ANTIFIBROTIC GENE THERAPY FOR VEIN GRAFT DISEASE

Assessment of the efficacy of anti-fibrotic gene transfer as a means of suppressing neointima formation in venous-arterial interposition grafts.

A thesis submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Faculty of Biology Medicine and Health

2018

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PREFACE

This thesis is presented in a traditional Masters Thesis style, and submitted to the University of Manchester for the degree of Master of Philosophy in the Faculty of Medical and Human Sciences. While initial stages of this project were involved in growing up of the viral vectors and purifying the solution needed to infect the vessels involved in the study, the latter part of the experiments were involving the pigs. I have obtained the Personal Licence (Home office Licence No-I04FFE420) issued by the Home Office to perform experiments on pigs.
ABSTRACT

Background
Diseases of the heart and circulatory system are the main causes of deaths in the UK, and accounted for almost 180,000 deaths in 2010. Coronary artery bypass grafting remains one of the viable modalities of coronary revascularization. Autologous Saphenous vein (SVG) is the most frequently used conduit for coronary artery bypass grafting. Its long-term success is however limited by progressive luminal loss, resulting from neointimal hyperplasia (NIH) and superimposed atherosclerosis. Due to the lack of effective pharmacological therapies and lack of long term benefits from percutaneous vein interventions described in the literature, vein graft disease following coronary artery bypass graft, is a potential target for novel approaches like gene therapy.

TGF-β1 is a key promoter of ECM deposition in the intima of the vessel by promoting phenotypic changes in the smooth muscle cells (SMC) of the media and stimulating ECM synthesis. Many preclinical studies have confirmed the role of TGF-β1 antagonists at inhibiting NIH in injured arteries but the same effect has not been studied in vein. The research group in the University of Manchester previously studied the effects of adenovirus-mediated delivery of anti-fibrotic transgenes like the Latency-associated peptide of TGF-β1 (LAP-β1) and of Fibromodulin in organ-cultured human long saphenous vein and the resulting suppression of neointimal hyperplasia ex-vivo. The next step was to demonstrate the same effect in-vivo where different factors like haemodynamic, rheological, inflammatory and immune responses may influence the effects seen already in organ-cultured human saphenous vein. The objective of this study was hence to develop a methodology for the saphenous vein interposition grafting in to internal carotid artery and harvesting of the same graft in pigs in Manchester.

Under appropriate conditions, long saphenous vein obtained from the pigs were infected with the virus Ad5-lacZ, expressing the marker gene β-galactosidase. These prepared veins were anastomosed as interposition grafts to the internal carotid arteries. These segments of venous-arterial interposition grafts explanted later were stained with 5-bromo-4-chloro-indolyl-D-galactopyranoside for expression of β-galactosidase present in vein segments infected with Ad5-lacZ. We obtained consistent results at repetition of experiments. This confirmed the testing of an in vivo system, paving pathway for further experiments. LAP-β1 gene transfer offers potential therapeutic benefit in the vein graft failure by reduction in NIH. The results of this work will thus help to establish whether these antifibrotic gene therapies will be suitable for clinical application to neointimal suppression in vein grafts in-vivo.
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DECLARATION

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Imthiaz Manoly
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DEDICATION

This work is dedicated to my soul mate Nishat, who has been a constant support during this period and always after we met.
ACKNOWLEDGEMENTS

I would like to thank all those who helped me to complete this project.

First and foremost, I would like to express my sincere gratitude to my supervisor, Dr. Paul Kingston, for his continuous support and motivation. His constant guidance made me feel at ease right from the time I joined University of Manchester till the completion of this project. I am very much obliged to his able supervision and support. I hope to liaise with him for future scientific endeavours.

This project wouldn’t have practically materialized without Dr. Salik Kakar, who has spent many hours with me advising, guiding and giving me constant feedback. I could not have asked for a better colleague than Dr. Salik Kakar.

I am also obliged to all the members of the laboratory and my friends at the University of Manchester. My special thanks to Joy Stewart, for being a nice human and helping me with my admission and reminding me of all the deadlines related to this Masters program.

Last but not the least, I wanted to thank my family especially my wife, Nishat, who managed our family efficiently, while I was busy with my clinical and university commitments. I am truly indebted to her for being my soul mate and my best friend.
ANGINA PECTORIS, commonly known as angina, is a chest pain due to lack of blood supply to the heart muscle, generally due to obstruction or spasm of the coronary artery.

ATHEROSCLEROSIS is the inflammatory fibro-proliferative lesion that develops on the inside surface of the walls of arteries, narrowing the vessel.

MYOCARDIAL INFARCTION (MI) is a medical condition that occurs when the blood supply to a part of heart muscle is disrupted, typically due to occlusion of a coronary artery. The resulting ischaemia causes damage to the heart tissue.

NEOINTIMAL HYPERPLASIA is the accumulation of smooth muscle cells and extracellular matrix in the intima of the vessel wall.

RESTENOSIS is the recurrent luminal narrowing at the site of angioplasty or stenting.

STENOsis is an abnormal narrowing of the blood vessel.

THE SAPHENOUS VEIN is the large superficial vein of the leg and thigh.

THROMBOSIS is the formation of a clot or thrombus inside a blood vessel, obstructing the flow of blood.
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<td>Adeno-associated virus</td>
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<tr>
<td>ACC</td>
<td>American college of cardiology</td>
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<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
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<td>Ad</td>
<td>Adenovirus</td>
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<td>AHA</td>
<td>American heart association</td>
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<td>ALK</td>
<td>Activin-like kinase</td>
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<td>ANOVA</td>
<td>Analysis of variance</td>
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<td>BMS</td>
<td>Bare-metal stent</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>CABG</td>
<td>Coronary artery bypass graft</td>
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<td>CAD</td>
<td>Coronary artery disease</td>
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<td>CAR</td>
<td>Coxsackie-adenovirus receptor</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>cDNA</td>
<td>Complementary DNA</td>
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<td>CIAP</td>
<td>Calf intestinal alkaline phosphate</td>
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<td>Co- Smad</td>
<td>Common-mediator Smad</td>
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<td>CPE</td>
<td>Cytopathological effect</td>
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<td>CTGF</td>
<td>Connective tissue growth factor</td>
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<td>CVD</td>
<td>Cardiovascular disease</td>
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<td>DAB 3</td>
<td>3’-diaminobenzidine</td>
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<td>DAP</td>
<td>4’, 6-diamindino-2-phenylindole</td>
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<td>DES</td>
<td>Drug-eluting stent</td>
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<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium 18</td>
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<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ds DNA</td>
<td>Double stranded DNA</td>
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<tr>
<td>ECs</td>
<td>Endothelial cells</td>
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<td>ECACC</td>
<td>European collection of animal cell cultures</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDRF</td>
<td>Endothelial-derived relaxing factor</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EDF</td>
<td>Epidermal growth factor</td>
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<td>Abbreviation</td>
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<td>EEL</td>
<td>External elastic lamina</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
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<td>ET-1</td>
<td>Endothelin-1</td>
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<tr>
<td>EtBr</td>
<td>Ethidium bromide</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>GPIIb/IIIa</td>
<td>Glycoprotein IIb/IIIa</td>
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<td>HDL</td>
<td>High-density lipoprotein</td>
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<td>Herpes simplex virus thymidine kinase</td>
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<td>I:M</td>
<td>Intima: media</td>
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<td>ICAM-1</td>
<td>Intercellular adhesion molecule-1</td>
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<td>Immunocytochemistry</td>
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<td>IEL</td>
<td>Internal elastic lamina</td>
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<td>IGF</td>
<td>Insulin-like growth factor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>I-SmaD</td>
<td>Inhibitory Smad</td>
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<td>ISR</td>
<td>In-stent restenosis</td>
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<tr>
<td>ITR</td>
<td>Inverted terminal repeat</td>
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<tr>
<td>IVBT</td>
<td>Intravascular brachytherapy</td>
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<tr>
<td>LAP</td>
<td>Latency-associated peptide</td>
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<td>LAR</td>
<td>Luciferase assay reagent</td>
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<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LITA</td>
<td>Left internal thoracic artery</td>
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<tr>
<td>LLC</td>
<td>Large latent complex</td>
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<tr>
<td>LTBP</td>
<td>Latent TGF-β binding protein</td>
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<td>M6P/IGF-2-R</td>
<td>Mannose-6-phosphat/insulin-like growth factor-2 receptor</td>
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<td>MCS</td>
<td>Multiple cloning site</td>
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<td>MEM</td>
<td>Minimum essential medium</td>
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<td>MI</td>
<td>Myocardial infarction</td>
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<td>Definition</td>
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<td>MLEC/PAI-L</td>
<td>Mink lung epithelial cells (plasminogen activated inhibitor-1)</td>
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<td>MIEmCMV</td>
<td>Major immediate-early murine cytomegalovirus enhancer/promoter/luciferase</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>NIH</td>
<td>Neointimal hyperplasia</td>
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<tr>
<td>NO</td>
<td>Nitric oxide</td>
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<td>OD</td>
<td>Optical density</td>
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<td>OPCABG</td>
<td>Off-pump CABG</td>
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<td>PAD</td>
<td>Peripheral artery disease</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor-1</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PCI</td>
<td>Percutaneous coronary intervention</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PDGF</td>
<td>Platelet-derived growth factor</td>
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<td>PECAM</td>
<td>Platelet/endothelial cell adhesion molecule</td>
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<td>PTCA</td>
<td>Percutaneous trans luminal coronary angioplasty</td>
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<td>RE</td>
<td>Rabbit (SM-MHC) enhancer</td>
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<tr>
<td>RLU</td>
<td>Relative luminosity unit</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>R-Smad</td>
<td>Receptor-activated Smad</td>
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<td>Sirolimus-eluting stent</td>
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<td>Small latent complex</td>
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<td>SM-αA</td>
<td>Smooth muscle α-actin</td>
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INTRODUCTION

Diseases of the heart and circulatory system are the main causes of deaths in the UK, and accounted for almost 180,000 deaths in 2010. One in five male deaths and one in ten female deaths were due to coronary artery disease (CAD), amounting to 80,000 deaths in 2010[1]. This is a burden to society due to loss of productivity, sickness, expenditure for treatment and benefits. However, in recent years, the death rate due to CAD has dropped significantly in patients over 55 years of age, and to an extent, in those less than 55 years. This is due to a combination of awareness and prevention of risk factors along with early intervention, both surgical and percutaneous, once the disease has been diagnosed[2].

1.1. Coronary artery disease

The two major arteries of the heart are the Left coronary artery (LCA) and the Right Coronary artery (RCA) (Figure 1). The LCA divides into the Left anterior descending (LAD) and Left Circumflex artery (LCx). The RCA further divides into Posterior descending artery (PDA) and Posterior Left Ventricular (PLV) branches.

Figure 1 – Coronary arteries and its branches (With permission from the illustrator -Patrick J. Lynch, medical illustrator derivative work: Fred the Oyster (talk) adaption and further labelling: Mikael Häggström - Coronary.pdf, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=9967381)
Coronary artery disease (CAD) is the most common type of heart disease in adults. CAD is a progressive disease, which occurs when the major blood vessels supplying blood to the heart gets stenosed or narrowed due to various reasons, primarily atherosclerosis. Coronary atherosclerosis is a progressive inflammatory disorder of the large and medium sized muscular arteries, due to the focal accumulation of lipids and subsequent hardening (sclerosis) of the vessel wall. A lot of factors are responsible for the pathophysiology of coronary atherosclerosis, which can be summarised as endothelial dysfunction leading to inflammation of the vascular wall followed by deposition of cholesterol, calcium and cellular debris. Atherosclerotic lesions do not occur randomly. Interaction of activated vascular endothelium by turbulent blood flow generates shear stress. These fluid shear stresses influence the phenotype of the endothelium, which occurs as a result of modulation of gene expression and regulation of activities of flow sensitive proteins. The segments of coronary arteries exposed to low shear stress undergo constrictive remodelling than those segments of high shear stress, which develop greater necrotic core and calcium progression, regression of fibro fatty tissue and expansive remodelling. The plaque formation and the subsequent vascular remodelling could result in acute and chronic luminal narrowing and obstruction of the coronary blood flow to the myocardium and hence diminished blood and oxygen supply to various organs. The nature of the atherosclerotic plaque and its rupture are primarily responsible for most of the clinical manifestations of the acute coronary syndrome. The spectrum of presentation of the coronary artery disease can be varied, as a result of myocardial ischaemia which could be stable or unstable angina, acute myocardial infarction with or without mechanical complications, congestive cardiac failure and cardiac arrest.

**The management of coronary artery disease:**

One of the strategies in the management of coronary artery disease includes identification of its’ risk factors which are categorised into irreversible and reversible risk factors. Irreversible risk factors are age, gender and genetic predisposition while reversible risk factors include diabetes, hypertension, hypercholesterolemia and smoking.
The management of CAD can be categorised in to:

1. **Medical management**: The goals of medical therapy are to relieve the symptoms of CAD and to prevent future cardiac events like angina, myocardial infarction or heart failure. Prevention and treatment of coronary atherosclerosis can be achieved by identification and management of modifiable risk factors, using anti-diabetics, anti-hypertensives, statins (HMG-CoA reductase inhibitors), and lifestyle modification including cessation of smoking. Beta-blockers, anti-platelets as well as nitrates are used for symptomatic management.

   a) **Anti-platelets** – This group of drugs include Aspirin, Clopidogrel, Ticagrelor, Prasugrel etc. The role of antiplatelet in the prevention of coronary atherosclerosis and treatment of acute coronary syndromes has been proven in many trials [3-5]. Currently both AHA and ESC guidelines recommend anti-platelets to be the first line drugs in the management of acute coronary syndrome [6, 7]. Though the role of Aspirin in the management of CAD was studied and reported in multiple trials, the role of second anti-platelet in addition to Aspirin has also been inculcated in the recent guidelines. What constitutes to be the second anti-platelet is still a debate with the recent studies favouring Ticagrelor to Clopidogrel along with Aspirin.

   b) **Statins or HMG-CoA reductase inhibitors** – Statins are used in the primary and secondary prevention of coronary atherosclerosis [8]. Results of the CASCADE trial confirmed that usage of statin along with Clopidogrel independently associated with improved graft patency, as demonstrated by coronary angiography and saphenous vein graft intravascular ultrasonography performed 12 months postoperatively[9].

   c) **Angiotensin-converting enzyme (ACE inhibitors)** – This group of drugs primarily help in the blood pressure reduction and their efficacy in the treatment of CAD was studied in the last two decades with equivocal results. The role of ACE in the prevention and regression of plaque formation was not clear. Though the initial studies reported no significant role in the prevention of CAD, their role in the plaque regression and dose-related proportional reduction in the intima-
media thickness of carotid artery was reported in the SECURE trial[10-12]. The Heart Outcomes Prevention Evaluation (HOPE) study reported direct involvement of Ramipril on atherosclerosis on 9297 subjects with history of CAD, stroke, peripheral vascular disease, or diabetes[13]

d) **Beta-blockers** - Beta-blockers tend to decrease myocardial oxygen demand by inhibiting sympathetic stimulation of the heart, reducing heart rate and contractility. In patients with CAD, they can hence prevent and relieve angina. They include Bisoprolol, Metoprolol, Carvedilol, Atenolol etc. The Current AHA guidelines recommend beta-blockers, unless contraindicated, in patients with stable angina who have had an ACS or who have left ventricular dysfunction (LVD).

e) **Calcium channel blockers** – As the name implies, they prevent entry of calcium into vascular smooth muscle cells and myocytes, which leads to coronary and peripheral vasodilatation and reduced contractility. They also provide negative inotropic effect; reduce systemic vascular resistance and arterial blood pressure.

f) **Nitrates** – They are commonly used in the management of acute anginal symptoms and work by causing systemic vasodilation and reduce myocardial oxygen demand. Used in combination with Beta-blockers and Calcium channel blockers, they are also effective in the management of chronic stable angina.

2. **Percutaneous coronary interventions (PCI)** – This is a non-surgical technique for treating obstructive CAD including unstable angina, acute myocardial infarction, and multi-vessel coronary artery disease. This includes balloon angioplasty, atherectomy (rotational, laser and directional) and stent deployment (bare metal and drug eluting). Further to the updated ESC guidelines in the management of CAD, PCI are one of the revascularisation procedures for both symptomatic myocardial ischaemia and also in stable patients with selective coronary atherosclerotic lesions [14]. The indication of PCI has now been extended to asymptomatic or mildly symptomatic patients, if there is an objective evidence of a moderate to large area of viable myocardium or moderate to severe ischemia on non-invasive testing. Long-term benefits from PCI are however, limited by late chronic restenosis of the vessel wall at the site of the procedure, which occurs in 10-20% of cases within 6 months. New advancements in pharmacological
therapy have resulted in significant improvement in clinical outcomes following PCI. The new generation drug-eluting stents, Glycoprotein IIb/IIIa receptor inhibitors and Bivalirudin produced significant reduction in restenosis, as well as decrease in ischaemic and bleeding complication rates. Contraindications for PCI include intolerance to anti-platelet agents or the presence of any significant comorbidity that could severely limit the lifespan of the patients.

3. Surgical management which are further divided into
   a. Surgical revascularisations, by means of coronary artery bypass grafting (CABG).
   b. Surgical management of mechanical complications of acute myocardial infarctions including repair of ischaemic ventricular septal rupture, ischaemic mitral repair, repair of left ventricular free wall rupture.

1.2. Coronary artery bypass grafting

![Coronary artery bypass grafting with various anastomosed conduits.](image)

Coronary artery bypass grafting (CABG) (Figure 1B) is one of the most commonly performed procedures in heart surgery. It has been demonstrated to be effective in angina relief, improving quality of life and prolonging survival in selected patients [15]. The conventional
CABG (Figure 2) involves splitting the sternum (sternotomy), followed by simultaneous harvesting of conduits including left internal mammary artery (LIMA) and long saphenous vein (LSV). It is followed by institution of the cardiopulmonary bypass circuit (Figure 2), and the heart is arrested in diastole using a high concentration of potassium solution complex along with other membrane stabilising constituents of the heart. The area of significant narrowing or occlusions of coronary arteries, identified earlier by coronary angiography are then directly assessed, and the harvested conduits are grafted distal to the occlusion. The proximal end of the graft is then anastomosed to the ascending aorta and the arrested heart is reversed to normal rate and rhythm, maintaining good haemostasis and haemodynamic stability.

Figure 3: Cardiopulmonary bypass (CPB) circuit and the usual institution of CPB

Majority of the cardiac surgeries are now performed with the institution of the cardiopulmonary bypass circuit (Figure 3). However, coronary artery bypass grafting can also be performed off-pump (popularly called as OPCAB or off-pump coronary artery bypassing) without the need for cardiopulmonary bypass circuit. The benefits and risks of off-pump cardiac surgery in comparison with on-pump surgery have been much debated, with the
former technique affecting the long-term benefits of CABG over PCI. Few prospective trials demonstrated the increased need for repeat revascularisation and reduced patency rates of the grafts with off-pump technique [16, 17]. However, with careful selection of patients, OPCAB has reduced some of the significant morbidities associated with on-pump CABG [18]. Though many studies have initially demonstrated the survival advantage of CABG over percutaneous coronary intervention (PCI) or medical management for three-vessel disease or equivalent (ostial /proximal) lesion [19-21], the gap has been narrowed now with the improved techniques of PCI[22]. Despite advancement in the technology for PCI including newer generation drug-eluting stents, laser atherectomies, and newer pharmacological agents like Glycoprotein IIb/IIIa receptor inhibitors and Bivalirudin, there still exist a subset of patients who would benefit from surgical revascularisation[23, 24]. The current ESC guidelines emphasise on this and have ranked both PCI and CABG as Class I indication for coronary revascularisation for three vessel disease as well as Left main stem disease with low syntax score[25].

Conduits for CABG

Figure 4: Conduits for CABG – Internal mammary artery (Image by Henry Vandyke Carter - Henry Gray (1918) Anatomy of the Human Body)
Figure 5: Conduits for CABG – Radial artery (A) and Long Saphenous Vein (B) (Image by Henry Vandyke Carter - Henry Gray (1918) Anatomy of the Human Body)

The selections of the coronary artery bypass graft conduits are influenced by various factors, and crucial for CABG outcomes, affecting survival, freedom from myocardial infarction, symptoms and re-interventions[26].

The first reported CABG using the IMA (Figure 4) in humans was performed by R. Goetz in 1960 [27]. Coronary artery revascularisation using saphenous vein Figure 5 B) was first performed by D. Sabiston in 1962[28]. In the same decade, the utilisation of the long saphenous vein as a graft conduit was reported by another prominent surgeon, Favaloro in 1968 [28]. Amongst the available graft conduits, the long saphenous vein, otherwise known as the great saphenous vein, is the most readily available and widely used conduit, followed by the left internal mammary artery. Different types of autologous conduits have been utilised subsequently, namely right internal mammary artery, radial artery and gastroepiploic artery. The choice of conduit used for the graft anastomosis is still at the discretion and experience of
the individual operators and the centres despite all the recommendation and guidelines [29]. The left internal mammary artery (LIMA) also known as the Left internal thoracic artery graft to bypass the left anterior descending artery (LAD) is the gold standard and, is the most important factor in improving survival and freedom from MACE in patients undergoing CABG[30]. The use of LIMA to LAD is also a Class I recommendation regarding CABG conduits in the latest ESC guidelines on myocardial revascularisation[25]. LIMA is a superior conduit in comparison with not only vein grafts but also to other arterial grafts used in CABG. This could be attributed to the lower tendency to vasospasm, enhanced endothelial function as well as nitric oxide and prostacyclin release in the LIMA[31]. LIMA can be called as a transition artery having some features of large vessels, such as the elasticity of the aorta, and other features from medium-sized vessels, such as the abundance of smooth muscle cells of coronary arteries. Over the last decade, there has been increased awareness of the utilisation of the arterial grafts namely radial artery and right internal mammary artery, due to poor long-term outcomes of CABG utilising saphenous vein graft for myocardial revascularisation [32-34]

1.3. Saphenous vein graft failure

Saphenous vein graft failure can be defined as complete occlusion, stenosis of more than 70% or diffuse narrowing of the vein graft on coronary angiogram. It is a complex, multifactorial event that occurs in a significant proportion of patients who had CABG. Despite improved surgical techniques and use of newer pharmacological agents including newer ADP antagonists including Ticagrelor, Glycoprotein IIb/IIIa receptor inhibitors, and newer statins, the long-term efficacy of CABG is still impacted by saphenous vein graft failure[35]. Within one year of CABG surgery, 10-15% of venous grafts were reported to occlude or have significant stenoses [35-37], and almost half of the venous graft conduits fail at 10 years. [38-40]. This can impose a significant challenge in the management of coronary artery disease both financially and clinically.

Several studies have reported better long-term outcomes of CABG using arterial grafts compared with long saphenous vein grafts [33, 41-43]. However, SVG is the most convenient graft conduit for surgical revascularisation mainly due to better accessibility [44]. It is also the preferred conduit used in emergency CABG due to its abundance. Hence it is imperative to study the factors responsible for the vein graft failure and know the preventive strategies that we could implement, for a better outcome in surgical revascularisation.
1.3.1. Factors affecting the long-term patency of the vein:

Factors affecting the long-term patency of a venous bypass graft can be categorised as:

A. **Patient factors** – Also called as systemic atherosclerotic risk factors, they produce local effects on vascular biology and promote a pro-atherogenic phenotype in the graft. These factors include age, diabetes, female sex, smoking and were found to be associated with both early and late graft failure due to either thrombosis or accelerated atherosclerosis. The association of diabetes and graft failure have been reported in few studies.[42, 45]. Diabetes mellitus is associated with platelet activation, endothelial dysfunction and accelerated intimal hyperplasia [46]. In the landmark RAPS trial comparing radial artery versus saphenous vein graft patency, diabetes was an independent predictor of graft occlusion and was significantly more with SVG compared to radial artery [47]. The same trial also reported occlusion rates for the SVG at 12.0% and 23.3%, respectively for men and women. Age (acceleration factor, 1.28; CI, 1.04–1.58; \( P=0.02 \)), Cholesterol levels (acceleration factor, 0.76; CI, 0.60–0.96; \( P=0.02 \)), and CCS II-IV (acceleration factor, 0.64; CI, 0.40–1.02; \( P=0.05 \)) were reported to be predictors of long-term graft status in the angiographic VA study [40]. The characteristics of the grafted vessel can also impact its long-term patency. This includes the size mismatch between the grafts and the target vessels and underlying pathology of the vein grafts.

B. **Operator or Technical factors** - Technical factors are very important in determining graft patency and hence the outcome of CABG. Endoscopic saphenous vein harvesting (EVH) and the off-pump (OPCAB) techniques have been debated and correlated with lower graft patency rates. Harvesting techniques including EVH and skeletonised vein graft have the potential to affect graft patency. Improvising surgical technique or use of specific devices may significantly improve SVG outcome. The no-touch harvesting of SVG with a fat pedicle and avoidance of overdistension, reported excellent long-term patency rates and comparable to those of the LITA patency[48, 49]
**Endoscopic versus open vein harvesting techniques** - Current evidence suggests that endoscopic harvesting could be associated with reduced patency of the vein graft though this has now been accepted as one of the alternative techniques in vein harvesting by National Institute of Health and Care Excellence (NICE)- IPG494. Subset analysis of the PREVENT IV trial reported that EVH was associated with a higher risk of SVG stenosis or occlusion compared with an open harvest (OVH) technique (p=0.0001)[37]. The one-year patency rate of SVG from EVH was significantly lower than that of SVG from OVH (74.5% versus 85.2%, P<0.001) in the landmark ROOBY trial [50]. However, a recent meta-analysis demonstrating angiographic results from 7929 patients and limiting only to randomised control trials (RCT), couldn’t reach statistically significant difference between the two groups (EVH vs. OVH) though the incidence of graft stenosis and occlusion was higher for endoscopically harvested saphenous vein grafts. The possible differences could be due to the higher degree of damage to the vascular wall induced by EVH [51]. A 5-year observational study of 235,394 patients, undergoing isolated CABG surgery at 934 surgical centres, participating in the Society of Thoracic Surgeons (STS) national database reported equivalent clinical outcomes in patients operated using an EVH versus OVH [52].

**On-pump versus Off-pump CABG** - It is still not very clear about the effect of on-pump versus off-pump surgery on the graft patency rates. The landmark ROOBY trial demonstrated lower patency rate and repeated revascularisation in the off-pump group. A recent meta-analysis of RCTs, looking at the angiographic data on 7011 grafts reported an Odds ratio of 1.51 for graft occlusion in the off-pump versus on-pump series. These differences could be due to the long learning curve and operator experience, study design, and enrolment criteria [53].

**C. Target vessel factors** – The characteristics of the target vessels have the potential to influence the long-term patency of bypass grafts. The current ESC guidelines emphasise bypassing only those coronary vessels that are significantly stenosed (60% for left main and 70% stenosis for the rest of the coronary vessels). The grafting of a vessel that is not significantly stenosed creates a competitive state, in which the flow through the conduit
and the native coronary artery compete. This is particularly true with the arterial grafts more than SVG. Of the different grafts analysed, radial artery was reported to be most affected by competitive flow because of their higher degree of contractility and vasospastic characteristics [54, 55]. Saphenous vein grafts lack the ability to regulate blood flow to the coronary territory and hence demonstrate reduced variations in graft flow. As SVG is a compliant vessel, the pressure at the distal graft anastomosis is nearly equal to the aortic pressure, with minimal risk of developing competitive flow. However, a crucial determinant maintaining the patency of the SVG is the diameter of the target vessel. In a ten year observational study, Goldman et al. found the patency of the SVG was 88% if the diameter of the target vessel was more than 2 mm compared to 55% if the diameter of the target vessel was less than 2 mm [40]. Similar results were reported in RCT and observational studies [45, 56].

In addition to the diameter of the target vessel, the extent of atherosclerotic burden and previous PCI, have the potential to influence graft patency. Diffuse target vessel was more associated with lower graft patency than focal disease [57] and hence the emphasis on the functional studies than the conventional angiographic evidence to determine the extent of stenosis. The patency of the graft was also affected in patients with previous stented target vessel [58].

1.3.2. Pathophysiology of vein graft failure

The pathophysiology of vein graft failure is complex process, involving many interlinking factors, which can be broadly categorised into thrombosis, neointimal hyperplasia and progressive atherosclerosis (Figure 6). Though the mechanism is different in each time frame, each one ultimately contributes to the graft failure. Based on the time of occurrence, early graft failure (defined as failure occurring in less than 30 days) is mainly due to acute thrombosis predominantly caused by the technical factors but also due to the mismatch of the conduit or patient-related factors like hypercoagulable state (Protein C or S deficiency, Thrombocytosis etc.), whereas mid-term and long-term failures are attributed to neointimal hyperplasia, pathological vascular remodelling and progression of atherosclerosis.
Figure 6: Pathophysiology of vein graft failure

The changes occur at different layers of the saphenous vein graft. The wall of the vein has three layers; the innermost layer is intima, which is composed of single layer of endothelial cells and basement membrane. The next layer is media, which contains vascular smooth muscle cells and extracellular matrix. The outermost layer, adventitia is the outermost of the three layers and contains connective tissue, nerves and vasa vasorum, which is micro vascular network outside the vessel.

Many of the systemic atherosclerotic risk factors produce local effects on vascular biology and promote a pro-atherogenic phenotype in the saphenous vein graft. These factors include age, diabetes, female sex, smoking and were found to be associated with both early and late graft failure due to either thrombosis or accelerated atherosclerosis. Of particular note, diabetes is associated with severe coronary artery disease and poor prognosis after cardiac surgery [59]. Recent studies have reported diabetes as an independent predictor for poor prognosis after coronary artery bypass grafting,[60, 61].
**1.3.3. Early graft failure**

Early graft failure occurs due to acute thrombosis, which is caused predominantly by technical factors and certain patient factors alike. Graft failure due to poor anastomosis, small target vessel size, pre-existing vein disease, and graft kinking and hypercoagulable state of the blood have already been reported and described earlier in the chapter. The pathological changes observed in the early phase of the autogenous SVGs, grafted as aorto-coronary vessels are denudation of the endothelium, platelet accumulation, sub-endothelial infiltrates in the intimal cells, smooth muscle proliferation and necrosis, and inflammation[62]

**Endothelial cells (EC)** are essential mediators and regulate platelet anticoagulant as well as pro-coagulant processes along with fibrinolytic functions. ECs facilitate circulation of blood; they also promote blood clotting and thrombogenesis following endothelial injury. Surgical harvesting and tissue handling play a critical role in the pathogenesis of acute or early graft failure. Vein harvesting leads to disruption and damage to the endothelium. The exposed basement membrane and extracellular matrix recruits platelets, neutrophils, monocytes and fibrin. This leads to limited release of anticoagulant and vaso-relaxing factors and increased secretion of pro-coagulant and vasoconstrictors. Also when the vein is dissected and cut in preparation for CABG, vasa vasorum, the micro vascular network, which provides oxygen and nutrition to the veins, gets disrupted and the endothelium of the vessel loses its protective factors including nitric oxide and prostacyclin [63]. Prostacyclin and nitric oxide secreted by the endothelium normally result in the relaxation of smooth muscle by increasing platelet and smooth muscle cell cyclic GMP (cGMP). Adenosine, on the other hand, also released by the endothelial cells induces smooth muscle cell relaxation by means of the adenosine-receptor coupled G protein and adenylate cyclase, by modulating the cyclic-AMP (cAMP) levels in the conduits [64]. Endothelial injury impairs these biochemical pathways, leading to loss of vasomotor function and contributing significantly to graft occlusion. Activated platelets recruited by damaged endothelium also secrete vasoactive substances such as adenosine diphosphate, adenosine triphosphate, 5-Hydroxytryptamine, thromboxane A$_2$, and platelet activating factor. These substances together, potentiate vasoconstriction and recruitment of more activation of platelets, leading to accelerated thrombogenesis and graft failure. Hypoxemia due to the disruption of vasa vasorum also leads to generation of oxygen free
radicals and can eventually lead to endothelial dysfunction [62]. There is also an increased expression of matrix metalloproteinases especially MMP-2 and MMP-9 [65] and hyperplasia [66].

Re-endothelialisation starts within 24 hours to replace the disrupted endothelium. It is, however, often incomplete [67] and along with other changes, results in activation of inflammatory mediators and eventually leads to intimal hyperplasia [68].

In brief, the acute graft failure (Figure 7) of the vein graft can be attributed to the response of the vessel to a combination of mechanical insult and the resulting physiological adaptation and transient ischaemia.
1.3.4. Midterm graft failure

Failure of complete re-endothelialisation, which results in inflammation, activation, or de-differentiation of vascular smooth muscle cells (VSMCs) and neointimal hyperplasia are responsible for mid-term vein graft failures (Figure 8).

Figure 8: Pathophysiology of vein graft failure (Image from Circulation. 136(18): 1749-1764, with permission from M Gaudino)

- Neointimal hyperplasia is the accumulation of fibrocellular content on the luminal aspect of the vessel, within the internal elastic lamina (IEL). It is the response of blood vessels to injury or denudation, and is predominantly due to phenotypic modulation of vascular smooth muscle cells of the media that migrate to the region between the endothelium and IEL, where they synthesise extracellular matrix.

- Smooth muscle proliferation in response to atherogenic stimuli is an important step in the formation of neointima [69]. The smooth cells of the media have a high proliferative index and extracellular matrix production (ECM) and produce chemoattractant
mediators of inflammation. The inflammatory mediators and markers produced in this layer include platelet-derived growth factors (PDGF), transforming growth factors (TGF), macrophage inhibitory factors (MIF), gamma interferon (G-IFN), monocyte chemotactic protein -1 (MCP-1), tumour necrosis factor (TNF), fibroblast growth factors (FGF) and insulin like growth factor (IGF). These mediators promote the proliferation of vascular smooth muscle cells (VSMC), migration of the smooth muscle cells and extracellular matrix deposition [70].

Smooth muscle cell proliferation and neointimal hyperplasia are not demonstrated in the normal endothelium of saphenous veins. However, within four to six weeks, nearly all SVGs subjected to trauma, mechanical forces and high arterial pressure, demonstrate a phenotypic modulation of the vascular smooth cells [71]. These synthetic or ‘de-differentiated’ vascular smooth cells (VSMCs), exhibit an increased rate of proliferation, migration, and synthesis of extracellular matrix components and have decreased expression of SMC- specific contractile markers, such as smooth muscle myosin heavy chain, smooth muscle actin, caldesmon, calponin, and smoothelin [71]. Upregulation of VSMC phenotype switching contributes to the development and progression of the intimal hyperplasia [72, 73].

Phenotypic modulation of the VSMC is influenced by various factors of which environmental factors play an important role in the intimal hyperplasia. The environmental factors include mechanical forces as mentioned earlier, interaction of extracellular matrix, release of cytokines, cell contact and cell adhesion [74, 75]. The other factors namely growth factors including platelet-derived growth factors, fibroblast growth factors and epidermal growth factors, also contribute in the phenotype switching of the smooth muscle cells [76, 77].

Various matrix glycoproteins are upregulated in response to the injury to the vessel wall. These glycoproteins present in the ECM also help in the phenotypic modulation of vascular smooth muscle cells, proliferation and migration. The reported glycoproteins include thrombospondin [78], fibronectin-1[79], Vitronectin[80], Tenascin-C [81, 82], Osteopontin[83], Hyaluronic acid [84]and collagen VIII [85]. Koyama et al. reported the expression of these glycoproteins to be elevated for up to two weeks after deposition of collagens, elastin and proteoglycans take place [86]. As a result of these modifications, there is a change in total collagen content from 25% of vessel wall protein in the normal vessel to almost 50% of total vessel wall protein in the
vessel wall 30 days after injury [87]. Adventitial fibroblasts similarly contribute to the neointimal hyperplasia by myofibroblast phenotype modulation with increased proliferative capacity. It is however observed only in the first few days. Although the exact mechanism is not sure, it is proposed that the migration of the adventitial fibroblasts to the intima contribute to ECM deposition [88, 89].

Smooth cell migration plays a pivotal role in the development of neointimal hyperplasia. Migration of the cells is regulated by cell attachment and detachment from the extra cellular matrix (ECM), contraction of muscle proteins and cytoskeletal plasticity. Cell migration requires extra cellular matrix degradation by a variety of proteinases, amongst which matrix metalloproteinase (MMPs) play an important role.

MMPs also known as matrixins are a group of glycopeptidases capable of degrading various components of extracellular matrix. Excess MMPs degrade the structural proteins of the blood vessel and hence dysregulation of the balance between MMPs and their inhibitors, Tissue inhibitor of metalloproteinases (TIMPs) is a characteristic of acute and chronic cardiovascular diseases [90]. MMPs are usually latent proenzymes and have to be activated in the extra cellular space by proteolytic cleavage of N-terminal propeptide domain[91]. Based on the structure and substrate specificity, they are classified into five groups: matrilysins, collagenases, stromelysins, gelatinases and the membrane type MMP (MT-MMP). Their main function is to degrade ECM and enhance the availability of other active growth factors and cytokines. Up-regulation and activation of matrix metalloproteinases especially MMP-2 and MMP-9, both also called as gelatinases, have been implicated in cellular migration[70]. The usual substrates of the gelatinases are type IV collagen and gelatin, and these enzymes are characterised by the presence of an additional domain inserted into the catalytic domain. Pre-existing high expression of matrix metalloproteinase-2 in tunica media of saphenous vein conduits is associated with unfavourable long-term outcomes after coronary artery bypass grafting [92].
1.3.4.1 Transforming growth factor

Transforming growth factor-beta 1 (TGF-β1) plays an integral role in the development of neointimal hyperplasia after vascular injury. There is strong association of transforming growth factor-β1 (TGF-β1) with extracellular matrix (ECM) deposition, and it is now identified as an important regulator of neointima formation [93]. TGF-βs are a group of growth factors or proteins that belong to the TGF-β superfamily of cytokines. Of the four TGF-β isoforms reported, only three isoforms are expressed in humans – TGF-β1, TGF -β2 and TGF-β3 [94]. TGF-β1 is the most extensively studied and is considered the prototype of TGF-β isoforms. TGF-β is produced as a large precursor, pre-pro-TGF-β, and requires sequential proteolytic digestion and activation for the release of active TGF-β from its pro-region. The pro-region is called the latency-associated peptide (LAP) [95]. LAP and mature TGF-β are held together by a non-covalent interaction in 1:1 ratio [96], preventing or inhibiting the mature TGF-β from interacting with its high affinity plasma membrane receptors [97]. Dimerization occurs between them to form, the small latent complex (SLC) [98]. The Small latent complex binds with another gene product called Latent TGF-β binding protein (LTBP) through disulfide bonds between cysteine residues in the LAP portion of SLC and specific cysteine residues in LTBP, forming the large latent complex (LLC) [99].

Activation of TGF-β1 requires the release of the LLC from its associated ECM, and/or subsequent release of mature TGF-β1 from the SLC [100, 101]. LAP is either proteolytically cleaved or undergoes conformational changes to release the mature TGF-β1. However, biological activation is a complex and regulated process.

The mature TGF-β1 is released from its latent form and exerts its biological effects through binding to cell-surface TGF-β1 receptors (Tβ-R). There are three TGF-β receptors in humans: Tβ-RI, Tβ-RII and Tβ-RIII. The Tβ-RI and Tβ -RII are serine/threonine kinase receptors having a cysteine-rich extracellular domain, a transmembrane domain and a cytoplasmic serine/threonine-rich domain [94]. In the absence of TGF-β1 ligand, Tβ-RI and Tβ-RII exist as homodimers on the cell surface. TGF-β1 ligand binds to Tβ-RII to form an initial ligand-receptor complex. This complex, in turn, recruits Tβ-RI making a bigger and complete ligand-receptor-receptor complex that transduces cell signalling.
Seven Tβ-RI and five Tβ-RII receptors have been identified in mammals [102]. The Tβ-RI is also known as activin-like kinase-5 (ALK5) having a characteristic Gly-Ser (G-S) sequence upstream from the kinase domains [103]. Activation of Tβ-RI, and consequent cell signalling, requires phosphorylation of its G-S domain by Tβ-RII. There are seven ALKs expressed in humans and of these, two ALKs are important in the vascular system: ALK1 and ALK5. They differ by using different pathways for signal transduction. ALK1 stimulates cell proliferation and migration through Smad1 and Smad5 signal pathway, whereas ALK5 activates Smad2 and Smad3 to counteract these effects. These different signalling pathways account for the diverse pleiotropic effects of TGF-β on proliferation and migration of cells and the state of the endothelium [103]. ALK1 is expressed in the endothelial layer of blood vessels, whereas ALK5 expression is localized to the media and adventitia and was undetected in the intimal layer [104].

TGF-β1 is a pleiotropic cytokine that is expressed by all cells and tissues within the body. It also regulates a number of cellular processes including proliferation, differentiation, migration, ECM synthesis and apoptosis. TGF-β’s effect on the cardiovascular system is both “protective” as well as pathological. Its effect on the anatomy of the vasculature is complex due to the TGF-β’s ability to stimulate other vasoactive substances including angiotensin-II, ET-1, platelet-derived growth factor (PDGF), thrombin and CTGF. TGF-β1 can be both growth promoting and growth inhibiting for vascular SMC. At low concentration (<0.1ng/mL), TGF-β1 potentiates growth of vascular SMC; while at higher concentration it diminishes growth, which may be associated with levels of PDGF-A and PDGF-β receptor [105]. The varying effects of TGF-β1 in vivo have been attributed to variations in the concentration and availability of its signalling receptors [106]. It has been suggested that the normal vessel contains Tβ-RII in abundance, which results in cell signalling that promotes contractile protein expression but not ECM production. Diseased vessels in contrast have higher Tβ-RI receptor levels that promote ECM production in response to TGF-β1 [106].

TGF-β1 exerts its effects on cell surface receptors that mediate intracellular signalling via Smad signal pathways. Smads are intracellular signalling proteins [103]. There are five receptor-activated Smads (R-Smads): Smad1, Smad2, Smad3, Smad5 and Smad8. Their activation and
consequent cellular responses depends upon the type of cell receptor that they are associated with. Tβ-RI. ALK5 activates Smad2 and Smad3, whereas, ALK1, ALK3 and ALK6 activate Smad1, Smad5 and Smad8 [102, 107, 108]. The R-Smads bind to common-mediator Smad (Co-Smad) Smad4 to form a heterotrimeric complex of two R-Smads and one Smad4, which is translocated into the nucleus [109]. Smad6 and Smad7 are inhibitory Smads (I-Smad) for R-Smads [94, 100, 103].

NIH in SVG has shown increased levels of expression of TGF-β1, LTBP-1 and Tβ-RII when compared to pre-bypass grafts. Similarly, although levels of TGF-β1 expression in internal mammary artery grafts are lower than those seen in SVG, their expression is significantly higher than in control internal mammary grafts [110].

Though a great structural homology is shared between TGF-β1 and TGF-β3, the functional effects of both are quite different. TGF-β3 has been shown to inhibit TGF-β1-induced ECM expression in fibroblasts, suppress phenotypic modulation of fibroblasts to myofibroblasts and decrease TGF-β1-induced scar formation. Similarly, TGF-β3 has been shown to down-regulate TGF-β1-induced collagen and TGF-β1 mRNA levels [93]. Unlike TGF-β1, adenovirus-mediated TGF-β3 gene transfer into porcine arteries significantly reduced luminal loss primarily through inhibiting constrictive remodelling [111]. Significant intimal thickening is seen at the site of reanastomosis of common carotid arteries in a goat model. Vessels infiltrated with TGF-β3 at the time of surgery demonstrated reduced vessel wall thickening at the site of the anastomosis three months post-surgery with reduced cellular content and decreased collagen VIII levels [112].

TGF-β1 hence has a significant influence on ECM production in injured vessels. It acts within the vessel wall to promote the formation of NIH. Extracellular matrix production comprises 90% of the bulk of the neointima, and antagonising TGF-β1 has shown to inhibit neointima formation.

Midterm graft failure of the saphenous vein graft can hence be summarised as a failure of the graft to remodel and to adapt to the new haemodynamics it is subjected to, after coronary artery bypass grafting [113-116].
Atherosclerosis is an important factor in the late graft failure, which is characterised by continued vessel wall remodelling and intimal fibrosis [117]. Atherosclerosis is a chronic inflammatory process, resulting from the interaction of oxidised cholesterol particles with monocytes derived macrophages, T-cells and normal cells of the vein wall, which results in the narrowing of the vessel. The pathogenesis of atherosclerosis is a result of a combination of inflammatory infiltration of the vessel wall, cellular proliferation, and fibrous plaque formation. Atheromatous plaques can be seen as early as one-year post-CABG[118]. These atherosclerotic plaques are similar to the native coronary artery atherosclerotic plaques, composed of mainly smooth muscle cells, foam cells, lymphocytes and giant cells. Peykar et al. however, have reported that the venous atheromatous plaques are more vulnerable to rupture [117]. This is due to the fact that the venous atheromata are more diffuse and concentric and have poorly developed fibrous caps[35].

The role of lipids as a significant risk factor in the pathogenesis of vein graft atherosclerosis has been reported [119]. Campeau et al reported elevated levels of apolipoprotein B and low levels of high-density lipoproteins to be predictive factors for the development of vein graft atherosclerosis[119]. Even though the Cholesterol Lowering Atherosclerosis Study (performed to test the effect of lipids on the progression of atherosclerosis in native coronary vessels versus vein grafts) reported a significant reduction of new lesions in the native coronary artery and vein graft [120], the treatment did not completely eliminate the disease, indicating that multiple factors were responsible for atherosclerosis[121].

Other factors, which may contribute to the progression of atherosclerosis in vein graft, include higher mean arterial pressure and smoking. Though not researched extensively, a small study group demonstrated a link to elevated homocysteine levels in the progression of atherosclerosis[122].

1.4. Strategies to prevent vein graft failure

Vein graft failure has the potential to lead to recurrence of angina, MI, death or repeat vascularisation along with their associated costs and morbidities. This imposes a major clinical and economic problem on society. In one retrospective analysis using the Duke Cardiovascular
Databank, Halabi et al. analysed baseline clinical and angiographic characteristics and clinical outcomes among patients who underwent catheterisation 1 to 18 months after their first CABG from 1986 to 2004. They reported that most events occurred immediately after catheterisation in patients with critical and occlusive SVG disease and were primarily repeat revascularisation. On multivariate analysis, critical, non-occlusive SVG disease was the strongest predictor of the composite outcome (hazard ratio 2.36, 95% confidence interval 2.00 to 2.79, \( p < 0.0001 \)).[123].

Different strategies were implemented in an attempt to prevent vein graft failure. Most of them were preventative measures aimed at halting or delaying the neointimal hyperplasia. These can be broadly categorised as:

- Pharmacological agents
- Surgical techniques - No touch technique in vein harvesting and anastomosis
- Mechanical support, including external stent
- Cardiovascular Gene therapy
- Stem cell therapy

**1.4.1 Pharmacological agents**

There are only a few pharmacological agents, which appears to have beneficial effects on early vein graft remodelling or subsequently accelerated atherosclerosis. Currently, the drugs which are used to prevent vein graft failure are lipid-lowering agents and antiplatelet drugs. Some other drugs which may be of specific benefit in preventing vein graft disease, but have not yet been reported in humans are angiotensin II converting enzyme (ACE) inhibitors [124, 125], endothelin-1 (ET-1) antagonist [126], antioxidants [127, 128], beta-adrenoceptor antagonists [129] and growth factor inhibitors.

**1.4.1.1. Statins**

The benefit of statins in improving cardiovascular outcomes after coronary artery bypass grafting has been observed in trials and observational studies [130, 131]. HMG-CoA reductase inhibitors (atorvastatin, fluvastatin, rosuvastatin, lovastatin, pravastatin and simvastatin), help in the inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase by blocking
substrate binding to the active site of the enzyme [132]. The latest ACC/AHA guidelines for CABG highlight the beneficial role of statins in LDL-lowering therapy for VGF prevention. The recommended optimal dose of statin is to maintain LDL target levels <2.5 mmol/l and even <1.9 mmol/l in high-risk patients [133]. The study which looked at the benefits of aggressive lipid-lowering agents in prevention of vein graft failure, The NHLBI Post CABG Clinical Trial, reported a 31% reduction in the obstructive changes of the vein graft over 5 year period, by maintaining the LDL cholesterol level to 2.4 – 2.5 mmol/l [134].

Statins significantly helps in reducing peri-operative mortality, stroke and atrial fibrillation [135]. Preoperative treatment with statins, even for the short term before CABG, leads to a rapid improvement of endothelial function in both arteries and veins [136, 137]. However, a meta-analysis of 21 randomised statin trials, using myocardial infarction as the primary endpoint after the procedure, revealed significant benefits of statins post PCI and non-cardiac procedures but not for CABG. This could be due to fewer trials looking into the benefits of statins in CABG [8]. A recent randomised trial by Shah et al. revealed significant benefits of aggressive statin treatment (atorvastatin 80 mg od vs 10 mg od) for repeat coronary revascularisation after CABG, with better control of LDL in the intensive treatment group [138].

1.4.1.2 Anti-platelet drugs

Platelets play an important role in the pathogenesis of early vein graft failure following vascular injury as a result of vessel wall handling and other mechanical forces acting on the vein graft endothelium [139]. Antiplatelets help in reducing vascular events related to the platelet aggregation. Chesebro et al. in their randomised controlled trial at the Mayo Clinic reported that the vein graft patency was significantly better with antiplatelet therapy (aspirin plus dipyridamole versus placebo) both at 1 month (98% versus 90%, aspirin and dipyridamole versus placebo, p < 0.0001) and 1 year (89% versus 77%, aspirin and dipyridamole versus placebo, p < 0.0001) post CABG. [140].

The two most commonly used antiplatelet agents after CABG are Aspirin and Clopidogrel. Though there is a consensus about the administration of antiplatelet drugs, especially aspirin, in reducing the risk of graft failure after CABG [19, 141], controversy remains over the dosage of anti-platelet and the addition of a second antiplatelet [142]. The current AHA / ACC guideline recommends Aspirin (100 to 325 mg) within six hours post coronary artery bypass grafting to
prevent vein thrombosis.[19]. There are only limited studies that looked at the benefits of post-operative Clopidogrel post-CABG probably due to the limited usage after CABG. Gurbuz et al. assessed the impact of Clopidogrel administration on the short-term outcomes of 591 off-pump CABG patients. They reported less symptom recurrence and less adverse cardiac events, including fatal or non-fatal MI or repeat revascularisation, in comparison with patients receiving Aspirin after CABG [143].

1.4.2 Surgical techniques

Surgical technique is a major determinant factor in preventing acute vein graft failure by preventing thrombosis. There are a few reports that a ‘no touch’ technique may improve the patency of the vessel by leaving intact, the fatty and muscular tissue along with the adventitia that immediately surrounds the saphenous vein [144]. In recently published randomised, longitudinal studies, no-touch saphenous vein grafts showed a significantly higher patency rate than radial artery grafts. Patency was comparable to the patency for left internal thoracic artery grafts both at a mean follow up of 6 and 16 years respectively [49, 145, 146].

Off-pump coronary artery bypass grafting which was reported to reduce renal impairment, stroke and atrial fibrillation in comparison with on-pump surgery, have been, however, reported to affect the vein graft patency. The graft patency was affected both in the early and midterm follow-up at angiography [16, 147] However a recent prospective multicentre randomised pilot study compared graft patency after on-pump and off-pump techniques and reported similar graft patency at 1 year [16, 148]. In addition to reducing the superficial surgical site infection the leg, endoscopic vein harvesting techniques have now been shown to be no worse than open vein harvesting, in terms of vein graft patency [51, 149].

1.4.3 Mechanical support including internal or external stent

Mechanical support in the form of PCI and stent deployment has always been implemented as a therapeutic modality in diseased vein grafts. Though the use of stents did offer some benefit immediately, stent expansion has resulted in worse outcomes, including increased myocardial infarction rates than in native coronary disease at one year[150].
A few pre-clinical studies have reported long-term inhibitory effects on neointimal and medial thickness following the placement of external sheaths (Figure 9) [149, 151, 152]. It is proposed that this external sheath could offer protection by forming a neoadventitia between the sheath and the graft and could thus preserve the endothelium cells, smooth muscle cells and elastic fibres. Different types of such sheaths have been tried experimentally on animal models, like polyester [153], Dacron [154], PTFE [155], biodegradable polyglactin [156], nitinol mesh [157] and drug eluting mesh [153, 158]. A recent clinical study looking at the effect of the external sheath on vein graft hyperplasia had to be abandoned, due to acute thrombosis caused by the rigid external sheath [159]. The long-term results of such sheath are still awaited.

1.4.4. Cardiovascular gene therapy

Due to the relative lack of efficacy of currently used therapy in the prevention of VGF, the pathogenesis mediating the development of neointimal formation of the vein graft at molecular and cellular level have been investigated. This quest has resulted in the identification of modifiable targets in the vein graft for implementation of gene therapy in an attempt to prevent vein graft failure.
Currently, the application of gene therapy for cardiovascular disease is most frequently used in clinical trials in angiogenesis (Table 1). The other areas where cardiovascular gene therapy could be potentially beneficial includes prevention of thrombosis, management of heart failure [160], hypertension [161] pulmonary hypertension [162] and regulation of pacemaker cells [163].

Vascular gene therapy is aimed at prevention of intimal hyperplasia of saphenous vein grafts and in-stent restenosis. Intimal hyperplasia occurs as a part of vascular healing response resulting from SMC proliferation and migration, and extracellular matrix deposition with superimposed atherosclerosis. Gene therapy strategies are hence to prevent thrombosis, enhance re-endothelialisation, decrease SMC proliferation and migration, and reduce ECM deposition and atherosclerosis. Vascular gene therapy in the prevention of vein graft failure requires careful selection of transgenes that will deliver the appropriate response or modification phenotypically at the right time period, selection of appropriate gene delivery vectors and identification of suitable cellular promoters which will enable transgene expression[164]. For CABG, saphenous veins after harvest from the leg are well-suited as a potential target for gene therapy ex-vivo, for the administration of vectors before they are anastomosed to the coronary artery and ascending aorta.

1.5. Vectors for gene delivery

Several vectors have been used, for preclinical studies, which include both viral and non-viral vectors, each having its advantages and disadvantages.

The ideal requirements of vectors for gene delivery are:

- The vectors should be able to transduce target cells with minimal effect on non-target cells.
- They should also be non-cytotoxic
- They should have minimal side effects
- They produce a clinical response by sustained transgene expression.[165].
1.5.1. Non-Viral Vectors for Cardiovascular gene therapy

Non-viral vectors have been employed in clinical studies for cardiovascular gene therapy [166]. Different forms of non-viral gene transfer have been tested, including naked plasmid DNA, polymer–DNA complexes, lipid-DNA, etc. Non-viral vectors are less immunogenic, can be produced in large quantities and are cheaper and comparatively safer. However, they are inefficient for in-vivo gene transfer in the myocardium. They also offer less transduction to the target cells and the expression is usually of relatively shorter duration [167].

Few techniques have been utilised to increase the efficiency of transduction with non-viral vectors, like electroporation [168] and ultrasound mediation in pre-clinical studies [169]. Akowuah et al. reported significant biological effects in a porcine model of saphenous vein grafting by non-viral TIMP-3 plasmid delivery [169]. Despite the pre-clinical results, both electroporation and ultrasound mediation were not used for clinical studies.

1.5.2. Viral Vectors for Cardiovascular gene therapy

The first study about the transfer of recombinant genes into the endothelium and vascular smooth muscle cells using retroviral vectors transfection was reported in the early 1990s [170]. Viral vectors, in contrast to non-viral vectors, can deliver genes to cells in order to provide either transient (e.g. Adenovirus, Vaccinia virus) or permanent (e.g. Retrovirus, Adeno-associated virus) transgene expression.

The most commonly used viral vectors, such as Adenovirus (Ad) or Adeno-associated viruses (AAV), however, have a propensity to transduce non-vascular cells as well as vascular cells, which poses a big challenge to its application in a clinical setting. Other viral vectors in use include Lentivirus [171], Retrovirus, Sendai virus [172], Herpes simplex virus and to a smaller extent, Baculo virus [173] and Semliki forest virus. Poor target cell selectivity, pre-existing antibodies and immunogenicity generated by the host tissue could, however, be potential problems in the application of viral vectors in the management of cardiovascular disease.

1.5.2.1. Adenoviruses

Currently, adenoviruses are the most widely used viral vectors in the clinical trials of cardiovascular gene therapy. They are naturally occurring viruses, with a naked, double-
stranded DNA genome. There are more than 50 serotypes identified which have been divided into seven species or groups (A – G). The species C serotype Ad 5 (Ad5) is the most commonly used serotype in experimental and clinical studies.

Serotype Ad 5 (Ad5) is mainly reliant on the primary surface receptor, Coxsackie and Adenovirus receptor (CAR), for binding to target cells. Coxsackie and adenovirus receptors have integrin as co-receptors [174]. Some independent studies in the last decade have also identified another co-receptor, heparin sulphate proteoglycans (HSPGs) that can mediate hepatocyte transduction of interactions of adenoviral serotypes with blood factors. [175-177]. The genes in the genome are organised as early (E1-E4) and late (L1-L5) regions.

Adenovirus efficiently transduces dividing and non-dividing cells. While this is advantageous, they carry a risk of non-target effects. As they are highly immunogenic, cells transduced by adenovirus-mediated gene transfer are eliminated in a short duration, as the pro-inflammatory antigens trigger neutralising antibodies. Their gene expression peaks at 7-14 days but is lost in about 28 days [178]. This feature could, however, be used in the management of conditions like acute PCI complications and vein graft failure whose pathology is mostly due to an acute denudation of vascular endothelium.

There are innate and acquired immune and inflammatory responses involved in the rapid clearance of adenovirus infected cells, which reduces the ability of gene expression at desired duration [179]. It is estimated that more than 90% of the general population have got neutralising antibodies against the adenovirus, especially serotype c, which is one of the prime reasons for the reduced infective efficacy [180]. ‘Detargetting’ and ‘retargeting’ strategies have helped to evade the host response to the viral vectors, thus preventing the early expiry [180, 181]. A recent study by George et al. reported that increased exposure to the target tissues (saphenous vein segment) lead to improved efficacy of the first generation vectors[182].

Recombinant vectors are normally derived from serotype 5, adenovirus (Ad5), subgroup C; to ensure replication deficiency of the virus, E1 region is deleted. First-generation vectors have usually had the E1 and E3 regions removed. Second-generation modifications include deletion of the E4 and E2A regions/ functional mutations in the E2A region [183, 184]. The second-generation modifications offer little benefit over first-generation vectors: a 4-fold increase in transgene expression was observed at days 10 after vessel infection, but no difference was
observed at day 3 or day 28[184]. With further removal of all wild-type adenovirus-coding sequences, has given third-generation recombinant Ads. This has expanded the cloning capacity to 35kb compared to 8kb first-generation vectors. In addition, helper-dependent Ads have demonstrated markedly reduced host immune responses and stable gene expression from day 14 to day 56 [185]. Despite these benefits there have been no clinical trials carried out using helper-dependent Ad vectors. This may be because of the difficulty in making these vectors in large quantity, and the fact that peak transgene expression has been reported to be only 10% of that observed with first-generation Ad vectors [186]. As a result, first-generation vectors have frequently been used in gene delivery due to ease of manufacture.

1.5.2.2. Adeno-Associated Virus (AAVs)

Adeno-Associated Virus (AAV) is a group of non-enveloped, non-pathogenic parvoviruses, which have potential as vectors for gene therapy. AAV belong to the genus Dependovirus, which require a helper virus, such as adenovirus or herpes simplex virus, to facilitate replication. They are not associated with any pathology in humans and lack toxicity in vivo. AAV has the ability of long-term gene expression through either episomal or integrative mechanism in the nucleus. There are 11 serotypes (all have been tested in pre-clinical studies) and more than 100 variants identified to date, of which, human adeno-associated virus serotype 2 (AAV-2) has gained popularity for almost two decades[187].

These viral vectors have been tested in preclinical studies for a variety of diseases such as Haemophilia, alpha-1 anti-trypsin deficiency, cystic fibrosis, Duchene muscular dystrophy, and rheumatoid arthritis. Recombinant AAV is very efficient in transducing cardiomyocytes. Their application in heart failure was first demonstrated in CUPID trial, which evaluated the effectiveness of a gene transfer vector based on adeno-associated virus 1 (AAV1) for delivery of SERCA2a complementary DNA, in 39 patients with advanced heart failure. [188].

1.5.2.3. Lentivirus

Lentiviruses are a subgroup of viruses from the retrovirus family. They are enveloped, spherical viruses and have a single-stranded RNA genome in a bilipid layer. They are derived from human immunodeficiency virus (HIV-1), which, unlike other retroviruses, can transduce dividing and non-dividing cells with long-lasting transgene expression. The lentiviral proteome consists of five major structural proteins and 3-4 non-structural proteins. The primary cell receptor for HIV
is the T-cell, CD4 receptor. The vesicular stomatis virus glycoprotein (VSV-G) is commonly incorporated into the vector envelope to change the virus tropism[189]. Lentiviruses have gag, pol and env genes, coding for viral proteins in the order: 5´-gag-pol-env-3´ like other retroviruses; in addition they two more regulatory genes tat and rev and few accessory genes. Five serogroups of lentiviruses are recognized, according to their association with the vertebrate hosts, which includes primates, sheep and goats, horses, domestic cats, and cattle.

The first generation Lentiviruses were made by replacing HIV envelope protein with more promiscuous envelope glycoprotein (G) of vesicular stomatitis virus (VSV). The stable glycoprotein of VSV allowed concentrating the viral particles released into the culture media from “producer” cells by ultracentrifugation and the infectious titre thus produced was hundred folds higher than previously produced and did demonstrate transduction of non-dividing cells[190]. Sequences encoding proteins needed for maintaining the HIV virulence, but with no apparent role in the transduction process were deleted from the packaging construct to generate second generation [191] (ΔVpr, Vif, Vpu, Nef) and third (ΔTat) generation [192] vector systems, ensuring removal of pathogenic features of the parental virus.

Lentivirus has been demonstrated to be superior to Adenovirus serotype 5 as transducers of coronary endothelial cells and vascular smooth cells in vitro and in rat carotid arteries. [193]. Dishart et al. demonstrated the potential for third-generation lentiviral vectors as efficient vascular gene delivery vectors. In their study, Dishart and colleagues tested third-generation lentivirus, derived from human immunodeficiency virus-1 (HIV-1), pseudotyped with (VSV-G), for transduction of both SMC and EC and compared this to permissive HeLa cells. In direct contrast to Adeno Associated Virus, lentiviruses efficiently transduced both EC and SMC. [194]. However, there have been reports of insertional oncogenic risk with retroviral vectors [195, 196]. The mutagenic potential of Lentivirus, though it is low, could pose a threat to its use in the clinical setting even though there have been no reports of recombinant lentivirus-induced mutation to date. With the advent of developing non-integrating lentiviral vectors, the risk of theoretical oncogenesis is alleviated. There are encouraging reports of both in vitro [197] and in-vivo studies [198] demonstrating stable transgene expression.
<table>
<thead>
<tr>
<th>Viral System</th>
<th>Size</th>
<th>DNA insert size</th>
<th>Max titre (particle s/mL)</th>
<th>Infection</th>
<th>Expression</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenovirus</td>
<td>36 kb (dsDNA)</td>
<td>8 kb</td>
<td>$1 \times 10^{13}$</td>
<td>Dividing and non-dividing cells</td>
<td>Transient</td>
<td>Elicits strong antiviral immune response</td>
</tr>
<tr>
<td>Retrovirus</td>
<td>7–11 kb (ssRNA)</td>
<td>8 kb</td>
<td>$1 \times 10^{9}$</td>
<td>Dividing cells</td>
<td>Stable</td>
<td>Insertional mutagenesis potential</td>
</tr>
<tr>
<td>Lentivirus</td>
<td>8 kb (ssRNA)</td>
<td>9 kb</td>
<td>$1 \times 10^{9}$</td>
<td>Dividing and non-dividing cells</td>
<td>Stable</td>
<td>Insertional mutagenesis potential</td>
</tr>
<tr>
<td>Adeno-associated virus</td>
<td>8.5 kb (ssDNA)</td>
<td>5 kb</td>
<td>$1 \times 10^{11}$</td>
<td>Dividing and non-dividing cells</td>
<td>Stable; site-specific integration</td>
<td>Requires helper virus for replication; difficult to produce pure viral stocks</td>
</tr>
<tr>
<td>Vaccinia virus</td>
<td>190 kb (dsDNA)</td>
<td>25 kb</td>
<td>$3 \times 10^{9}$</td>
<td>Dividing cells</td>
<td>Transient</td>
<td>Potential cytopathic effects</td>
</tr>
</tbody>
</table>

Table 1. The key properties of viral vector system
1.6. Preclinical and clinical trials looking at potential genes for prevention of vein graft failure.

The potential therapeutic genes, include neuronal nitric oxide synthase (NOS)[199], Tissue inhibitors of matrix metalloproteinases (TIMP -1, 2, 3) [169], p53 [200, 201], Glucagon like peptide[202], Antisense to transforming growth factor-beta(TGF-Beta) [203] and VEGF[204].

<table>
<thead>
<tr>
<th>Preclinical/ Clinical target</th>
<th>Animal Model</th>
<th>Transgene studied</th>
<th>Trials</th>
<th>Endpoint</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH</td>
<td>Saphenous vein graft, pig</td>
<td>TIMP-3</td>
<td>Akowuah <em>et al.</em> (2005) [169]</td>
<td>Increased luminal area</td>
</tr>
<tr>
<td>NIH</td>
<td>Vein graft, Dog</td>
<td>betaARKct peptide</td>
<td>Petrofski et al.[205]</td>
<td>Decreased NIH, Decreased VSMC proliferation</td>
</tr>
<tr>
<td>NIH</td>
<td>Human SVG</td>
<td>NOS</td>
<td>Cable DG 1999 [206]</td>
<td>Intima/media ratio</td>
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<tr>
<td>NIH</td>
<td>Vein graft, Dog</td>
<td>NOS</td>
<td>Matsumoto 1998 [207]</td>
<td>Decreased NIH</td>
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<tr>
<td>NIH</td>
<td>Jugular vein graft, Rabbit</td>
<td>NFkB decoy</td>
<td>Miyake 2006 [208]</td>
<td>Decreased NIH</td>
</tr>
<tr>
<td>NIH</td>
<td>Jugular vein, rabbit</td>
<td>COX-1</td>
<td>Eichsteadt 2008 [209]</td>
<td>Lumen size and blood flow</td>
</tr>
<tr>
<td>NIH</td>
<td>Jugular vein, rabbit</td>
<td>Superoxide dismutase</td>
<td>Turunen 2006 [210]</td>
<td>Decreased NIH</td>
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<tr>
<td>NIH</td>
<td>Jugular vein, rabbit</td>
<td>Polyethylene glycolated superoxide dismutase</td>
<td>Huynh 1999[211]</td>
<td>Decreased NIH, Decreased malondialdehyde concentration.</td>
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<td>NIH</td>
<td>Vascular graft, rabbit</td>
<td>NOS</td>
<td>West <em>et al.</em> (2001) [199]</td>
<td>Reduced neointimal hyperplasia</td>
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<tr>
<td>Thromboresistance</td>
<td>IVC, rats</td>
<td>Thrombomodulin</td>
<td>Tabuchi[212]</td>
<td>Maintenance of thromboresistance</td>
</tr>
<tr>
<td>Vein graft disease</td>
<td>E2F decoy</td>
<td>Mann <em>et al.</em> (PREVENT I) [213]</td>
<td>Safety and occlusions</td>
<td></td>
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<td></td>
<td></td>
<td>Grube <em>et al.</em> (PREVENT II)</td>
<td>Occlusion, vessel wall thickness</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Conte <em>et al.</em> (PREVENT III) [214]</td>
<td>Reintervention</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Alexander <em>et al.</em> (PREVENT IV) [215]</td>
<td>Angiographic failure</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Preclinical and clinical trials looking at potential genes for prevention of vein graft failure.
1.7. Gene Therapy Strategies to prevent VGF

The strategies involved in preventing vein graft failure are

- Anti-proliferative
- Anti-migratory
- Pleiotropic
- Improvement in re-endothelialisation and endothelial function strategies.

Though the above-mentioned strategies have been studied in both arteries and veins, we will be concentrating on those studied on veins.

1.7.1. Anti-proliferative strategies

Smooth cell proliferation subsequent to vascular endothelial injury occurs due to phenotype switching of vascular smooth muscle cells present in the media of saphenous vein graft. Smooth cell proliferation involves various mediators including cytokines and growth factors. The proliferating cells in the intima consist mainly of monocyte/macrophage in contrast to vascular smooth muscle cells, which is the most prevailing cell type in the media[69].

Anti-proliferative strategies have been an area of interest for many investigators in gene therapy for the management of vein graft failure[216]. Anti-proliferative strategy is achieved by inhibiting various steps in the cell cycle (cytostatic) or by inducing death of the injured cells (cytotoxic).

In general, the anti-proliferative strategy for the treatment of experimental cardiovascular diseases can be grouped into:

A) Inactivation of the positive cell cycle regulators, e.g., CDK/cyclins, proto-oncogenes, E2F, growth factors

B) Forced overexpression of negative regulators of cell growth, e.g., CKI, p53, pRb, GAX, GATA-6.

Retinoblastoma protein (Rb) is one of the key regulators of the cell cycle which can modulate the activity of the E2F family of transcriptional regulators, required for S-phase progression of the cell cycle [217]. In the presence of any vascular injury, the Rb protein is phosphorylated at multiple sites by cyclin/cdk complexes to generate hyperphosphorylated Rb, which is unable to repress E2F-dependent transcription. Some cyclin dependent kinase inhibitors that act to prevent Rb hyperphosphorylation and thereby maintain cells in the G1 phase regulate Rb
kinase activity. In studies carried out on rat carotid artery, virus-mediated transfer of a non-phosphorylable form of Rb resulted in significant reduction in smooth muscle cell proliferation [218]. In another study by Chang et al. it was reported that a non-phosphorylable, active form of murine Rb protein could inhibit vascular smooth cell proliferation and reduce neointima formation in the injured rat carotid artery and porcine femoral artery models [219]. The majority of studies attempting to modify the process of accelerated atherosclerosis have been performed in arterial injury models and hence described here.

The cell cycle regulatory proteins p21 is otherwise known as senescent cell-derived inhibitor-1. Plasmid-mediated delivery of p21 into the jugular vein of rabbit with subsequent grafting as an interposition graft in the internal carotid artery demonstrated a significant reduction in cellular proliferation and reduction in intima: media ratio at 14 days [220].

George et al. showed that wild-type p53 gene transfer inhibits neointima formation in human saphenous vein segments by modulation of smooth muscle cell migration and induction of apoptosis [200]. At one week post-surgery, the same group demonstrated that the neointima thickness had reduced by 70 % in saphenous vein grafts treated with p53; however the effect had diminished to 30 % at 28 days [200].

### 1.7.2. Anti-migrational strategies

Smooth muscle cell migration plays a pivotal role in the development of neointimal hyperplasia. Migration of cells is regulated by cell attachment and detachment from extra cellular matrix, contraction of muscle proteins and cytoskeletal plasticity. The role of MMP in the in the degradation and remodelling of extracellular matrix has been described earlier in the chapter.

Selective gene-silencing of either MMP-2 or MMP-9 have been reported to have reduced the invasive nature of cultured human saphenous vein smooth muscle cells(SV-SMC), indicating that these MMPs may play distinct roles in SV-SMC invasion in vitro [221]. Expression of Tissue inhibitors of metalloproteinases (TIMPs 1-3) can result in the inhibition of matrix metalloproteinases [91]. Adenovirus-mediated transfer of TIMP-1 [222] and TIMP-2[223] have
demonstrated to suppress NIH in injured rat carotid arteries, and TIMP-3 gene transfer from an adenovirus-coated coronary stent has shown to reduce NIH in porcine coronary arteries[224].

George and colleagues have reported series of studies on the influence of three different TIMPs (TIMP 1-3) on neointima formation in human saphenous vein and porcine internal carotid artery. In one of their studies, the effects of expression of TIMP-3 were assessed in the ex-vivo model of human saphenous vein organ culture before being compared with TIMP-2 in a porcine carotid artery interposition graft model in-vivo[225]. Exposure of human vein segments to TIMP-3, mediated by adenovirus was associated with an 86% reduction in neointimal thickness, and with inhibition of MMP activity and increased levels of apoptosis. Expression of TIMP-3 also resulted in reductions of approximately 50% in the neointimal area and intima: media ratio in porcine interposition vein grafts at 28-day post-surgery. In comparison with TIMP -1 and TIMP- 2, TIMP-3 was more effective in reducing the neointimal thickness by reducing the degradation of extracellular matrix and cellular migration. The greater efficacy of TIMP-3 appears to be derived from two factors: its pro-apoptotic effects and the fact that it is highly matrix bound [182, 225, 226]. TIMP-3 was also studied in porcine interposition vein grafts following ultrasound-enhanced plasmid-mediated gene transfer. Though the lumen area of vessels receiving TIMP-3-expressing plasmid was significantly greater than in control groups, the neointimal area was not affected, as the process of ultrasound enhancement of plasmid gene transfer appeared to promote NIH.[169, 227].

1.7.3. Pleiotropic strategy and Endothelialisation

There are some transgenes demonstrating multiple effects, which can, through their expression, result in a range of effects to prevent the mechanisms involved in vein graft failure. Sugimoto et al. have identified the pleiotropic effects of nitric oxide synthase, which generates nitric oxide (NO)[228]. Nitric oxide has a wide range of effects on the vessel wall, including platelet inhibition, inhibition of SMC proliferation and migration, promotion of endothelialisation and endothelium-dependent vasodilation [229]. Adenovirus-mediated gene transfer of neuronal nitric oxide synthase (NOS) has been reported to improve endothelial cell function [230], as well as inhibition of smooth muscle cell proliferation following vein grafting. Intra-operative transfer of NOS results in the reduction of inflammation and endothelial activation [231]. Overexpression of nitric oxide synthase results in a more differentiated
phenotype of intimal smooth muscle cells, which are modulated with less superoxide production. This may help in preventing the vein graft from reactive oxygen-related damage [199].

Endothelialisation, stimulated by vascular endothelial growth factor (VEGF), can in addition, also increases the vascular permeability with the help of nitric oxide [232]. The significance of this gene has been studied in many vascular models and is the only gene to be studied in a clinical trial of neointimal hyperplasia [233]. In a small animal study, stainless steel stents, coated with human umbilical vein endothelial cells (HUVECs) transfected with VEGF, reduced neointimal hyperplasia, promoted endothelialisation, and reduced in-stent restenosis [233].

1.8. Animal Models used to study vein graft failure and interventions

Animal models have been used to study vein graft failure and therapeutic interventions to prevent vein graft failure, which includes pharmacological, surgical and cardiovascular gene therapy [149, 234, 235]. They act as preliminary tools wherein our understanding of the pathology, prevention and intervention in vein graft failure can be explored. Some of the most commonly used animal models used to study vein graft failure and interventions to prevent vein graft failure are described below:

1) Mouse – Very popular experimental animal. Its main advantages are that they are easy to breed, readily available in large quantities, do not require large space for habitation and can be used to administer gene therapy of interest. Also, due to its small size, the time taken to complete anastomosis or any procedure is relatively shorter compared to larger animals like pigs. Mice have been extensively studied in the animal experiments to investigate vein graft failure and, it is very popular to make ‘knockouts’ and ‘knockins’ of genes of interest on mouse models [236-238].

2) Rat – Historically, rats have been used for studying the grafting methods. Both veins and bio-artificial grafts have been studied on rat models. Few notable studies include quantitative study of revascularization and its relationship to intimal hyperplasia by McGeachie et al. [239], and the study of vascular remodelling during vein arterialisation in the rat models by Borin et al. [240] .
3) Rabbit – Rabbits have been used in studying vein graft failure from the late nineteen eighties. Zwolak et al. evaluated the kinetics of smooth muscle cell (SMC) and endothelial cell (EC) replication on rabbit jugular vein segments, which were transplanted into the carotid arterial circulation in order to define the mechanism of cell proliferation in this process [139]. Wong et al. observed expansive remodelling in vein graft of rabbits. They wanted to determine the changes in lumen remodelling in arterialisled vein grafts, including the events at the cellular and extracellular levels, using reversed jugular vein-to-common carotid artery interposition grafts [241]. A similar study on vein graft remodelling induced by shear stress on rabbits was also carried out by Tran-Son-Tay et al. who established a relationship between neointimal thickness, time and shear stress within the graft.[242].

4) Pig – Pig model is considered to be the most clinically relevant due to similar vascular anatomy and haemodynamics of the pig to that of humans. The porcine vessels employed have the similar histological feature to that of human vessels. It is therefore believed that the response of the vein to the surgical preparation and the distribution of endoluminally administered virus vectors within the wall of the vein will be similar to human vein grafts as well. Different strategies to prevent vein graft failure were studied in pig models. By using the saphenous vein into carotid artery interposition (end-to-end) grafts in experimental porcine models, it was possible to demonstrate the luminal thickness and intimal hyperplasia of the vein graft after the administration of Folic acid [243]. The same model was also used to assess the effectiveness of several anti-proliferative agents on graft cells, like cytochalasin D, paclitaxel and rapamycin [244].

Recently, pig model was studied to assess the effectiveness of gene therapy in prevention of acute thrombosis by using tissue plasminogen activator gene therapy [235]. Surgical intervention, like the placement of external stents in the juvenile pig vein graft model [149], simulation of the surgical techniques including different graft anastomosis, and the institution of cardiopulmonary bypass to assess the effectiveness of different strategies in the prevention of vein graft failure were experimented in pig models [245, 246]. The vascular anatomy and haemodynamics of larger animals is preferred to smaller animals, due to the ease of access and also the availability of more
tissue, thereby enabling researchers to perform more experiments on a single animal [247]. The large animals, however, do present with their limitations including, being more expensive to maintain, requiring larger holding pens and needing greater quantities of relevant drugs and materials.

### 1.9. Current Clinical Trials for Gene Transfer in vein graft disease and coronary in-stent stenosis

<table>
<thead>
<tr>
<th>Trial</th>
<th>Therapeutic Target</th>
<th>Therapeutic Agent</th>
<th>Result</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>KAT</td>
<td>Angiogenesis In CAD</td>
<td>Pvegf165 (Liposome Complex)</td>
<td>Negative</td>
<td>(Hedman et al., 2003)[248]</td>
</tr>
<tr>
<td>Prevent IV</td>
<td>Vein graft failure in CAD</td>
<td>Edifoligide (an E2F transcription factor decoy)</td>
<td>Negative</td>
<td>(Alexander et al., 2005)[249]</td>
</tr>
<tr>
<td>REVASC</td>
<td>Angiogenesis In CAD</td>
<td>Ad-VEGF121</td>
<td>Positive</td>
<td>(Stewart et al., 2006)[250]</td>
</tr>
<tr>
<td>Euroinject One</td>
<td>Angiogenesis In CAD</td>
<td>Pvegf165</td>
<td>Negative</td>
<td>(Kastrup et al., 2005)[251]</td>
</tr>
<tr>
<td>Genasis</td>
<td>Angiogenesis In CAD</td>
<td>Pvegf-2 (VEGF- C)</td>
<td>Negative At Interim (Stopped In 2006)</td>
<td>Unpublished</td>
</tr>
<tr>
<td>Northern</td>
<td>Angiogenesis In CAD</td>
<td>Pvegf121</td>
<td>Negative</td>
<td>(Stewart et al., 2009)[252]</td>
</tr>
<tr>
<td>AGENT-2</td>
<td>Angiogenesis In CAD</td>
<td>Ad5-FGF-4</td>
<td>Positive</td>
<td>(Grinesene et al., 2003)[253]</td>
</tr>
<tr>
<td>AGENT-3</td>
<td>Angiogenesis In CAD</td>
<td>Ad5-FGF-4</td>
<td>Negative</td>
<td>Unpublished (Clinicaltrials.GOV, 2006)</td>
</tr>
<tr>
<td>AGENT-4</td>
<td>Angiogenesis In CAD</td>
<td>Ad5-FGF-4</td>
<td>Negative</td>
<td>Unpublished (Clinicaltrials.GOV, 2005)</td>
</tr>
<tr>
<td>Italics</td>
<td>In-Stent Restenosis In CAD</td>
<td>Antisense Oligonucleotide Of C-Myc</td>
<td>Negative</td>
<td>(Kutryk et al., 2002)[254]</td>
</tr>
</tbody>
</table>

Table 3 Clinical trials for gene transfer in vein graft disease and coronary artery disease (copied with permission from Dr Salik Kakar)
The only clinical studies of gene therapy for the prevention of vein graft disease, till date, are the PREVENT Trial series. These studies investigated edifoligide, an oligonucleotide decoy that bound to and inhibited E2F transcription factors, thereby preventing cellular proliferation. Using the strategy of E2F decoy oligodeoxynucleotides, PREVENT trial 1 (a single-centre, prospective, randomised controlled trial of ex vivo therapy for human vascular bypass grafts) showed a significant reduction in vascular cell proliferation in E2F decoy oligodeoxynucleotides treated vein grafts [213]. Although the phase 1 trial (PREVENT Trial) showed positive results, subsequent studies were not promising. The phase III, multicentre, randomised, double blind, placebo-controlled PREVENT IV trial of over 3000 patients undergoing CABG, found no significant difference in any outcome between treatment and placebo groups [214, 215].

Currently, the only published clinical study of gene transfer to prevent coronary in-stent stenosis is the KAT Trial [255]. Initial balloon angioplasty was followed by immediate delivery of placebo, a VEGF-A-expressing adenovirus, or plasmid/liposome complexes expressing VEGF-A. At 6-month follow-up, no effect was observed on angiographic restenosis. In the ITALICS trial, the effect of delivery of antisense oligodeoxynucleotides (ODN) against the nuclear proto-oncogene c-myc was studied. This study looked at neointima formation or ISR in the coronary arteries after 10mg of ODN or saline were infused into the coronary arteries immediately after stent delivery. No effect was observed on neointima formation or ISR at six months [254].
1.10. Research Aims and Objectives

Saphenous vein grafts are ideal targets for therapeutic gene transfer. After harvesting from the patient’s leg, the bypass conduits are prepared *ex vivo* for thoracic implantation. There is a period, of around one hour, between the completion of vein harvesting and the beginning of conduit anastomosis, after the institution of the cardiopulmonary bypass circuit. During this period, vein segments can be exposed to gene transfer agents with the intention of inhibiting neointimal hyperplasia in the early post-implantation phase, thereby delaying the occlusion of the vein graft.

Two novel anti-fibrotic transgenes have been identified, which are effective suppressors of neointima formation in surgically prepared human saphenous vein segments: the small, leucine-rich proteoglycan Fibromodulin, and the latency-associated peptide of transforming growth factor-β1 (LAP-β1). The intention of the research group is to investigate the effect of the latency-associated peptide of transforming growth factor-β1 (LAP-β1) and Fibromodulin on neointima formation in saphenous vein graft in-vivo. As part of that work, the objective in this project was to verify the possibility of performing the porcine saphenous vein interposition graft procedure in Manchester.

The specific objectives were

1. To establish an in-vivo system using porcine saphenous vein graft as an interposition graft into the internal carotid artery of the pig, as part of our research in the University of Manchester. A different research group for a different study outside Manchester already established this porcine saphenous model of vessel bypass.

2. To confirm that it was possible to transduce the vein grafts using a recombinant adenovirus vector expressing the marker transgene *lacZ* (expressing E.coli β-galactosidase).
2. MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Cell Culture Media and Solutions

The cell culture procedures were performed in a tissue culture safety flow cabinet (class II) while maintaining full sterility. Cell cultures were incubated in a humidified condition with 5% carbon dioxide (CO$_2$) at 37°C. All materials were obtained from Sigma Biosciences Ltd unless stated otherwise. The composition of cell culture media for each cell line is listed in the appendices. For all cell cultures, care was taken not to disturb the attached monolayer while removing and dispensing media from the tissue culture flasks.

2.1.2. Recombinant Adenovirus vector

The viral vector used for the experiments were called Ad5-PREP-lacZ, which was provided by the project supervisor. [256]. It was reported to elicit between 50 and 100-fold more transgene expression in vascular smooth muscle cells than the hCMV based viruses that are typically used.

2.1.3. AD-293Cells

For propagation of recombinant adenoviruses, AD-293 cells were used, which were obtained from Stratagene (Cat. No 240085). These cells are the derivatives of HEK-293 cell (human embryonic kidney) line, with improved adherence and plaque formation. HEK-293 cells were obtained from the culture of HEK cells transfected with the adenovirus DNA that had been sheared by repeated passage through a needle [257]. The transformed cells (HEK-293) are susceptible to infection with adenovirus type 5 (Ad5) and other human adenovirus serotypes. Stratagene has further transformed the HEK-293 cell line that has rendered improved properties like cell adherence and plaque formation; these are AD-293 cells [258]. AD-293 cells, like HEK-293, provide the E1A region of the Ad5 genome in trans, thus allowing propagation of recombinant Ad5 virus particles from which the E1A region has been deleted.

2.1.3.1. Growing Up AD-293 Cells from Frozen Stock

A cryo-vial of AD-293 cells was rapidly warmed in a 37°C water bath until only a small ice pellet was visible. The contents of the vial were transferred to a sterile 15-mL Falcon tube containing 9mL of pre-warmed 293-Medium. Cells were centrifuged at 500x g for 10 minutes, and the
supernatant was discarded. The pellet was resuspended in 6mL of 293-Medium and transferred to a 25-cm² tissue culture flask and placed in an incubator. The cells were observed under the microscope after 24 hours of incubation for cell adhesion to the base of the flask. If viable cells were seen, the cells were rinsed with 6mL of Phosphate buffered saline (PBS) and fresh medium was added before returning the flask to the incubator. Cells were monitored daily and, the medium was changed every 72 hours. Cells were passaged when the culture was at 80-90% confluency.

2.1.3.2. Passaging AD-293Cells

The medium was aspirated from the tissue culture flask, and the cell monolayer was washed with Dulbecco’s PBS. The PBS was aspirated and, Trypsin-EDTA 1x solution was added in sufficient quantity to just cover the monolayer. Typically it is 0.5mL for a 25-cm² tissue culture flask, 1mL for 75-cm², and 2mL for a 175-cm² tissue culture flask. The flask was gently tilted to ensure that entire cell monolayer was exposed to trypsin. The flask was returned to an incubator for 5 minutes to maintain the 37°C temperature. Once the monolayer had detached, trypsin was inactivated by addition of the same amount of 293-Medium that was aspirated initially. The cell suspension was poured over the base of the flask to gently wash off any remaining cells attached to the surface, and to break any cell clumps. AD-293 cells were split at a 1:5 ratio with fresh 293-Medium in a new flask and were returned to the incubator.

2.2. PURIFICATION OF ADENOVIRUS

For the purification and concentration of adenovirus (from Ad5 strains), viral purification kit with syringe filters from a company called Sartobind filters was used. The viral purification kit was used according to manufacturer’s instructions. The figures of the purification are copied from the Instruction Manual (Catalogue # 240243) with their permission. The Sartobind filters contain an ion exchange membrane-adsorber that binds adenoviral particles selectively. The viral particles can normally be concentrated and purified and obtained in 2–3 hours, in contrast to traditional Cesium Chloride (CsCl) gradient centrifugation, which typically takes 12–48 hours. The adjuncts to this kit are described in Table 5 in the Appendix. 60 ml of cell culture (3 × 15-cm² plates) purified using this method yield approximately 1–3 × 10¹² viral particles.

2.2.1. Virus Culture
10 flasks of 75-cm² (each flask containing 10 mL of 293 cell medium) were seeded with AD-293 cells and incubated at 37°C with 5% CO₂. When the cell monolayer reached a confluency of about 60–80%, the cells were infected with an Ad5 type adenovirus stock at a multiplicity of infection (MOI) of 5. The infected cells were then cultured at 37°C with 5% CO₂ for approximately 3 days until the majority of the cells showed cytopathic effects. Ideally, the cells should round up and detach, but occasionally, it may be necessary to detach adhered cells using a cell scraper.

### 2.2.2. Sample Preparation

Once the virus culture was ready, infected cells and medium were pooled. Cell pellets were obtained from the sample by centrifugation at 3,500 × g for 15 minutes. The supernatant was decanted to a sterile container and stored at 4°C (referred to as ‘reserved supernatant’). The cell pellet was then resuspended in 4 ml of the reserved supernatant. Once the volume was measured following suspension, an equal volume of Arkline P (1,1,2-trichlorotrifluoroethane) was added. The sample was mixed by repeated inversion for 10 minutes and centrifuged at 1000x g for 15 minutes. The upper pink/opalescent layer was removed without disturbing the cell debris layer and added to the reserved supernatant. 12.5 U of Benzonase nuclease for each millilitre of supernatant was added, for a final concentration of 12.5 U/ml. The samples were mixed by repeated inversion and incubated at 37°C for 30 minutes.

![Filter equipment assembly](Images copied with permission from AdEasy Viral purification kit Instruction Manual – Catalog #24023)
0.45 μm Filter Clarification of Viral Supernatant

A 50-ml syringe was attached to a tube set, and this entire set was clamped to a retort stand (Figure 7A). With the help of a feeding tube, which is immersed into the supernatant, some liquid was drawn up first into the syringe (Figure 10A). This liquid and the air in the syringe were now pushed back in the container until all the air was removed from the syringe. The syringe was then filled with supernatant, treated with Benzonase nuclease and a 0.45μm filter was attached to the syringe assembly. (See Figure 10B) The whole volume was filtered into a fresh container (This unit was called Unit A). A small volume (around 1 – 2 mls) of supernatant in the syringe was left on each cycle to avoid air entering the filter unit. Once the desired volume was obtained, the filter was removed.

2.2.3 Sartobind Filter Purification of Adenovirus

A fresh sterile 10-ml syringe was filled with approximately 10 ml of PBS. A Sartobind unit was attached to the 10 ml syringe (Figure 11). (This connected unit was called Unit B).

Figure 11: Sartobind Filter Equipment Assembly. (Images copied with permission from AdEasy Viral purification kit Instruction Manual – Catalog #24023)

The unit was rinsed with the pre-filled PBS. As before, the air from the unit was removed by pushing and pulling the plunger of the syringe, and ensuring that some volume of PBS was left in the syringe (around 1-2 mls) of PBS). The outlet of the unit was covered with a cap, and the unit was kept upright (Figure 11).
2.2.4. Sample Loading

Unit A with previously prepared 50-ml syringe and tube set was taken. The end of the feeding tube was placed into the sample solution ensuring that air was not trapped in the syringe or valve (Figure 12) and the 50-ml syringe was filled with supernatant. Once we were sure that there was no air trapped in the connection between Unit A and Unit B, the sample solution was passed through Unit B at an optimal flow rate (approximately 10 ml/minute, which is 20 x 10 drops). As before, a small amount of liquid was left in the 50-ml syringe.

![Figure 12: Sample Loading](Images copied with permission from AdEasy Viral purification kit Instruction Manual – Catalog #24023)

2.2.5. Sample Washing

The volume of original culture was now substituted with washing buffer, which was poured into the sample container. The buffer was passed to the Sartobind units as well making sure there was no air entering the units.

2.2.6 Elution

A fresh 10-ml syringe was filled with 5 mls of Elution Buffer (Unit C). The Sartobind unit (Unit B) was now detached from Unit A and attached to Unit C. The elution tip was connected to the outlet of the Sartobind unit, which made the flow of elution easier. Now 1 ml of elution buffer was very slowly passed through Unit B. This was then collected in a separate sterile 15-ml tube (Figure 13A). The remaining 4 mls of elution buffer, was left in the syringe, connected to Unit B.
and incubated for 10 minutes at room temperature. After 10 minutes, the remaining 4 mls of Elution Buffer was also passed through the Sartobind Unit very slowly as before.

**Figure 13A: Elution set-up.** (Images copied with permission from AdEasy Viral purification kit Instruction Manual – Catalog #24023 Figures 13A and 13B)

### 2.2.7. Viral Eluate Concentration and Buffer Exchange

The eluate, obtained by above steps was transferred to a Centrifuge Concentrator (Figure 13B). The rotor was counterbalanced with a second concentrator, by filling an equal volume of PBS or water. Centrifugation for five minutes at room temperature at 3,000x g, normally brings 5 mLs of eluate to 1 mL. The volume was adjusted by either centrifuging for adjusted time if the volume was higher or by adding storage buffer if the volume dropped below 1 mL. The concentrated virus was now recovered with the help of a pipette. Finally the concentrate was divided into aliquots and stored at -80°C.

**Figure 13 B:**
Centrifuge concentrator
2.3. Gene Transfer

2.3.1 Animal model - Large white pigs weighing between 25 and 35 kg were used. The demographics of the pigs are described in the Appendices. Such animals are of optimal weight for ease of handling and have adequate vessel size. Four pigs were used for the study with three more pigs as a reserve, if there were any unforeseen technical issues. All animals received humane care according to the United Kingdom Home Office (Scientific Procedures) Act of 1986. The Home office licence (Personal Licence I04FFE420) for conducting experiments on pigs and sheep was obtained. All the photographs of the experiments published here were taken by the project supervisor, while they were performed.

2.3.2 Materials used

1. Vein bathing solution: 0.9% saline with 2 IU/ml of heparin and 50µg/ml GTN.

2. Infection dishes: Any pre-sterilized plastic dish can be used (minimum 15 cm x 15 cm).

3. Crocodile clips.

4. 18 gauge 32-mm catheter needle.

5. Lockable 3-way stopcock.

6. Recombinant adenovirus.

2.3.2.1. Anaesthetic Requirement

1. Blease’s anaesthetic machine with circle circuit and O₂ and N₂O supply (Figure 14).

2. Direct Laryngoscope with a straight blade.

3. Isoflurane

4. Endotracheal tube 5.0–8.0 mm cuffed

5. Endotracheal connector
2.3.2.2. Surgery

General Requirement
- Operating room with an adjustable table.
- Autoclave machine
- Diathermy unit pads and handle.
- 3.0x magnifying operating surgical loupes (UKLOUPES, Bristol)

2.3.2.3 Surgical Instruments
- Scalpel blade holder with scalpel blade No 11 and 15 (Swann Morton, UK).
- Travers and West self-retaining retractor.
• Scissors - Mayo and Metzenbaum.
• Tissue forceps – DeBakey, Adsons (Toothed and non-toothed).
• Potts fine forehand and backhand scissors.
• Spencer Wells’s and Halstead artery forceps.
• DeBakey angled vascular clamps- No. 9 and No. 10.
• Fine Needle holder - Castro and Ryder.
• Backhaus Towel Clips.
• Crocodile clips.

Most surgical instruments were bought from Anetic Aid, UK.

2.3.2.4 Disposables

• Syringes – 1-ml, 2-ml, 5-ml, 10-ml, and 50-ml.
• Needles – 18-gauge, 21-gauge, and 27-gauge.
• Winged butterfly needle.
• Endotracheal tape (50 cm) and Masking tape.
• Sterile surgical gloves (Biogel)
• White (10 x 10 cm) and blue swabs.
• Disposable drapes and gowns.
• 30-ml plastic Gallipots and Kidney dish.
• Mersilk ties
• Surgipro sutures with 9-mm needle (Size 7/0, 6/0 - Covidien).
• 2/0 Polysorb sutures (Auto Suture, Dagford, UK).
2.3.2.5 Solutions

- Videne Surgical Scrub with antiseptic solution (7.5% Iodine)
- Heparin used according to the body weight (100 U / kg body weight)
- 1 mL glyceryl trinitrate (1mg/mL)
- 500 mL 0.9% Sodium chloride
- 1 mL Buprenorphine (400µg/mL)
- Pentobarbitone solution 20%

2.3.3. Methods

2.3.3.1. Induction of Anaesthesia

Oral feeds were avoided six hours prior to the induction of anaesthesia to minimize the risk of aspiration. Pigs were not sedated. The theatre operator achieved anaesthesia by mask inhalation of Isoflurane (4 % isoflurane and 4 litres/ minute oxygen and nitrous oxide) until stage three (surgical level, based on the assessment of state of muscle relaxation) is achieved. The respiration was maintained at 10–12 breaths/min and there was no response to pain. A butterfly intravenous needle was inserted into the ear lobe vein to gain intravenous access. This was mainly to administer intravenous heparin and maintain adequate hydration.

The pig was transferred to the surgical table in prone position. With the help of an assistant, the jaws were held open while the operator inserted the endotracheal tube (size 7.0 / 8.0 adult) using a long flat-blade direct laryngoscope, and connected it to the oxygen source. Anaesthesia was maintained using a closed circuit with 3% Isoflurane, 3 litres of oxygen, and nitrous oxide in equal parts at flow rates of 2 L/per minute. Vital parameters were monitored including oxygen saturation with pulse oximetry, heart rate (LifetrackECG) and end tidal carbon dioxide, which enabled to gauge the depth of anaesthesia (Figure 15). Once in position, the pig was secured, turned supine, with the limbs and the head held in place with tape if needed. The skin of the legs and the neck were disinfected with an iodine-based solution (Betadine) and draped to provide a sterile operating field. We used monopolar diathermy for vessel cauter.
2.3.3.2. Harvesting and preparation of Long saphenous vein

After positioning each hind limb to permit adequate exposure, an incision was made 1 cm posterior to the lateral malleolus and extended proximally to the knee posteriorly (Figure 16). The subcutaneous layer and fascia were dissected till the saphenous vein was exposed. Unlike human saphenous vein, the vein was handled as minimally as possible to avoid any spasm of the vessel, which is prone to occur. ‘Non-touch-technique’ (described this technique earlier in this chapter), was used for the vein graft harvesting. This was to maintain the integrity of the vessel wall and also helped subsequently to assess the lumen size, during anastomosis to the internal carotid artery.
Once the appropriate length of the vein, which was for required for grafting two interposition grafts (about 5 cm each) was exposed, the vein was carefully dissected to identify the tributaries (usually 4-10 in number), ligated the tributaries with 4-0 silk ties and cut (Figure 17).
Care was taken to identify the proximal and distal ends of the vein segment, as most veins have unidirectional valves. Despite all efforts to get grafts with appropriate length and lumen size, there were size mismatches of the lumen size between the saphenous vein and the internal carotid artery during anastomosis. Bevelling the harvested vein graft at both ends and spraying the graft with nitrate solution helped in reducing the size mismatch. In coronary artery bypass grafting, Papaverine or nitrate solutions were normally used after harvesting the internal mammary artery.

Figure 18: The harvested long saphenous vein with the adenovirus solution (Image size scale – 128%×187%)
The vein was then placed into sterile bathing media containing Phosphate-buffered saline for about 10-15 minutes. In a sterile environment, two 18 gauge intravenous cannulas were taken. The sheath of the cannulas was trimmed to approximately 2cm long. One end of the vein was cannulated and the bathing medium was injected into the vein to ensure that there was unobstructed flow through its entire length.

Once unobstructed flow had been confirmed, the sheath of the second cannula was inserted into the other end of the vein (the proximal end). Crocodile clips were used at both ends of the graft to fix the cannula to the vein (Figure 18). A three-way tap was then attached to both ends of vessel cannulas. 100 µL of Ad5-PREP-lacZ solution at 2.5 x 10^8 pfu (plaque forming units)/mL was taken in a sterile 1-mL syringe and the contents injected from the distal end of the vein making sure that the vein was not overly distended. Once the lumen of the vein was filled, the three-way tap of the distal end was closed. The solution was kept in the vein for 60 minutes. After the required time, the Ad5-lacZ solution was flushed through with the phosphate-buffered saline making sure that the vein was not contaminated externally with the viral solution. Adenovirus negative control was not used in this study as it was the preliminary study to establish a porcine vein graft model.

2.3.3.3. Preparation of the carotid artery and interposition graft anastomosis of saphenous vein into internal carotid artery.

The next stage now was to expose the carotid arteries on both sides in order for the vein to be anastomosed. The veins were anastomosed as interposition grafts into both internal carotid arteries.

After positioning the neck to the side of surgery, an incision was made from the lower angle of the mandible to the upper level of the shoulder, which was approximately 15cm long. After dividing the skin and the subcutaneous fat, the platysma muscle was divided to expose the submandibular glands. Once the strap muscles were identified, the plane between the submandibular gland and the muscles could lead to the carotid sheath. The carotid pulsation was regularly felt to direct the dissection and to avoid any damage to the vessels. With the help
of a Travers or West retractor (Figure 19), the submandibular gland was retracted laterally, to prevent it from coming in the way of the carotid artery.

With careful dissection, the internal carotid artery was exposed making sure that there was reasonable length proximally and distally (about 10 – 12 cms) to apply the vascular clamps. The internal jugular vein (IJV) was very close, and vascular slings were used to retract IJV away from the internal carotid artery. The pigs were heparinised with 100 IU/kg heparin via the internal jugular vein, using a fine-gauge needle.

![Figure 19: Exposure of internal carotid artery with the surrounding structures (Image scale-221% x 248%-Magnification x 2.5)](image)

The virus instillate was left in the saphenous vein segment for an hour. Once the vein was prepared and ready to use, the carotid artery was clamped proximally and distally with two vascular clamps isolating about 10cm of the artery (Figure20). A segment of the artery, approximately 5 cm long, was now removed and the edges of the remaining vessel were bevelled in preparation for the anastomosis.
The proximal end was anastomosed first, with the reverse saphenous vein (due to the presence of unidirectional valves in the vein) in a continuous way using 7/0 Surgipro sutures making sure that there was unobstructed flow. The vein was then anastomosed to the distal end of the internal carotid artery. Just before completing the anastomosis, with only couple of stitches to spare, the distal clamp was slowly released so as to washout any debris or clot within the lumen through the wound. Just before tying the knot, the proximal clamp was also released so as to ensure a good pulsating flow and washout of any further clots. Haemostasis was maintained throughout the procedure and the flow was pulsatile. In case any arterial spurt was found due to gaps in the anastomosis, interrupted sutures were used to secure the anastomosis. The procedure was repeated on the other side (Figure 21). After the completion of IP grafts, Mersilk tie slings around the internal carotid artery proximally and distally were kept, to locate in harvesting the interposition grafts later. This was done after a couple of interposition grafts harvests became very difficult to identify.

![Image of surgical procedure](image.png)

**Figure 20:** The harvested long saphenous vein is anastomosed to the proximal end of the internal carotid artery (Size scale – 12% x 15% of the actual size).

After confirmation of good pulsatile flow in the grafted vessel proximally and distally, the wounds were closed with 2.0 Vicryl suture for the subcuticular layers and skin in the neck and
leg. Prophylactic antibiotics (200 mg of Amoxicillin intramuscularly) and analgesia (0.3 mg of Buprenorphine intramuscularly) were given. 

After the procedure, all the disposables were disposed of, abiding by the sterile regulations and the reusable ones were again sent for autoclaving or appropriate sterilisation. Once the pigs were extubated, they were returned to the pen for slow recovery taking care of their feeding and checking for any secondary haemorrhage in the wounds. The pigs would constantly lick the leg wound and hence subcuticular sutures to close the skin were used. Post-operatively, the wounds were regularly checked for haematoma in the neck, as it was prone to occur, and for the healing of the neck wounds and leg wounds. So far, there were no wounds re-explored or any extra doses of antibiotics were given.

Figure 21: The long saphenous vein anastomosed as an interposition graft to the internal carotid artery. The blood flow on palpation was pulsatile. (Image scale – 75% x 100%)

2.3.3.4. Vein Graft Harvesting

The grafted saphenous vein was harvested again in a week’s time. The procedures to anaesthetise the pig were the same as described previously. Once the interposition grafts were exposed after careful dissection of the neck, the grafts with a segment of internal carotid
-arteries on both ends (approximately 8-10 cm in total) were explanted, by ligating the internal carotid artery both proximally and distally, away from the anastomosis, and cut.

The previously operated fields were likely to be severely adhered and it was very difficult to dissect to get to the right plane, especially after a week due to inflammation and resultant fibrosis. Carotid artery pulsation was used to direct our dissection. After a couple of graft harvests, Mersilk suture slings were placed around the internal carotid artery proximally and distally, after IP anastomosis and these silk suture were brought to the subcuticular plane to locate the vessels, during re-harvesting. After retrieval of all grafts, the pig was euthanized with 20 mL (4 g) of Pentobarbitone sodium intravenously. Aseptic precautions and regulations were abided in transportation and disposal of the pig.

2.3.3.5. X-gal staining of the explanted segments of saphenous vein-internal carotid artery

After a week, Ad5-lacZ infected segments of long saphenous vein - internal carotid artery, explanted by the above method was washed in PBS for 5 minutes and then fixed with 4% paraformaldehyde for 20 minutes at room temperature. The segment was washed with PBS three times to remove excess fixative. It was then permeabilised with 0.2% Triton X-100 for 10 minutes. The Triton was aspirated and the vein was washed three times with PBS. The explant was opened by cutting longitudinally with iridectomy scissors and pinned down with lumen side up on polyester mesh gauze in a glass-culture dish (Cat. No. PDS-110-030J, Schott Glass).

The glass-culture dish had a layer of pre-formed Sylgard resin in its base, and polyester mesh gauze was placed on top to support the vein segment. The segments were pinned to this support by 0.1 x 10mm stainless steel pins (Cat. No. E6901, Watkins and Doncaster). Then 10ml of freshly prepared X-gal staining solution (5-bromo-4-cholor-indolyl-β-D-galactopyranoside) (See Appendix for the contents of the solution) was applied to the vein segment, and left in a dark place at 37°C to incubate for 1-3 hours. After incubation, the vein segment was washed three times with PBS and air-dried.
CHAPTER 3 - RESULTS

Seven interposition grafts using porcine vein grafts into the internal carotid arteries were performed after they were harvested from the respective pigs. Four pigs in total were used to perform bilateral interposition grafts on each pig, except the first pig. The results of the experiments are described for individual pigs separately, from the beginning of the procedure including harvest of the saphenous vein graft, till the end which includes explantation of the harvested graft. This is to get a clear picture of the flow of the experiments. The analysing of the vein segments for X-gal staining is then explained on a separate heading.

The results of this study are described in the following order:

3.1 Establishment of an in-vivo system with porcine saphenous vein interposition (IP) grafts into the internal carotid arteries
3.2 To demonstrate transgene expression in the vein interposition grafts after the anastomoses using a marker gene in vivo.

3.1 Establishment of in-vivo system in pigs using IP vein grafts

3.1.1- Experiment 1

A. Harvest of long saphenous vein: The pig saphenous veins, unlike human saphenous veins, were very small in calibre and delicate to handle. The harvest of the first saphenous vein was extremely difficult as it was in spasm even before dissection had started, and hence the other leg had to be opened (Figure 22). By handling the vein to the minimum, and grasping only the surrounding tissues around the long saphenous vein, an appropriate segment of around 6 to 7 cm of vein, was obtained. The lumen of the vein was less than 3 mm diameter and hence infusion of the Ad5-lacZ solution into the vein was extremely difficult, consuming more than an hour in preparation of the vein.
B. **Preparation of the long saphenous vein with the Ad5-lacZ vein solution:**

The infection of the vein graft with recombinant adenovirus following the harvest was technically easier but time consuming due to precautions maintained during procedure. Preparation of the vein graft for anastomosis was carried out with sterile techniques. It took approximately 30 minutes to prepare a segment of vein, which included keeping the vein in the bathing medium, flushing the vessel lumen with Phosphate buffered solution, and cannulating the vein at both ends before the Ad5-lacZ solution was instilled into the luminal surface. This prolonged the total procedural time, as dissection and exposure of the internal carotid arteries had to be done simultaneously or immediately after the preparation of the vein graft. After the first anastomosis, it was decided that after harvesting the vein, preparation of vein and dissection of the internal carotid artery, could be done simultaneously by two personnel, and in this experiment, the project supervisor helped to infect the harvested saphenous vein with the Ad5-lacZ solution. This had two benefits:

1) Improved efficiency
2) It enabled the research team to simulate the real time setting in CABG. Normally in humans, there are approximately 30 minutes to an hour after harvesting and preparing the long saphenous vein for using the same conduit as a bypass graft in CABG. So time will be very limited to prepare the vein graft, to get exposed to the vein solution containing the viral vector and the transgenes, to prevent the physiological changes it may undergo after distal and proximal anastomosis in a normal setting of CABG.

C. **Anastomosis of LSV as an interposition graft in to the internal carotid arteries of the pigs:**

There was only one graft anastomosis performed in the first pig. The small lumen size of the saphenous vein and it’s going into spasm made it difficult to anastomose it, as an interposition graft. There was also a significant size mismatch between the ICA and the saphenous vein. Bevelling the ends of the saphenous vein more than the internal carotid artery and topical application of the glycercyl trinitrate solution on the vein helped to an extent to overcome this situation.

D. **Explantation of the first interposition grafts:**

This was done seven days post-grafting. There was dense adhesion of tissues around the interposition graft anastomosis site and the fact that the first graft was thrombosed made it difficult to access the right plane of dissection due to non-pulsatility of the vein graft. The direction of the pulsation of the grafted vessel for dissection could not be used in the first graft, simulating a redo CABG with occluded vessel. A great amount of time was spent for dissection on getting in to the plane eventually demonstrated a thrombosed vessel. It was however explanted.

3.1.2 - **Experiment 2**

**A. Harvest of long saphenous vein:** The second harvest of saphenous vein was slightly better than the first one, mainly because it was a better calibre vessel. However, like the first vein harvest, it went into total spasm once the bathing solution was infused in to the vein segment. By this time, an external research team was contacted, who had established an in-vivo system using porcine saphenous vein into the internal carotid artery. An appropriate literature to use the ‘no touch technique’ (already described about this technique earlier) while vein harvesting, was implemented. Local application of glycercyl trinitrate solution on
the vein graft also helped in preventing the spasm of the vessel. ‘No touch technique’ in vein harvesting is not to handle vein directly during dissection and harvest. Some amount of surrounding tissues with the vein was left, and by not stripping the vein, off all the protective factors and microvascular network surrounding the graft, the vein had better patency this time. Monopolar diathermy (In this type of diathermy, electric current passes from one electrode near the tissue to be treated to the other fixed indifferent electrode, elsewhere in the body. Usually this type of electrode is placed in contact with buttocks or around the leg), was also avoided directly on the vein. These manoeuvres helped in overcoming the difficulty of vein harvest, in the next few occasions (Figure 23).

Figure 23: The effect of non-touch technique on the saphenous vein graft (Actual size of the image)

B. Preparation of the long saphenous vein with the Ad5-lacZ vein solution:  
The infection of the vein graft with recombinant adenovirus, following the harvest was technically easier than the first time due to better calibre vessel and due to two personnel working simultaneously. Preparation of the vein graft for anastomosis was carried out with sterile techniques.
C. **Anastomosis of second and third interposition graft in to the internal carotid arteries of the pigs:**

The subsequent two IP anastomoses were dealt with precaution to avoid both spasm and mismatch. The vein was marked at the distal end with a ligaclip (LIGACLIP® EXTRA Ligating Clips LT100) to confirm the distal end of the vein. The dosage of heparin was calculated according to the body weight (100 units/kg body weight). The heparin was injected into the internal jugular vein before the start of the anastomosis. The IP anastomoses were performed three to five minutes after the administration of heparin for it to circulate. Bevelling the ends of the vein, minimal handling of the tissue and Glyceryl trinitrate solution to the graft improved the overall results of second and third IP anastomosis. The subsequent grafts were not thrombosed and had good pulsatile flow.

D. **Explantation of the second and third interposition grafts:** Like the first anastomosis, a way to get to the right plane without injuring the vessel was challenging, before explanting the interposition graft. With previous experience of a thrombosed IP graft, it was anticipated that the subsequent grafts could also occlude though every precaution was maintained to prevent it. Though not described elsewhere, mersilk loops around the internal carotid arteries both proximal and distal to the anastomosis were kept and the ends of the sutures were brought to the subcutaneous plane. Though there were adhesion in the previous surgical site, it was relatively easier to direct the dissection to the IP graft. The time required to explant these IP grafts were better than the first graft. Even though the second IP graft explantation took longer than third graft in the same pig, this could be attributed to the learning curve and apprehension about injuring the vessels during dissection.

### 3.1.3 - Experiment 3 and Experiment 4

Both the experiments were similar and hence mentioned together

A. **Harvest of long saphenous vein:** Saphenous vein graft harvest for the next two pigs were not difficult due to various precautions taken as mentioned above. An approximate length of 10 cm was harvested from each pig, which was of good calibre and less spasmodic. The length of vein was sufficient enough to perform two IP anastomoses each making a total of four IP grafts.
B. **Preparation of the long saphenous vein with the Ad5-lacZ vein solution:** The infection of the vein graft with recombinant adenovirus following the harvest got better, both in terms of time and from the technical point of view. Preparation of the vein graft for anastomosis was carried out with sterile techniques.

C. **Anastomosis of interposition grafts in to the internal carotid arteries of the pigs:** The next four IP anastomoses were straightforward and did not have any major concerns. As advised by an external research team, some tissues were left along with the harvested saphenous vein, which the research team believed would have contributed in preventing the spasm. After the anastomoses, once the vascular clamps were released, there were good pulsatile flow in the grafts which was also confirmed by the project supervisor.

D. **Explantation of the interposition grafts:**
   a. **Fourth and Fifth IP anastomosis:** The explantation of the next two grafts was not too difficult thought it was time consuming due to dense adhesion. Multiple factors could have contributed to this including the healing process, infection and general condition of the pig. The pulsatility of fifth IP anastomosis was lesser in comparison with the fourth graft though a specific reason could not be attributed. The explanted graft however, did not demonstrate any thrombus within the lumen.
   b. **Sixth and Seventh IP anastomosis:** The explantations of the next two IP grafts were easy as it had a big lumen and the pulsatility was never an issue. The dissection of the grafts did take time as expected but the grafts were not injured and getting in to the plane directed by the Mersilk sutures helped. Both the grafts did not demonstrate any thrombus and were taken for further testing.
3.2 Assessment of Ad5-lacZ-mediated transgene delivery by X-Gal staining

The efficiency of Ad5-mediated gene transfer of β-galactosidase into the walls of veno-arterial graft segment was assessed 7 days after intra-luminal delivery of $10^8$IU of Ad5-lacZ. The saphenous vein graft infected with Ad5-lacZ encodes the protein β-Galactosidase which can break down lactose into glucose and galactose. Expression of β-Galactosidase in segments of saphenous vein and internal carotid artery, infected with Ad5-lacZ, were confirmed by staining with X-gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside) medium. β-Galactosidase generates an intense blue pigment from the X-Gal medium (Figure 24) as a result of breaking the bond of the medium. Macroscopically, the X-gal staining showed intense blue colouration on the luminal surface of the vein segment exposed to Ad5-lacZ (Figure 25) due to the presence of galactose.

Figure 24: Ad-lacZ infected saphenous vein-internal carotid artery segment after explanting and staining with X-gal solution (Image size – Magnification x 3)
Figure 25: Gene transfer to vein grafts (Graft number 7) illustrating the carotid artery (CA) anastomotic sites (large arrows), and vein graft (VG). Blue colours (dark cells) are due to virus-infected cells within the vein graft anastomotic site.
Coronary artery bypass grafting (CABG) is the most commonly performed cardiac surgical procedure in the adult. The statistics have reported that more than 400,000 CABGs are performed annually in the United States alone. The current American Heart Association and European Society of Cardiology guidelines recommend CABG as Class I indication for the management of complex three-vessel disease and Left main or Left main equivalent disease. However, it is the complexity of the coronary lesions that determine the selection of intervention, as the same guidelines also recommend PCI with drug eluting stents as Class I indication, for the above-mentioned lesions with favourable anatomy [25].

Despite many reports favouring total arterial revascularisation in the recent literature [43, 47], autologous reverse saphenous vein graft (SVG) is the most frequently used conduit for the anastomosis in the surgical management of three-vessel coronary artery disease [259]. SVGs are easily available, require reduced harvesting time and have sufficient lengths to enable multiple grafts with adequate flow. Its main drawback however, includes high incidence of graft failure, which has an impact on the long-term results of CABG as a revascularisation strategy. According to Dashwood et al. about half of all vein grafts get occluded by 10 years post-CABG, and the remaining vein grafts demonstrated evidence of disease progression[260].

Several strategies have been implemented in an attempt to prevent vein graft failure, which was reported in the ‘Introduction’. Gene therapy of the saphenous vein ex-vivo is a newer technique to condition the conduit and hence prevent it from failing. Several therapeutic transgenes have now been studied in experimental models [81, 88, 92] of saphenous vein graft
disease. The research group, as part of which the work presented was performed, has investigated the potential use of specific antagonists of TGF-β1 including Latency associated peptide and Fibromodulin, as a means of suppressing neointima formation in human saphenous vein segments in-vitro. This was a pilot study, as the isoforms of latency-associated peptides TGF-β other than TGF-β1 have never previously been investigated as potential antagonists of TGF-β. While this was a useful preliminary model of vein graft neointima formation; it, however, doesn’t equate to the in-vivo setting due to the absence of physiological conditions like pulsatile arterial-pressure flow in the vessel and host inflammatory responses that might affect levels of transgene expression. It was thus necessary to assess these antagonists of TGF-β1 in vivo. In order to achieve this assessment, it was initially necessary to set up a porcine model of vessel bypass and undertake preliminary work in the gene transfer systems, which formed the objective of this project.

This thesis described the possibility of an in-vivo model of pig saphenous vein-internal carotid artery interposition graft, which was performed in Manchester and also demonstrates the transfer of Adenovirus and transduction of vein grafts. This study could well represent an extension of the research done by the same team, as mentioned earlier.

For this investigation, recombinant replication-defective adenoviruses giving rise to the expression of the lacZ have been developed and characterised. After performing the adenovirus mediated gene transfer into the pig venous-arterial graft, the macroscopic appearance and the result of X Gal staining of the venous-arterial graft were studied, explanting the interposition graft 7 days post anastomosis. X-gal staining of cryostat-sections of Ad5-lacZ-infected venous-arterial interposition graft revealed β-galactosidase expression principally along the luminal surface. This finding could suggest that the luminal delivery of
Ad5-mediated gene transfer was viable and enabled gene expression predominantly in the neointimal/intimal area. However, further confirmation is required by microscopic examination and immunohistochemistry.

Along with the specific objective, the technical and other challenges faced during the procedure were assessed and reported. The small sizes of the saphenous vein, its spasmodic nature on handling, and the venous-arterial mismatches during anastomosis are to name a few. It has to be emphasised that while, in the real-time setting, theatre scrub nurse, two assistants and a surgeon will be there to carry out the procedure but here the work was single-handedly managed. This definitely prolonged the procedure and it took almost 4-6 hours to complete a single procedure in one pig.

Every precaution was taken to ensure proper delivery of the Ad5-lacZ solution into the luminal surface of the vein graft only. There was no contamination of the vein solution outside the vein (Figure 18) as it could induce a response including infection of the surrounding tissues in the neck, after the interposition graft anastomosis. Though the initial transfer was done in a sterile environment, further experiments were done with aseptic precaution and not completely sterile. While this wouldn’t have affected the results, it will be expected that all the future delivery of the Ad5-lacZ solution are to be done with all sterile precautions. For further experiments, an assistant, will be required, who could take care of the instruments, assist the surgeon and finally help in the delivery of genes into the vein.

There existed a significant vein-to-artery mismatch, making it technically challenging for the IP anastomose. Surgical advice from an experienced external source was sought to overcome this challenge. However, the explantation of the grafted vessel a week post anastomosis was also difficult till some measures were adopted using the mersilk suture loop. Though the
other groups have not mentioned this in the literature, this technique has been effective at least for now. The concern of this maneuver was that, this suture could be a potential source of infection or sinus formation, if the graft has to be explanted 28 days post grafting.

Despite all efforts, the desired results were not achieved initially, which could be attributed to early thrombosis, poor anastomotic technique or narrowing due to spasm. With improved proficiency in anastomosis and minimal handling of the vessels to prevent spasm, the desired result was gradually achieved with pulsatile flow in the interposition grafts at the time of explantation. The X-gal staining of vessels exposed to $10^8$IU of Ad5-lacZ has confirmed the transgene delivery macroscopically. The dose of the Ad vector was lesser compared to the usual dose reported, which could well be due to regulatory elements including major immediate-early murine cytomegalovirus (MIEmCMV) promoter and the enhancer element RE from the rabbit smooth muscle myosin heavy chain promoter, in the vector [256]. Since this was conducted in-vivo, a balance between the optimum doses required to transduce the vein and elicit minimal host inflammatory response was mandatory. The result showed luminal expression of $\beta$-galactosidase after Ad5-lacZ-mediated gene transfer.

Lac Z is a part of the gene family called the lac operon, which by itself is a system of three genes composed of a promoter, terminator, operator and regulator along with it. Though there are three genes present in the lac operon namely lac Z, lac Y and lac A; the latter two do not have the same function as lac Z. Lac Z encodes for the protein, beta-galactosidase, which can break the bond between galactose and glucose in lactose molecules. It is this characteristic feature, which is used in many microbiology experiments involving research into the expression of a particular gene of interest or promoter because it allows researchers to quickly determine that the gene of interest is being expressed. When Adenovirus 5 that
have the lac Z gene introduced into them, and are grown on a particular type of media called X-gal, only the colonies expressing lac Z and thus beta-galactosidase are capable of breaking the bond in the X-gal molecule to produce galactose and a blue dye. Therefore the virus, properly expressing the gene of interest appears blue (Figure 26).

Figure 26: Expression of β-galactosidase with X-gal staining on colonies with lac Z.

Our research group previously used lac Z gene as a control when comparing the effect of antifibrotic genes like LAP-β1 and Fibromodulin after Ad5-LAP-β1-and Ad5-Fmod mediated gene transfer to the human SVG culture model. However, in this study, lac Z was used as a marker gene only to prove that transgene expression is possible on the vein graft model in-vivo which could be confirmed macroscopically and hence helped our group to decide on the dose or dilution of the Adenovirus vector that could produce the said effect.
As neointimal hyperplasia (NIH) develops on the luminal surface in the intima, gene transfer by this method will effectively target the site of NIH in the vessel wall. A previous study at our centre has demonstrated the expression of LAP-β1 and Fibromodulin after Ad5-LAP-β1-and Ad5-Fmod mediated gene transfer to the human SVG culture model respectively [261]. The expression was also confirmed immunologically on the luminal surface and in the periluminal surface of the infected vessel.

The majority of gene therapy studies have been positive in the human saphenous vein organ culture model to prevent neointimal hyperplasia. Our initial results have demonstrated that in-vivo models of saphenous vein-internal carotid artery graft and virus expression in it are possible. This paves the way to study the effect of LAP-β1 and Fibromodulin gene transfer in vivo, which could be effective as one of the strategies to prevent vein graft disease due to neointimal hyperplasia.
FURTHER RESEARCH

Our next objective will be to demonstrate the gene expression of LAP-β1 and Fibromodulin in veins infected with the optimum dose of Ad5-LAP-β1 and Ad5-Fmod. The effects of Ad5-LAP-β1 and Ad5-Fmod on neointima formation at 7 and 28 days post-grafting could then be assessed. These effects will have to be assessed more comprehensively by different mechanisms like Immunohistochemistry (IHC), qRT-PCR, MLEC9PAI/L assay.

In the clinical setting, the application of gene therapy on human saphenous vein graft ex-vivo, post-harvesting could be challenging due to the short window for vector mediated gene transfer, prior to being anastomosed to the coronary vessels and aorta. Hence the need to focus on any gene-delivery enhancing technique would be very helpful in clinical translation of pre-clinical therapies.

Recently, few strategies have emerged including fibre switching or pseudotyping [189]and modification of Ad5 fibres with targeting peptides. Although the vast majority of studies were done on gene therapies in the treatment of cancer, there are reported methods of enhancing the vector mediated gene expression, which could be applied in the transduction of vein grafts [171]. Once successful in establishing and confirming the gene expression by the described mechanisms, the next stage for the research team would be to proceed to clinical studies on human saphenous vein in-vivo.
### APPENDICES

**Table 4 – 293 Medium**

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<tr>
<td>MEM non-essential amino acids 1x</td>
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**Table 5 – X-GAL STAINING SOLUTION**

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Make to 20 mls in PBS
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<tr>
<td>10× Loading Buffer</td>
<td>25 ml</td>
</tr>
<tr>
<td>Washing Buffer</td>
<td>120 ml (1× concentration)</td>
</tr>
<tr>
<td>Elution Buffer</td>
<td>20 ml</td>
</tr>
<tr>
<td>Tubing Set (Masterflex® L/S 16–size tube)</td>
<td>2</td>
</tr>
</tbody>
</table>

**TABLE 6 - The Ad Easy Virus Purification**

<table>
<thead>
<tr>
<th>Pig</th>
<th>Type</th>
<th>Weight</th>
<th>Age</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Large white</td>
<td>35 kg</td>
<td>NS</td>
</tr>
<tr>
<td>2</td>
<td>Large white</td>
<td>37 kg</td>
<td>NS</td>
</tr>
<tr>
<td>3</td>
<td>Large white</td>
<td>35 kg</td>
<td>NS</td>
</tr>
<tr>
<td>4</td>
<td>Large white</td>
<td>37 kg</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS – Not specified

**TABLE 7 – Demographics of the pigs used.**
Serial-Dilution Table and Corresponding Concentrations

These calculations are based on the assumption that, as 1300µL of the $10^{-2}$ dilution stock contains 13µL of the virus stock, 100 µL of the diluted stock contains the equivalent of 1µL of the undiluted stock. Naturally, this applies to any volume of virusstock that is diluted 1:100 (i.e. $10^{-2}$ dilution) and then has 0.1mL (100µL) applied to the AD-293 cells as $0.1 \times 10^{-2} = 10^{-3}$.

<table>
<thead>
<tr>
<th>Well</th>
<th>Dilution</th>
<th>IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^{-2}$</td>
<td>$10^3$</td>
</tr>
<tr>
<td>2</td>
<td>$10^{-3}$</td>
<td>$10^4$</td>
</tr>
<tr>
<td>3</td>
<td>$10^{-4}$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>4</td>
<td>$10^{-5}$</td>
<td>$10^6$</td>
</tr>
<tr>
<td>5</td>
<td>$10^{-6}$</td>
<td>$10^7$</td>
</tr>
<tr>
<td>6</td>
<td>$5\times10^{-7}$</td>
<td>$2\times10^7$</td>
</tr>
<tr>
<td>7</td>
<td>$2.5\times10^{-7}$</td>
<td>$4\times10^7$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Well</th>
<th>Dilution</th>
<th>IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>$1.25\times10^{-7}$</td>
<td>$8\times10^7$</td>
</tr>
<tr>
<td>9</td>
<td>$6.25\times10^{-8}$</td>
<td>$1.6\times10^8$</td>
</tr>
<tr>
<td>10</td>
<td>$3.13\times10^{-8}$</td>
<td>$3.2\times10^8$</td>
</tr>
<tr>
<td>11</td>
<td>$1.56\times10^{-9}$</td>
<td>$6.4\times10^8$</td>
</tr>
<tr>
<td>12</td>
<td>$7.81\times10^{-9}$</td>
<td>$1.28\times10^9$</td>
</tr>
<tr>
<td>13</td>
<td>$3.91\times10^{-9}$</td>
<td>$2.56\times10^9$</td>
</tr>
<tr>
<td>14</td>
<td>$1.95\times10^{-9}$</td>
<td>$5.12\times10^9$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Well</th>
<th>Dilution</th>
<th>IU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>$9.77\times10^{-10}$</td>
<td>$1.02\times10^{10}$</td>
</tr>
<tr>
<td>16</td>
<td>$4.88\times10^{-10}$</td>
<td>$2.05\times10^{10}$</td>
</tr>
<tr>
<td>17</td>
<td>$2.44\times10^{-10}$</td>
<td>$4.10\times10^{10}$</td>
</tr>
<tr>
<td>18</td>
<td>$1.22\times10^{-10}$</td>
<td>$8.19\times10^{10}$</td>
</tr>
<tr>
<td>19</td>
<td>$6.10\times10^{-11}$</td>
<td>$1.64\times10^{11}$</td>
</tr>
<tr>
<td>20</td>
<td>$3.05\times10^{-11}$</td>
<td>$3.28\times10^{11}$</td>
</tr>
</tbody>
</table>

**TABLE 8 - Serial Dilution Table and Corresponding Virus Titres. IU=infectious units.**
REFERENCES


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