonstrated in response to both innate immune system activation as well as cell stress associated with hypoxia. Downstream effects of PLCγ activation demonstrated appear important in pathways of intimal hyperplasia formation: phenotypic alteration and proliferation.

Such pathways have not been investigated in VSMC of human origin, therefore this thesis will present novel data as to the potential role of PLCγ in proliferative pathways of human VSMC proliferation as a potential common pathway of activation for both growth factors and cell stressors hypoxia and pathways of innate immunity.
1.8 Overall Summary

Intimal hyperplasia is the cause of the majority of graft failure in infrainguinal bypass surgery. Attenuation of this process could have significant implications in improving the survival of infrainguinal bypass grafts. Pilot data from this laboratory has demonstrated that ischaemia can influence vascular smooth muscle cell proliferation and migration, two key cellular aspects of intimal hyperplasia. Additionally it has been demonstrated in arterial models that hypoxia alone can cause intimal hyperplasia. The influence of hypoxia alone upon cellular aspects of intimal hyperplasia will be investigated.

Activation of innate immunological processes via Toll Like Receptors have been linked to atherosclerosis formation and arterial intimal hyperplasia, but have never been studied in a model of vein graft failure. The presence and functionality of Toll Like Receptors in human saphenous vein of ischaemic and non-ischaemic origin will be determined in this project. Additionally links between cell signalling pathways activated by hypoxia and those of PLCγ will be explored.

Phospholipase Cγ (PLCγ) has been associated with alteration of vascular smooth muscle cells from quiescent to contractile phenotype, though has not been investigated in a venous model of intimal hyperplasia. PLCγ signalling pathways have been linked to both rac and MAP Kinase activation, processes which have been implicated in downstream signalling of TLR’s and hypoxia. The influence of PLCγ upon VSMC proliferation will be assessed.
1.9 Original Hypothesis.

The original hypothesis of this project is as follows:

- Ischaemia and Hypoxia enhance key cellular processes involved in intimal hyperplasia formation.
- Toll Like Receptor activation in grafted vein tissue causes intimal hyperplasia by activating proinflammatory pathways.
- PLCγ activation is a key part of the pathway in cellular processes of intimal hyperplasia.
- PLCγ inhibition can reduce VSMC.
- Hypoxia and TLR’s activate pathways sharing common signalling pathways: MAP kinases, PLCγ and rac.

1.10 Aims of Project

Using vein tissue from amputated limbs as a source of ischaemic veins and smooth muscle cells and smooth muscle cells and those used for coronary artery bypass as a source of non-ischaemic veins and smooth muscle cells, the aims of this project are as follows:

1. Determine the presence and functionality of Toll Like Receptors in Human Veins.
2. Determine the presence and functionality of Toll Like Receptors in Vascular Cells.
3. Determine if stimulation of TLR’s can enhance proliferation of Vascular Smooth Muscle Cells and subsequent cell signalling pathways.
4. Determine if Hypoxia influences the Proliferation of Vascular Smooth Muscle Cells.
5. Determine the presence of PLCγ in Human Veins.
6. Determine if PLCγ inhibition has functional effects under both hypoxic and normoxic conditions.
CHAPTER 2
MATERIALS AND METHODS
2.1 Ethical Approval.

Ethical approval for the use of patient material was gained from the Glasgow Royal Infirmary Research and Ethics Department covering the collection of vein tissue from patients undergoing amputation and patients undergoing coronary artery bypass graft surgery. (REC reference number 07/S0704/48.)

A Material Transfer Agreement was also obtained to allow for the transfer of vein tissue between Greater Glasgow Health Board and Strathclyde University (CLO File Ref: GT10/60 MP/SW)

Further Agreement was obtained to allow transfer of tissue between the Golden Jubilee National Hospital and Strathclyde University.

Informed consent was obtained from all patients entered into the study.
2.2 Patient Groups and Tissue Collection.

2.2.1 Patients Undergoing Amputation.

Patients undergoing lower limb amputation at hospitals within Greater Glasgow Health Board were identified by consultant surgeons and made known to the researcher. All patients required amputation due to critical ischaemia of the lower limb. Informed consent was obtained from all patients, who were given a patient information sheet and consented to be involved in the study using a separate form for that of the procedure, which was filed in their case notes. Data was recorded on the patients age, past medical history, drug history and smoking status in an anonymised form. The only exclusion criteria for this patient group was mental incapacity (inability to give informed consent), or amputation for reasons other than critical limb ischaemia.

2.2.2 Patients Undergoing Coronary Artery Bypass Grafting.

Patients undergoing Coronary Artery Bypass Grafting at Glasgow Royal Infirmary were identified in conjunction with consultant surgeons and made known to the researcher. Informed consent was obtained from all patients who were given a patient information sheet and the consent form filed in the patients notes. Data was recorded on the patients age, past medical history, drug history and smoking status. Ankle:Brachial Pressure Index was also recorded as part of the exclusion criteria. All data was kept in an anonymised form.

Exclusion criteria from this patient group was an APBI < 0.85, previous vascular surgery or inability to give informed consent.
2.2.3 Collection of Tissue from Amputated Limbs.

After the operating surgeon had amputated the limb, it was placed on a trolley with a sterile drape. Using a sterile scalpel blade and instruments sterilised in 70% ethanol, the long saphenous vein was identified and dissected with a surrounding cuff of tissue. It was then placed in a small volume of sterile culture medium (50:50 Waymouths:F12 with 1% penicillin/streptomycin) and stored at 4 degrees for transfer to Strathclyde University Institute of Pharmacy and Biomedical Sciences.

2.2.4 Collection of Tissue following Coronary Artery Bypass Surgery.

After the completion of coronary artery bypass surgery, leftover long saphenous vein was kept in a sterile solution of 0.9% normal saline and the researcher contacted by telephone. The vein was then transferred to a small volume of sterile culture medium (50:50 Waymouths:F12 with 1% penicillin/streptomycin) and stored at 4 degrees for transfer to Strathclyde University Institute of Pharmacy and Biomedical Sciences.
2.3 Processing of Veins.

All veins were returned to Strathclyde University where using a Class 2 Sterile Cell Culture Hood and instruments sterilised in 70% ethanol the surrounding tissue was dissected. The vein was then dissected into 2 to 4 mm segments and placed into a 6 well plate along with 4ml of 50:50 Waymouths:F12 with 15% Foetal Calf Serum. They were stored in an incubator at 37 degrees and the media replaced every 48 hours. Spontaneous explantation of vascular smooth muscle cells would occur after 14 to 21 days. Following explantation, the vein rings were removed and the vascular smooth muscle cells maintained as before. When each well reached confluency, the cells were transferred to culture flasks (either T25 or T75 depending upon cell volume) for further culture.

VSMC explantation occurred readily from veins obtained from patients undergoing CABG. In specimens from amputated limbs, VSMC culture was difficult and no significant yield could be obtained. All cell culture experiments were performed using VSMC obtained from non-ischaemic sources only. Methodological changes in collection including dissection technique (skeletonising vein at time of retrieval versus just prior to cell culture), different transportation medium and various concentrations of foetal calf serum for culture did not improve the yield.

All leftover human tissue was disposed of in accordance with the material transfer agreement.

2.3.1 Transfer of Vascular Smooth Muscle Cells.

Once vascular smooth muscle cells had been grown to confluency, the culture medium was aspirated and replaced with a small volume of TrypLE Express (Gibco 12604). The cells were returned to the incubator for 10 minutes or until they had visibly detached from the base of the culture flask. A small volume of standard media was then added before the suspension transferred to 10ml centrifuge tubes and centrifuged at 10000 rpm for 4 minutes. The supernatant was aspirated and disposed of and standard media added to resuspend the cells in media. The suspension was then transferred as required to further culture plates or flasks.
2.4 Immunohistochemistry Materials and Methods.

2.4.1 Preparation of Vein Segments.

Vein rings of both ischaemic and non ischaemic origin were used. All veins were left in 10% buffered formalin for 24 hours before being processed for wax embedding using a Thermo Shandon Citadel 1000 tissue processor. The cycles were as follows:

70% ethanol ➔ 90% ethanol ➔ Histo-clear:ethanol 50:50 mix ➔ Histo-clear 100% ➔ hot wax.

Following completion of processing, specimens were wax embedded (using a Leica EG1140C) and correctly orientated before being cut into 4μM segments (using Leica RM2125RTE), floated in a warm water bath and mounted on salinated slides (salinated by placing in acetone followed by acetone with APES (3-aminopropyl triethoxysilane), a running water wash then covered and allowed to dry for 48 hours).

When the specimen was mounted, slides were placed in a 65 degree centigrade oven for 1 hour and allowed to cool prior to processing.

2.4.2 Primary Antibodies.

PLC-γ1: 1249 sc-81 rabbit polyclonal IgG available commercially from Santa Cruz Biotechnology but a gift from Prof Graeme Nixon, Aberdeen University.

TLR 2: rabbit antihuman sc-10739 IgG, commercially available from Santa Cruz Biotechnology

TLR4: rabbit antihuman sc-10742 IgG commercially available from Santa Cruz Biotechnology.
Both TLR antibodies were gifted by Dr Clett Erridge, Strathclyde University.

2.4.3 Immunohistochemistry Staining Protocol.

Slides to be used for the immunohistochemistry protocol were rehydrated using a Thermo Shandon Varistain 24-4 for 5 minutes in histoclear x 3, alcohol x2 and water. Endogenous peroxidases were then quenched using Me OH with 0.3% H₂O₂ for 10 minutes before being washed in phosphate buffered saline.

Antigen retrieval was then performed using a preheated TRIS/EDTA buffer, with the specimens cooked under pressure for 5 minutes, depressurised, allowed to cool and then washed in phosphate buffered saline (PBS).

The vein samples were then incubated with 20% normal goat serum (Vector Laboratories) in 100ml for 20 minutes to block any non-specific primary binding and following this, the primary antibody for 18 hours at 4 degrees. Following incubation the slides were washed in PBS before the secondary antibody was added (polyclonal goat anti-rabbit Ig, DakoCytomation) for 20 minutes, followed by another PBS wash and addition of Horseradish Peroxidase (HRP) (Vector Laboratories) for 20 minutes. The reaction was completed by addition of DAB (DAB Chromagen and Substrate, DakoCytomation) for 9 minutes before counterstaining with haemotoxylin, again using Thermo Shandon Varistain 24-4 (sequence for counterstain: haemotoxylin 12 minutes → acidic alcohol 1 minute → water rinse 1 minute → Scots Tap Water Substitute 2 minutes → water rinse 1 minute → absolute alcohol 2 minutes x3 → histoclear 2 minutes x4) and addition of a coverslip for storage of specimen.
2.4.4 Immunohistochemistry Quantification Method.

Images from the processed slides were obtained using Media Cybernetics Cool Snap Pro RS Photometric Adapter and Cool Snap Pro Software. Using this arrangement the white balance and colour calibration was corrected in conjunction with the intensity of the backlight and remained constant for each slide photographed in every group.

Slides were photographed 4 times to cover the complete vein segment or as close to complete as possible. The exposure time was fixed (20ms) as was magnification (10x eyepiece; 20x objective). Images obtained were saved in .tiff format.

Image Pro Plus 6.0 was used for image quantification. Initially segmentation of each image was performed using Hue, Saturation and Intensity to mask all but the positive staining on each slide. The areas which were not stained were masked by black colouring with an intensity value of zero. The overall stained area could then be calculated in pixels (by easily excluding all pixels with intensity value of zero) along with the total intensity of the area. The mean intensity of the stain could then be calculated by dividing the intensity with the area.

2.4.5 Statistical Analysis.

Quantification outcomes between the two groups (ischaemic and non-ischaemic) were compared using a student’s t-test using SPSS for windows. Outcomes of $P < 0.05$ were deemed significant.
2.5 Toll-Like Receptor Challenge: Materials and Methods.

2.5.1 Toll Like Receptor Ligands.

The following established TLR Ligands were a kind gift from Clett Erridge, University of Strathclyde: Pam$_3$CSK$_4$, PolyI:C, Bacillus subtilis flagellin, loxoribine, single stranded RNA and LPS-free bacterial CpG DNA (originally purchased from invivogen), LPS of Escherichia coli R1 (NCTC 13114) and Porphyromonas gingivalis (originally gifted University of Edinburgh). They were repurified prior to use by phenol extraction to remove contaminating lipopeptides and used at the concentrations listed below in all experiments.

<table>
<thead>
<tr>
<th>TLR</th>
<th>Ligand</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR 2</td>
<td>Pam$_3$CSK$_4$</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>TLR 3</td>
<td>PolyI:C</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>TLR 4</td>
<td>LPS of e.coli</td>
<td>100 ng/ml</td>
</tr>
<tr>
<td>TLR 5</td>
<td>Bacillus subtilis flagellin</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>TLR 6</td>
<td>loxoribine</td>
<td>1 mM</td>
</tr>
<tr>
<td>TLR 8</td>
<td>Single stranded RNA</td>
<td>5 µg/ml</td>
</tr>
<tr>
<td>TLR 9</td>
<td>CpG DNA</td>
<td>5 µg/ml</td>
</tr>
</tbody>
</table>

Table 2.1 List of TLR Ligands

2.5.2 Preparation of Vein Tissue and Ligand Application.

Saphenous vein from both ischaemic and non-ischaemic veins was dissected into 1mm rings, washed with blank media and added to a 24 well plate with 270µL of media supplemented with 0.1% FCS. The Ligands of Toll Like Receptors 2,3,4,5 and 9 were added to each well at the concentration listed above. The vein segments were incubated for 18 hours at 37 degrees before the supernatant was withdrawn and frozen at –20 degrees.
2.5.3 Preparation of Vascular Smooth Muscle Cells and Ligand Application.

Vascular smooth muscle cells of non-ischaemic origin were obtained by explant method and transferred to T75 culture flasks as detailed in section 2.3. Once confluency had been reached, the cells were split according to section 2.3.1 and transferred to a 96 well plate at a density of $1 \times 10^4$ cells per well.

Once transferred to the 96 well plate, the cells were stimulated with ligands for TLR 2,3,4,5,7,8 and 9 at the concentration indicated or medium alone, and then incubated either in a LEEC automatic incubator ($O_2$ 20%; $CO_2$ 5%; temperature 37 degrees). Each experiment was performed in triplicate. After 24 hours, the experiment was terminated and the supernatant withdrawn for interleukin 8 assay.

2.5.4 Preparation of Human Umbilical Vein Endothelial Cells and Ligand Application.

Human Umbilical Vein Endothelial Cells (HUVECS), were gifted by Clett Erridge, University of Strathclyde (originally purchased Cascade Biologics). They were grown to confluency in T75 culture flasks in M200 Medium with low serum growth supplement as per manufacturer’s instructions. Once confluency was reached, the cells were split using trypsin/EDTA and transferred to a 96 well plate, where the ligands to TLR 2,3,4,5,7,8 and 9 were added at the concentrations indicated, or medium alone as control. Following this the HUVEC’s were incubated) in a LEEC automatic incubator ($O_2$ 20%; $CO_2$ 5%; temperature 37 degrees). Each experiment was performed in triplicate. After 24 hours, the experiment was terminated and the supernatant withdrawn for interleukin 8 assay.
2.5.5 Interleukin 8 Measurement.

Interleukin 8 secretion was measured from supernatants using IL-8 ELISA kit from R&D and the supplied protocol.

The capture antibody was diluted in PBS, added to 96 well plate at 100μL per well and incubated for 24 hours at room temperature.

Following incubation, each well was washed three times with 400 μL of the supplied wash buffer. After removal of the wash buffer the plates were blocked by adding 300 μL of the supplied blocking buffer per well and left to incubate for one hour. Each well was then washed again 3 times with 400 μL of wash buffer.

100 μL of supernatant was added per well and allowed to incubate for 2 hours followed by another wash step. 100 μL of the supplied detection antibody was then added to each well and incubated for another 2 hours. The wells were again washed out as before, before addition of HRP for 20 minutes, followed by another wash step and addition of the supplied substrate solution for 20 minutes. The solution was removed and a stop solution added before the 96 well plate was transferred to a 96 well plate reader. Readings were made at absorbance of 450nm and compared to the standard curve which had been obtained using the supplied standard of human IL-8 (140 ng/ml) dissolved in 0.5 ml of distilled water, read in 2 fold serial dilutions.

2.5.6 Statistical Analysis.

A students t-test was used to compare each TLR agonist to that of control value. All experiments were performed in triplicate and incorporated into the statistical analysis.
2.6 Determination of Oxygen Levels in Media.

2.6.1 Preparation.

The lid of a standard 6 well plate was drilled to permit the sensor of Strathkelvin Instruments Oxygen Meter Model 781 to pass into the media.

2.6.2 Calibration of Oxygen Meter.

The oxygen meter was calibrated. Firstly, the zero value was obtained by placing the electrode in a beaker with 1 cm depth of sodium borate to which a pinch of sodium sulphite had been added and swirled to partially dissolve. The meter was allowed to equilibrate and then set to zero as appropriate.

The higher point of the expected range was calculated by using air saturated water at 37 degrees. The pO₂ expected from this was calculated from 20.93/100 x (b – vp), where b is barometric pressure for that geographical location and time and vp the vapour pressure of water at 37 degrees. The reading on the meter was set to this calculated value.

2.6.3 Measurement of Oxygen Levels in Media.

4ml of media with 15% FCS was added to one well of the 6 well plate, the oxygen sensor placed in it and the apparatus was then placed in a Biotech Galaxy Oxygen Control incubator. The incubator was set to deliver 5% O₂ and 5% CO₂ at 37 degrees and left for 24 hours. Recordings of oxygen meter reading were made at regular intervals and correlated with the percentage of oxygen as recorded by the O₂ monitor fitted in the incubator.
2.6.4 Results of Calibration.

Using a media-based probe it took approximately 6 hours for the oxygen levels measured in the media to equilibrate with those in the hypoxic incubator. The hypoxic incubator reached 5% oxygen in approximately 20 minutes.

Figure 2.1 Results of Hypoxic Chamber Calibration: Note the time taken for oxygen levels in cell culture medium to equilibrate with that of the chamber.
2.7 Proliferation Assay Materials and Methods

2.7.1 Preparation of Vascular Smooth Muscle Cells.

Vascular smooth muscle cells were grown to 60% confluency in a 24 well plate in 50:50 Waymouth: F12 Ham solution with 15% Foetal Calf serum. Once 60% confluency was achieved the cells were quiesced using media with 0.1% foetal calf serum for 24 hours.

2.7.2 Proliferation Assay.

After 24 hours of quiescence, cells were stimulated with the addition of media containing 15% foetal calf serum. Control wells were left unstimulated with media containing 0.1% FCS. For investigation of the effect of hypoxia upon proliferation, intermediate concentrations of FCS were also used (1% and 0.5%).

At this point, U-73122 was added at concentrations of $10^{-7}$, $10^{-6}$ and $10^{-5}$ M. A vehicle containing an equal volume of ethanol was added as a control at 5μl. For proliferation experiments using LPS, this was again added at this point at a concentration of 100ng/ml.

After stimulation with the appropriate FCS concentration, half of the replicates for each experiment were transferred into a Biotech Galaxy Oxygen incubator and maintained at conditions of either 10% Oxygen and 5% CO$_2$ or 5% Oxygen and 5% CO$_2$ at 37 degrees for the remainder of the experiment, only being removed to facilitate the necessary steps to allow completion. The other set of replicates of vascular smooth muscle cells of non-ischaemic origin were kept under normoxia in the LEEC automatic incubator (O$_2$ atmospheric%; CO$_2$ 5%; temperature 37 degrees).
For proliferation experiments using LPS, all replicates were incubated in the LEEC automatic incubator.

2.7.3 Addition of Radiolabelled Thymidine.

A solution of $^3$H labelled thymidine was prepared by adding 28 μL of 3H to 972μl of culture medium. This contained a total activity of 1081 kBq. 10μL of this solution was added to each well of the 24 well plate 18 hours following the addition of 15% FCS. This ensured that the replicating cells were in S phase of the cell cycle and allowed incorporation of the thymidine.

2.7.4 Assay Termination.

At 24 hours following the addition of FCS, the plates were visualised to ensure that the vascular smooth muscle cells were still present and then the assay terminated by placing the 24 well plates on ice. Cell membranes were permeabilised by the addition of 10% Trichloroacetic acid (TCA). Between 4 and 6 washes of (15 minutes duration) were required for this, which was confirmed by visual inspection of the cells. After membrane permeabilisation, 250μL of sodium lauryl sulphate was added to each well to lyse the cells. This was left in the incubator at 37 degrees overnight before processing.

2.7.5 Scintillation Counting.

Beta vials were loaded with 2ml of liquid scintillation counting fluid before the 250μL of sodium lauryl sulphate/ cell lysis product was removed from each well and added. Vials were numbered
and transferred to a scintillation counter (Wallac 1409 DSA Liquid Scintillation Counter) where disintegrations per minute (DPM) were recorded for a period of one minute.

2.7.6 Statistical Analysis.

One Way Analysis of Variance was performed with Dunnett’s post-hoc test to compare significance of values to maximum stimulation.

For comparison of proliferation under hypoxic and normoxic conditions and single concentrations of foetal calf serum, a paired t-test was performed.

SPSS for Windows was used for all statistical processing. P values of < 0.05 were considered significant.
2.8 Drugs, Reagents and Solutions Used.

2.8.1 Drugs and Reagents by Supplier.

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Reagent/Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Amersham Biosciences, Buckinghamshire, UK.</strong></td>
<td>[methyl-$^3$H]Thymidine</td>
</tr>
<tr>
<td><strong>Bioquote Ltd, York, UK.</strong></td>
<td>Cytoskeleton G-LISA Rac Activation kit</td>
</tr>
<tr>
<td><strong>Cambrex Bioscience Wokingham Ltd., Berkshire, UK.</strong></td>
<td>Penicillin/Streptomycin.</td>
</tr>
<tr>
<td><strong>DakoCytomation, Cambridgeshire, UK</strong></td>
<td>Antibody Dilutent Solution.</td>
</tr>
<tr>
<td><strong>Invitrogen Ltd., Paisley, UK.</strong></td>
<td>DAB Substrate and Buffer.</td>
</tr>
<tr>
<td></td>
<td>Polyclonal Goat Anti-rabbit Ig, biotinylated</td>
</tr>
<tr>
<td></td>
<td>F12 Ham Solution</td>
</tr>
<tr>
<td></td>
<td>Waymouths Solution + L-Glutamine</td>
</tr>
</tbody>
</table>
| Supplier | Reagents
<table>
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<th></th>
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</thead>
<tbody>
<tr>
<td>Perkin-Elmer, Beaconsfield, Bucs, UK</td>
<td>Liquid Scintillant</td>
</tr>
<tr>
<td>R&amp;D Systems, Abingdon, UK</td>
<td>IL-8 ELISA Kit</td>
</tr>
<tr>
<td>Santa Cruz Biotechnology, Middlesex, UK</td>
<td>PLC Gamma Antibody</td>
</tr>
<tr>
<td></td>
<td>TLR2 Antibody</td>
</tr>
<tr>
<td></td>
<td>TLR4 Antibody</td>
</tr>
<tr>
<td>Sigma – Aldrich Co Ltd., Poole, UK</td>
<td>Absolute Alcohol</td>
</tr>
<tr>
<td></td>
<td>Acetone</td>
</tr>
<tr>
<td></td>
<td>APES</td>
</tr>
<tr>
<td></td>
<td>Foetal Calf Serum</td>
</tr>
<tr>
<td></td>
<td>Hydrogen Peroxide 30% w/w</td>
</tr>
<tr>
<td></td>
<td>Lipopolysaccharide of e.coli 055:B1</td>
</tr>
<tr>
<td></td>
<td>Platelet Derived Growth Factor-AB</td>
</tr>
<tr>
<td></td>
<td>Simvastatin</td>
</tr>
<tr>
<td></td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td></td>
<td>Trichloroacetic Acid</td>
</tr>
<tr>
<td></td>
<td>TrypleE Express</td>
</tr>
<tr>
<td></td>
<td>U73122</td>
</tr>
<tr>
<td>Vector Laboratories, Peterborough, UK</td>
<td>Horseradish Peroxidase</td>
</tr>
<tr>
<td></td>
<td>Normal Goat Serum</td>
</tr>
</tbody>
</table>

Table 2.2 Drugs and Reagents by Supplier

2.8.2 Formula of Solutions Used.

2.8.2.1 *SDS: Sodium Lauryl Sulphate*

Sodium Dodecyl Sulphate: 1g

Sodium Hydroxide: 6g
in 500ml Distilled Water

2.8.2.2 PBS: Phosphate Buffered Saline.

Sodium Chloride: 16g
Potassium Chloride: 0.2g
DiSodium hydrogen Orthophosphate (NA2HPO4): 2.88
Potassium Dihydrogen Orthophosphate (KH2PO4): 0.48

In 2 litres distilled water and adjusted to pH 7.5

2.8.2.3 Tris/EDTA Buffer.

Ethylenediaminetetraacetic acid (EDTA): 0.37g
Trizma Base: 0.55g

In 1 Litre distilled water

2.8.3 Plasticware & Glassware by Supplier.

<p>| Corning Inc, Hackney Rd, London, UK | 50ml Centrifuge Tubes. |
| ELKAY Ltd, Hampshire, UK          | Beta Scintillation Vials |</p>
<table>
<thead>
<tr>
<th>Supplier</th>
<th>Items</th>
</tr>
</thead>
<tbody>
<tr>
<td>Greiner Bio-one Ltd, Stonehouse, UK</td>
<td>6 well plates</td>
</tr>
<tr>
<td>IWAKI Ltd via Sterilin Ltd Caerphilly, UK</td>
<td>T25 Culture Flasks</td>
</tr>
<tr>
<td></td>
<td>T75 Culture Flasks</td>
</tr>
<tr>
<td></td>
<td>24 Well Plates</td>
</tr>
<tr>
<td>Poulten &amp; Graf Ltd, Barking, Essex, UK</td>
<td>230mm Pasteur Pipettes</td>
</tr>
<tr>
<td>Sarstedt, Leicester, UK</td>
<td>10ml Serological Pipettes</td>
</tr>
<tr>
<td>TPP Switzerland</td>
<td>15ml Centrifuge tubes</td>
</tr>
<tr>
<td></td>
<td>24 well plates</td>
</tr>
<tr>
<td>VWR International, Leicestershire, UK</td>
<td>Super Premium Microscope Slides 1 to 1.2 mm thick, twin frosted.</td>
</tr>
<tr>
<td></td>
<td>Nitrile Latex Free Gloves</td>
</tr>
</tbody>
</table>

Table 2.3: Plasticware & Glassware by Supplier
CHAPTER 3
RESULTS
3.1 Characteristics of Patients who Donated Tissue

3.1.1 Introduction.

The intention of this study was to investigate pathways of intimal hyperplasia formation in infrainguinal bypass surgery. Vein samples were collected from two sources: patients undergoing amputation and patients undergoing coronary artery bypass surgery.

To perform infrainguinal bypass surgery, the ipsilateral vein to the diseased leg is used, with the vein having suffered the ischaemic conditions which have necessitated the bypass graft. Vein from patients undergoing amputation for ischaemia has been conditioned under similar circumstances, therefore accurately represents the vein used for infrainguinal bypass graft.

Saphenous vein utilised for coronary artery bypass surgery was identical to that in patients undergoing amputation other than that it had not been exposed to ischaemic pre-operatively (as was confirmed by screening with APBI prior to collection). The patient characteristics between these groups was also similar, making it an excellent choice of control tissue for immunohistochemistry experiments.

Vascular Smooth Muscle Cells for proliferation and cell signalling experiments were obtained from non-ischaemic veins only.

3.1.2 Patient Characteristics.

A total of 20 patients donated tissue and were included in the study. 11 Patients donated ischaemic vein tissue and 9 patients donated non-ischaemic vein tissue. Patient characteristics
were broadly similar between the two groups. Table 3.1 illustrates the patients involved in the study along with key demographic data.

<table>
<thead>
<tr>
<th>Ischaemic Saphenous Vein</th>
<th>Non-Ischaemic Saphenous Vein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age</td>
</tr>
<tr>
<td>ILSV1</td>
<td>72</td>
</tr>
<tr>
<td>ILSV2</td>
<td>70</td>
</tr>
<tr>
<td>ILSV3</td>
<td>87</td>
</tr>
<tr>
<td>ILSV4</td>
<td>68</td>
</tr>
<tr>
<td>ILSV5</td>
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<tr>
<td>ILSV6</td>
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</tr>
<tr>
<td>ILSV7</td>
<td>63</td>
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<td>IVSL8</td>
<td>71</td>
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<tr>
<td>ILSV9</td>
<td>64</td>
</tr>
<tr>
<td>ILSV10</td>
<td>75</td>
</tr>
<tr>
<td>ILSV11</td>
<td>61</td>
</tr>
</tbody>
</table>

Table 3.1: Summary of patients involved in study with key demographic data.

3.1.3 Comparison of Patient Groups.

There were no statistically significant differences between age and number of medications between each group. Figures 3.1 and 3.2 represent the age distribution of the patients involved in the study and distribution of number of medications.
Using a chi squared test there was no statistically significant differences between sex distribution, current smoking status (actively smoking or not smoking) nor the number of diabetic patients in each group. Table 3.2 demonstrates the distribution of all key demographic data.

Figure 3.1.1 Age distribution of patients: The age of patients in each group, represented as complete range, 25th to 75th percentile and median. (p > 0.5 Mann-Whitney Test).

Figure 3.1.2 Number of medications prescribed in patients of each group: The number of medications prescribed in each patient group represented as range, 25th to 75th percentile and median (p > 0.1 Mann-Whitney Test).
## Table 3.2: Distribution of key demographic data between the ischaemic and non-ischaemic tissue groups. No statistically significant differences between the groups: * M:F ratio, current smoking status and diabetic status p > 0.5 chi squared test. #1 Age range p > 0.5 Mann-Whitney test. #2 Number of Medications, p > 0.1, Mann-Whitney test. #3,#4 Number of patients on antiplatelet agents and statin p > 0.5 chi squared test.

### 3.1.4 Discussion.

Broadly both groups of patients shared similar characteristics, which was expected when the project was conceived. There were slightly more diabetic patients in the ischaemic group, however there was no statistically significant difference demonstrated.
Statistically both groups appear equally matched in terms of key demographics. When acting as a control for ischaemic saphenous vein tissue in immunohistochemistry experiments, non-ischaemic vein is suitable.

Non-ischaemic vein cells only were used for proliferation and cell signalling studies. For these studies, the cells acted as their own control by virtue of the experimental design.
3.2 Toll Like Receptors in Vein Graft Failure.

3.2.1 Introduction.

Toll Like receptors activate inflammatory processes in response to pathogen associated molecular proteins (PAMPs) as part of the innate immune system. Atherosclerosis formation has been linked to TLR activation, as has intimal hyperplasia in an arterial model, where epidemiologically TLR phenotypes are linked to increased rate of reintervention following angioplasty. The influence of TLR on saphenous vein grafts has not been extensively investigated and no reports exist on the influence of ischaemia upon expression of TLR’s in human veins.

The aims of this work are twofold. The first will be to determine the presence of TLR2 and 4 (those with the strongest links to cardiovascular disease) in saphenous vein tissue.

The second aim, having demonstrated the presence of TLR’s will be to determine functional aspects of TLR’s. TLR function will be assessed in both vein tissue and vascular cells in terms of response to known ligands. Finally, ligands with functional effects will be tested to determine if they have actions on vascular cells that could underlie the stimulation of intimal hyperplasia.

3.2.2 Methods

3.2.2.1 Immunohistochemistry.
Immunohistochemistry was performed as detailed in methods section 2.4 on vein segments of both ischaemic and non-ischaemic origin. Antibodies for TLR2 and TLR4 were used to determine the presence of these receptors in human vein tissue and if ischaemia alters the level of expression. Stain intensity was then quantified according to the protocol in section 2.4.4.

3.2.2.2 TLR Ligand stimulation in Vein Tissue and Vascular Cells.

Ligands for TLR2 to 9 were applied to both vein tissue as well as vascular smooth muscle cells and HUVEC’s according to methods section 5 and the IL-8 levels in response to these ligands measured using the IL-8 ELISA kit as per methods 2.5.

3.2.2.3 The effect of Lipopolysaccharide on Vascular Smooth Muscle Cell Proliferation.

Proliferation assay was performed in accordance with methods section 2.7 in response to stimulation with LPS at concentrations of FCS 15%, 1%, 0.5% and unstimulated. Counts were read in DPM using a scintillation counter.

3.2.3 Results.

3.2.4 Quantitative Immunohistochemistry Results.

3.2.4.1 Toll Like Receptor 2 Quantitative Immunohistochemistry.

Immunoreactivity for TLR 2 was similar in both ischaemic and non-ischaemic veins, with staining in the endothelium and smooth muscle cells (see figure 3.2.1 and 3.2.2). Human infant lung tissue was used as a positive control. The infants had died of respiratory infection and had
significant leucocyte infiltration which was positive for TLR2 and 4. For negative control, tissue was processed in the absence of primary antibody with normal rabbit serum. Positive and negative controls are presented in figure 3.2.3

Quantification of the level of staining intensity was performed, and correlated with visual inspection, with no significant difference in quantitative stain level between the two groups. (see figure 3.2.4).

Figure 3.2.1: Immunohistochemistry for TLR2 in Human Saphenous Vein: TLR2 Antibody binding is seen in both endothelial and smooth muscle cells and intensity of staining is similar in both A: ischaemic and B: non-ischaemic veins (TLR 2 at concentration 1:100)
Figure 3.2.2: Immunohistochemistry for TLR2 in Human Saphenous Vein: High Magnification micrograph demonstrating TLR2 antibody binding of both A: endothelium and B: vascular smooth muscle cells in vein tissue.

Figure 3.2.3 Positive and Negative Controls for TLR2 in Human Saphenous Vein: Positive and negative controls for TLR2 immunoreactivity (TLR2 at 1:100). A: Human infant Lung tissue (positive control) B: Human infant lung tissue (negative staining of positive control) C: Human LSV section (negative control)
Figure 3.2.4: Quantitative Immunohistochemistry for TLR2 in Human Saphenous Vein:
Quantitative stain intensity of TLR2 uptake in both endothelial cells and vascular smooth muscle
cells of ischaemic and non-ischaemic veins. No significant difference between two groups using
students t-test (p>0.05).

3.2.4.2 Toll Like Receptor 4 Quantitative Immunohistochemistry.

Immunoreactivity for TLR 4 was again similar in both ischaemic and non-ischaemic veins, with
antibody binding in the endothelium and smooth muscle cells (see figure 3.2.5 and 3.2.6).
Positive and negative controls were used as previously described and are presented in figure
3.2.7.

Quantification of the level of staining intensity was performed, and correlated with visual
inspection, with no significant difference in quantitative stain level between the two groups. (see
figure 3.2.8).
Figure 3.2.5: Immunohistochemistry for TLR4 in Human Saphenous Vein: Antibody binding for TLR 4 is seen in both endothelial and smooth muscle cells. Intensity of staining is similar in both A: ischaemic and B: non-ischaemic veins (TLR 4 at concentration 1:200).

Figure 3.2.6: Immunohistochemistry for TLR4 in Human Saphenous Vein: High Magnification micrograph demonstrating TLR4 antibody binding of both A: endothelium and B: vascular smooth muscle cells in vein tissue.
Figure 3.2.7 Positive and Negative Controls for TLR4 in Human Saphenous Vein: Positive and negative controls for TLR4 immunoreactivity (TLR4 at 1:200). A: Human infant Lung tissue (positive control) B: Human infant lung tissue (negative control) C: Human LSV section (negative control).

Figure 3.2.8: Quantitative Immunohistochemistry for TLR4 in Human Saphenous Vein: Quantitative stain Intensity of TLR4 uptake in both endothelial cells and vascular smooth muscle cells of ischaemic and non-ischaemic veins. No significant difference between two groups using students t-test (p>0.05).
3.2.5 Toll Like Receptor Ligand Results.

3.2.5.1 TLR Ligand Application in Human Vascular Smooth Muscle Cells and Production of IL-8.

TLR Ligands from 2 to 9 were applied to cultured vascular smooth muscle cells, with the intention of demonstrating a picture of the functional response of vascular cells to the TLR ligands. Control levels of IL-8 secretion were low, and relative to this the IL-8 secretion in response to the ligands of TLR2, 5, 7, 8 and 9 was also low, with no significant increases in secretion. Incubation with both PolyI:C (TLR3) and LPS (TLR4) however led to significantly increased levels of IL-8 secretion by vascular smooth muscle cells. This is demonstrated in figure 3.2.9

Figure 3.2.9: IL-8 response of Human Vascular Smooth Muscle Cells to TLR Ligands. Note the significant increase of IL-8 production in response to Ligands for TLR 3 and 4. (n=2 assays in triplicate. * p < 0.01 vs. cells cultured in medium alone (control) using students t-test. The same control level was used for comparison of all TLR Ligands)
3.2.5.2 TLR Ligand Application in Human Umbilical Vein Endothelial Cells and Production of IL-8.

TLR Ligands from 2 to 9 were then applied to HUVEC’s. It was felt important that a ‘profile’ of response to TLR ligands by all vascular cells was obtained. Vascular endothelial cell response to TLR stimulation had not previously been documented, and subsequent experiments were planned using whole vein tissue. It was necessary to determine in advance alternative potential origins of IL-8 secretion in tissue experiments.

Control levels of IL-8 secretion were low, and relative to this the IL-8 secretion in response to the ligands of TLR2, 5, 7, 8 and 9 was also low, with no significant increases in secretion. Incubation with both PolyI:C (TLR3) and LPS (TLR4) however led to significantly increased levels of IL-8 secretion by HUVEC’s. This is demonstrated in figure 3.2.10. Compared to VSMC’s the background IL-8 levels were similar, however the response of VSMC to PolyI:C and LPS appeared more exaggerated, with maximal IL-8 levels of 1700 pg/ml recorded compared with 400 pg/ml.
Figure 3.2.10: IL-8 response of Human Umbilical Vein Endothelial Cells to TLR Ligands. Note the significant increase of IL-8 production in response to Ligands for TLR 4. (n=2 assays in triplicate. * p < 0.01 vs. cells cultured in medium alone (control) using students t-test. The same control level was used for comparison of all TLR Ligands)

3.2.5.3 TLR Ligand Application in Human Saphenous Vein Rings and Production of IL-8.

Unlike in vascular cells, there was no consistent response in IL-8 production from TLR ligand application from vein rings. The panels below represent the results obtained from incubation of 1mm³ vein tissue with the TLR ligand named according to section 2.5. Each panel represents vein from a different patient and for each vein the assay was performed in triplicate. The responses of each vein varied significantly as can be seen in figure 3.2.11.

Background levels of IL-8 expression in control experiments (with no TLR Ligand) varied widely in the veins tested, with some veins expressing almost no background IL-8 at all and others expressing as much as 40,000 pg/ml. The response to the TLR ligands was also inconsistent between veins, with further wide variation in IL-8 output either when considered relative to the control IL-8 or as an individual value. IL-8 production was not upregulated in ischaemic veins compared to those of non-ischaemic origin.

TLR2 showed a particular unpredictable response when considered relative to control IL-8 output. In the ischaemic group, with some veins increased IL-8 output in response to Pam3CSK4 and others had no upregulation or lower values relative to control. Whilst both non-ischaemic veins had increased IL-8 output, the magnitude of increase varied significantly.
Incubation with PolyI:C (TLR3) did not upregulate IL-8 output relative to control in all but one of the veins tested, which was non-ischaemic.

For TLR4, incubation with LPS increased IL-8 output relative to control in 2 of the ischaemic veins and 1 of the non-ischaemic veins. In the other veins it remained around the level of the background. Again no obvious response pattern was evident either in general or between veins of ischaemic and non-ischaemic origin.

Incubation with *Bacillus subtilis* flagellin led to IL-8 levels either equal to or less that of control levels, except in one vein, where it appeared significantly increased.

Incubation with CpG DNA similarly led to no consistent response from the vein tissue. 2 veins (one ischaemic and one non-ischaemic) showed large increases in IL-8, one ischaemic vein showed a moderate increase in IL-8 secretion and the remaining 3 veins had reductions in IL-8 secretion relative to controls.

From these studies no clear response of vein tissue to Ligands of TLR2, 3, 4, 5 and 9 could be seen, independent of the origin of tissue. 4 veins of ischaemic origin were tested and 2 veins of non-ischaemic origin. Figure 3.9 below demonstrates the results from each vein in individual panels, grouped by whether they were of ischaemic or non-ischaemic origin.

To ensure that errors secondary to differing tissue mass did not occur, the results were then adjusted to the mass of tissue in each well and normalised to the control level. Even with this adjustment there was no clear pattern, with significant variation in standard error as can be seen in figure 3.2.12. This data was considerably more variable than the effect seen from individual
cell types, possibly due to a variable content of leucocytes within tissue samples (see discussion), and therefore it was not thought valuable to continue this study into a full group.
Figure 3.2.11: TLR ligand stimulation in Human Vein Rings. Each figure represents the result from the vein ring of an individual patient, with ELISA’s performed in triplicate. Column A: Veins of Ischaemic Origin, Column B: Veins of Non-Ischaemic Origin.
Figure 3.2.12: Results for A: ischaemic and B: non-ischaemic veins expressed as percentage difference relative to control. Note the wide error bars, indicating significant variability of response.
3.2.6 TLR4 Ligand Stimulation and VSMC Proliferation.

3.2.6.1 General Observations of the Effect of Foetal Calf Serum on Proliferation.

FCS induced proliferation of VSMC, with highest stimulation occurring using 15% FCS. 15% FCS has been demonstrated in previous studies using vascular smooth muscle cells to promote maximal levels of proliferation (ref Paul). Levels of proliferation at maximal stimulation in all experiments were greater than 10 times that of unstimulated (background) levels of proliferation. This indicates that the experiment was successful, and in keeping with degrees of proliferation demonstrated previously in the laboratory. LPS at 100ng/ml had no significant effect on proliferation at these concentrations of FCS.

![Graph showing proliferation of VSMC in 15% FCS and unstimulated state and the effect of LPS. Data has been normalised to percentage deviation relative to proliferation at 15% FCS. LPS at 100ng/ml had no significant effect on proliferation at maximal or minimal stimulation (n=6).](image-url)
3.2.6.2 The Effect of LPS on VSMC Proliferation at Intermediate Concentrations of FCS.

As demonstrated in figure 3.2.14, LPS appears to produce a modest increase in the proliferation of VSMC when utilised at a concentration of 100ng/ml. This increase was only statistically significant at 1% FCS.

Figure 3.2.14: Proliferation at 1% and 0.5% FCS of VSMC treated with 100ng/ml LPS compared to untreated VSMC. Data is normalised relative to proliferation at 15% FCS and expressed as deviation from this value. LPS produces modest but statistically significant increases in proliferation at 1% FCS. ( * p < 0.05 using paired students t test, n=6).

3.2.7 Discussion of Results.

Toll Like Receptors have been demonstrated to play a role in both atherosclerosis formation as well as intimal hyperplasia formation in an arterial model. TLR4 deficient mice have reduced atherosclerotic plaque size$^{185}$ and TLR2 activation has been demonstrated to induce arterial
intimal hyperplasia following balloon injury. TLR4 expression has also been shown to increase in balloon models of arterial injury. Significantly less data exists on both the presence and function of the human TLR’s in human vein tissue and the potential impact on intimal hyperplasia formation in vein when it is used as a grafting conduit.

These results initially demonstrate the presence of both TLR2 and 4 in human vein tissue in both endothelial and smooth muscle cells. Previous work using PCR has demonstrated TLR4 to be present in human vein tissue, with TLR2 expressed to a lesser degree. Previous immunohistochemistry studies agree with the findings from this research that TLR4 is present in human saphenous vein and is expressed by both endothelial cells and smooth muscle cells. The presence of TLR2 as demonstrated by these results is more contentious. Despite small amounts being detected using PCR in human vein tissue, functional experiments using human endothelial cells demonstrate responses only from those of arterial origin and not venous, refuting the functionality but not presence of TLR2 at low levels in vein tissue. Certainly a higher concentration of antibody was required to achieve staining (no TLR2 antibody binding was achieved at concentrations < 1:100), and, while the experiment was not designed to quantitatively compare TLR2 with TLR4 antibody binding, mean intensity per pixel for staining was less even at this higher antibody concentration for TLR2 when compared with TLR4, which would be in-keeping with these previous observations. Overall this data suggests that TLR2 has a less significant functional role in vein tissue and potentially contribute less to pathways inducing intimal hyperplasia formation.

There was no difference in expression of either TLR2 or TLR4 in veins of ischaemic origin compared to non-ischaemic origin. This was the first time that a comparison of expression between two such groups has been made. Ischaemia can induce inflammatory processes in peripheral tissue as early as 1 day in animal models of limb ischaemia that persists at 4 weeks. TLR expression could potentially be upregulated as part of this process as the vein tissue itself suffers from the ischaemic conditions of the limb via disruption in vasa vasorum nutrient supply. When used for infrainguinal bypass grafting, the ipsilateral vein is used which has been exposed
to the ischaemic conditions which necessitated the bypass – therefore ischaemia prior to use as a conduit is clinically relevant. If expression of TLR’s was increased by these processes, the vein may be more vulnerable to subsequent activation by PAMP’s and potentially more vulnerable to intimal hyperplasia formation. Using quantitative immunohistochemistry, no such difference in expression was determined between these two types of vein, indicating that ischaemia does not affect the expression of TLR2 and TLR4.

Next the functionality of various TLR’s was assessed in whole vein tissue of both ischaemic and non-ischaemic origin. Previous work using LPS on vein segments had demonstrated upregulation of IL-8 release from vein segments in response to LPS at doses 0.1, 1 and 10 ng/ml which reached statistical significance, though the authors had similar problems with wide variation in background IL-8 levels. This is the first work which attempts to quantify the response of vein tissue to an array of TLR ligands in addition to this and compare functional differences between ischaemic and non-ischaemic veins. Disappointingly no clear response to any of the TLR Ligands in human vein tissue could be demonstrated, and when data was compared relative to control and adjusted by weight of tissue, there were large fluctuations in the response of not only TLR4 but all TLR ligands.

There a number of potential reasons for this. Firstly there may have been variability in the conditions to which the vein was exposed prior to use in the experiment. The ischaemic veins were all taken from amputated lower limbs. Whilst none of the amputations performed were for lower limb infection, subclinical infection could still have been present in the limb tissue, with potential pre-excitation by LPS and other TLR ligands prior to experimentation. This could have significantly affected the response when the TLR challenge was performed. Similarly, whilst veins are harvested for coronary artery bypass graft under sterile conditions, there remains the possibility again of exposure to subclinical infective agents or PAMP’s during the harvesting process or transfer and processing of the specimen.
In addition ‘contamination’ with other cell groups could also potentially explain the variability of results in this section. Infiltration, particularly of the ischaemic vein segments, with leucocytes as part of an inflammatory response would alter the cellular profile of the vein, with monocyte infiltration in particular likely to lead to exaggerated IL-8 secretion in response to PAMP’s. Variability in leucocyte content is the most likely explanation for the variability of vein segment response compared to that when using vascular cells alone.

The response of vascular cells in the responses to TLR Ligands gave a significantly clearer picture of the responses of vein to TLR Ligands. Previously IL-8 secretion had been demonstrated in response to TLR4 ligands in arterial endothelial cells. Human arterial and vein VSMC’s have also been shown to be respond to LPS by producing Prostaglandin E2 when stimulated at high concentrations of LPS (10 μg/ml), however this is a concentration in excess of what would be found even in systemic sepsis and not clinically relevant to the processes potentially at work in contributing to intimal hyperplasia, where much lower LPS concentrations have been associated with atherosclerotic processes. No previous studies have considered the response of vein smooth muscle cells to LPS at lower concentrations (100ng/ml).

The response to TLR2 ligands of venous endothelial cells has previously been tested, however a ‘profile’ of the response of vein VSMC and endothelial cells to ligands for all known TLR receptors does not exist, therefore it was felt important to include ligands for other TLR’s also in the experiment. This would exclude the possibility of non-specific effects on other TLRs (LPS can also activate TLR2) by the ligands. This data presents a profile of the responses of human vein cells to the TLR Ligands 2, 3, 4, 5, 7, 8 and 9 and demonstrates that those with functional action are TLR3 and TLR4.

In both HUVEC’s and human vein VSMC’s, IL-8 production was seen in response to TLR3 and TLR4 ligands only. IL-8 levels in response to these ligands were significantly higher in VSMC
than HUVEC’s, suggesting that activation of TLR3 and 4 in VSMC may have a more significant role to play in intimal hyperplasia formation.

Demonstrating that TLR3 is functionally active in VSMC and Endothelial Cells of venous origin is an important step in investigating intimal hyperplasia of vein graft failure. TLR3 functions in the recognition of viral nucleic acids\textsuperscript{246} and while it has been demonstrated to be present in VSMC and endothelial cells of arterial origin, its functionality in cells of venous origin had not been tested. In arterial VSMC TLR3 activation by PolyI:C has been demonstrated to induce a proinflammatory and proliferative phenotype.\textsuperscript{191} Seropositivity for both cytomegalovirus or herpes simplex virus has also been epidemiologically linked to atherosclerosis formation.\textsuperscript{247} This potentially implicates viral infection, or encounter of vascular cells with viruses as a potential contributory factor in intimal hyperplasia formation: TLR3 is functionally active in vascular cells and activation can induce VSMC to exhibit behaviour seen in intimal hyperplasia formation. Viral infection is a known stimulant in neoplastic processes such as cervical carcinoma. Indeed a vaccine against Human Papilloma Virus has been demonstrated to reduce the risk of cervical cancer.\textsuperscript{248}

The function of TLR4 in both vein VSMC and HUVEC’s in response to LPS stimulation is important as while this has been demonstrated in cells of arterial origin, this confirms the functionality in human venous origin cells at LPS concentrations of clinical relevance. TLR4 expression is increased by LPS and is associated with elevated balloon injury intimal hyperplasia in arterial models.\textsuperscript{192} The finding that TLR4 is both present and functionally active in venous cells implies that activation of similar pathways could be relevant in intimal hyperplasia in vein grafts.

Incubation of VSMC with LPS at 100ng/ml, in addition to enhancing production of IL-8 also produced modest, but significant increases in VSMC proliferation. These increases in proliferation were only statistically significant when the cells were also stimulated with 1% FCS,
indicating that LPS has a small, additive effect to pathological mechanisms associated with intimal hyperplasia. It is unclear if this effect was due to LPS directly enhancing proliferation of VSMC, or via LPS inducing secretion of mediators from VSMC which subsequently stimulated proliferation. IL-8 has also been demonstrated to cause proliferation of VSMC in a concentration dependent manner, ranging from $1 \times 10^{-10}$ to $1 \times 10^{-12}$. These concentrations encompass a range similar to those measured in response to TLR Ligands in both vascular cells and whole vein tissue, therefore LPS may be an indirect stimulant of proliferation.

The presence and functionality of TLR4 in human vein VSMC and endothelial cells as well as the functionality of TLR3 in human vein VSMC and endothelial cells has been demonstrated by these experiments, along with evidence that TLR4 stimulation effects VSMC proliferation, a key cellular element in intimal hyperplasia. Currently, it is unclear how these pathways would become activated in a model of vein graft failure. Direct stimulation may be a potential mechanism. When saphenous vein is utilised as a conduit it is dissected free of the surrounding tissue, handled by the surgeon and anastomosed to the appropriate vessels. This is done under sterile conditions, and clinical infection rates of saphenous vein grafts are very low. It is still possible that during this period the vein is still exposed to low levels of toxins and bacteria, which while not enough to cause clinically relevant infection can still challenge and activate these innate immunological pathways, initiating the downstream effects which contribute to intimal hyperplasia formation. No studies exist to determine if bacteria can be cultured directly from failed saphenous vein grafts, however in studies of atherosclerotic plaque, viable bacteria have only rarely been cultured, indicating that ongoing infection is an unlikely cause. Transient challenging of the innate immune system however (of which vascular cells, by virtue of their TLR expression are part of) may stimulate TLR3 and TLR4, and having activated downstream effects such as alteration of VSMC into a proliferative state initiate processes contributing to intimal hyperplasia formation.

It is also possible that circulating endotoxin could also contribute to processes initiated by TLR4. Even healthy individuals can be demonstrated to have low levels of circulating endotoxin, with
higher levels being associated with enhanced risk of atherosclerosis. It has also been demonstrated that femoral-popliteal bypass grafting, by virtue of ischaemia-reperfusion injury, is associated with increased intestinal permeability, peaking at day 3 post-operatively. Other forms of surgery, as well as trauma have also been associated with this phenomenon. This potentially allows translocation of enteral bacteria and endotoxin. Whilst this may not have any immediate clinical sequale in terms of sepsis, low concentrations may be enough to stimulate TLR’s in the newly grafted vein and initiate intimal hyperplasia. Circulating endotoxin following femoral popliteal bypass grafting was also measured by Edrees et al. Whilst no significant rises were detected, the objective of the paper was investigating sepsis following surgery and infrequent sampling meant that the authors potentially missed smaller, subtle rises in LPS that could contribute to intimal hyperplasia formation.

Another attractive explanation is that PAMP’s accumulate within the vein graft via preferential recruitment of activated monocytes which have already phagocytosed foreign bacteria. DNA sequencing of carotid atherosclerotic plaque revealed that up to 17 different bacterial types could be detected. Accumulation of PAMP’s via monocyte recruitment would explain why the proximal third of the vein graft is preferentially affected by intimal hyperplasia formation, with haemodynamic changes in flow in this area increasing MCP-1 secretion and recruitment of monocytes. Once these monocytes are recruited to this area they become part of an early plaque, potentially presenting the phagocytosed PAMP’s to the vascular cells and enhancing the inflammatory response contributing to intimal hyperplasia formation. Analysis of failed vein grafts for the presence of bacteria has not been performed as per carotid plaques, but potentially similar findings could be made, with multiple different bacterial types being present. If such a situation exists, it could make the modest increases in proliferation demonstrated here significantly more clinically relevant. Chronic stimulation of TLR4 by PAMP’s present within the vein (recruited preferentially to the proximal third) over a period of weeks to months could contribute significantly to the development of clinically relevant levels of intimal hyperplasia formation.
3.3 The Influence of Oxygen Level on Vascular Smooth Muscle Cell Proliferation.

3.3.1 Introduction.

Hypoxia, by occlusion and formation of thrombus within the vasa vasorum causes accelerated intimal hyperplasia in porcine and murine arterial models. In only one study was an attempt to measure oxygen levels following vasa vasorum occlusion made, with tunica media oxygen levels decreasing by approximately 20%, though technical difficulties limited the amount of readings obtained in this study. Histologically the lesions that formed in response to vasa vasorum occlusion consisted of large numbers of VSMC’s. 213,253,254

Hypoxia alone has also been shown to increase the proliferation of vascular smooth muscle cells of human pulmonary artery at 5% oxygen.198. Human saphenous vein cells from one patient were analysed as part of this study and 5% oxygen also appeared to enhance proliferation in these cells. VSMC Proliferation is a key cellular event in intimal hyperplasia formation, and when vein graft is used for infrainguinal bypass surgery it is exposed to hypoxic conditions as the ipsilateral vein of the diseased leg is used. Transcutaneous oxygen levels in patients with Fontaine III/IV disease have been quantified at 16.1+15 mmHg, compared to normal subjects in which it is 54.5 + 7 mmHg.255 Dissection of the vein that disrupts vasa vasorum supply can further increase hypoxia in the vascular cells.

The influence of the vasa vasorum and normal nutrient supply is important to consider as a potential cause of hypoxia of long saphenous vein both during Fontaine III/IV disease of the limb, where arterial flow is compromised and following dissection for use as a conduit
It is generally accepted that human lower limb veins have the most anatomically significant vasa vasorum supply when compared to upper limb veins and arteries. In human saphenous vein, the vasa vasorum is derived from arterial feeding vessels, supplying the vein at intervals of approximately 15mm. These feeder vessels are the basis of a nutrient supply that supplies both the tunica adventitiae and the outer two thirds of the tunica media. The intima and inner third are supplied by diffusion of blood from the long saphenous vein lumen. Debate exists as to the presence of vasa vasorum venules opening directly into the lumen. Some studies claim to have identified the existence of such venules whereas others have failed to identify them in long saphenous vein segments. This is important as when the saphenous vein is used as a conduit, ‘reverse flow’ through such putative venules could continue to supply blood of arterial oxygen concentrations through the vasa vasorum even if the normal arterial supply has been abolished. Whilst this matter has yet to be concluded (some authors claim reverse flow occurs readily), in practical terms it has been demonstrated that pressurising the lumen of saphenous vein at 300mmHg does not flush erythrocytes from the vasa vasorum circulation, and when combined with the obvious sparsity of such venules if they are in existence, indicates that saphenous vein blood supply can potentially be considered as via distinct routes: arterial origin vasa vasorum and diffusion from saphenous vein lumen.

Histologically the tunica media has been shown to consist of an inner part, with 3 loose layers of smooth muscle cells, and an outer layer having inner and outer circularly orientated layers surrounding a middle, longitudinally arranged layer. The outer layers have significantly more, densely packed VSMC, arranged in 5 to 6 layers and the vasa vasorum arterial supply does not extend beyond this area. Thus within the media of the saphenous vein there are potentially two distinct populations of VSMC. A larger (in terms of number) group that is maintained under oxygen levels similar to arterial oxygen concentrations and a smaller group that is maintained at oxygen concentrations found in saphenous vein.
Thus in Fontaine III/IV disease, by decreasing PO$_2$ in the limb to a significant degree by compromised arterial inflow, the vasa vasorum arterial supply is reduced. Subsequently the outer two thirds (and largest population) of VSMC’s are exposed to conditions that are significantly hypoxic compared to their normal state.

Disruption of the vasa vasorum during preparation is also likely to leave the larger population of VSMC’s previously conditioned at arterial levels of oxygen in a state of relative hypoxia. Following grafting, ‘reverse flow’ of arterial blood via vasa vasorum venules is unlikely to significantly contribute to maintaining blood flow in the vasa vasorum at arterial concentrations, leaving this population of VSMC’s in a relatively hypoxic state. When combined with the potential pre-existing hypoxia suffered by the vein as part of the ischaemic process requiring bypass grafting, hypoxia of VSMC is likely to be ongoing in saphenous vein grafts and could be a potent stimulus of intimal hyperplasia formation in infrainguinal bypass grafts by promoting proliferation of VSMC’s. Reversal of these processes is unlikely to occur until a new vasa vasorum is established. This occurs between 3 days and 1 month in animal models.$^{262}$ but has been demonstrated to be erratic in its formation (intruding into the inner third of media, though not into any lesions forming in the intima).$^{259}$

Figure 3.3.1 illustrates the changes in oxygenation that occur in saphenous vein compartments A: endothelium and inner third of media (supply: diffusion from lumen) and B: Outer two thirds of media and adventitae (supply: vasa vasorum) when used as a conduit in infrainguinal bypass surgery.
Figure 3.3.1. The changes in oxygen level of blood supplying A: tunica intima and inner third of media (via diffusion from lumen) and B: outer two thirds of media and adventitae (via vasa vasorum) when used as a conduit. Note in healthy vein A is supplied by blood with relatively low oxygen levels whereas B is supplied by blood of arterial origin. In ischaemic limb saphenous vein, although the vasa vasorum is intact, limitation of the flow of arterial blood in the large vessels of the limb reduces the oxygen level of supplying blood to B. In Saphenous Vein following graft insertion, the situation is reversed compared to healthy vein if the vasa vasorum is disrupted. Arterial blood is now flowing in the lumen, therefore the supply to A: the endothelium and inner third of media now has a high oxygen concentration whereas lack of flow in the vasa vasorum combined means it continues to suffer from relative hypoxia.

From a clinical perspective however, the role of the vasa vasorum in contributing to infrainguinal bypass graft failure has been doubted. Infrainguinal bypass grafting can be performed using either a ‘reversed’ technique, where the saphenous vein is dissected from its anatomical position (disrupting the vasa vasorum), an in-situ technique, whereby the vein is left in its anatomical
position and anastomosed to the appropriate arterial points (largely preserving vasa vasorum) or non-reversed, a technique similar to in-situ but with skeletalization of the vein (disrupting vasa vasorum). For in-situ and non-reversed grafts a valvulotome is required to break down the valves of the saphenous vein to allow arterial flow. Valvulotomes have not been demonstrated to injure the vasa vasorum, however they cause significant trauma to the endothelium.263

Follow up of all these types of grafts has demonstrated no differences in long term patency in above knee grafts,264,265 and superiority of the in situ graft demonstrated in femoral-distal bypass grafts.266 Non-reversed saphenous vein grafts have similar long term patency to in situ grafts,260 suggesting that in practice arterial flow, or lack of, in the vasa vasorum does not effect intimal hyperplasia formation to a significant degree.

In cardiac surgery, where the saphenous vein has not been subjected to ischaemic conditions prior to use as a conduit a ‘no-touch’ technique that preserves vasa vasorum has been demonstrated to have superior long term patency to skeletalized saphenous vein, approaching that seen with the internal mammary (gold standard) for coronary artery bypass surgery.267 The technique used in this paper has been associated with minimum damage to vasa vasorum compared to others utilised in ‘no touch’ processes.268

Taken together this suggests that hypoxic processes play a role in intimal hyperplasia formation. In infrainguinal surgery, the ischaemia present prior to grafting could have already initiated pro intimal hyperplasia pathways, making subsequent oxygenation levels irrelevant, whereas in cardiac surgery, preventing the VSMC of outer two thirds of media being exposed to hypoxic conditions prevents initiation of such pathways, improving long term patency. From an infrainguinal bypass perspective then, the potential role of hypoxia and ischaemia remains important, as despite technical and procedural improvements, hypoxia prior to use as a conduit cannot be avoided. The processes and pathways initiated by hypoxia thus become important therapeutic targets in infrainguinal surgery.
3.3.2 Methods.

Vascular Smooth Muscle Cells were acquired from non-ischaemic veins by the explant method and used between passages 1 and 3. Thymidine incorporation was used to determine the proliferation of VSMC according to the protocol in methods section 2.7.

The primary objective of this experiment was to compare proliferation of VSMC under hypoxic and normoxic conditions. Each concentration of FCS used represented a paired observation from individual cell lines (of the same passage and experimental timing) to its response under both hypoxic and normoxic conditions. In total an n of 6 was obtained with all assays performed in triplicate. A paired t-test was used to compare statistical significance between proliferation under hypoxic and normoxic conditions at the paired concentrations of FCS.

3.3.3 Proliferation of Vascular Smooth Muscle Cells at 10% Oxygen Compared to Normoxia.

3.3.3.1 General Observations of the Effect of Foetal Calf Serum on Proliferation.

FCS induced proliferation of VSMC, with highest stimulation occurring using 15% FCS. 15% FCS has been demonstrated from previous laboratory data using vascular smooth muscle cells to promote maximal levels of proliferation. Levels of proliferation at maximal stimulation in all experiments were greater than 10 times that of unstimulated (background) levels of proliferation. This indicates that the experiment was successful, and in keeping with expected degrees of proliferation.

3.3.3.2 The Effect of 10% Oxygen on Vascular Smooth Muscle Cell Proliferation.
As demonstrated in figure 3.3.2, when exposed to 18 hours at 10% oxygen, vascular smooth muscle cell proliferation significantly increased at FCS concentrations of 1% and 0.5% when compared with controls incubated for 18 hours under atmospheric oxygen. A paired student’s t-test confirmed this observation between groups with n=6 in each group (10% oxygen and atmospheric oxygen).

Figure 3.3.2 Proliferation of Human Vascular Smooth Muscle Cells at 15% FCS, 1% FCS 0.5% FCS and unstimulated at both 10% Oxygen and atmospheric oxygen. Hypoxia significantly increased VSMC proliferation at FCS concentrations of 1% and 0.5%. (* p < 0.05 paired t-test, n=6).

3.3.4 Proliferation of Vascular Smooth Muscle Cells at 5% Oxygen compared to Normoxia.

3.3.4.1 General Observations of the Effect of Foetal Calf Serum on Proliferation.
FCS induced proliferation of VSMC, with maximal stimulation occurring using 15% FCS. Levels of proliferation at maximal stimulation in all experiments were greater than 10 times that of unstimulated (background) levels of proliferation. This indicates that the experiment was successful.

3.3.4.2 The effect of 5% Oxygen on Vascular Smooth Muscle Cell Proliferation.

As can be seen in figure 3.3.3, when VSMC’s were exposed to 5% Oxygen for 18 hours, proliferation significantly increased, even at maximal stimulation by 15% FCS when compared to controls incubated at atmospheric oxygen levels. The apparent difference in proliferation at 1% FCS did not reach statistical significance, however differences at 15% FCS and 0.5% reached p < 0.05 using a paired t test.
Figure 3.3.3: Proliferation of Human Vascular Smooth Muscle Cells at 15% FCS, 1% FCS, 0.5% FCS and unstimulated at both 5% Oxygen and atmospheric oxygen. Hypoxia significantly increased VSMC proliferation at FCS concentrations of 15% and 0.5%. (* p < 0.05 paired t-test, # p < 0.01 paired t-test, n=6). (~ns p = 0.053)

3.3.5 Discussion.

Hypoxia has been demonstrated to induce proliferation of human VSMC’s. Most models of hypoxia-induced proliferation are using human VSMC’s obtained from pulmonary artery with the intention of modelling pulmonary hypertension. Experimental models to investigate hypoxia in intimal hyperplasia have been limited to arterial models only.

In pulmonary hypertension, chronic hypoxia is thought to be a trigger of the disease process, causing proliferation and hyperplasia of the VSMC which subsequently effects the arterial tone, increasing pulmonary vascular resistance and causing progression of the disease. The lesion of pulmonary hypertension is distinct from vein graft intimal hyperplasia in that it is characterised by fibromuscular intimal hypertrophy most commonly of precapillary arteries, however in common is the key cellular element of VSMC proliferation. When vein is used as a conduit for grafting not only has the vasa vasorum been stripped and nutrient supply lost (which has been demonstrated to cause intimal hyperplasia in arterial models), but the vein has been exposed to chronic hypoxic conditions as part of the disease process necessitating infrainguinal bypass grafting. Thus the vascular cells have been exposed to relative hypoxia, and potentially similar processes in VSMC of the vein have been induced to those that cause proliferation of VSMC of the pulmonary artery in hypoxia. It is important to clarify at this point that although saphenous VSMC are part of the venous system, the outer two thirds of the media, containing the largest volume of VSMC is normally supplied via the vasa vasorum by arterial blood. This arterial supply is compromised when the vasa vasorum is disrupted by dissection or if the limb tissue is generally ischaemic as would be the case prior to infrainguinal bypass grafting.
This data demonstrates increases in proliferation of human saphenous vein VSMC when exposed to 10% oxygen and 5% oxygen, conditions which would be hypoxic for the majority of VSMC of the saphenous vein compared to normal conditions. This has potentially significant implications for development of intimal hyperplasia within the vein graft. Pilot data from this laboratory has demonstrated enhanced proliferative and migratory responses of VSMC explanted from ischaemic veins compared to those explanted from non-ischaemic veins, the implication being that veins of ischaemic origin will be more susceptible to development of intimal hyperplasia.\textsuperscript{196} The increases in proliferation in this study appeared larger than those that were apparent in this experimental group. There are several potential reasons for this: 

First the VSMC in this ischaemic group were obtained from saphenous veins of amputated limbs. These limbs were exposed to severe levels of hypoxia and ischaemia prior to amputation of the limb. Data from pulmonary artery studies indicates that at 1% oxygen, proliferation of VSMC is not enhanced.\textsuperscript{198} The larger increases in proliferation at 5% and 10% oxygen could therefore be due to the VSMC in the ischaemic group being conditioned at levels closer to 1% rather than 5% or 10% oxygen studied here. Another explanation could be that the VSMC in this ischaemic group were explanted over 14 days at atmospheric oxygen conditions, with the effect of hypoxia/ischaemia having diminished by this point.

It is interesting to note that at 10% oxygen, proliferation was enhanced only at low concentration of FCS, however at 5% oxygen proliferation increased even at concentrations of FCS normally deemed to cause ‘maximal’ stimulation of VSMC’s. Potentially 10% oxygen enhances proliferative pathways already induced by FCS whereas at 5% oxygen alternative pathways are activated, further supplementing VSMC proliferation.

The baseline levels of proliferation at normoxia differed by a factor of 10 in the experiments conducted at 5% oxygen and 10% oxygen. Whilst all vascular smooth muscle cells were used at an early passage, cells from different patients were used for each experiment. It is possible that such individual variation contributed to this disparity. The experimental design meant that each patients cells acted as their own individual control, with levels of proliferation at 10% and 5%
oxygen being compared to the level of normoxia. Whilst this variation in proliferation was significant, it did not affect the outcome or analysis of the experiment.

The differences in proliferation demonstrated by these experiments is in keeping with the degrees of increase that have previously been published for human VSMC. Pulmonary Artery VSMC have been demonstrated to respond similarly to 5% oxygen, and as part of this study similar increases in saphenous vein VSMC proliferation was demonstrated although over a longer time period and using VSMC from only one patient.\textsuperscript{198,210} One study has reported conflicting results on the effect of hypoxia on diabetic infrapopliteal artery VSMC’s by demonstrating that 1% and 5% oxygen reduced proliferation of VSMC in response to insulin, however the effect of hypoxia on proliferation in the absence of insulin was not reported.\textsuperscript{270} Diabetes has been demonstrated to cause local hypoxia within arterial walls and hyperglycaemia can inhibit Hypoxia Inducible Factor 1 (HIF-1) expression within vascular smooth muscle cells.\textsuperscript{271} The conflicting results of the effect of hypoxia on proliferation to those achieved here could be explained by chronic hyperglycaemia in their patient group downregulating factors involved in the proliferative response to hypoxia. Another explanation is that diabetes has led to local hypoxia within the vessel, conditioning the cells to this level of oxygen. The ‘hypoxic’ group has actually been maintained at an oxygen concentration similar to their usual environment while the ‘normoxic’ group have been exposed to a hypoxia-reoxygenation cycle that has further promoted proliferation. This methodology has been shown to induce oxidative stress and proliferation in VSMC’s.\textsuperscript{272} While tissue samples in this study were taken from both diabetic and non-diabetic patients, there was significant difficulty in cultivating cells from diabetic patients veins therefore the proliferation assays here included only non-diabetic patients. It would be interesting to examine saphenous VSMC explants from diabetic and non-diabetic patients to compare both baseline proliferation as well as the response to hypoxia.

These results demonstrate the response of saphenous vein VSMC at both 10% and 5% oxygen with statistically significant increases in proliferation demonstrated in both groups using a large number of patients ($n=6$). This confirms that the hypoxic processes at work when saphenous vein
is used as a conduit for infrainguinal bypass grafting (pre-graft ischaemia and vasa vasorum blood supply loss) are potentially deleterious to the long term survival of the conduit, with key cellular processes of intimal hyperplasia being potentially enhanced.

The mechanism of action by which hypoxia stimulates proliferation of VSMC has still to be clarified. Hypoxia could stimulate the release of mitogenic factors from the VSMC (or from endothelial cells or adventitial cells when considered in whole tissue), enhance the response of the VSMC to such mitogens or directly stimulate pathways of proliferation in the VSMC. Identification of the signalling pathways responsible for enhancements in VSMC proliferation under hypoxic conditions is a target for future research.
3.4 The role of Phospholipase Cγ in intimal hyperplasia formation.

3.4.1 Introduction.

Phospholipase Cγ (PLCγ) has been linked to cell processes associated with intimal hyperplasia formation such as change of vascular smooth muscle cells from quiescent to contractile phenotype, a change which occurs in association with vascular smooth muscle cell proliferation in the lesion of intimal hyperplasia. Cell stress has also been associated with PLCγ activation.

Initially the presence of PLCγ in human long saphenous vein was determined using quantitative immunohistochemistry. Differences in expression of PLCγ between ischaemic and non-ischaemic veins was also determined using this technique. Subsequently the effect of PLCγ inhibition upon vascular smooth muscle cell proliferation was determined using U73122, a PLCγ inhibitor.

3.4.1.1 U73122 (1-[(6-[(17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione): A PLCγ inhibitor.

Figure 3.4.1 Structure of U73122.
U73122 is used as an inhibitor of PLCγ. It inhibits the hydrolysis of PPI to IP₃ leading to a decrease in cytosolic calcium. IC₅₀ values for proliferation in human vascular smooth muscle cells are not published but for platelet aggregation in response to thrombin and collagen it is 0.6 μM and 5 μM. Inhibition of proliferation of rat pulmonary artery smooth muscle cells occurs with concentrations of 10 μM. An IC₅₀ of 0.25 μM is reported in rat portal vein VSMC for inhibition of the Calcium response. 10 μM has been used successfully to inhibit proliferation of other cell types, including colorectal cancer cells. 5 μM has also been successfully used to inhibit cell proliferation in astrocytes in response to thrombin stimulation.

U73122 has been used in these experiments to determine if inhibition of PLCγ results in inhibition of cellular processes contributing to intimal hyperplasia, namely vascular smooth muscle cell proliferation. As hypoxia can stimulate signalling pathways that are known to be downstream of PLCγ signalling processes, it was also determined if PLCγ inhibition had preferential effects under hypoxic conditions.

3.4.2 Methods.

3.4.2.1 Immunohistochemistry.

Immunohistochemistry was performed as detailed in methods section 2.4 on vein segments of both ischaemic and non-ischaemic origin. PLCγ antibody was used to determine the presence of PLCγ in human vein tissue and if ischaemia alters the level of expression. Stain intensity was then quantified according to the protocol in section 2.4.4.
3.4.2.2 Proliferation Assay.

Proliferation assay was performed in accordance with methods section 2.7 in response to stimulation with FCS at 15%, and no stimulation. Counts were read in DPM using a scintillation counter.

3.4.3 Results.

3.4.3.1 PLC\(\gamma\) Expression in Ischaemic and Non-ischaemic vein Tissue.

Immunostaining for PLC Gamma was similar in both ischaemic and non-ischaemic veins, with uptake in the endothelium and smooth muscle cells (see figure 3.4.2 and 3.4.3). Quantification of the level of staining intensity was performed, and correlated with visual inspection, with no significant difference in quantitative stain level between the two groups. (See figure 3.4.3).

Figure 3.4.2: Immunohistochemistry for PLC\(\gamma\) in Human Long Saphenous Vein: Uptake of PLC\(\gamma\) is seen in both endothelial and smooth muscle cells. Intensity of staining is similar in both ischaemic and non-ischaemic veins (PLC Gamma at concentration 1:100)
3.4.3.2 The Effect of U73122 on Vascular Smooth Muscle Cell Proliferation.

U73122 reduced Vascular Smooth Muscle Cell proliferation in a concentration dependent manner at foetal calf serum concentration of 15% (maximal stimulation) over 24 hours. The vehicle (ethanol) did not have a significant effect on vascular smooth muscle cell proliferation, and for each experiment unstimulated basal levels of proliferation were low.
Figure 3.4.4: The effect of U73122 on Vascular Smooth Muscle Cell Proliferation. There is a concentration dependent inhibition of VSMC Proliferation exhibited by U73122. (# p < 0.05; * p < 0.01 using One Way ANOVA and post-hoc Dunnett’s for comparison with the 15% FCS alone group. (n=6).

3.4.3.3 The Effect of U73122 on Vascular Smooth Muscle Cell Proliferation at 5% Oxygen.

Again, U73122 reduced Vascular Smooth Muscle Cell proliferation in a concentration dependent manner at foetal calf serum concentration of 15% (maximal stimulation) over 24 hours, though the reduction in proliferation at $10^{-6}$ M was not statistically significant, reductions at other concentrations of U73122 were. The vehicle (ethanol) did not have a significant effect on vascular smooth muscle cell proliferation, and for each experiment unstimulated basal levels of proliferation were low.
Figure 3.4.5: The effect of U73122 on Vascular Smooth Muscle Cell Proliferation at 5% Oxygen. n=6, * p < 0.05 using one-way Anova with post hoc Dunnett’s test.

Statistical analysis was performed as 3.4.3.2. One-way analysis of variance showed the difference in the means to be statistically significant (p = 0.0001) and Bartlett’s test of equal variance demonstrating significant difference in variances (p = 0.0139).

Dunnett’s post hoc test was performed to compare each concentration against the control group (15% FCS). Reductions in proliferation at all concentrations of U73122 were statistically significant as summarised.
3.4.3.4 Comparison of Reduction in Proliferation at 5% Oxygen.

Figure 3.4.6 demonstrates the comparison of reduction in proliferation by U73122 at normoxic conditions compared with 5% oxygen. In both cases, reduction of proliferation occurred in a concentration-dependent manner; however the volume of reduction under hypoxic conditions was less at concentrations of $10^{-5}$ and $10^{-6}$. This was statistically significant at a concentration of $10^{-6}$.

Figure 3.4.6: The effect of U73122 at 5% oxygen compared to normoxia. n=6, p < 0.05 students t-test.
3.4.4 Discussion.

PLCγ has been previously demonstrated to have effects on VSMC that may be important in intimal hyperplasia formation. Little data exists on the potential effects on intimal hyperplasia formation in infrainguinal bypass grafts.

These results initially demonstrate the presence of PLCγ in human vein tissue in both endothelial and smooth muscle cells. PLCγ has been previously demonstrated in arterial human VSMC by western blot,276 human umbilical vein endothelial cells277 and human vein VSMC of various origin.278 These immunohistochemical experiments confirm the presence of PLCγ in human vein endothelial and smooth muscle cells.

Comparison of PLCγ expression between ischaemic and non-ischaemic vein tissue did not demonstrate any significant difference in expression as judged by quantitative immunohistochemistry. The effect of ischaemia on PLCγ expression has been shown in cultured mouse brain endothelial cells. In response to ischaemia, a transient rise in PLCγ activation occurs,279 peaking at around 30 minutes. In one model of hind limb ischaemia, a more prolonged activation of PLCγ was identified in myocytes, being present greater than 24 hours following activation with vascular endothelial growth factor.280 In models of chronic disease, renal arteriolar vascular smooth muscle cells from spontaneously hypertensive rats have been shown to have increased PLCγ expression by western blot, however in this paper differences in expression were only seen in cells used between passages 3 and 8, limiting the physiological application.281 It is possible that there are acute increases in PLCγ expression in human vein tissue, which subsequently normalised in the chronic condition of critical limb ischemia not detected by this experimental method. No differences in PLCγ expression was demonstrated between veins of ischaemic and non-ischaemic origin.
Next the influence of PLCγ inhibition on proliferation of VSMC was assessed under normal conditions and under hypoxic conditions. Previous data indicated that VSMC proliferation is enhanced at 5% and 10% oxygen. This experiment was not set up to provide direct comparison of proliferation under 5% oxygen and normoxia. Hypoxia has been demonstrated to stimulate intracellular calcium release in astrocytes which is inhibited by U73122. U73122 inhibition can prevent hypoxia-induced apoptosis in myocytes and has also been the signalling mechanism implicated in hypoxic vasoconstriction of arterial myocytes. No previous work has focussed on the response of human VSMC derived from saphenous vein to U73122 under normoxic and hypoxic conditions in a model of infrainguinal bypass graft failure.

These results demonstrate that inhibition of PLCγ by U73122 inhibits VSMC proliferation in a concentration dependent manner at normal oxygen concentrations. This is in-keeping with published results in other areas, where rat aortic VSMC proliferation stimulated by BST406, a Benzylideneacetophenone, a class of drugs known to have antiproliferative effects has been shown to be via inhibition of PLCγ signalling pathways. Similarly PDGF-stimulated proliferation of rat and human aortic vascular smooth muscle cells has recently been shown to be inhibited by U73122 in a model of atherosclerosis.

U73122 inhibited vascular smooth muscle cell proliferation in vascular smooth muscle cells cultured at 5% oxygen, however the magnitude of inhibition was significantly reduced compared with reductions under normal oxygen concentrations, when used at a concentration of $10^{-6}$M and appeared reduced at a concentration of $10^{-5}$M, though this did not reach statistical significance. There was no apparent difference when a lower concentration of U73122 was used. No significant difference may have been apparent at this concentration as the magnitude of reduction was less.

Signalling mechanisms implicated in VSMC proliferation in response to mitogenic stimuli are ERK1/2. Under conditions of cell stress p38 and JNK MAP kinases have been shown to be
activated. Under hypoxic conditions, protein kinase C occurs in addition to ERK 1/2 proliferation. HIF-1 is also upregulated in hypoxic VSMC. In addition to direct induction of signalling pathways, hypoxic VSMC release further pro-inflammatory mediators such as IL-1, IL-6 and IL-8. These proinflammatory mediators further enhance pathways of VSMC proliferation.

PLCγ is a downstream signalling mechanism of protein kinase C pathways, and HIF-1 activation has been shown in vascular smooth muscle cells to be dependent upon PLCγ activation. Activation of other stress-related cell signalling pathways to promote proliferation could explain the loss of magnitude of effect of PLCγ inhibition under hypoxic conditions. Whilst cross-talk between these pathways can occur, there may be other factors influencing proliferation outwith PLCγ-dependent pathways under hypoxic conditions.

These experiments used U73122, a recognised PLCγ inhibitor. Whilst use of U73122 is suggestive that PLCγ has a significant role in VSMC proliferation, this research cannot exclude that reductions in proliferation are via other potential non-specific effects of U73122. In platelets, in addition to inhibition of PLCγ pathways, U73122 also interfered with tyrosine protein phosphorylation and also antagonised PLCβ. In rat portal vein vascular smooth muscle cells, the inhibition of hydrolysis of PPI to IP₃ by U73122 has been confirmed, however the non-specific effect of inhibition of voltage-dependent L-type Ca²⁺ channel in a concentration-dependent manner was also seen. Further experimental research would be required to confirm that the U73122-induced inhibition is via pathways of PLCγ inhibition. Initially western blot analysis of PLCγ concentrations in stimulated vascular smooth muscle cells and those treated with U73122 should be performed.

PLCγ appears to have an active role in proliferation of human VSMC, however it is not present in greater quantities in ischaemic human vein tissue, and under hypoxic conditions, while reductions in VSMC proliferation are seen, the magnitude of reduction is not as great as under
normoxic conditions. This suggests that in a model of hypoxia/ischaemia in infrainguinal bypass grafting, PLCγ may not be a final common pathway in inhibition of proliferation. Further research is required to confirm the specificity of U73122 inhibition in human VSMC, and cell signalling experiments are required to confirm correlation with VSMC proliferation and PLCγ activation.
CHAPTER 4
GENERAL DISCUSSION
4 General Discussion.

12,000 infrainguinal bypass grafts are performed annually in the United Kingdom,292 and outcomes remain suboptimal: 20% of above-knee vein grafts require intervention to maintain patency by 3 years.293 At present there are little pharmacological interventions demonstrated clinically to improve patency. Only antiplatelet agents77 and warfarin therapy in grafts at high risk of occlusion have any demonstrated efficacy in randomized control trials.294 80% of graft failure is caused by neointimal hyperplasia formation, and this process has been the target of research aimed at improving graft patency.

The aim of this work was to investigate pathways of neointimal hyperplasia formation, and explore the influence of ischaemia/hypoxia, the potential of PLCγ as a key signalling pathway and the role of Toll Like Receptors.

Initially the presence of TLR 2 and 4 was demonstrated in human long saphenous vein by immunohistochemistry. While TLR2 appeared to be present in saphenous vein, functional experiments demonstrated that only TLR4 was active in vascular smooth muscle cells and endothelial cells in response to specific ligands. Furthermore LPS, the TLR4 ligand was shown to induce modest, but significant increases in VSMC proliferation. To enhance clinical relevance, inhibition of TLR4 pathways should be demonstrated to inhibit LPS-induced vascular smooth muscle cell proliferation in a laboratory setting, and use of an inhibitor reduce intimal hyperplasia formation, initially in an animal model. To date, TAK-242 a cyclohexane derivative, has been shown to inhibit TLR4 activation in vivo in mouse immune cells transfected with human TLR4 and in vivo in a mouse model of sepsis in response to LPS,295 and is now commercially available as a TLR4 inhibitor. TAK-242 has also undergone phase 1 clinical studies which confirmed its safety, however a randomized trial of its use in adults with sepsis was halted early as insufficient cytokine suppression was seen in subjects.296 Eritoran is a lipid A
analogue which has been shown to be a LPS antagonist with efficacy in human trial test subjects. In phase II clinical trials in patients with severe sepsis, a trend towards lower mortality (56% VS 33% in those treated with eritoran) was noted. This demonstrates that specific TLR4 inhibition is a safe in-vivo treatment, and if further evidence for the role of TLR4 activation in intimal hyperplasia formation was demonstrated, in-vivo treatments are in place that could be considered for clinical trial.

There is further evidence that statins, agents with established use in peripheral vascular disease, have inhibitory effects on TLR4 signalling pathways. Dose-dependent decreases in TLR4 mRNA can be seen in human monocytes incubated with simvastatin, and in-vivo, treatment with atorvastatin reduced TLR4 expression in 12 volunteers by 36%. Reductions in TLR4 expression in response to LPS in healthy volunteers treated with high dose simvastatin have also been documented. Treatment with simvastatin has previously been demonstrated to reduce proliferation and migration of human vascular smooth muscle cells in this laboratory and in two retrospective reviews been shown to improve graft survival. Further investigation into the potential inhibitory effects of statins upon demonstrated LPS-enhanced proliferation of vascular smooth muscle cells warrants further investigation.

Subsequently the role of hypoxia in VSMC proliferation was investigated. Proliferation of VSMC was enhanced at 5% oxygen and 10% oxygen when compared with normoxia. Enhancements in proliferation were greatest at 5% oxygen. When saphenous vein is harvested for infrainguinal bypass grafting, it has been exposed to ischaemic conditions by virtue of the disease process of critical limb ischaemia. In addition, various harvesting techniques may interrupt the saphenous vein blood supply via the vasa vasorum, and render the tissue further hypoxic.

Recent evidence using an oxygen sensitive probe has demonstrated that pig saphenous vein rapidly becomes hypoxic once it is harvested, and remains so up to a month after implantation.
Prolonged hypoxia, in addition to directly enhancing VSMC proliferation also stimulates endothelial cells to produce VSMC mitogens as well as inducing inflammatory processes including secretion of IL-6 and IL-8. New techniques in cardiac surgery have evolved, using Dacron external graft supports. In porcine models of cardiac bypass surgery these have reduced saphenous vein intimal hyperplasia formation, an effect attributed to allowing rapid neovascularization of the vasa vasorum and restoration of normoxia. A ‘no touch’ technique in saphenous vein harvest has also been developed with the aim of preserving the vasa vasorum and minimizing the requirement for vessel disruption. This again has shown to improve patency rates in cardiac surgery. Such techniques are not prevalent in peripheral vascular surgery for infrainguinal bypass grafting, however evidence for the critical role of ischaemic and hypoxic pathways in a laboratory setting could form the basis of further research in an infrainguinal bypass grafting model in vivo.

The pre-existing ischemia that the saphenous vein is exposed to prior to use as a conduit for infrainguinal bypass grafting is not a factor that can be easily modified, however identification that it is a potential significant contributing factor to intimal hyperplasia formation and vein graft failure could guide future therapies. In addition VSMC proliferation, hypoxia has been demonstrated to induce HIF-1α. Stimulation of this pathway appears to promote proliferation in human VSMC obtained from the pulmonary artery, this being via telomerase reverse transcriptase (TERT). The PREVENT trial demonstrated that gene therapy can be successfully performed in vivo in infrainguinal bypass grafting as an incubation step prior to implantation, and specific gene therapy to inhibit TERT has been developed. Identification of the key role of hypoxic pathways in intimal hyperplasia formation could provide the basis for gene therapy trials aimed at improving graft survival.

Subsequently, PLCγ inhibition by U73122 was demonstrated to reduce vascular smooth muscle cell proliferation under both normoxic and hypoxic conditions. Reductions under hypoxic conditions appeared to be less, though were not significantly different. PLCγ has been associated with a variety of cellular processes critical to VSMC, including change of VSMC from quiescent
to contractile phenotype. Additionally in rat pulmonary artery VSMC similar reductions in proliferation to those demonstrated in this project have been demonstrated. This novel preliminary data presents the first evidence that PLCγ may have a critical role in human saphenous vein VSMC proliferation and subsequent intimal hyperplasia formation. The magnitude of reduction seen in proliferation appeared reduced at conditions of 5% oxygen, though statistically significant reductions were still observed. It is possible that under hypoxic conditions, stimulation of other pathways of proliferation occur that are not dependent upon PLCγ activation. Further research is required to confirm the specificity of U73122 to PLCγ dependent pathways in human VSMC. Subsequent cell signalling work is also required to confirm that reductions in proliferation correlate with PLCγ inhibition under both normoxic and hypoxic conditions.

PLCγ inhibition has been achieved by U73122 as well as various other compounds, however none have been found suitable for development for use clinically, however recent high-throughput screening has identified further compounds which are specific to PLCγ inhibition that may be suitable for clinical development, the most potent being in the micro-molar range. The lack of a pharmacological product with human safety and efficacy at present limits translation of this research into clinical application.

The original aim of this project was to determine the influence of Toll-Like receptors in intimal hyperplasia formation and subsequently assess the influence of hypoxia and PLCγ inhibition. It was demonstrated that TLR4 stimulation has functional effects in human vascular smooth muscle cells and that stimulation of VSMC with lipopolysaccharide enhanced proliferation. Similarly hypoxia was shown to enhance VSMC proliferation, and treatment with U73122, a PLCγ inhibitor reduced this proliferation. A subsequent aim of the project and area for future research is to determine if any links exist between these pathways in VSMC. Initially it was felt that PLCγ may be a common pathway of activation of proliferative responses to both TLR activation and hypoxia. The effect of TLR4 activation by lipopolysaccharide can be attenuated by inhibition of PLCγ in a mouse model of sepsis in cultured myocytes, and PLCγ inhibition has also been
demonstrated to reduce cytokine release from mouse macrophages in response to lipopolysaccharide.\textsuperscript{234}

Future research will initially determine if PLCγ signalling is upregulated in vascular smooth muscle cells in response to lipopolysaccharide and if so, determine if inhibition can reduce the enhancements of proliferation induced by in vascular smooth muscle cells. Furthermore experimental evidence of PLCγ upregulation in response to hypoxia by western blot will be obtained and correlated with existing data.

Subsequent research is proposed in the form of an animal model of intimal hyperplasia, using an interposition vein graft in rabbit carotid artery (an established model in this laboratory). Initially sequential immunohistochemistry would be performed to determine if upregulation of TLR4 and PLCγ occurs in association with intimal hyperplasia formation. Correlated with the cell culture results obtained, a model is proposed whereby a ‘pre-incubation’ step is performed with the vein prior to use as an interposition conduit; with a TLR4 inhibitor and PLCγ inhibitor. Subsequent blinded sequential measurements of intimal hyperplasia of vein tissue exposed to a pre-incubation step would be measured against control veins. This would correlate with potential clinical application, where an incubation step with a pharmacological inhibitor prior to implantation is feasible method of local drug delivery, with potentially minimal systemic effects.

This research has yielded novel data demonstrating the role of innate immune pathways, hypoxia and the potential central role of PLCγ in proliferative pathways of human vascular smooth muscle cells. Key areas for future research with the aim of improving outcomes in infrainguinal bypass grafting have been demonstrated both in the laboratory and potentially at a clinical level.
5. Bibliography


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