DECLARATION

I declare that this thesis is the result of my own work. It includes nothing that is the outcome of work done in collaboration, except where indicated in the text. I confirm that this thesis has not been previously submitted for any other degree.

_________________________
Susan Mary Gallogly

2016
ABSTRACT

Rationale and Objectives

New therapeutic approaches that promote repair of the vasculature following injury are necessary to improve the clinical outcomes in patients undergoing angioplasty and stenting for coronary artery disease. I sought to establish a new cellular model to study the coronary endothelium in patients with acute myocardial infarction, and to utilise this model to identify novel polymer substrates that promote endothelialisation of coronary stents. Furthermore, I evaluated the potential of a novel cyclin-dependent kinase (CDK) inhibitor (AT7519) to resolve the acute neutrophil-mediated inflammatory response to stenting without adversely affecting the function and integrity of the endothelium.

Methods

In 49 patients with acute myocardial infarction atherothrombotic specimens were isolated from the coronary circulation, dissected and cultured to obtain coronary endothelial outgrowth (CEO) cells. These cells were phenotyped, underwent assessments of proliferation and function (attachment, wound closure and tubule formation) in vitro and the capacity for angiogenesis in vivo was investigated. For comparison, endothelial cells from peripheral blood (late outgrowth endothelial cells [EOCs]) and human umbilical veins (HUVECs) were used. Polyacrylate (PA) and polyurethane (PU) polymers with high attachment of CEO cells but low attachment of neutrophils and monocytes were identified by high-throughput polymer microarrays. Polymers that facilitated CEO cell attachment under steady-state flow in an IBIDI chamber and supported endothelial cell proliferation were selected for further testing in a Badimon chamber to quantify thrombus formation as well as platelet and leucocyte attachment. Optimal concentrations of AT7519 to induce neutrophil apoptosis were assessed by morphological assessments of pyknosis. The effect of these concentrations on endothelial cell function and viability was assessed in vitro.
Results

CEO was obtained from 27/37 (73%) atherothrombotic specimens and gave rise to cells with a cobblestone morphology and high expression of CD146 (94±6%) and CD31 (87±14%). CEO cells had lower proliferative capacity to umbilical vein endothelial cells (23.0±6.8 versus 55.5±5.3 cumulative population doubling level, P<0.001) but not other cells. CEO cells had a similar potential to control endothelial cells in other assays of endothelial cell function (cell attachment, migration and tubule formation, P>0.05 for all) in vitro. Unlike HUVECs and EOCs, CEO cells did not incorporate into new host vessels in vivo.

Polymer microarray libraries (337 polymers) identified PA309, an amine-functionalized methacrylate-based co-polymer, as having high endothelial cell attachment (~1,000 nuclei/mm²) with low inflammatory cell attachment (<200 nuclei/mm²). PA309 and PA318 supported endothelial cell attachment, migration and retention to the same extent as the collagen or 8G7 control (P>0.05). PA309, but not PA319, supported CEO cell proliferation compared to collagen (3.5±1.3 versus 4.5±0.2 cumulative population doubling level [CPDL]). In ex vivo studies, PA309 (2,242±3,200μm²) and PA318 (3,043±4,054μm²) reduced thrombus formation compared to control (11,851±8,118μm²) (P<0.01 and P<0.001 respectively). Attachment of platelets and leucocytes was equivalent to conventional bare metal stents in clinical use.

AT7519 at a concentration of 0.1μmol/L induced neutrophil apoptosis compared to vehicle control (11±1 versus 1±1 nuclei pyknosis, P<0.05) but did not inhibit CEO cell function (proliferation: 2.7±0.4 versus 2.8±0.5 fold-increase, attachment: 32±23 versus 57±5% attachment, wound closure: 94±8% versus 70±19%, tubule formation: 44±6 versus 58±11 tubule structures, P>0.05 for all). Apoptosis and cytotoxicity was not observed in CEO cells at this concentration.

Conclusions

Coronary endothelial outgrowth cells can be reliably isolated and cultured from thrombectomy specimens and represent a novel and relevant model to study endothelial cell function. Polymer PA309 promotes coronary endothelial cell attachment and expansion in vitro with similar potential to collagen and inhibits thrombus formation ex vivo. Furthermore, the CDK inhibitor AT7519 selectively induces neutrophil apoptosis with no adverse effect on endothelial cells at a defined concentration in vitro. Controlled elution
of AT7519 and a PA309-coated coronary stent has major potential to promote the resolution of neutrophilic inflammation and promote endothelialisation, which could improve the clinical outcome for patients following coronary angioplasty and stenting for coronary artery disease.
ACKNOWLEDGEMENTS

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<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>degree Celsius</td>
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<tr>
<td>ACE</td>
<td>angiotensin-converting enzyme</td>
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<td>ACS</td>
<td>acute coronary syndrome</td>
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APC</td>
<td>allophycocyanin</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>bFGF</td>
<td>basic fibroblast growth factor</td>
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<td>BMS</td>
<td>bare metal stents</td>
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<tr>
<td>CABG</td>
<td>coronary artery bypass grafting</td>
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<td>CAD</td>
<td>coronary artery disease</td>
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<td>CD</td>
<td>cluster of differentiation</td>
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<td>CDK</td>
<td>cyclin-dependent kinase</td>
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<td>CEO</td>
<td>coronary endothelial outgrowth</td>
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<tr>
<td>CPDL</td>
<td>cumulative population doubling level</td>
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<tr>
<td>DAB</td>
<td>3,3'-Diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DES</td>
<td>drug-eluting stents</td>
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<tr>
<td>Dil</td>
<td>1,1'-dioctadecyl-3,3,3'tetramethylindocarbocyanine</td>
</tr>
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<td>ECG</td>
<td>electrocardiogram</td>
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<tr>
<td>ECM</td>
<td>extra cellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic</td>
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<tr>
<td>EPCs</td>
<td>endothelial progenitor cells</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>EOC</td>
<td>endothelial outgrowth cell</td>
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<td>FBS</td>
<td>foetal bovine serum</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>-----------------------------------------------</td>
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<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
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<tr>
<td>GFR</td>
<td>growth factor reduced</td>
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<td>GMP</td>
<td>good manufacturing practice</td>
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<tr>
<td>HCAEC</td>
<td>human coronary artery endothelial cell</td>
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<td>HPCA</td>
<td>hematopoietic progenitor cell antigen</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cell</td>
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<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>IMDM</td>
<td>iscove's modified Dulbecco's medium</td>
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<tr>
<td>KDR</td>
<td>kinase domain receptor</td>
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<tr>
<td>LCA</td>
<td>leucocyte common antigen</td>
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<td>LDH</td>
<td>lactate dehydrogenase</td>
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<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>MACS</td>
<td>magnetic activated cell sorting</td>
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<td>MCAM</td>
<td>melanoma cell adhesion molecule</td>
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<td>MI</td>
<td>myocardial infarction</td>
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<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
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<td>MNCs</td>
<td>mononuclear cells</td>
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<td>mTOR</td>
<td>mammalian target of rapamycin</td>
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<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NGS</td>
<td>normal goat serum</td>
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<tr>
<td>NMP</td>
<td>N-Methyl-2-pyrrolidone</td>
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<tr>
<td>NO</td>
<td>nitric oxide</td>
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<tr>
<td>NF</td>
<td>nuclear factor</td>
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<td>OPT</td>
<td>optical projection tomography</td>
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<tr>
<td>PA</td>
<td>polyacrylate</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PCI</td>
<td>percutaneous coronary intervention</td>
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<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<td>Acronym</td>
<td>Full Form</td>
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<td>PDT</td>
<td>population doubling time</td>
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<td>PE</td>
<td>phycoerythrin</td>
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<td>PECAM</td>
<td>platelet endothelial cell adhesion molecule</td>
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<td>paraformaldehyde</td>
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<td>platelet rich plasma</td>
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<td>PTPRC</td>
<td>protein tyrosine phosphatase receptor type C</td>
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<td>polyurethane</td>
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<tr>
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<td>reactive oxygen species</td>
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<td>STEMI</td>
<td>ST segment elevation myocardial infarction</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<td>tetrahydrofuran</td>
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<td>tumour necrosis factor</td>
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<td>United Kingdom</td>
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<td>United States of America</td>
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<td>ultraviolet</td>
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<td>VCAM</td>
<td>vascular cell adhesion molecule</td>
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<td>vascular endothelial growth factor</td>
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<td>vascular smooth muscle cell</td>
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<td>vWF</td>
<td>von Willebrand Factor</td>
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CHAPTER 1

INTRODUCTION
1.1 OVERVIEW

Coronary artery disease has reached epidemic proportions in Europe with 1 in 3 adults affected\(^1\). Atherosclerotic lesion rupture and thrombus formation is life threatening and the immediate restoration of blood flow is required to preserve myocardial function. The mainstay of clinical treatment is percutaneous coronary intervention (PCI), facilitating revascularisation through balloon angioplasty and the adjunct therapy of coronary stent implantation.

There is a substantial need for new therapeutic approaches that promote repair of the vasculature following PCI. Adverse clinical outcomes, such as restenosis and thrombosis, arise from disruption to the endothelium during PCI and adverse inflammatory reactions to the implanted stent biomaterials. Thus, promoting endothelialisation and the suppression of inflammation are of utmost important to the next generation of coronary stents as this could improve the clinical outcome for patients following acute myocardial infarction (MI).

1.2 CORONARY ARTERY DISEASE

Coronary artery disease (CAD) is the most common cause of premature death worldwide. In Europe, CAD is responsible for 39% of all deaths, equating to more than 4 million deaths per year\(^1\). Epidemiological studies, metabolic studies and genetic studies have identified various lifestyle (cigarette smoking, diet, physical inactivity, stress), biological (hypercholesterolaemia, hypertension, diabetes mellitus) and personal (age, gender, family history) risk factors that contribute to disease state\(^2\). The pathology underlying CAD is atherosclerosis\(^3\).

1.2.1 Atherosclerosis

Atherosclerosis is a chronic inflammatory condition that becomes a clinical event when an atherosclerotic lesion ruptures. The prevalence of atherosclerosis is high. A study of 105 Vietnam war combat deaths
indicated that 45% of subjects had atherosclerosis and 5% had severe CAD despite the mean age of 22\textsuperscript{4}. And while the Global Burden of Disease from 2010 stated that the global age-standardised mortality rates for atherosclerosis has fallen, atherosclerosis is still the leading cause of premature adult mortality worldwide\textsuperscript{5}.

Atherosclerosis is a disease of the vessel wall. The vessel wall is composed of three layers: the intima, the media and the adventitia. The innermost layer of the artery is the intima, which is composed of endothelial cells sitting on a basement membrane and internal elastic lamina. Over this lies the media, which is made up of vascular smooth muscle cells (VSMCs) sitting on a basement membrane, interstitial extracellular matrix, and external elastic lamina. The outermost layer is the adventitia, which is made up of perivascular nerves, fibroblasts and connective tissue that act as a support element. Atherosclerotic lesions are composed of lipids, leucocytes, and connective tissue elements\textsuperscript{6}. They manifest beneath the endothelium within the intima of the vessel wall and occur as sites of predilection related to disturbed laminar flow such as branch points with low and oscillatory shear stress\textsuperscript{7}. Atherosclerotic lesions develop as a result of a dysfunctional endothelium, an imbalanced lipid metabolism and a maladaptive immune response\textsuperscript{7}.

**Atherogenesis**

Atherosclerotic lesions can be initiated when risk factor stimuli render the intact endothelium permeable leading to the sub-endothelial accumulation of low-density lipoprotein (LDL) in the vessel wall (**Figure 1.1**). LDL entry is concentration dependent and does not require receptor-mediated endocytosis. Animal studies have demonstrated that atherosclerosis can be induced by a mutation in a single gene of the LDL receptor indicating that the key step in lipid aggregation is LDL association with collagen\textsuperscript{8}. Animal studies have also shown that LDL rapidly associates with collagen within the extracellular matrix (ECM) and that concentrations of apolipoprotein-B (the major protein of LDL) are higher in the arterial wall than in plasma indicating that LDL becomes trapped within subendothelial space\textsuperscript{9}.
As LDL accumulates in the sub-endothelial space, arterial cells secrete oxidative products initiating lipid oxidation and structural alterations in the size and surface topography of LDL. The mild oxidation of LDL activates endothelial cells leading to monocyte cell tethering, activation and attachment with increased expression of P-selectin\textsuperscript{10}. This leads to monocyte entry to investigate the pathological stimuli\textsuperscript{11}. On arrival to the vessel wall, monocytes convert to macrophages and further oxidise LDL. This leads to a shift in receptor recognition and cellular uptake of LDL by receptors that are not regulated by the cholesterol content of cells resulting in mass accumulation in the sub-endothelial space. Macrophages also phagocytose the oxidised LDL causing focal collections of lipid-laden macrophages (foams cells) and the appearance of fatty streaks.

Fatty streaks progress to more complex lesions as foam cells die with inefficient clearing leading to pooled lipids that provoke further inflammation and the formation of a necrotic core\textsuperscript{12}. The presence of inflammatory released cytokines (interleukin [IL]-1, IL6, and tumour necrosis factor [TNF]-\(\alpha\)) and growth factors (platelet-derived growth factor [PDGF] and basic fibroblast growth factor [bFGF]) increase the production of matrix metalloproteinases (MMPs) in the vessel wall catalysing the removal of basement membrane and the proliferation and migration of VSMCs to the intima\textsuperscript{13, 14}. In turn, VSMCs deposit ECM (such as interstitial collagen and elastin) that encase the necrotic core in a fibrous cap and contribute to the progression of atheroma. Lesions grow by the entry of new mononuclear cells that enter at the shoulder region of lesions, by the proliferation and migration of VSMCs, by the production of ECM, and by the accumulation of extracellular lipids\textsuperscript{12}. Enlarging pools of necrotic lipids can dominate the central part of the intima. These pools can grow out toward the media until a critical point is reached at which point the lesion encroaches on the lumen\textsuperscript{15}. 
Figure 1.1 Atherogenesis

Low-density lipoprotein (LDL) enters the artery wall and becomes oxidised. Monocytes arrive to investigate the pathological stimulus. They covert to macrophages, phagocytose oxidised LDL causing focal collections of lipid-laden macrophages (foam cells) and the appearance of fatty streaks. Fatty streaks progress to more complex lesions as foam cells die with inefficient clearing provoking further inflammation and the formation of a necrotic core. The excess presence of cytokines (Interleukin [IL]-1, IL-4 and tumour necrosis factor [TNF]-α) and growth factors (platelet-derived growth factor [PDGF] and basic fibroblast growth factor [bFGF]) induce proliferation of vascular smooth muscle cells (VSMCs) from the intima. VSMCs encase the necrotic core in a fibrous cap and contribute to the progression of the atheroma.
1.2.2 Clinical manifestation of atherosclerosis

The protrusion of atherosclerotic lesions into the lumen of the artery can cause decreased blood flow in various vascular beds including the coronary and peripheral circulation. The protrusion of atherosclerotic lesions can also lead to plaque rupture, which may result in an acute coronary syndrome (ACS) or stroke. Atherosclerotic lesions often rupture at the shoulder regions of eccentric lesions due to a combination of internal and external pressures. Internally, the up-regulation of proteolytic enzymes (such as MMPs) can degrade ECM in the fibrous cap leading to sites of structural weakness; A thin fibrous cap less than 65μm thick is vulnerable to rupture\(^\text{16}\). Externally, mechanical forces can erode the endothelium and fibrous cap\(^\text{17, 18, 19}\). ACS is classified as ST-segment elevation myocardial infarction (STEMI), non-ST-segment elevation myocardial infarction (NSTEMI) or unstable angina depending on the consequence of plaque rupture and electrocardiograph at presentation. NSTEMI occurs when there is occlusion of a minor artery or a partial occlusion of a major artery resulting in ST-segment depression with partial damage to the myocardium. STEMI occurs when there is complete occlusion of a coronary artery with ST-segment elevation indicative of damage to the entire thickness of the myocardium. It is indicative of atherosclerotic lesion rupture with occluding thrombus formation and immediate medical intervention is required to restore blood flow and preserve myocardial function.

1.2.3 The role of the endothelium in atherosclerosis

1.2.3.1 Endothelial cell function

A single layer of endothelial cells lines the entire vascular system. This monolayer of endothelial cells, or endothelium, consists of approximately $1 \times 10^{13}$ cells forming an almost 1 kg organ\(^\text{20}\). The endothelium sits on a basement membrane and internal elastic lamina within the vasculature. From this position, the endothelium forms a semi-permeable barrier between circulation and vessel wall and it controls the passage of large and small molecules into the vessel wall. The endothelium is a multi-functioning paracrine and endocrine organ. It regulates inflammation, haemostatic processes, mitogenic and
contractile activities of the vessel wall through mechanical and chemical cues received from the circulation and it influences underlying VSMCs and overlying blood elements such as leucocytes and platelets20.

The endothelium regulates the extravasation of leucocytes between endothelial cells to sites of inflammation by the expression of selectins and integrin on its surface. Under basal conditions, the endothelium discourages inflammation in the vessel wall by regulating leucocyte adherence and extravasation21. Endothelial cells prevent leucocyte accumulation within the vessel wall by low-level expression of selectins and integrin on its surface for leucocyte trafficking.

The endothelium also regulates the delicate haemostatic balance between pro-thrombotic and anti-thrombotic states by secretions of regulatory molecules that control platelet phenotype. Under basal conditions, the endothelium is anti-thrombotic and anti-coagulant. Endothelial cells prevent thrombus formation and platelet activation by a number of mechanisms. First of all, they constitutively express the anti-aggregate 13-hydroxyoctadecadienoic acid on their cell surface22. Secondly, endothelial cells release nitric oxide (NO) and prostacyclin from arachidonic acid via the cyclooxygenase pathway in response to pro-coagulant and pro-thrombotic mediators to control platelet phenotype. Third, endothelial cells degrade aggregating agents (adenosine diphosphate [ADP] and adenosine triphosphate [ATP]) by membrane ectonucleotidases21. Fourth, endothelial cells constitutively express the thrombin receptor thrombomodulin. Thrombomodulin converts thrombin (a potent aggregating agent) from a pro-coagulant enzyme to an anti-coagulant enzyme. Lastly, endothelial cells express tissue factor pathway inhibitor as well as cell surface glycosaminoglycans (heparin-like molecules) to prevent coagulation, and they release pro-fibrinolytic activators such as tissue-type plasminogen activator21.

Furthermore, the endothelium regulates mitogenic activities within the vessel wall and it regulates vasotone. It regulates the mitogenic activities and the growth of surrounding connective tissue by the secretion of growth factors that encourage proliferation and migration of VSMCs within the media (e.g. platelet derived growth factor [PDGF], basic fibroblast growth factor [bFGF], endothelin, anti-
proliferative NO and anti-proliferative transforming growth factor [TGF]-β). Because of this, it plays a protective role by suppressing abnormal proliferation of VSMCs following various pathological situations including atherosclerosis. The endothelium regulates vascular tone by the production and release of a number of vasodilator and vasoconstrictor substances, which regulate vascular tone. The most potent vasodilators released by the endothelium are NO and prostacyclin which can be released in response to the inflammatory mediator bradykinin. Potent vasoconstrictors include endothelin and angiotensin II.

### 1.2.3.2 Endothelial dysfunction

Endothelial dysfunction is the critical determinant of atherosclerosis. Endothelial dysfunction can manifest in a number of ways and has been characterised by impaired production or release of NO, fibrinolysis, or through defects in the interactions between endothelial cells and inflammatory cells. Endothelial dysfunction occurs in response to stimuli from risk factors, such as cigarette smoking or hypercholesterolaemia, although the exact mechanism through which these factors induce endothelial dysfunction is not well defined. Many predisposing risk factors for atherosclerosis have been associated with endothelial dysfunction.

Pathological and mechanical cues received from the circulation can lead to dysfunction and activation of the endothelium contributing to the development and progression of atherosclerotic lesions. One such mechanical cue imposed by circulating blood is shear stress. Shear stress is a key regulator of endothelial cell function and it can confer protection or predilection to atherogenesis. High shear stress is athero-protective inducing a ‘quiescent’ endothelial cell phenotype resistant to inflammatory stimuli. While low shear stress is atherogenic, inducing an ‘activated’ endothelial cell phenotype with increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, increased expression of leucocyte adhesion molecules, increased reactive oxygen species (ROS), and increased scavenging of NO with activation of nuclear factor (NK)-κB. As such, atherosclerotic lesions co-localise with regions of low shear stress throughout the arterial tree such as at branches, bends and bifurcations. Thus, the carotid artery
bifurcation, the coronary arteries, the infrarenal artery and femoral artery are geometrically predisposed to lesion formation. In vascular diseases, endothelial dysfunction is defined as a systemic pathological state of the endothelium. It can be broadly defined as an imbalance between the vasodilating and vasoconstricting substances produced by (or acting on) the endothelium. It is commonly characterised by a decrease in the bioavailability of NO (the most potent vasodilator) and/or a failure of the physiological balance between NO and its counterpart endothelin (a potent vasoconstrictor). NO is synthesized by the endothelial NO synthase (eNOS) from the precursor L-arginine. The endothelium can produce NO through stimulation by acetylcholine or bradykinin, and through flow-mediated shear stress. Diminished levels of NO may result from a decrease in NO synthesis or an increase in NO inactivation due to increased production of ROS. The reduced bioavailability of NO is the earliest clinical manifestation of atherosclerosis preceding angiographic evidence of atherosclerotic lesions. Endothelial dysfunction is measured as an impairment of endothelium-dependent vasodilation through the infusion of vasoactive medicines using forearm venous occlusion plethysmography or following ischemia-reperfusion (flow-mediated dilatation) by ultrasound of the brachial artery. The role of NO in atherogenesis is supported by several studies in apolipoprotein-E knockout mice, where the inhibition of endothelial NO production accelerated atherosclerotic lesion formation. NO prevents leukocyte adhesion, platelet activation and it inhibits the release of plasminogen activator inhibitor. The disruption in the delicate balance of NO and endothelin is also implicated in the atherogenesis process as endothelin is a strong chemo-attractant for monocytes leading to over secretion of inflammatory mediators. Endothelin also stimulates VSMC migration, and it stimulates platelet attachment though increased expression of P-selectin.

Endothelial dysfunction results in an endothelium that is pro-inflammatory. As atherosclerosis is a chronic inflammatory disorder, the passage of leukocytes from the circulation to the vessel wall is one of the leading factors in lesion formation. The movement of leukocytes from the circulation through the vessel wall is a multi-step cascade involving the capture (or tethering), rolling and arrest of leukocytes, followed by their transmigration. With prolonged pathological stimuli in endothelial dysfunction, the endothelium translocates P-selectin from Weibel–Palade bodies to the cell surface and it up-regulates the
expression of E-selectin encouraging leucocyte attachment and rolling. Selectins are transmembrane type I glycoproteins that were named after the cells where they were first discovered: E-selectin, L-selectin and P-selectin for endothelium, leucocyte and platelets, respectively. Slow rolling leucocytes become activated by endothelial chemokines, leading to conformational changes in integrins, a superfamily of adhesion receptors expressed by the leucocytes. The leucocyte integrins interact with their ligands on endothelial cells, which are CD31 (also known as platelet–endothelial cell adhesion molecule [PECAM-1]), CD106 (also known as vascular cell adhesion molecule [VCAM-1]) and CD54 (also known as intercellular adhesion molecule [ICAM-1]). This interaction leads to firm arrest of leucocytes, leading to transmigration through the endothelial cell barrier via paracellular (inter-endothelial junctions) or transcellular routes. These sustained interactions contribute to the immune response and chronic inflammation leading to the entry and attachment of leucocytes into the vessel wall. This contributes to the formation of foam cells as leucocytes engorge on oxidized LDL, leading to foam cell-rich fatty streak development and complex atherosclerotic lesion formation.

Endothelial dysfunction is associated with disruption of the delicate haemostatic balance between pro-thrombotic and anti-thrombotic states leading to an environment predisposed to thrombus formation. In response to thrombin (and protease-activated receptors) the endothelium releases von Willebrand factor (vWF) from storage in cytoplasmic Weibel–Palade bodies into the circulation resulting in complexes with Factor VIII and platelet–endothelial cell interactions. In response to ADP, ATP and serotonin- the endothelium also releases platelet-activating factor, leading to platelet attachment and the release of thromboxane A₂, which encourage thrombus formation. The endothelium also expresses thrombin receptor protease-activated receptor-1, which enhances the release of vWF and prevents fibrinolysis by inhibiting tissue plasminogen activator.
1.2.4 Modelling the endothelium *in vitro*

For *in vitro* vascular modelling of the coronary endothelium in atherosclerosis, studies employ endothelial cells from other sources or distal locations, as there is no easy and reliable method to isolate endothelial cells from the diseased coronary vascular bed. The tacit assumption is that these endothelial cells are similar to endothelial cells from the coronary arteries. This assumption has been disproved with the concept of endothelial heterogeneity now widely established across the vascular tree\(^{32}\). Studies employing gene expression profiling have shown significant endothelial cell diversity at different sites along the vascular tree as endothelial cells perform many site-specific functions\(^{33}\). A well-known anatomical and physiological distinction between endothelial cells exists within the arteries and veins. Generally speaking, the arteries are smaller in diameter and have thicker walls. They carry afferent circulation rich in oxygen, do not contain valves and the endothelium that line these vessels are long and narrow due to their alignment in the direction of undisturbed high-shear blood flow. Veins carry efferent circulation low in oxygen, they contain valves and the venous endothelium is short and wide due to low-pressure blood flow. These differences, in particular the differences in fluid dynamics\(^{34}\), make the arterial endothelium more susceptible to atherosclerosis. And while the morphological and functional adaptation of venous endothelial cells in the arterial circulation has been reported\(^{35}\), evidence suggests that the identity of arterial and venous cells is established by genetic factors before the onset of circulation during embryonic development\(^{36}\) indicating an inherent bias for endothelial cell placement.

In the laboratory, large vessel endothelial cells from the veins and arteries are used for studies of coronary artery endothelial cells in atherosclerosis. Endothelial cells from the umbilical veins\(^{37}\) are largely used despite arguments that this cell type is not relevant for arterial disease modelling\(^{38}\) and cadaveric human coronary artery endothelial cells are used, but little information relevant to the health of these arterial beds is supplied. With the recognition of current vascular cell inadequacies, new methods for obtaining primary endothelial cells through endovascular biopsy of the superficial forearm vein and the radial artery of patients have been developed\(^{39}\). Recently, late endothelial outgrowth cells (EOCs) have been employed to study arterial disease\(^{40}\). These cells are derived from endothelial progenitor cells and are widely
believed to be representative for the mature endothelium in vitro however the origin of these cells still remains unknown raising the question of their relevance in arterial disease modelling.

1.3 CLINICAL INTERVENTION FOR CORONARY ARTERY DISEASE

Patients with CAD receive lifestyle modification and risk factor control advice (primary prevention), as well as pharmacotherapy to manage and reduce adverse clinical outcomes associated with angina and ACS.

Statins (or 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors) reduce LDL synthesis in the liver and suppress inflammation reducing the incidence of atherothrombosis. Low dose aspirin and/or clopidogrel (dual antiplatelet therapy) are also prescribed to reduce the risk of platelet aggregation and tissue factor release which reduces the risk of thrombus formation in patients with ACS. Dual antiplatelet therapy is a very effective treatment to prevent stent thrombosis following MI, with a rate of <2% for recurrent MI in patients following 12 months of treatment. Patients also receive antihypertensive medication such as angiotensin-converting enzyme (ACE) inhibitors and beta blockers to regulate blood pressure and increase the lumen size to improve blood flow.

1.3.1 Revascularisation

Revascularisation is used in patients with stable angina or ACS to re-establish blood flow for symptomatic relief. In patients with complete vessel blockage (such as thrombus formation resulting in ST segment elevation myocardial infarction) and incomplete vessel blockage (such as severely narrowed arteries resulting in non ST segment elevation myocardial infarction), reperfusion can be accomplished through PCI or thrombolytic agents (such as tissue plasminogen activator, streptokinase, and urokinase) which stimulate fibrinolysis to destroy blood clots. In patients with complete vessel blockage, PCI is preferred over thrombolitics as reduced ischaemia and increased survival have been reported. If PCI is
unsuccessful or if multiple vessel occlusions are diagnosed, coronary artery bypass grafting (CABG) may be performed but this is associated with a higher risk of perioperative morbidity and mortality.

1.3.2 Percutaneous coronary intervention

PCI encompasses balloon angioplasty and the adjunct therapy of coronary stent implantation. It is a routine procedure that is performed in patients presenting with STEMI, NSTEMI, unstable and stable angina. In asymptomatic patients, PCI can be considered in patients with moderate to severe ischemia on non-invasive testing. From January to December 2014, a total of 96,143 patients received PCI procedures in the UK48.

Balloon angioplasty was first described in 196449. During this procedure, a flexible wire is passed through the radial or femoral artery into the narrowed segment of the coronary artery, over which a balloon catheter is guided (Figure 1.2 A and B). The balloon is then inflated to stretch the vessel segment and compress the atherosclerotic lesion against the vessel wall leading to revascularisation of the lumen and the re-establishment of coronary blood flow (Figure 1.2 C). PCI may be accompanied by atherothrombectomy to remove the occluding thrombus if it is substantial in size (Figure 1.2 D). However this technique is only recommended in a subset of patients as it is not more effective than angioplasty50, 51.

The long-term benefit of balloon angioplasty was limited by abrupt vessel closure, which was occurring in 6-8% and 30-50% of low-risk and high-risk patients respectively52. While the systemic administration of pharmacotherapy reduced thrombus formation and the incidence of abrupt vessel closure44, it had little impact on the incidence of restenosis (angiographically demonstrated reduction of vessel diameter by at least 50%)53-56. Improved results were observed by the insertion of hollow metal mesh tube mechanical supports (stents) to stabilise and widen the compromised vessel segment. The first balloon-expandable coronary stent was deployed over 30 years ago (the Palmaz-Schatz stent)57. The proportion of PCI
procedures using stents rose from 10% in 1993 to 80% in 1999. It continued to increase to 94% in 2005 and today stents are used in 99% of PCI procedures\textsuperscript{58}. Patients may receive a bare metal stent (BMS) or a drug eluting stent (DES) during PCI. Randomised studies have demonstrated that the 6 month rate of reoccurrence of stenosis is 32%-42% after balloon angioplasty and 16%-32% after the implantation of a BMS\textsuperscript{59}. The 6 month rate of reoccurrence of stenosis after the implantation of a DES is less than 10% but there is a slight increase in the risk for thrombosis (~2\%\textsuperscript{60}). The underlying physiologically mechanism for restenosis following balloon angioplasty is due to the proliferation and migration of VSMCs (neointimal hyperplasia), elastic recoil and negative arterial remodelling. Stent implantation prevents both elastic recoil and negative arterial remodelling therefore the predominant underlying physiologically mechanism for restenosis following stent implantation is neointimal hyperplasia. Clinicians avoid percutaneous revascularisation with stents if the patient is at high risk of restenosis, such as patients with diabetes mellitus or in those with small vessels (<2.5 mm), or if there are contraindications to prolonged anti-platelet therapy (e.g. high bleeding risk).

![Figure 1.2](image.png)

**Figure 1.2 Revascularisation of the coronary artery to re-establish blood flow.**

(A) Angiography of the coronary artery demonstrating complete occlusion and filling defect consistent with acute thrombosis. (B) During percutaneous coronary intervention, a balloon catheter is inserted into the narrowed segment of vessel and the balloon is inflated to stretch the narrowed segment of vessel (C) and re-establish coronary blood flow. (D) If the obstruction is substantial in size, balloon angioplasty may be accompanied by thrombectomy with aspiration of the atherothrombectomy specimen.
1.3.3 Stents

**Bare metal stents**

BMS are hollow metal mesh cylindrical mechanical supports. They were designed to stabilise the comprised region of the vessel segment to prevent vessel recoil, vessel collapse and abrupt vessel closure following balloon angioplasty\(^6\). BMS are made from surgical grade metallic alloys such as stainless steel (surgical grade 316L), cobalt-chromium and platinum-chromium. These metals are strong to prevent elastic recoil and maintain radial pressure after implantation, they are flexible so they can be deformed from the fully annealed state, they are malleable to reduce the stent cross-sectional diameter in the lumen to reduce vascular trauma after implantation (endothelial cells denudation, internal elastic lamina fracture, media injury, and adventitial injury), and they possess good radio-opacity for tracking through lesions by radiographic imaging\(^6\). Platforms made with thinner struts may result in less arterial injury and reduce the risk of restenosis and thrombosis. For this purpose, future research is focusing on platinum-chrome alloys to further improve radial strength and conformability.

**Drug-eluting stents**

DES are hollow mesh cylindrical mechanical supports that elute pharmacological agents that interact with the coronary vascular environment. The platform for DES is traditionally made from metal and these platforms are coated with efficacious pharmacological agent eluted at a controlled rate by a persistent or biodegradable polymer. These agents inhibit cellular proliferation and the complex cascade of events that lead to neointimal formation following PCI. DES are ideal delivery systems as they allow the local delivery of active agent to areas of vascular injury, averting the need for high systemic dosing. The first proof of concept study for DES (the RAVEL study\(^6\)) showed that patients randomly assigned to the control arm of the trial had 26% angiographic restenosis, whereas those with a DES (rapamycin) had a restenosis rate of 0%. In addition, the SIRIUS study\(^6\) (with more complex lesions at higher risk of restenosis) supported this study showing restenosis was 3% which was less than the control group at 35.4%.
The pharmacological agents used in the first generation of DES were Paclitaxel and Rapamycin. Paclitaxel (Taxol) is a lipophilic diterpenoid and a microtubule-stabilising agent that increases tubulin polymerisation preventing mitotic spindle formation during cell division leading to cell cycle arrest\(^64\). Rapamycin (Sirolomous) is a macrocyclic lactone that inhibits cell proliferation and cell migration through interference with cell cycle regulators\(^65\). Rapamycin binds to the FK-binding protein 12 and inhibits the mammalian target of rapamycin (mTOR) leading to cell cycle arrest and immune-modulatory properties as it also blocks proliferation of T and B cells.

Second generation DES use Everolimus and Zotarolimus. Everolimus is an analogue of Rapamycin. It was previously used as an immunosuppressant to prevent allograft rejection after organ transplantation and as an anti-mitotic treatment for cancer. The everolimus-FKBP12 complex inhibits the FKBP12-rapamycin associated protein inhibiting downstream cell cycle signaling pathways. As a consequence, mRNAs that code cell-cycle proteins are impaired leading to cell cycle arrest in the late G1 phase. In addition to its anti-mitotic properties, animal studies have also shown that Everolimus selectively clears macrophages in rabbit atherosclerotic plaques by autophagy indicating it could reduce the burden of atherosclerotic plaques\(^66\). In randomized trials, Everolimus-eluting stents improved clinical outcomes compared with Paclitaxel-eluting stents. They also demonstrated increased efficacy and safety by reducing the risks of repeat revascularisation, myocardial infarction, and stent thrombosis\(^67\). Indeed, a meta-analysis showed that Everolimus-eluting stents reduced the risk of stent thrombosis over the long term and myocardial infarction compared to Rapamycin-eluting stents\(^68\).

Zotarolimus is also an analogue of Rapamycin. It was designed for use in stents by substituting a tetrazole ring in place of the native hydroxyl group at position 42 in Rapamycin. This substitution induced a structural change without impairing biological activity leading to an extremely lipophilic compound. This substitution was necessary as hydrophilic Rapamycin did not distribute uniformly through the media and adventitia, resulting in arterial drug concentration more than an order of magnitude above the applied level leading to necrosis within the vessel wall\(^69\). The lipophilic nature of Zotarolimus makes it ideal for sustained low-dose release of drug from the stent at the required therapeutic concentration. And it also
allows for increased permeability thought the vessel wall. In randomised trials, Zotarolimus-eluting stents have shown equivalence to Rapamycin-eluting stents for the prevention of stent thrombosis at 3 years, however these studies demonstrated a higher risk for repeat revascularisation procedures with the Zotarolimus-eluting stent. The Zotarolimus-eluting stent has also been compared with the Everolimus-eluting stent in large-scale trials. While some studies have shown similar risks of cardiac death, myocardial infarction, repeat revascularisation, and stent thrombosis throughout a 2-year period, meta-analysis of studies with longer follow up suggest reduced safety with Zotarolimus-eluting stent perhaps favouring the use of Everolimus-eluting stents instead.

**Bioresorbable scaffolds**

Bioresorbable scaffolds are hollow mesh cylindrical mechanical supports that absorb or dissolve in the body. These scaffolds provide transient vessel support and the platform may elute pharmacological agents if desired. Bioresorbable scaffolds are generally made from polymers (long chain molecules consisting of small repeating units) such as poly-l-lactic acid or polylactic acid. These polymers have high elastic modulus, non-toxic by-products and they are compatible with computed tomography and magnetic resonance imaging for follow-up non-invasive imaging. Also, these stents do not restrict future revascularisation procedures, as the stent platform does not persist.

**1.3.4 Adverse events following percutaneous coronary intervention**

Whilst PCI improves myocardial perfusion and relieves the symptoms of myocardial ischaemia, adverse events continue to be problematic. Disruption of the endothelial monolayer, delayed endothelialisation, and adverse reactions to implanted biomaterials can cause adverse clinical outcomes such as restenosis and stent thrombosis. These conditions increase patient morbidity and mortality, necessitating the need for further revascularisation with economic consequences due to increased hospital stays.
1.3.4.1 Disruption of the endothelium

During PCI, the treated vessel segment endures significant mechanical trauma. The high-pressure balloon inflation of balloon angioplasty and the implantation of rigid stent struts disrupt the endothelium disturbing vascular function and initiating inflammation. Immediately following endothelial cell loss, activated platelets adhere to the de-endothelialised segment recruiting neutrophils to mediate the acute inflammatory response. If endothelial cell loss is minimum, inflammation resolves within a couple of hours with macrophage efferocytosis of apoptotic neutrophils and release of IL10 and TGF-β which activates a pro-resolution M2 macrophage phenotype. If endothelial cell loss is extensive or if endothelialisation is delayed by the implantation of a biomaterial, there is prolonged release of cytokines and chemokines from neutrophils. This primes macrophages to orchestrate the chronic inflammatory response.

1.3.4.2 Adverse reactions to implanted biomaterials

The implantation of foreign material into the coronary artery initiates inflammation as the body mounts an attack against the newly implanted biomaterial. The classic foreign body reaction to the biomaterial involves absorption of water and ions followed by adsorption of plasma proteins such as albumin and immunoglobulin onto the stent surface. This can lead to adhesion of platelets and inflammatory cell recruitment. If the biomaterial elicits minimal inflammation, fibrosis and the eventual encapsulation of the implant by fibrous tissue proceed quickly and inflammation desists. However, the inflammatory response may persist for weeks, months and even years depending on the stent material used.
1.3.4.3 Restenosis and neointimal hyperplasia

Restenosis is the re-narrowing of vessel segments by vascular remodelling and neointimal hyperplasia following PCI. It is an adverse wound healing response that results in lumen loss precipitating the need for further revascularisation.

The predominant mechanism for restenosis after balloon angioplasty is the migration of VSMCs, thrombus formation, elastic recoil and geometric arterial remodelling (inward remodelling) as a compensatory response to vessel stretch and the increasing tissue mass caused by the synthesis of ECM by VSMCs. Vascular remodelling is a spectrum of structural changes whereby the vascular wall responds to changes in its hemodynamic environment. Geometric vascular remodelling is a compensatory response of blood vessels to arterial injury and it occurs in two phases- the artery enlarges after 24 hours for 1 month after angioplasty (probably due to elastic recoil), and then it inward remodels for 1-6 months. While the exact mechanism for remodelling is unknown, it is believed to occur in response to the overstretched vessel wall and in response to the increasing tissue mass caused by the synthesis of ECM by the temporarily dysfunctional or ‘stunned’ VSMCs over a period of weeks to months while they regain their function.

The predominant mechanism for restenosis after balloon angioplasty with stent implantation is neointimal hyperplasia as stent implantation supports the lumen and prevents both elastic recoil and negative arterial remodelling. Animal studies in swine have demonstrated that while neointimal hyperplasia starts at the same time following stent implantation and angioplasty alone, the peak of activity for neointimal hyperplasia differs between the two procedures. This study demonstrated that with balloon angioplasty, VSMCs remained in the intima for up to day 14. While with stent implantation, VSMCs remained present in the intimal nestled around stent struts for a longer period of time. The longer time period for accumulation of VSMCs correlated with an increase in inflammatory cells provoking VSMC proliferation.
Neointimal hyperplasia is driven by inflammation following endothelial cell denudation and adverse reaction to implanted stent materials⁸¹ (Figure 1.3). It is an adverse wound healing response mediated by VMSCs. Neointimal hyperplasia begins when the high pressure inflation of balloon angioplasty and the implantation of rigid stent struts leads to endothelial cell denudation, the rupture of the internal elastic membrane with exposure of underlying myointima, and injury to the underlying vascular cells by media stretch. Immediately, platelets attach to the injured segment and release a plethora of active substances (PDGF, transforming growth factor [TGF]-β, bFGF) to induce vasoconstriction and promote cell growth. They also release endoglycosidase, which cleaves heparin proteoglycan from the surface of endothelial cells and VSMCs rendering them more susceptible to the influence of growth factors. Meanwhile the newly released heparin actively binds to PDGF further increasing the concentrations of growth factors. Within a few hours, monocytes arrive and secrete factors that further encourage cell growth activating VSMCs to a synthetic phenotype. As endothelial cells cover the wound’s surface, VSMCs switch phenotype from contractile to synthetic and they synthesize new ECM components (hyaluronic acid and proteoglycans). The presence of excess cytokines and the production of MMPs further activate VSMCs causing migration from the media to the intima (within 6 weeks of revascularisation³⁵⁴), the deposition of ECM³, and the formation of neointimal hyperplasia with restenosis of the vessel wall. VSMCs produce large amounts of proteoglycan (chondroitin sulfate and dermatan sulfate proteoglycans) which replaces fibronectin as the major ECM component⁸¹. As endothelial cells cover the injured area, they cease proliferation and synthesise heparin proteoglycan which inhibits VSMC proliferation, but this does not prevent VSMC producing ECM⁸¹. Notably, if endothelialisation of the surface is not complete then VSMCS are 50x more proliferative than their underlying counterparts. Thus, VSMCs may encroach in the lumen, thickening the artery wall, reducing myocardial perfusion and inducing the symptoms of myocardial ischaemia.
Figure 1.3 Development of neointimal hyperplasia

(A) Platelets (blue circles) cover the segment of de-endothelialised vessel wall secreting platelet-derived growth factor (PDGF), transforming growth factor (TGF)-β and basic fibroblast growth factor (bFGF). Platelets also secrete endoglycosidase, which cleaves heparin proteoglycan from the surface of vascular cells. Free-floating heparin proteoglycan binds to growth factors and makes them more potent.

(B) Monocytes arrive and further secrete cytokines and growth factors.

(C) Vascular smooth muscle cells (VSMCs) are activated due to the excess amount of cytokines and growth factors. They change from a contractile phenotype (pink VSMCs) to a synthetic phenotype (purple VSMCs) with excess proliferation and migration to the intima. VSMCs also synthesize extracellular matrix components leading to the formation of neointimal hyperplasia and restenosis of the vessel wall.
1.3.4.4 Stent thrombosis

Stent thrombosis may result in the abrupt closure of the stented artery. Although uncommon in practice\textsuperscript{82}, the consequences are severe with high risk of MI (70%) and death (20%)\textsuperscript{83}. Stent thrombosis can occur by the contact activation pathway and/or the tissue factor pathway\textsuperscript{84} and it may occur acutely (within 24 hours of stent implantation), sub-acutely (up to 30 days after stent implantation), late (after 30 days) or very late (after 12 months). While the exact pathogenesis of stent thrombosis is not fully understood, disruption of the coronary endothelium with delayed endothelialisation, adverse inflammatory reactions to implanted biomaterials\textsuperscript{85,86}, and procedure-related factors (dissections left untreated, perforation of necrotic core and stent malapposition [separation of a stent strut from the intima with evidence of thrombus behind the strut]) as well as a combination of patient and lesion-related factors are all understood to increase the risk of stent thrombosis\textsuperscript{87,88}. Acute stent thrombosis is viewed as a problem originating from the biomaterial or procedure while late stent thrombosis is associated with premature cessation and resistance to antiplatelet therapy and incomplete endothelialisation/insufficiencies of drug-eluting stents.

1.4 IMPROVING THE CLINICAL OUTCOME FOLLOWING PERCUTANEOUS CORONARY INTERVENTION

1.4.1 The limitations of current strategies

The main factors underlying the deficiencies in stents are related to the biomaterial and the drug for elution. Currently, there is no biomaterial that fulfils the desired requirement for a BMS or a bioresorbable scaffolds. While precision engineering and the generation of new metal alloys have paved the way for thinner stent struts with improved radial strength and conformability of the metallic platform for BMS, these biomaterials elicit inflammation with the subsequent development of restenosis and stent thrombosis. Also, while there have been improvements in polymer composition, biodegradation, thickness, biocompatibility and thrombogenicity, there been no polymer biomaterial of the tensile strength of metal to mimic the mechanical properties of BMS for bioresorbable scaffolds. DES employ
anti-proliferative drugs to prevent VSMC proliferation for the prevention of restenosis but these drugs adversely affect the vasculature creating an inflammatory and thrombotic environment poised for thrombus formation.

**Bare metal stents (BMS)**

BMS were traditionally designed as cylindrical mechanical supports to stabilise the compromised region of the coronary artery. With the introduction of these stents there was a 13% reduction in the incidence of restenosis and a 10% reduction in subsequent target vessel revascularisation compared to angioplasty alone. The first metal alloys used to produce BMS were fundamentally incompatible with the vasculature eliciting inflammation and stent thrombosis. Hypersensitivity to metallic ions that predispose to a higher frequency of restenosis was reported, but no clear links between metal allergy and restenosis were ever fully established. The metal alloys used in second generation BMS (cobalt-chromium and platinum-chromium) have somewhat reduced the inflammatory responses and third generation BMS are coated in polymer in an effort to increase biocompatibility and decrease hypersensitivities to traditional bare metal platforms. While these efforts have increased biocompatibility, the implantation of BMS is not recommended following PCI as high levels of neointimal hyperplasia occur. Drug-eluting stents are recommended as these stents have reduced the need for subsequent target vessel revascularisation by 53% in comparison to BMS.

**Drug-eluting stents (DES)**

Drug-eluting stents (DES) were developed to inhibit neointimal hyperplasia following stent implantation. These stents have a metal platforms with an efficacious anti-proliferative drug (Rapamycin, Paclitaxel, Everolimus and Zotarolimus) encased in biodegradable polymer targeting VSMC proliferation for the prevention of neointimal hyperplasia. The pharmacological agents used in the first generation of DES were Paclitaxel and Rapamycin. While second generation DES use Everolimus and Zotarolimus.

While DES have reduced the morbidity associated with BMS implantation, they have offered little in
terms of safety with mortality rates equal to BMS. This is due to the increased risk of very late thrombosis (<2 years) with DES. Although effective at reducing neointimal hyperplasia, anti-proliferative drugs adversely affect the vasculature increasing the incidence of life-threatening late stent thrombosis. This is attributed to the inflammatory and pro-thrombotic environment created by reduced endothelial cell proliferation with reports of a 40% reduction in endothelialisation of stent struts at 6 months. There have been slight improvements in levels of endothelialisation with second generation DES (in particular Everolimus eluting stents), but these drugs have been linked to increased tissue factor expression. As tissue factor is an important transmembrane protein that forms a complex with Factor VIIa initiating the formation of thrombin from the zymogen prothrombin in the extrinsic blood coagulation cascade, it has been suggested that increased levels of tissue factor could predispose patients to thrombosis as increased thrombosis has been reported in animal studies. Also, the current low rates of stent thrombosis (2%) are predicted on adherence and tolerance to anti-thrombotic medication, which is not feasible for patients requiring surgery or at high risk of gastrointestinal bleeding.

To prevent thrombosis occurring, patients are required to use antiplatelet drugs (such as aspirin or clopidogrel) after the implantation of a stent. Following data published in 2006, the FDA recommends the duration of anti-platelet drugs to be extended in patients receiving a DES due to the increased risk of stent thrombosis. Very late stent thrombosis (>2 years) can occur from underlying excessive neointimal hyperplasia and from neoatherosclerosis, but it is unclear why it occurs at >2 years when drug delivery is complete after 6 months of implantation. The most widely held theory is that late stent thrombosis occurs in stents that have not been fully opposed to the vessel wall (malapposition), often due to fibrocalcific plaque disease, where stent struts are not able to effectively endothelialise. This is more likely to occur in drug eluting stents, where the drug inhibits cell migration during the early phase of repair.

Currently, it is unknown whether very late stent thrombosis can be prevented with an extended course of dual anti-platelet therapy. Delayed arterial healing and more specifically delayed re-endothelialisation after the elution of anti-mitotic drugs are cited as the precipitating and predisposing factors for very late stent thrombosis. Abnormal vascular response such as hypersensitivity reactions have also been suggested as a possible mechanism for very late stent thrombosis. The persistence of the polymer
coating after drug release has also been implicated as the eroded polymer surface could provide a substrate for platelet deposition and thrombus propagation. In this context, it has been suggested that the stent platform and the polymer drug carrier is the area to target to reduce the incidence of very late stent thrombosis following implantation of DES.

Bioresorbable scaffolds

Bioresorbable scaffolds were developed to provide transient vessel support with drug-delivery capability. These stents were designed to reduce restenosis associated with BMS by the elution of drugs and to reduce late stent thrombosis associated with DES by the removal of the foreign implant material74. While this technology is still in its infancy, initial reports of bioresorbable scaffolds without drug elution have demonstrated high levels of restenosis with 40% of patients requiring revascularisation at their 4 month follow-up appointment103. This has been attributed to problems mimicking the mechanical properties of the metallic cage103. However, reports of bioresorbable scaffolds with drug elution have demonstrated stable lumen dimensions and low levels of restenosis at 5 years. Whilst encouraging, early clinical experience with first generation bioresorbable scaffolds has been mixed. Indeed, these products have been withdrawn in a number of countries following recent and emerging evidence that they are associated with higher rates of early and late device failure or thrombosis104.

1.4.2 The importance of the endothelium

The endothelium is anti-inflammatory and anti-mitogenic with anticoagulant properties. During PCI, the high-pressure balloon inflation of balloon angioplasty and the forceful apposition of comparatively rigid stent struts denude endothelial cells leading to segments of vessel with no endothelium. This exposes sub-endothelial matrix to flowing blood, instigating inflammation and contributing to the development of restenosis and stent thrombosis.
Endothelialisation over denuded vessel segments and the surface of coronary stents surfaces can re-instate vascular homeostasis with regulation of inflammation, the phenotype of VSMCs, and haemostatic processes. Endothelialisation can also suppress adverse inflammatory reactions to implanted biomaterials. Human autopsy studies have demonstrated that complete re-endothelialisation can take up to 3 months following stent implantation. It is during this time that vascular injury triggers local and systemic responses leading to the secretion of cytokines and growth factors that encourage VSMC proliferation and neointimal hyperplasia. Without the endothelium, anti-mitotic secretions to control VSMC growth (NO and TGF-β) and haemostatic regulatory molecules (such as prostacyclin and nitric oxide) are lost facilitating the development of restenosis and thrombosis. Indeed, studies have shown that VSMCs are 50 times more proliferative than their counterparts in the de-endothelialised segment. Thus, rapid re-endothelialisation in the early post-injury period can prevent neointimal hyperplasia despite the temporal differences between the two processes.

In experimental animal models it has been demonstrated that endothelial cell loss correlates with the severity of inflammation and that endothelial cell loss correlates with neointimal hyperplasia. Human studies have demonstrated that the severity of inflammation (demonstrated by C-reactive protein) correlates with the risk of stent thrombosis. Human studies have also demonstrated that endothelialisation of stent struts is a powerful histological predictor of stent thrombosis.

1.4.2.1 Endothelialisation following percutaneous coronary intervention

The mature endothelium is typically quiescent (47-23,000 days turnover rate) however pathological stimuli (such as endothelial cell loss during PCI) can stimulate in-situ endothelialisation by chemotaxis gradients with growth of coronary endothelium. Complete endothelialisation takes approximately three months following BMS implantation. With DES, endothelialisation may not be complete after 2 years.

Rapid endothelialisation may reinstate vascular homeostasis reducing adverse outcomes following PCI.
Restoration of the endothelium has been implicated in the suppression of neo-intima hyperplasia in rat and rabbit animal models of balloon injury and restoration of the haemocompliant endothelium is known to deter thrombus formation. This seems to occur despite that fact that the endothelium remains immature after endothelial cell regeneration with compromised barrier integrity and functionality characterised by impaired endothelium-dependant vasodilation. Autopsy studies have demonstrated that endothelialisation is related to clinical outcomes, but currently there is no method available to determine the extent of endothelialisation following stent implantation in practice. Optical coherence tomography is a promising technology that has excellent spatial resolution and can be performed in the coronary circulation. Research using this method may help improve our understanding of the injury and repair response to stent deployment.

Endothelialisation was previously thought to occur through the migration and proliferation of endothelial cells from regions adjacent to endothelial cell denudation. However, isolated islands of endothelial cells in the centre of intra-prosthetic Dacron hubs and intravascular devices as well as the discovery of endothelial progenitor cells challenged the concept that the sutured ends of the endothelium were the only source of cells. It is now accepted that neighbouring/local endothelial cells and circulating endothelial progenitor cells contribute to the intima lining of stent surfaces with Tie2-LacZ reporter mice demonstrating re-endothelialisation by endothelial cells from two localities following PCI.

**Endothelial progenitor cells (EPCs)**

The term ‘endothelial progenitor cell’ (EPC) has been applied to many cell types involved in the regeneration of endothelial cells. These cells were first isolated described by Asahara in 1997 as a population of circulating cells that could differentiate into mature endothelial cells *ex vivo*. EPCs can be isolate from the mononuclear cell fraction of peripheral blood and cord blood. They are believed to play a crucial role following vascular injury by direct incorporation to de-endothelialised vessel and indirectly by the secretion of angiogenic factors encouraging resident endothelial cell proliferation and migration. Because of this, EPCs have generated great interest as a mechanism to promote and repair the
endothelium and have been trialled in numerous clinical trials in an attempt to promote vascular regeneration.

The identification of the EPC in blood has remained controversial due to the phenotypic overlap between EPC, haematopoietic progenitor cells and mature endothelial cells. EPCs in blood were originally identified by expression of CD34 (a cell surface glycoprotein for cell adhesion) and kinase domain receptor (KDR) (a receptor for vascular endothelial growth factor). Subsequent studies have identified circulating EPCs on the basis of co-expression of CD133 with CD34. Most recently, our laboratory had demonstrated that EPCs reside in the CD34+ CD133- and CD146+ cell fraction of mononuclear cells. Circulating levels of EPCs have traditionally been used to assess vascular health and function in patients with CAD with low circulating concentrations found in patients with traditional cardiovascular risk factors for CAD (such as hypercholesterolaemia, hypertension, physical inactivity) and established atherosclerosis disease.

In Ashara’s original description, EPCs were defined as a population of circulating mononuclear cells that could differentiate into endothelial-like cells when cultured on endothelial cell-specific medium. Today, phenotypic studies have demonstrated two functionally distinct EPC subtypes within blood. The first population, termed myeloid-derived EPCs or CFU-Hill colony forming cells, has a genome signature similar to monocytes and is not believed to be the progeny of EPCs. These cells positively regulate angiogenesis by the secretion of cytoprotective or proangiogenic factors in a paracrine manner to encourage the survival and proliferation of resident endothelial cells. The second population, termed late endothelial outgrowth cells (EOCs) have robust proliferative potential and are believed to be the true progeny of an EPC because these cells uniformly express endothelial cell but not hematopoietic cell specific surface antigens, possess clonogenic potential, and contribute to a endothelium when implanted in vivo.

At present, no specific marker can differentiate between an EPC and a mature differentiated endothelial cell, and thus, the origin of circulating EPCs has remained controversial. Numerous articles have been published in an attempt to clarify the origin and function of EPCs with limited success. It was
originally believed that EPCs reside in the bone marrow and mobilise to sites of endothelial denudation and ischaemia where they facilitate re-endothelialisation and contribute to new-vessel formation. But as EOCs can be readily isolated from umbilical cord and peripheral blood but not bone marrow, new evidence suggests EPCs are not derived from the bone marrow. In this study (unpublished from our laboratory), EOCs were derived from the culture of circulating mononuclear cells in male patients who had received allogeneic bone marrow transplants from female donors. It was determined that EOCs capable of clonal expansion had a recipient genotype indicating circulating EPCs do not originate from the bone marrow. This study and others, raise the possibility that EPCs in circulation are derived from a resident stem cell niche within the vascular endothelium.

EPCs have generated great interest as a mechanism to encourage repair of the endothelium in vascular artery disease, and over 2,000 patients have received autologous bone transfers in an attempt to relieve ischemia. It has been shown that CD34+ cells from autologous bone marrow can increase collateral perfusion in patients with bilateral leg ischemia128. It has also been shown that intra-myocardial injection during CABG surgery129 and intracoronary infusion 130 can improve cardiac perfusion, left ventricular function and regional wall motion following MI.

1.4.3 Next generation strategies

Adverse clinical outcomes, such as restenosis and thrombosis, arise from disruption of the endothelium during PCI and adverse inflammatory reactions to the implanted stent biomaterials. Rapid endothelialisation following disruption can inhibit restenosis and thrombosis. Furthermore, the resolution of inflammation may further inhibit restenosis, as neointimal hyperplasia is an inflammatory driven process. Next generation stents seek stent coatings that support growth of the coronary endothelium and inhibit inflammation as this might address the inadequacies with current stents.
1.4.3.1 Endothelialisation following percutaneous coronary intervention

The unsatisfactory performance of both BMS and DES has led to continued research into stent coatings and compounds to promote endothelialisation following PCI. There have been multiple studies that have attempted to encourage re-endothelialisation of stents. The micro-patterning of cobalt chromium stent surfaces to manipulate the attachment and migration of endothelial cells has demonstrated increased endothelialisation at 3 days (81.3% versus 67.5% endothelialisation of stent struts) and reduced restenosis at 28 days (21.1% versus 40.8%) in comparison to stainless steel stents in pig coronary arteries.

Bioactive peptide coatings (such as the Arg-Gly-Asp peptide) mimic the ECM and promote endothelialisation but with an increased risk of platelet binding. The elution of local growth factors, such as VEGF from stent platforms have brought interesting and very controversial results, with some studies reporting beneficial effects and others reporting non-beneficial effects in animal models.

Studies have also assessed polymer substrates to identify those that will promote endothelialisation in vivo. A previous study in our lab assessed the angiogenic and thrombotic properties of polymers with the aim to accelerate re-endothelialisation by applying a thin layer of the polymer to the surface of intra-vascular devices. This study identified 8G7 (a polyacrylate) as capable of promoting the attachment, differentiation and expansion of both EPCs and mature endothelial cells in vitro while minimizing platelet attachment in vivo.

The most advanced surface modification to promote attachment of endothelial cells has been the use of CD34-monoconal antibodies in a bio-engineered stent (Genous™), which has progressed to clinical trials. The bio-engineered Genous™ stent consists of a polysaccharide intermediate with CD34 antibodies immobilised to its surface. It was coated in CD34 antibodies in an attempt to capture circulating EPCs (0.005%-0.01% of all leucocytes) and it was anticipated that the binding of EPCs would result in the formation of a confluent endothelium on stent struts lowering the occurrence of very late stent thrombosis and the need for long-term antiplatelet therapy. In patients with symptomatic CAD (n=98), the Genous™ stent was well tolerated (3.1% versus 2.1% death/MI) but associated with increased levels of restenosis (12.2% versus 8.4% target lesion revascularisation) at 1 year compared to Paclitaxel coated stents.

The reason for increased restenosis was attributed to indiscriminate binding of cells that express CD34 (as...
this glycoprotein is associated with a number of blood-borne stem cells such as vascular smooth muscle stem cells\(^{139}\). As anti-platelet drugs were used for only 1 month after implantation of the Genous\(^{TM}\) stent compared to 6 months for the Paclitaxel eluting stent, it was suggested that the Genous\(^{TM}\) stent might be a useful alternative in patients with active bleeding or a contraindication to prolonged anti-platelet use.

### 1.4.3.2 Suppressing inflammation following percutaneous coronary intervention

Stent coatings have also been explored in an attempt to suppress inflammation mediated against the implanted biomaterial following PCI. Third generation BMS are coated in polymers to increase biocompatibility and decrease hypersensitivities to traditional stent platforms. In the past, poly(dimethyl)siloxane (silicone) and polyethylene terephthalate (dacron) were the material of choice for medical devices due to their excellent track record in prosthetic devices. But these materials are not used in modern day stent coatings as increased inflammation\(^{140}\) and foreign body reactions\(^{141}\) were reported in animal models. Today, novel synthetic polymers and natural polymer coatings are explored to discourage inflammation. Synthetic polymers such as polyzene have been assessed for passivity with thrombo-resistant surface properties reported \textit{in vitro}\(^{142}\) and safety and patency have been reported in first-in-man clinical trials at 6 months (0% death/MI with 9% target revascularisation \(n=55\))\(^{143}\). Natural polymer coatings such as phosphorylcholine have also been explored. Phosphorylcholine is a naturally occurring phospholipid polymer believed to mimic the phospholipase on the outer surface of cells providing a highly compatible surface. This coating demonstrated equivalence to stainless steels in terms of safety (0% \textit{versus} 1% death/MI) and effectiveness (17.7% \textit{versus} 20% binary restenosis) at 6 months in patients with symptomatic CAD (\(n=313\))\(^{144}\). To date, passive coatings have demonstrated safety and patency but they have offered little benefit compared to BMS. Given these inadequacies, pharmacological agents have been explored in an attempt to actively suppress inflammation in the coronary artery. The elution of corticosteroids (such as Dexamethasone\(^{145}\) and Methylprednisolone\(^{146}\)) and statins (such as Cerivastatin\(^{147}\)) from the stent platform have reduced inflammation with a reduction in neointimal hyperplasia compared to BMS, but there has been little reduction in restenosis compared to anti-proliferative agents eluted from DES.
Given these outcomes, it is clear that new compounds are required to reduce the inflammation associated with the implantation of BMS and DES. Recently, cyclin dependent kinase (CDK) inhibitors have gained attention as possible compounds to reduce inflammation following stent implantation. CDK inhibitors are negative regulators of the cell cycle\textsuperscript{148-150}. They are currently under investigation as novel anti-inflammatory agents as they activate neutrophil apoptosis in the presence of powerful survival factors \textit{in vitro} promoting the resolution of neutrophilic inflammation \textit{in vivo}\textsuperscript{151-153}. It has recently been demonstrated AT7519, a novel 2nd generation CDK inhibitor with selective inhibition of CDK 1, 2, 4, 6 and 9\textsuperscript{154}, reduces atherosclerotic plaque size in mice (unpublished) suggesting AT7519 may have a role in resolving the chronic inflammatory response to coronary stenting.

\section*{1.5 SCOPE OF THESIS}

Within this thesis, I explore strategies to improve the clinical outcome for patients following percutaneous coronary intervention. To aid in this process, I develop a novel method to reliably isolate coronary endothelial cells from atherothrombotic specimens and I establish a new cellular model to study the coronary endothelium in patients with acute myocardial infarction. Using this cell model, I identify novel polymer substrates to promote endothelialisation of coronary stents and I evaluate the potential of a novel cyclin-dependent kinase (CDK) inhibitor (AT7519) to resolve neutrophil-mediated inflammatory and the effect this would have on the function and integrity of the coronary endothelium.
1.6 HYPOTHESES AND AIMS

There is a substantial need for new therapeutic approaches that promote repair of the vasculature following percutaneous coronary intervention as this could improve the clinical outcome for patients with coronary artery disease. The identification of stent coatings that promote endothelialisation could prevent adverse events following angioplasty and stenting as this could inhibit neointimal hyperplasia reducing the risk of stent thrombosis. Uncovering novel compounds to resolve inflammation without adversely affecting the endothelium could further prevent adverse events, as this could reduce neointimal hyperplasia reducing the risk of restenosis following angioplasty and stenting. A new *in vitro* model of the coronary endothelium would aid in these investigations, as it would better reflect the coronary environment for stent implantation in patients. This thesis is comprised of three studies that explore the following hypotheses and aims.
ISOLATION AND CHARACTERISATION OF CORONARY ENDOTHELIAL CELLS FOLLOWING THROMECTOMY FOR ACUTE MYOCARDIAL INFARCTION (CHAPTER 3)

HYPOTHESIS

Coronary endothelial outgrowth cells can be isolated from atherothrombectomy specimens and will have impaired function compared to endothelial cells derived from other sources.

1.7 AIMS

1. Isolate and characterise coronary endothelial outgrowth cells from atherothrombectomy specimens obtained from patients receiving thrombectomy for acute myocardial infarction.

2. Compare the phenotype and functions of these cells by measures of growth kinetics, cell attachment, wound closure and tubule formation in vitro with endothelial cells derived from other sources.

3. Compare the function of these cells by measures of angiogenesis in vivo with endothelial cells derived from other sources.
POLYMER SUBSTRATES FOR ENDOTHELIALISATION OF CORONARY STENTS (CHAPTER 4)

HYPOTHESIS
Defined polymer substrates will promote endothelial cell attachment, expansion and retention compared to controls collagen and 8G7 (a polymer previously characterised for high endothelial cell attachment), without increasing thrombosis or platelet and leucocyte attachment.

AIMS
1. Identify polymer substrates with high attachment of endothelial cells and low attachment of inflammatory cells using high-throughput polymer microarrays.

2. Identify polymer substrates that facilitate endothelial cell attachment, expansion and retention under dynamic flow conditions in vitro.

3. Ensure polymer substrates do not aggravate thrombus formation or platelet and leucocyte attachment compared to bare metal stents in an ex vivo model of thrombosis.
CYCLIN-DEPENDENT KINASE INHIBITORS FOR THE RESOLUTION OF NEUTROPHILIC INFLAMMATION FOLLOWING PERCUTANEOUS CORONARY INTERVENTION (CHAPTER 5)

HYPOTHESIS

The cyclin-dependent kinase inhibitor AT7519, will selectively induce neutrophil apoptosis without adversely affecting endothelial cell function or viability in vitro.

AIMS

1. Determine the concentrations of AT7519 that induce neutrophil apoptosis in vitro.

2. Assess the effect of these concentrations on endothelial cell proliferation, function and viability in the presence or absence of pro-inflammatory growth factor (vascular endothelial growth factor) in vitro.

3. Assess the effect of these concentrations on coronary artery vascular smooth muscle cell proliferation and viability in the presence or absence of pro-inflammatory growth factor (platelet-derived growth factor-BB) in vitro.
CHAPTER 2

MATERIALS AND METHODS
2.1 CELL ISOLATION AND MAINTENANCE

All patients recruited for these studies provided informed written consent and ethical approval was obtained from the Lothian Research Ethics Committee. Studies were conducted in accordance with the declaration of Helsinki. Experimental numbers are biological repeats for all except human coronary artery endothelial cells (HCAECs) and human coronary artery vascular smooth muscle cells (VSMCs), which are results of three independent experiments performed at different time points from a commercially available cell line.

2.1.1 Primary cultures generated in-house

2.1.1.1 Endothelial cells

For studies, endothelial cells were cultured on tissue culture plastics pre-coated with type I rat-tail collagen (Cat no. 356400; SLS, UK) and maintained in endothelial growth medium (EGM™ medium (‘EGM™-2’; Cat no. CC-3162; Lonza, UK) supplemented with 10% HyClone™ foetal bovine serum (FBS) (Cat no. 12379802; Fisher Scientific, UK) unless otherwise stated. Upon reaching confluence, endothelial cells were washed with phosphate buffered saline (PBS) (Cat no. 10010023; Thermofisher Scientific, UK) and detached by incubation with 0.25% ethylenediaminetetraacetic (EDTA)-trypsin for 5 minutes at 37°C (Table 2.1). Subsequently, an equal volume of EGM™-2 medium was added. The cells were then counted with a haemocytometer, centrifuged for 5 minutes at 300g, and resuspended in the required volume of EGM™-2 medium for experimental use or further cell culture. Medium was changed 3 times a week and all cell lines were treated in an identical manner. Cells were incubated at 37°C/5% CO₂/95% relative humidity.
Table 2.1 Volumes for cell detachment and cell seeding densities for in vitro culture.

<table>
<thead>
<tr>
<th>Tissue culture plastic</th>
<th>Surface area (mm²)</th>
<th>0.25% EDTA trypsin (ml)</th>
<th>Seeding number</th>
</tr>
</thead>
<tbody>
<tr>
<td>6-well plate (1 well)</td>
<td>950</td>
<td>1</td>
<td>50000</td>
</tr>
<tr>
<td>12-well plate (1 well)</td>
<td>380</td>
<td>0.5</td>
<td>40000</td>
</tr>
<tr>
<td>24-well plate (1 well)</td>
<td>190</td>
<td>0.25</td>
<td>20000</td>
</tr>
<tr>
<td>T75 flask</td>
<td>7500</td>
<td>5</td>
<td>200000</td>
</tr>
</tbody>
</table>

Typical volumes of 0.25% ethylenediaminetetraacetic (EDTA)-trypsin used for detachment of cells and typical seeding densities into tissue culture plastics.

Coronary endothelial outgrowth (CEO) cells

Atherothrombotic specimens were washed with PBS and manually disaggregated into smaller pieces with a sterile surgical scalpel. Tissue explants were seeded into collagen-I coated 6-well plates and maintained under standard cell culture conditions. After 24 hours, tissue explants, non-adherent cells, and debris were aspirated. Medium was changed every other day until the first passage of coronary endothelial outgrowth cells. Cells were left in culture 15-28 days (dependent on proliferative activity) prior to first passage. For functional experiments, endothelial cells of passage 2-4 were used. Further details are provided in Section 3.5 (chapter 3 methods).

Endothelial outgrowth cells (EOCs)

Venous blood from patients with MI (100ml) was drawn from the median cubital vein and placed in 8ml of 3.8% sodium citrate (Cat no. 25116; Sigma Aldrich, UK). Late EOCs were isolated from the mononuclear cell (MNC) fraction of whole blood by buoyant density centrifugation over Ficoll-Paque PLUS (Cat no. 17-1440-03; GE Healthcare, Sweden). Without further cell subpopulation enrichment procedures, 10 x 10⁶ MNCs were resuspended in 1 well of a 6-well tissue culture plate as previously described¹². After 24 hours, cells were washed with PBS, non-adherent cells were aspirated and fresh
media was added. Late EOCs were characterised by their cobblestone morphology and cells were maintained for 21 days in culture prior to first passage.

2.1.1.2 Polymorphonuclear and mononuclear leucocytes

Polymorphonuclear and mononuclear leucocytes were incubated on uncoated tissue culture plastics (Cat no. 3738; Sigma Aldrich, UK) and maintained in Iscove's modified Dulbecco's medium (IMDM) with 10% autologous serum unless otherwise stated. Cells were used immediately for experiments.

Polymorphonuclear leucocytes

40ml aliquots of peripheral blood from healthy volunteers were drawn from the median cubital vein into 4ml of 3.8% sodium citrate. Aliquots were centrifuged (350g, acceleration 0, deceleration 0, for 20 minutes) and overlying platelet rich plasma (PRP) was removed and allocated for autologous serum conversion. Dextran (Cat. 31392; Sigma Aldrich, UK) (6mls of 6% w/v) was added to each aliquot and the final volume of each tube was adjusted to 50ml with pre-warmed saline (Cat no. UKF7124, Baxter, UK) before solutions were mixed and incubated at room temperature for 25 minutes to encourage erythrocyte sedimentation. The upper leucocyte-rich fraction was then removed, washed and pelleted by centrifugation (350g, acceleration 9, deceleration 9 for 6 minutes) and mononuclear and polymorphonuclear leucocytes were separated by buoyant density centrifugation over isotonic percoll gradients. Briefly, percoll (Cat no.17-0891-02, GE healthcare, UK) was diluted to 90% by the addition of 10x PBS (Cat no. D1408; Sigma Aldrich, UK) (without Ca2+/Mg2+ ions) to a make a stock solution. Gradients of 81%, 70%, 55% were achieved by the addition of 8.1ml stock to 1.9ml PBS, 7ml stock to 3ml PBS, and 5.5 ml stock to 4.5ml PBS, respectively. The 70% solution (3ml) was laid over the 81% solution (3ml), and the 55% solution (3ml) mixed with cells was laid over the 70% solution. Gradients were centrifuged (720g, acceleration 0, deceleration 0, for 20 minutes) and MNCs were isolated at the 55%/70% interface while granulocytes were harvested at the 70%/81% interface. Cell population purity was assessed by flow cytometry profiles of side scatter area and forward scatter area. Purified
granulocytes/neutrophils were re-suspended at 2 x 10^6 cells per ml in IMDM medium. Purified mononuclear cells were re-suspended at 4 x 10^6 cells per ml in IMDM medium without autologous serum and allocated for CD14\(^+\) monocyte isolation.

**CD14\(^+\) mononuclear leucocytes**

10 x 10^6 MNCs were centrifuged at 300g for 10 minutes and resuspended in 80μl of magnetic activated cell sorting (MACS) buffer consisting of PBS, 0.5 % human albumin serum (Cat. PL10673/0031; Octapharam, UK) and 2mM EDTA (Cat. ED-100g; Sigma Aldrich, UK). CD14 microbeads (20μl) (Cat. 130-050-201; Miltenyi Biotec, UK) were added and cells were incubated for 30 minutes at 4°C prior to washing and resuspension in 500μl MACs buffer. Cells were then passed through a magnetic MS column (Cat. 130-042-201; Miltenyi Biotec, UK) and magnetically attached cells were collected by pressure generated when a plunger was applied to the MS column. Purified CD14\(^+\) cells were washed and resuspended in IMDM medium with 10% autologous serum. Purity was assessed by flow cytometry profiles of side scatter area and forward scatter area. Cells were used immediately for experiments.

**Autologous serum**

Overlying platelet rich plasma (PRP) isolated by centrifugation of peripheral blood was converted to autologous serum by the addition of 220μl 1M calcium chloride per 10ml PRP. The PRP was then heated to 37°C in a water-bath to encourage platelet aggregation. Serum was filtered through at 5μm sterile wire mesh and stored at 4°C.
2.1.2 Primary culture commercial cells

**Endothelial cells**

Researchers often encounter phenotypic variation amongst batches of commercially acquired human umbilical vein cells (HUVECs) due to donor-donor variation and the method of isolation used (e.g. type of collagenase). Where possible experiments were undertaken using pooled rather than single donor HUVECS to minimise the potential impact of phenotypic variation. Pooled HUVECs were commercially acquired from Lonza, UK (Cat. CC-2519) and Promocell, Germany (Cat. C-12203), while single donor HUVECs were commercially acquired from Invitrogen, UK (Cat. C-003-5C). Single donor human coronary artery endothelial cells (HCAECs) were commercially acquired from Lonza (CC-2585).

Endothelial cells were cultured on tissue culture plastics pre-coated with type I rat-tail collagen and maintained in EGM™-2 medium unless otherwise stated. Medium was changed 3 times per week and all cell lines were treated in an identical manner to endothelial cells generated in house. For functional experiments, endothelial cells of maximum passage 2-4 were used.

**Vascular smooth muscle cells**

Single donor human coronary artery vascular smooth muscle cells (VSMCs) were obtained commercially from Lonza (UK). Cells were cultured on uncoated tissue culture plastics and maintained in Clonetics™ SmGM™-2 Bullet kit Smooth Muscle Growth Medium-2 (Cat. CC-3182; Lonza, UK) unless otherwise stated. VSMCs were cultured, seeded and trypsinised at identical densities and volumes to endothelial cells and incubated at 37°C/5% CO₂/95% humidity (*Table 2.1*). For experiments, cells above passage three were used to ensure a synthetic phenotype instead of a contractile phenotype\(^{155}\).
2.2 PHENOTYPIC CHARACTERISATION OF ENDOTHELIAL CELLS IN VITRO

2.2.1 Flow cytometry

Endothelial cells cultured on type I collagen (Chapter 3) or endothelial cells cultured on 19mm polymer-coated coverslips for 7 and 28 days (Chapter 4) were trypsinised. Aliquots of $1 \times 10^5$ cells were resuspended in 100μl EGM™-2 medium and incubated for 30 minutes at room temperature with pre-conjugated monoclonal antibodies: CD146-PECy7, CD31-FITC, CD105-APC, CD144-PE, CD54-PE, CD34-APC/Cy7, KDR-PE, CD133-APC, CD117-PE, CD106-FITC and CD45-V450 (Table 2.2, 2.3 and 2.4). For cytoplasmic antigens, $1 \times 10^5$ cells were permeabilised with BD Cytofix/Cytoperm™ Fixation/Permeabilisation solution (Cat no. 554714; BD Biosciences, UK) prior to incubation with monoclonal pre-conjugated antibodies: vWF-FITC and eNOS-PE (Table 2.2, 2.3 and 2.4). After washing, cells were fixed with BD FACSTM Lysing Solution (Cat no. 349202; BD Biosciences, UK). Cells were washed and analysed with a BD LSRFortessa™ II cell analyser. Unstained cells were used as controls. Spectral overlap and compensation was calculated and verified manually for each antibody with anti-mouse BD™ CompBead (Cat no. 552843, BD Biosciences, UK). A minimum of 5,000 events in the relevant gate was collected for data analysis with FlowJo version 10.0.6 (TreeStar Inc., Switzerland).
Table 2.2 Antigens expressed by endothelial cells.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Alternative name</th>
<th>Endothelial cell function</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD146</td>
<td>MCAM</td>
<td>Cell adhesion molecule located at endothelial cell junctions. Facilitates endothelial cell-cell interactions.(^\text{156})</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>Cell adhesion molecule. A dynamic regulator of endothelial cell behaviour by modulation of tyrosine phosphorylated b-catenin(^\text{157}).</td>
</tr>
<tr>
<td>CD105</td>
<td>Endoglin</td>
<td>Accessory receptor for TGF-β receptor. Important for endothelial migration and survival during inflammation(^\text{158}).</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>Cell adhesion molecule. Facilitates transmigration of leucocytes through the endothelium(^\text{11}).</td>
</tr>
<tr>
<td>CD144</td>
<td>VE-Cadherin</td>
<td>Cell adhesion molecule (cadherin). Regulates endothelium permeability(^\text{159}).</td>
</tr>
<tr>
<td>CD34</td>
<td>HPCA-1</td>
<td>Cell adhesion molecule. Modulates leucocyte cellular adhesion at portal entry sites on the endothelium(^\text{160}).</td>
</tr>
<tr>
<td>KDR</td>
<td>CD309</td>
<td>Receptor for vascular endothelial growth factor. Modules angiogenesis(^\text{161}).</td>
</tr>
<tr>
<td>CD133</td>
<td>Prominin-1</td>
<td>Pentaspan trans-membrane glycoprotein. Function in the endothelium is unknown(^\text{162}).</td>
</tr>
<tr>
<td>CD117</td>
<td>c-Kit</td>
<td>Receptor for stem cell factor. Function in the endothelium is unknown.</td>
</tr>
<tr>
<td>CD106</td>
<td>VCAM-1</td>
<td>Cell adhesion molecule. Facilitates leucocyte attachment to the endothelium (expression only upon cytokine stimulation)(^\text{11}).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Alternative name</th>
<th>Endothelial cell function</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWF</td>
<td></td>
<td>Protein. Important in haemostasis and the regulation of thrombogenesis and fibrinolysis.</td>
</tr>
<tr>
<td>eNOS</td>
<td></td>
<td>Enzyme. Generates nitric oxide to regulate vasodilation and vasoconstriction.</td>
</tr>
</tbody>
</table>

CD=cluster of differentiation; eNOS=Endothelial nitric oxide synthase; ICAM=intracellular adhesion molecule; KDR=kinase domain receptor; MCAM=melanoma cell adhesion molecule; PECAM=platelet endothelial cell adhesion molecule; TGF=transforming growth factor; VCAM=vascular cell adhesion molecule; VE=vascular endothelial; vWF=von Willebrand factor.
Table 2.3 Antibodies for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Flurochrome</th>
<th>Conc.</th>
<th>Cat no.</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell surface</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>V450</td>
<td>1:100</td>
<td>560368</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>LIVE/DEAD® Stain</td>
<td>V525</td>
<td>1:1000</td>
<td>L23105</td>
<td>ThermoFisher</td>
</tr>
<tr>
<td>CD31</td>
<td>FITC</td>
<td>1:100</td>
<td>555445</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD42a</td>
<td>FITC</td>
<td>1:20</td>
<td>558810</td>
<td>BD Pharmingen</td>
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<tr>
<td>CD106</td>
<td>FITC</td>
<td>1:100</td>
<td>551146</td>
<td>BD Biosciences</td>
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<td>CD54</td>
<td>PE</td>
<td>1:100</td>
<td>555511</td>
<td>BD Pharmingen</td>
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<tr>
<td>CD62p</td>
<td>PE</td>
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</tr>
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<td>1:200</td>
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<tr>
<td>eNOS</td>
<td>PE</td>
<td>1:100</td>
<td>560103</td>
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APC=allophycocyanin; Cat no=catalogue number; CD=cluster of differentiation; Conc=concentration; eNOS=endothelial nitric oxide synthase; FITC=fluorescein isothiocyanate; PE=phycoerythrin; vWF=von Willebrand factor
Table 2.4 Antibody panels for flow cytometric characterisation of endothelial cells.

<table>
<thead>
<tr>
<th>Cell surface</th>
<th>Cytoplasmic</th>
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<tbody>
<tr>
<td>Panel 1</td>
<td>Panel 2</td>
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<tr>
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<td>CD31-FITC</td>
<td>CD106-FITC</td>
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<tr>
<td>KDR-PE</td>
<td>CD54-PE</td>
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<tr>
<td>CD133-APC</td>
<td>-</td>
</tr>
<tr>
<td>CD34-APC/Cy7</td>
<td>-</td>
</tr>
<tr>
<td>CD146-PE/Cy7</td>
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</table>

APC=allophycocyanin; CD=cluster of differentiation; eNOS=endothelial nitric oxide synthase; FITC=fluorescein isothiocyanate; PE=phycoerythrin; vWF=von Willebrand factor.

2.2.2 Immunocytochemistry

Endothelial cells (Passage 1-4) were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) (Cat. PF101; FD Neutro technologies Inc., USA) for 10 minutes at room temperature. Cells were permeabilised with 0.1% Triton™ X-100 (Cat. T9284; Sigma Aldrich, UK) for 5 minutes, and then incubated with 2% normal goat serum (NGS) (Cat no. S-1000; Vector Laboratories Inc., USA) in PBS for 30 minutes to block non-specific staining. Cells were incubated overnight at 4°C with monoclonal antibodies: rabbit anti-human CD31, mouse anti-human von Willebrand Factor (vWF) antibodies (Table 2.5) and isotype controls for mouse primary antibody (Cat no. 086599; Invitrogen, UK) and rabbit primary antibody (Cat no. 086199; Invitrogen, UK). Cells were washed and incubated with fluorescent-labelled secondary antibodies for 30 minutes: Alexa Fluor® 488 goat anti-rabbit or Alexa Fluor® 647 goat anti-mouse to detect CD31 and vWF, respectively (Table 2.3). Cells were washed in PBS, rinsed in tap water and mounted with ProLong® Gold Antifade Reagent with 4′,6-diamidino-2-phenylindole (DAPI) (Cat no. P36935; Invitrogen, UK). Mountant and coverslips were applied and slides were dried at room temperature in the dark for 1 hour prior to visualisation with a Zeiss Axio Observer fluorescent microscope (Zeiss, Germany).
**Table 2.5** Antibodies for immunohistochemistry and immunocytochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
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<th>Reactivity</th>
<th>Conc.</th>
<th>Cat No.</th>
<th>Supplier</th>
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<td>Alexa Fluor® 488</td>
<td>Goat</td>
<td>Rabbit</td>
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<td>A11034</td>
<td>Life Technologies</td>
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<tr>
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<td>Horse</td>
<td>Mouse</td>
<td>1:250</td>
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Cat no=catalogue number; CD=cluster of differentiation; Conc=concentration; IgG=immunoglobulin G; vWF= von Willebrand factor.
2.3 FUNCTIONAL CHARACTERISATION OF ENDOTHELIAL CELLS IN VITRO

2.3.1 Growth kinetics and proliferation

Growth kinetics

For Chapter 3, endothelial cells (2 x 10⁵ cells) were cultured on BD BioCoat Collagen I-coated T75s until senescence. For Chapter 4, endothelial cells (4 x 10⁴ cells) were cultured on 19mm polymer-coated coverslips placed in 12-well plates for 28 days. At the second and subsequent passages, seeding density (C₀), days in culture (t) and harvested cell number (Cₜ) were recorded. Population doubling time (PDT) was calculated as the average time taken by a cell population to double in the log-phase/exponential phase according to the equation: PDT= \( \frac{\log(Cₜ/C₀)}{t} \) as previously described¹²⁵. Cumulative population doubling levels (CPDL) (the sum of all population doublings) were also calculated.

Proliferation

For Chapter 5, endothelial cells (1 x 10⁴ cells) were cultured on BD BioCoat Collagen I-coated 24-well plates. The next day, cells were washed three times with PBS, replenished in their respective medium and incubated with AT7519 for 48 hours at 37°C/5% CO₂/95% relative humidity. Mosaic images of the each well were captured with a Zeiss Axio Observer microscope (Carl Zeiss, Germany) at 0 hours and 48 hours. Cell number was calculated by automatic cell counts with Image J Software and fold-increase was calculated as the number of cells at 48 hours divided by the number of cells at 0 hours.
2.3.2 Cell attachment

2.3.2.1 Endothelial outgrowth cell colony formation on polymer substrates

Venous blood from healthy volunteers was drawn from the median cubital vein and MNCs were isolated as described in Section 2.1.1.1. EOC colony formation was assessed by cell seeding $5 \times 10^6$ MNCs onto a 19mm$^2$ polymer-coated coverslip placed in an uncoated 12-well plate tissue culture plate. After 24 hours, non-adherent cells were aspirated and fresh EGM™-2 was added. Numbers of colonies were assessed at day 21.

2.3.2.2 Cell attachment in static conditions

For Chapter 3, endothelial cells were trypsinised and aliquots of $4 \times 10^4$ cells were resuspended in 1ml of EGM™-2 medium and seeded into 1 well of a BD BioCoat Collagen I-coated 6-well plate for 30 minutes. For Chapter 4, endothelial cells were trypsinised and aliquots of $2 \times 10^4$ cells were resuspended in 500μl of EGM™-2 medium and seeded onto 19mm polymer-coated coverslips placed in 12-well plates for 24 hours. For Chapter 5, endothelial cells were grown to confluence on Collagen-I and incubated with or without AT7519 for 48 hours at 37°C. Endothelial cells were then trypsinised and aliquots of $2 \times 10^4$ cells were resuspended in 500μl of EGM™-2 medium with and without AT7519 and seeded into 1 well of a BD BioCoat Collagen I-coated 12-well plate for 24 hours. After the allocated time, wells were gently washed to remove non-attached cells and mosaics of digital images across the centre of the well were captured. Attachment within a defined region of each well was manually quantified and expressed as a percentage of seeded cell number or expressed as the number of attached cells per mm$^2$ area.
2.3.3 Wound closure migration assay

For *Chapter 3*, endothelial cells were grown to confluence on BD BioCoat Collagen I-coated 6-well plates. For *Chapter 4*, endothelial cells were grown to confluence on 19mm polymer-coated coverslips placed in 12-well plates. For *Chapter 5*, endothelial cells were grown to confluence on Collagen I-coated 6-well plates. Cells were rendered quiescent by incubation with serum-free EGM™-2 medium with AT7519 (*Chapter 5*) or without AT7519 for 24 hours at 37°C.

A linear vertical wound was created across the diameter of the well with a sterile pipette tip. Cells were then washed and replenished with or without AT7519 in serum-free EGM™-2 medium. Sets of digital images were taken at 0 hours and 24 hours. To quantify wound closure, the width of the wound was measured at the start time (0 hours) and the area of wound closure across the vertical stroke at 24 hours was quantified and expressed as a percentage of wound closure.

2.3.4 Tubule formation

For *Chapter 3*, endothelial cells were grown to confluence on BD BioCoat Collagen I-coated 6-well plates. For *Chapter 5*, endothelial cells were grown to confluence on Collagen I-coated 6-well plates and AT7519 was added for 24 hours.

BD Matrigel™ basement membrane matrix (Cat no. 365231; BD Pharmingen, UK) was thawed on ice and 150μl was used to coat each well of a Corning® Costar® 48-well, flat bottom cell culture plate (Cat. CLS3548; Sigma Aldrich, UK) and incubated for 30 minutes at 37°C. After gelification, endothelial cells were trypsinised and 2 x10^4 cells in 100μl complete EGM™-2 medium were seeded onto the Matrigel™. Wells were topped up with 500ul EGM™-2 with and without AT7519 and incubated for 24 hours. Mosaics of digital images across the entire well were captured and tubule formation was quantified as the number of complete spherical tubule structures present in the 48-well plate.
2.4 IMAGE CAPTURE

Atherothrombotic specimens and neutrophil images were captured with the Olympus Provis AX-70 microscope (Olympus, USA) equipped with Zen software (Zeiss, Germany). Cell and fluorescent images were captured with the Zeiss Axio Observer microscope (Zeiss, Germany) with AxioVision 4.8 software (Carl Zeiss, Germany). Temperature control of the microscope was used for retention of endothelial cells to polymer substrates. Images of polymer microarrays was carried out with a high content screening platform (Nikon 50i fluorescence microscope with an X-Y-Z stage), equipped with Pathfinder™ software (IMSTAR S.A., France). Polymer coatings on endothelium-denuded porcine aorta and stents were viewed with a Hitachi S-4700 scanning electron microscope.

2.5 STATISTICS

Data are shown as mean ± standard deviation. Categorical data were compared using the chi-squared with Fisher’s exact test. More than two groups were compared using a one-way ANOVA with Bonferroni post-test or a Kruskal-Wallis test with Dunn’s post-test where appropriate. For two-way tests of more than two groups, an ANOVA with Bonferroni post-test was used. The Mann-Whitney test were used to compare Chalkley counts between vehicle control- and cell-infiltrated sponges. Statistical significance was assumed if a null hypothesis could be rejected at P<0.05. Statistical analysis was performed with GraphPad Prism (version 6) (GraphPad Software Inc., USA).
CHAPTER 3

ISOLATION AND CHARACTERISATION OF CORONARY ENDOTHELIAL CELLS FOLLOWING THROMBECTOMY IN ACUTE MYOCARDIAL INFARCTION
5.1 ABSTRACT

**Rationale:** Endothelial dysfunction is central to the pathogenesis of coronary artery disease, but it is challenging to study the endothelium in the coronary circulation due to inherent difficulties in isolating vascular bed specific endothelial cells.

**Objective:** I sought to isolate and characterise coronary endothelial outgrowth cells from atherothrombectomy specimens isolated from patients receiving thrombectomy for acute myocardial infarction and to compare these cells to endothelial cell derived from other sources.

**Methods:** Forty-nine patients (62±12 years; 76% male) underwent percutaneous coronary intervention (PCI) with thrombus aspiration. Specimens were dissected and cultured on type I collagen to isolate coronary endothelial outgrowth (CEO) cells. CEO cells and endothelial cells isolated from the coronary arteries, peripheral blood (late outgrowth endothelial cells) and umbilical veins were phenotyped and underwent assessment of proliferation, attachment, wound closure and tubule formation *in vitro* as well as angiogenic potential *in vivo*.

**Results:** CEO was obtained from 27/37 (73%) atherothrombotic specimens and gave rise to cells with a cobblestone morphology and high expression of CD146 (94±6%), CD31 (87±14%) and von Willebrand factor (100±1%). CEO cells had lower proliferative capacity to umbilical vein endothelial cells (23.0±6.8 versus 55.5±5.3 cumulative population doubling level, P<0.001) but not other cells. CEO cells had a similar potential to control endothelial cells in other assays of endothelial cell function (attachment, migration and tubule formation, P>0.05 for all) *in vitro*. Unlike endothelial outgrowth cells and umbilical vein endothelial cells, CEO cells did not incorporate into new host vessels *in vivo*.

**Conclusions:** Coronary endothelial cells can be reliably isolated and cultured from atherothrombectomy specimens isolated from patients receiving thrombectomy for acute myocardial infarction. These cells have lower proliferative activity *in vitro* and do not incorporate into new
vessels in vivo compared to endothelial outgrowth cells and umbilical vein endothelial cells. CEO cells may model the dysfunctional coronary endothelium in patients with coronary artery disease and they may help identify novel therapeutic targets to enhance endothelial function for the prevention of acute myocardial infarction.
5.2 INTRODUCTION

Atherothrombosis is characterised by atherosclerotic plaque rupture with thrombus formation, and is the major cause of acute coronary syndromes and cardiovascular death. Disruption of the endothelial cell monolayer has been implicated in the onset, progression and clinical manifestations of atherothrombosis. This monolayer acts as a non-adhesive surface for platelets and leucocytes, and produces important factors in the regulation of inflammation, thrombosis and blood flow. It is now widely recognised that a variety of cardiovascular risk factors including cigarette smoking and hypercholesterolaemia cause endothelial dysfunction.

Our understanding of the cellular mechanisms of endothelial dysfunction in coronary artery disease (CAD) has been hampered by our inability to isolate and study endothelial cells from diseased vessels in the coronary circulation in man. Most of our observations have been based on an assessment of blood flow in the forearm or from measurement of surrogate biomarkers in plasma, such as vWF and soluble thrombomodulin. Whilst the number of circulating endothelial cells can be quantified in blood, and novel methods have been developed to isolate endothelial cells from superficial veins and arteries, our understanding of the cellular mechanisms of endothelial dysfunction in acute MI are largely inferred from the study of endothelial cells from more readily available sources. The study of commercially obtained cadaveric human coronary artery endothelial cells (HCAECs) from vascular beds of unknown health, late endothelial outgrowth cells (EOCs) from peripheral blood whose origin is unclear, or human umbilical vein endothelial cells (HUVECs) from vessels that do not develop atherosclerosis, provide limited insight into the pathogenesis of CAD.
5.3 HYPOTHESIS

Coronary endothelial outgrowth cells can be isolated from atherothrombectomy specimens and will have impaired function compared to endothelial cells derived from other sources.

5.4 AIMS

4. Isolate and characterise coronary endothelial outgrowth cells from atherothrombectomy specimens obtained from patients receiving thrombectomy for acute myocardial infarction.

5. Compare the phenotype and functions of these cells by measures of growth kinetics, cell attachment, wound closure and tubule formation in vitro with endothelial cells derived from other sources.

6. Compare the function of these cells by measures of angiogenesis in vivo with endothelial cells derived from other sources.
5.5 METHODS

Study population
Coronary atherothrombotic specimens (n=49) were obtained from patients receiving percutaneous coronary intervention (PCI) with thrombectomy for the treatment of acute myocardial infarction (MI). Venous blood (n=3) was obtained from patients with prior MI. The study protocol was approved by our local Research Ethics Committee and all subjects provided written informed consent.

Cellular characterisation of atherothrombotic specimens

Flow cytometry
Freshly isolated whole atherothrombotic specimens were washed with PBS and manually disaggregated into smaller pieces with a sterile surgical scalpel. A single cell homogenate was achieved following enzymatic disaggregation in PBS containing 1mg/ml type I collagenase (Cat. 17018-029; Life Technologies, UK) in a 37°C shaking water bath at 180 revolutions per minute for 60 minutes, followed by passing cells through a 100μm sterile nylon mesh. Cells were recovered by centrifugation and resuspended in EGM™-2 medium. Cell suspensions (100μl) were incubated for 30 minutes at room temperature with monoclonal antibodies conjugated to specific fluorochromes: LIVE/DEAD® Fixable Dead Cell Stains-V525, CD42a-FITC, CD45-V450, CD146-PE/Cy7 and CD31-PE (Table 2.2 and 2.4). After washing with PBS, cells were fixed and erythrocytes lysed simultaneously with BD FACSTM Lysing Solution (Cat no. 349202; BD Biosciences, UK). Samples were washed and then analysed with a BD LSRFortessa™ II cell analyser (BD Biosciences, UK). Unstained samples were used as controls. Spectral overlap and compensation was calculated and verified manually for each antibody with anti-mouse BD™ CompBead (Cat no. 552843, BD Biosciences, UK). A minimum of 200,000 events was collected for data analysis with FlowJo version 10.0.6 (TreeStar Inc., Switzerland). Endothelial cells were identified by the placement of population gates based on the unstained control. Following scatter selection and doublet discrimination, viable cells were selected by exclusion of LIVE/DEAD® Fixable Dead Cell Stain-V525, CD42a and CD45.
positive cells were excluded to avoid platelets, hematopoietic cells, and leucocytes. Endothelial cells were identified by expression of CD146 and/or CD31 (Figure 3.1).

Figure 3.1 Gating strategy for the identification of endothelial cells in atherothrombotic specimens.

(A) Doublets were excluded from the analysis by selecting cells with a proportional relationship of area scaling for side scatter-area (SSA-A) against side scatter-height (SSA-H). (B) The cell population was identified from cellular debris by characteristic light scatter signal for SSA-A against forward scatter area (FSC-A). From this population, (C) viable cells with intact cell membranes were identified by the exclusion of LIVE/DEAD® Stain-V525. Viable cells expressing (D) CD42a-Fluorescein isothiocyanate (FITC) and (E) CD45-v450 were identified by positive displacement to the right compared to unstained control. These cells were gated out of the analysis to avoid identification of platelets, hematopoietic cells and leucocytes. Remaining viable cells positive for (F) CD146 Phycoerythrin (PE) Cy7 and/or (G) CD31-PE were identified as endothelial cells within the atherothrombotic specimen.
**Immunohistochemistry**

Immunohistochemistry was performed on atherothrombotic specimens using anti-human CD146 antibody to detect endothelial cells (*Table 2.2 and 2.5*). The epitopes were retrieved by incubating specimens in 1mM EDTA solution for 20 minutes in a boiling water bath prior to endogenous peroxide activity quenching by 3% hydrogen peroxide. Specimens were treated with Endogenous Avidin/Biotin Blocking Kit (Cat no. Ab3387; Abcam, UK) according to the manufacturers’ instructions and non-specific antibody binding was prevented by incubating specimens with 10% normal horse serum (Cat no. S-200; Vector Laboratories, Inc., USA) in PBS. Specimens were incubated with monoclonal anti-human CD146 in PBS with 2% normal horse serum overnight at 4°C, washed in PBS, and incubated with biotinylated horse anti-mouse secondary antibody (*Table 2.5*) for 30 minutes at room temperature. The Vectastain Elite ABC Peroxidase kit (Cat no. PK-4000; Vector Laboratories, Inc., USA) was used for secondary antibody detection and visualisation occurred with 3,3’-Diaminobenzidine (DAB) as the substrate (Cat no. SK-4100; Vector Laboratories, Inc., USA). Specimens were counterstained with Mayer’s hematoxylin for 1 minute prior to washing and application of coverslips.

**Cell isolation**

Atherothrombotic specimens from patients receiving emergency PCI and manual thrombus aspiration for acute ST segment myocardial infarction (STEMI) were collected into complete EGM™-2 and transported to the laboratory for processing within 48 hours. Specimens were washed with PBS and manually disaggregated into smaller pieces with a sterile surgical scalpel. Tissue explants were seeded into 1 well of a 6-well tissue culture plate and maintained under standard cell culture conditions. After 24 hours, tissue explants, non-adherent cells, and debris were aspirated. Medium was changed every other day until first passage of coronary endothelial outgrowth (CEO) cells. Cells were left in culture 15-28 days (dependent on proliferative activity) prior to first passage. For functional experiments, endothelial cells of passage 2-4 were used (*Figure 3.2*).
EOCs were isolated by the *in vitro* expansion of PBMCs. HCAECs and HUVECs were commercially obtained from single and pooled donors.

**Figure 3.2 Processing of atherothrombotic specimens.**

Atherothrombotic specimens were placed in EGM™-2 and then manually disaggregated into smaller pieces using a sterile surgical scalpel. Disaggregated specimens were seeded into collagen-I coated plates with EGM™-2 for outgrowth of cells. Cells were incubated at 37°C/5% CO2/95% relative humidity.

**Phenotypic characterisation of endothelial cells**

For immunocytochemistry, cells were fixed in 4% PFA and stained with anti-human monoclonal antibodies and fluorochrome-conjugated secondary antibodies. Cells were phenotyped at each passage for flow cytometric expression of endothelial cell surface antigens, endothelial cytoplasmic antigens, and leucocyte antigens.
To determine if cells could uptake 1,1′-dioctadecyl-3,3,3′tetramethylindocarbocyanine (Dil)-labelled acetylated LDL (a phenotypic characteristic of endothelial cells), confluent monolayers of endothelial cells were incubated for four hours with Dil-labelled acetylated LDL (Cat no. L-3484; Life Technologies, UK) at 37°C. They were then visually inspected using a Zeiss Axio Observer fluorescent microscope (Zeiss, Germany).

**In vitro functional characterisation of endothelial cells**

For growth kinetics, population doubling times (PDT) and cumulative population doubling level (CPDL) were calculated. Cell attachment was quantified by seeding cells into a 6-well collagen I-coated plate for 30 minutes. Plates were washed and attachment within a defined region of each well was quantified and expressed as a percentage of seeded cell number. For the wound closure migration assay, cells were cultured to confluence, starved in serum-free media, and wounded by a linear vertical stroke across the diameter of the well with a P1000 pipette tip (time zero). The width of the wound was imaged at zero hours and at 24 hours and the percentage of wound closure was calculated. For tubule formation, cells were seeded into a 48-well plate pre-coated with Matrigel™ basement membrane matrix and tubule structures were quantified at 24 hours.

**In vivo angiogenesis assay**

The subcutaneous sponge implantation assay for *in vivo* angiogenesis was used to assess endothelial cell function. This model is commonly used to assess the influence of cells on vascularisation of subcutaneous sponges. All animal experiments were carried out in accordance with the British Home Office Animals (Scientific Procedures) Act 1986 in accordance with institutional guidelines. The experiments were designed and planned by Susan Gallogly. The surgical procedure was based on Barclay *et al.*, 2012 and was performed by Dr. Takeshi Fujisawa and Dr. Elizabeth Skinner, with assistance from Susan Gallogly, at the University of Edinburgh.
**Sponge implantation**

Male NOD-SCID gamma mice (NOD.Cg-PrkdcscidIil2rtg1Wj1/SzJ) (Stock no. 005557; Charles River, UK) aged 10-12 weeks (CEO cells and EOCs n=12 each, 3 biological repeats; HUVECs n=4, 1 biological repeat) were purchased and maintained in the Biomedical research facility at Edinburgh University. This mouse strain was chosen due to its excellent track record in xenogeneic cell engraftment. Engraftment of xenogeneic cells occurs as this mouse strain has numerous immunological dysfunctions such as impaired cytokine signalling and functional incompetent T-lymphocytes, B-lymphocytes and natural killer cells. The study design was powered based on previous studies from our laboratory that have used this model.

Endothelial cells were trypsinised and aliquots of 1 x 10^5 cells were resuspended in 100µl of 1:1 EGM™-2/phenol free GFR Matrigel™ (Cat 356231; BD Biosciences, UK). Sterilised sponge cylinders (0.5cm/1cm) (Caligen Foam, UK) were soaked with this suspension in a 1.5ml eppendorf and incubated for 30 minutes at 37°C. EGM™-2/phenol free-GFR Matrigel™ sponges were prepared as matched controls. Mice were anaesthetised intraperitoneally with Domitor containing Medetomidine (Orion Pharma, UK) and Vetalar containing Ketamine (Pfizer, UK); both were administered at 0.1ml per 10g body weight. Upon sedation, an analgesic, Vetergesic containing buprenorphine (Alstoe Animal Health, UK) was administered subcutaneously at 0.1mg per kg body weight. Matrigel™ sponges (vehicle control) were implanted subcutaneously into the left flank and cell-embedded sponges were implanted in the right flank. The sides of the wound were apposed with forceps and closed with skin staples (9mm Autoclips; Cat no. NC9050532; Fisher Scientific, USA). After surgery, a reversal agent, Antisedan-containing Atipamezole (Orion Pharma, UK), was administered subcutaneously at 0.05ml per 10g body weight and the mice observed in a cage on a heat mat until recovery. The wound was observed daily throughout the experiment and after 7 days the staples were removed. Mice were sacrificed by cervical dislocation and the sponges removed after 21 days. Sponges were fixed in 4% PFA overnight, then transferred to 70% ethanol and stored at 4°C.
**Vessel density analysis (Chalkley count)**

Fixed sponges were paraffin-embedded and cut transversely into 4μm section. Sections were deparaffinised, rehydrated and stained with haematoxylin and eosin by the histology department in SuRF at the University of Edinburgh. Vessel density within sponges was quantified with the Chalkley count, as previously described\(^{122, 169}\). The three most vascular areas (hot spots) with the highest number of micro-vessel profiles were chosen subjectively from each slide (3 slides per sponge per animal). Using a x20 objective, a 25-point Chalkley eyepiece graticule (NG52 Chalkley Point Array 26mm; Cat no. 01B26257; Pyser-SGI Ltd, UK) was applied to each hot-spot area and oriented to permit the maximum number of dot-perfusing vessel intersections (which were defined by the presence of red blood cells). The Chalkley count was determined by the sum of points coinciding with a vessel.

![Figure 3.3 The Chalkley count](image)

A Chalkley graticule was placed into the eyepiece of the microscope to superimpose a 25-point grid onto histologically sectioned murine sponges. The graticule was oriented to permit the maximum number of dot-vessel intersections in the grid. The Chalkley count was determined by the number of dots coinciding with a vessel. Image A demonstrates a Chalkley count of one as only 1 dot coincided with a vessel, while image B demonstrates a Chalkley count of five as 5 dots coincided with a vessel.

**Immunocytochemistry**

The presence of human cells within murine vessels was determined with human specific monoclonal antibodies to endothelial antigens. Paraffin embedded 4μm sections of sponges were deparaffinised and rehydrated prior to antigen retrieval with Tris based buffer at 100°C for 20 minutes. Specimens
were cooled for 40 minutes at room temperature, then washed in PBS, permeabilised and incubated with 10% NGS. Specimens were incubated overnight at 4°C with monoclonal antibodies: human-specific mouse anti-CD146 and cross-reactive rabbit anti-CD31 (Table 2.3) as previously reported. Specimens were washed and incubated with fluorescent-labelled secondary antibodies: Alexa Fluor® 488 goat anti-mouse and Alexa Fluor® 568 goat anti-rabbit to detect CD146 and CD31, respectively (Table 2.3). Slides were then rinsed in tap water and mounted as before. The EGM™-2/phenol free-GFR Matrigel™ only sponges were used as negative controls for human cells. Human fibroid tissue was used as a positive control for primary antibody immunoreactivity and specificity. Mouse tissue was used as a negative control. Using a x20 objective, three hotspot regions were chosen subjectively from each slide based on visual scanning for cross-reactive CD31 positive vessels (red). Composite multicolour images of each region were generated, CD31-positive and human-specific CD146-positive vessels (green) were counted, and the percentage of CD146-positive vessels was calculated.
5.6 RESULTS

Coronary atherothrombotic specimens (n=49) were collected from patients with acute MI with successive specimens fixed for histology (n=8), flow cytometry (n=4) or manually disaggregated for cell culture (n=37). Patients were 62±12 years old (76% male) with typical risk factors for acute coronary syndrome (Table 3.1).

Table 3.1 Clinical and procedural characteristics of all patients undergoing thrombectomy for ST-segment myocardial infarction.

<table>
<thead>
<tr>
<th>Total population (n=49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
</tr>
<tr>
<td>Gender, male</td>
</tr>
<tr>
<td><strong>Medical history and risk factors</strong></td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
</tr>
<tr>
<td>Previous PCI/CABG</td>
</tr>
<tr>
<td>Current smoker</td>
</tr>
<tr>
<td>Ex-smoker</td>
</tr>
<tr>
<td>Hypertension</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
</tr>
<tr>
<td>Family history of premature CAD</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td><strong>Medication on admission</strong></td>
</tr>
<tr>
<td>Aspirin</td>
</tr>
<tr>
<td>Clopidogrel</td>
</tr>
<tr>
<td>B-blockers</td>
</tr>
<tr>
<td>ACE-inhibitors</td>
</tr>
<tr>
<td>Statins</td>
</tr>
<tr>
<td><strong>Acute coronary syndrome</strong></td>
</tr>
<tr>
<td>Troponin I concentration, micrograms/L</td>
</tr>
<tr>
<td><strong>Culprit vessel</strong></td>
</tr>
<tr>
<td>Left anterior descending artery</td>
</tr>
<tr>
<td>Circumflex artery</td>
</tr>
<tr>
<td>Right coronary artery</td>
</tr>
</tbody>
</table>

Values are number (%) or mean ± standard deviation. ACE=angiotensin converting enzyme; CABG=coronary artery bypass grafting; CAD=coronary artery disease; PCI=percutaneous coronary intervention.
Atherothrombotic specimens

Histological examination of atherothrombotic specimens identified platelets, erythrocytes, leucocytes, and fibrin with atherosclerotic plaque containing cholesterol clefts (Figure 3.4 A-E). Clusters of CD146⁺ cells were visualised (Figure 3.4 F) but no microvessels were found. Flow cytometry analysis identified CD146⁺ and CD31⁺ endothelial cells in disaggregated atherothrombotic specimens (CD45⁻CD42a⁻CD146⁺ = 0.18±0.36% and CD45⁻CD42a⁻CD31⁺ = 0.11±0.12% of viable cells with 0.008±0.01% expressing both, CD45⁻CD42a⁻CD31⁻CD146⁺ cells) (n=4).
Figure 3.4 Coronary atherothrombotic specimens from patients undergoing treatment for ST-segment elevation myocardial infarction.

(A) The border zone between cholesterol cleft rich atheroma and thrombus is evident (arrows) with Carstairs staining. Atherothrombotic specimens were composed of (B) platelets (blue), (C) erythrocytes (red), (C) leucocytes (purple) and (E) cholesterol clefts encased in fibrin (pink). (F) Large cells with circular nuclei that stain positive for CD146 (brown) were also present. These cells were indicative of endothelial cells. Original magnification x10 for A, x20 for B-E, and x40 for E. Scale bars 100μm.
Isolation and characterisation of coronary endothelial outgrowth cells

Outgrowth from atherothrombotic specimens was obtained from 27/37 (73%) specimens (Table 3.2). Outgrowth was isolated more frequently in atherothrombotic specimens from the right coronary artery (21/23, 91%) than the left anterior descending artery (4/12, 33%).

Colonies emerged after 2-15 days in culture forming a median of 2 colonies per specimen (interquartile range of 6 colonies) (Figure 3.5 A and B). Cells were first passaged after 21 days (range 15-28 days) and continued to proliferate to form a confluent monolayer (Figure 3.5 C) with 21/27 (78%) cell lines proliferating for multiple passages (range 6-17).

Outgrowth cells had typical endothelial cell morphology with a centrally located circular nucleus. Cells stained for vWF with a typical granular pattern in the cytoplasm and had uniform cell surface CD31 expression (Figure 3.5 D). Cells took up fluorescent-labelled acetylated-LDL (Figure 3.5 E), formed tubule structures on a Matrigel™ membrane matrix (Figure 3.5 F) and were defined as coronary endothelial outgrowth (CEO) cells. These cells strongly expressed endothelial cell surface and cytoplasmic antigens with high expression of CD146, CD31, CD105, CD54 (intercellular adhesion molecule-1, ICAM-1), vWF and eNOS (>68% for all). A proportion of CEO cells expressed CD34 and KDR (Figure 3.5 G).

While the majority of endothelial antigens were unchanged during culture, CD34 expression diminished (P<0.05) and CD105 expression increased (P<0.05) during culture. Expression of pan-leucocyte antigen CD45 remained low (<3%) (Table 3.3).
Table 3.2 Clinical characteristics of patients with and without coronary endothelial outgrowth following thrombectomy for ST-segment elevation myocardial infarction.

<table>
<thead>
<tr>
<th>Total population (n=37)</th>
<th>Outgrowth</th>
<th>No outgrowth</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=27</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td>Age, years</td>
<td>60 ± 11</td>
<td>61 ± 11</td>
<td>0.885</td>
</tr>
<tr>
<td>Gender, male</td>
<td>19 (70%)</td>
<td>7 (70%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Medical history and risk factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Previous myocardial infarction</td>
<td>4 (15%)</td>
<td>1 (10%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Previous PCI/CABG</td>
<td>4 (15%)</td>
<td>0 (0%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Current smoker</td>
<td>14 (52%)</td>
<td>3 (30%)</td>
<td>0.287</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>3 (11%)</td>
<td>1 (10%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Hypertension</td>
<td>4 (15%)</td>
<td>1 (10%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Hyperlipidaemia</td>
<td>7 (26%)</td>
<td>2 (20%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Family history of premature CAD</td>
<td>13 (48%)</td>
<td>2 (20%)</td>
<td>0.153</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>2 (7%)</td>
<td>1 (10%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Medication on admission</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>5 (19%)</td>
<td>1 (10%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>2 (7%)</td>
<td>0 (0%)</td>
<td>1.000</td>
</tr>
<tr>
<td>B-Blockers</td>
<td>3 (11%)</td>
<td>0 (0%)</td>
<td>0.548</td>
</tr>
<tr>
<td>ACE-Inhibitors</td>
<td>4 (15%)</td>
<td>2 (20%)</td>
<td>0.653</td>
</tr>
<tr>
<td>Statins</td>
<td>7 (26%)</td>
<td>2 (20%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Myocardial injury</td>
<td></td>
<td></td>
<td>0.749</td>
</tr>
<tr>
<td>Troponin I concentration, micrograms/L</td>
<td>27.9 ± 20.0</td>
<td>30.3 ± 14.9</td>
<td></td>
</tr>
<tr>
<td>Culprit vessel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left anterior descending artery, n=12</td>
<td>4</td>
<td>8</td>
<td>0.041*</td>
</tr>
<tr>
<td>Circumflex artery, n=2</td>
<td>2</td>
<td>0</td>
<td>1.000</td>
</tr>
<tr>
<td>Right coronary artery, n=23</td>
<td>21</td>
<td>2</td>
<td>0.002**</td>
</tr>
</tbody>
</table>

Values are number (%) or mean ± standard deviation. ACE=angiotensin converting enzyme; CABG=coronary artery bypass grafting; CAD=coronary artery disease; PCI=percutaneous coronary intervention. Numeral data were compared using the student’s t-test. Categorical data were compared using the chi-squared with Fisher’s exact test *P<0.05, **P<0.01.
Figure 3.5 Outgrowth and characterisation of coronary endothelial outgrowth cells from atherothrombotic specimens.

(A) Outgrowth of coronary endothelial cells from dissected atherothrombotic specimen at 24 hours, (B) colony formation at 7 days, and (C) confluent cobblestone morphology at first passage. (D) Cells stain positive for cytoplasmic expression of granular vonWillebrand factor indicative of Weibel–Palade bodies (pink) and cell surface expression of CD31 (green) with 4',6-diamidino-2-phenylindole (DAPI) as a nuclei counterstain (blue). (E) Uptake of fluorescent-labelled Dil-acetylated-low density lipoprotein (red) with DAPI as a counterstain. (F) Tubule structure formation on Matrigel™. Scale bar 100μm. (G) Coronary endothelial outgrowth (CEO) cells strongly express cell surface antigens CD146, CD31, CD105 and CD54 and cytoplasmic antigens von Willebrand factor (vWF) and endothelial nitric oxide synthase (eNOS), but do not express the pan-leucocyte antigen CD45. Unstained (grey) and stained cells (black).
Table 3.3 Phenotypic characterisation of coronary endothelial outgrowth cells during *in vitro* culture.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Alternative name</th>
<th>Passage 1-2</th>
<th>Passage 3-4</th>
<th>Passage 5-6</th>
<th>Passage 7-8</th>
<th>Passage 9-10</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell surface</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>MCAM</td>
<td>94.5 ± 5.7</td>
<td>92.6 ± 12.8</td>
<td>88.8 ± 13.4</td>
<td>96.7 ± 5.0</td>
<td>88.9 ± 15.1</td>
<td>0.700</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>86.7 ± 14.4</td>
<td>87.4 ± 14.7</td>
<td>92.2 ± 11.9</td>
<td>94.8 ± 6.1</td>
<td>87.5 ± 14.9</td>
<td>0.534</td>
</tr>
<tr>
<td>CD105</td>
<td>Endoglin</td>
<td>92.7 ± 5.8</td>
<td>94.1 ± 6.0</td>
<td>99.6 ± 0.2</td>
<td>99.4 ± 0.3</td>
<td>99.7 ± 0.2</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>78.0 ± 36.7</td>
<td>84.6 ± 11.3</td>
<td>80.3 ± 11.5</td>
<td>68.6 ± 9.2</td>
<td>86.5 ± 16.8</td>
<td>0.646</td>
</tr>
<tr>
<td>CD144</td>
<td>VE-Cadherin</td>
<td>53.3 ± 39.4</td>
<td>49.1 ± 39.4</td>
<td>55.8 ± 31.3</td>
<td>52.7 ± 24.5</td>
<td>56.4 ± 36.4</td>
<td>0.998</td>
</tr>
<tr>
<td>CD106</td>
<td>VCAM-1</td>
<td>1.1 ± 0.5</td>
<td>3.0 ± 5.5</td>
<td>2.9 ± 4.0</td>
<td>3.7 ± 3.6</td>
<td>1.1 ± 0.4</td>
<td>0.653</td>
</tr>
<tr>
<td>CD34</td>
<td>HPCA-1</td>
<td>59.0 ± 34.3</td>
<td>51.5 ± 34.3</td>
<td>38.2 ± 28.4</td>
<td>29.1 ± 28.5</td>
<td>27.3 ± 24.9</td>
<td><strong>0.030</strong></td>
</tr>
<tr>
<td>KDR</td>
<td>CD309</td>
<td>74.4 ± 17.5</td>
<td>78.4 ± 22.1</td>
<td>90.8 ± 8.8</td>
<td>87.2 ± 26.1</td>
<td>97.2 ± 2.8</td>
<td>0.220</td>
</tr>
<tr>
<td>CD133</td>
<td>Prominin-1</td>
<td>43.3 ± 37.7</td>
<td>27.0 ± 32.9</td>
<td>19.8 ± 32.2</td>
<td>3.7 ± 3.1</td>
<td>0.8 ± 0.4</td>
<td>0.052</td>
</tr>
<tr>
<td>CD117</td>
<td>c-Kit</td>
<td>2.1 ± 1.3</td>
<td>2.5 ± 1.3</td>
<td>2.7 ± 1.4</td>
<td>2.3 ± 2.2</td>
<td>2.4 ± 2.2</td>
<td>0.992</td>
</tr>
<tr>
<td><strong>Cytoplasmic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td></td>
<td>99.8 ± 0.5</td>
<td>100.0 ± 0.0</td>
<td>99.8 ± 0.5</td>
<td>100 ± 0.0</td>
<td>100.0 ± 0.0</td>
<td>0.443</td>
</tr>
<tr>
<td>eNOS</td>
<td></td>
<td>74.3 ± 24.2</td>
<td>68.5 ± 41.5</td>
<td>70.4 ± 35.1</td>
<td>90.7 ± 13.2</td>
<td>69.6 ± 27.5</td>
<td>0.738</td>
</tr>
<tr>
<td><strong>Leucocyte</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>PTPRC or LCA</td>
<td>1.2 ± 0.7</td>
<td>1.0 ± 0.5</td>
<td>2.2 ± 1.5</td>
<td>1.6 ± 0.7</td>
<td>1.3 ± 0.6</td>
<td>0.237</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation for percentage antigen expression measured by flow cytometry (n=6-10). CEO=coronary endothelial outgrowth; eNOS=endothelial nitric oxide synthase; HPCA=hematopoietic progenitor cell antigen; ICAM=intercellular adhesion molecule; KDR=kinase domain receptor; MCAM=melanoma cell adhesion molecule; LCA=leucocyte common antigen; PECAM=platelet endothelial cell adhesion molecule; PTPRC=protein tyrosine phosphatase receptor type C; VCAM=vascular cell adhesion molecule; VE=vascular endothelial; vWF=von Willebrand factor. One-way analysis of variance (ANOVA).
Phenotypic and functional comparison of endothelial cells

Antigen expression
CEO cells had a similar phenotype to control endothelial cells (EOCs, HCAECs, and HUVECs) during early passage (1-4), although CEO cells had lower levels of CD45 expression compared with EOCs (P<0.01) and higher levels of CD133 and lower levels of CD34 expression compared with HCAECs (P<0.05 for both; Table 3.4).

Proliferation
Proliferative activity was lower in CEO cells (population doubling time [PDT]=2.5±1.0 days) compared to HUVECs (PDT=1.2±0.3 days; P<0.001) (ANOVA<0.001). (Figure 3.6 A). Also, CEO cells had a lower cumulative population doubling level (23.0±6.8) during prolonged culture (8 passages) than HUVECs (55.5±5.3) (P<0.05) (ANOVA=0.005) (Figure 3.6 B).

In vitro attachment, wound closure migration assay and tubule formation assay
CEO cells had a similar capacity to adhere to a collagen substrate (81±18%) than EOCs (83±19%), HCAECs (93±6%) and HUVECs (88±4%) (ANOVA=0.741) (Figure 3.6 C and E). The migratory capacity of CEO cells (29±20% wound closure) across a linear wound did not differ compared to EOCs (31±19% wound closure), HCAECs (4±4% wound closure) and HUVECs (85±19% wound closure). Migration of HCAECs was reduced compared to HUVECs (P<0.01) (ANOVA=0.003) (Figure 3.6 D and F).
Table 3.4 Coronary endothelial outgrowth phenotype during early passage (1-4) in vitro culture compared with endothelial cells derived from the coronary arteries, peripheral circulation (endothelial outgrowth cells) and the umbilical veins.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>CEO cells</th>
<th>HCAECs</th>
<th>EOCs</th>
<th>HUVECs</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=8</td>
<td>n=3</td>
<td>n=3</td>
<td>n=3</td>
<td></td>
</tr>
<tr>
<td>Cell surface</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>93.4 ± 10.3</td>
<td>99.1 ± 0.2</td>
<td>90.3 ± 6.8</td>
<td>97.4 ± 1.0</td>
<td>0.305</td>
</tr>
<tr>
<td>CD31</td>
<td>87.1 ± 14.0</td>
<td>95.2 ± 8.1</td>
<td>95.7 ± 2.0</td>
<td>94.1 ± 5.0</td>
<td>0.642</td>
</tr>
<tr>
<td>CD105</td>
<td>93.4 ± 5.5</td>
<td>99.3 ± 0.3</td>
<td>96.2 ± 5.7</td>
<td>98.9 ± 1.0</td>
<td>0.023</td>
</tr>
<tr>
<td>CD54</td>
<td>81.8 ± 23.9</td>
<td>97.2 ± 0.3</td>
<td>92.0 ± 13.0</td>
<td>95.1 ± 6.3</td>
<td>0.087</td>
</tr>
<tr>
<td>CD144</td>
<td>51.2 ± 36.5</td>
<td>37.9 ± 30.7</td>
<td>60.9 ± 36.2</td>
<td>75.7 ± 29.3</td>
<td>0.467</td>
</tr>
<tr>
<td>CD34</td>
<td>63.5 ± 24.5</td>
<td>98.0 ± 0.3*</td>
<td>43.7 ± 9.4</td>
<td>48.9 ± 12.5</td>
<td>0.015</td>
</tr>
<tr>
<td>KDR</td>
<td>76.8 ± 19.8</td>
<td>42.6 ± 28.4</td>
<td>80.0 ± 31.0</td>
<td>92.1 ± 10.8</td>
<td>0.076</td>
</tr>
<tr>
<td>CD133</td>
<td>33.5 ± 34.6</td>
<td>0.1 ± 0.1*</td>
<td>3.0 ± 1.9</td>
<td>10.1 ± 15.0</td>
<td>0.012</td>
</tr>
<tr>
<td>CD117</td>
<td>2.3 ± 1.2</td>
<td>0.6 ± 0.3</td>
<td>2.3 ± 1.0</td>
<td>2.0 ± 0.4</td>
<td>0.052</td>
</tr>
<tr>
<td>CD106</td>
<td>2.2 ± 4.2</td>
<td>1.7 ± 0.3</td>
<td>1.1 ± 0.3</td>
<td>1.3 ± 0.7</td>
<td>0.225</td>
</tr>
<tr>
<td>Cytoplasmic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vWF</td>
<td>99.9 ± 0.4</td>
<td>99.0 ± 0.1</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
<td>0.036</td>
</tr>
<tr>
<td>eNOS</td>
<td>71.4 ± 31.6</td>
<td>37.4 ± 18.0</td>
<td>26.0 ± 39.7</td>
<td>87.3 ± 5.2</td>
<td>0.013</td>
</tr>
<tr>
<td>Leucocyte</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>1.1 ± 0.6</td>
<td>1.0 ± 0.2</td>
<td>2.6 ± 0.2**</td>
<td>1.9 ± 1.1</td>
<td>0.046</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation for percentage antigen expression measured by flow cytometry. CEO=coronary endothelial outgrowth; eNOS=endothelial nitric oxide synthase; EOC=endothelial outgrowth cell; HCAECs=human coronary artery endothelial cells; HUVEC=human umbilical vein endothelial cell; KDR=kinase domain receptor; vWF=von Willebrand factor. Kruskal-Wallis analysis of variance. P-values for Dunn’s post-test: *P<0.05, **P<0.01 versus CEO cells.
Figure 3.6 Proliferation, attachment and wound closure of endothelial cells in vitro.

(A) Coronary endothelial outgrowth (CEO) cells had a higher population doubling time during the first 9 passages and a (B) lower cumulative population doubling level when maintained long term in culture compared to human umbilical vein endothelial cells (HUVECs).

(C & E) CEO cells had a similar capacity to adhere to a collagen substrate compared to endothelial outgrowth cells (EOCs), human coronary artery endothelial cells (HCAECs) and HUVECs.

(D & F) Following the infliction of a linear wound in the endothelial cell monolayer (black continuous line), CEO cells had a similar capacity to control endothelial cells for migration at 24 hours (yellow dotted line) but migration of HCAECs was impaired compared to HUVECs. Error bars are standard deviation. Kruskal-Wallis analysis of variance (ANOVA). P-values for Dunn’s post-test: *P<0.05, **P<0.01, ***P<0.001. Scale bar 100μm.
**In vivo angiogenesis assay**

Implantation of CEO cells in a subcutaneous sponge did not increase vessel density compared to vehicle control (Chalkley count 7±1 versus 6±1, P=0.484) (**Figure 3.7 A**), nor did CEO cells incorporate into vascular structures (0 vessels) (**Figure 3.7 A-C and J**). HUVECs, but not EOCs, increased vessel density compared to vehicle control (11±1 versus 7±1 and 7±1 versus 7±2, P=0.028 and P=0.074 respectively; **Figure 3.7 B and C**), and both were found to incorporate into murine vessels (HUVECs: 25±19%; EOCs: 25±19% vessels with human cells; ANOVA=0.022) (**Figure 3.8**).

![Figure 3.7 Angiogenic potential of endothelial cells in vivo.](image)

Sponges embedded with growth factor reduced (GFR)-Matrigel™ (vehicle control) and GFR-Matrigel™ with (A) coronary endothelial outgrowth (CEO) cells, (B) endothelial outgrowth cells (EOCs) or (C) human umbilical vein endothelial cells (HUVECs) were subcutaneously implanted into male NOD-SCID gamma mice. CEO cells and EOCs did not increase the vessel density compared to vehicle, whereas HUVECs did increase vessel density. Error bars are standard deviation. Mann-Whitney test. P-values for paired student’s t-tests: *P<0.05.
Figure 3.8 Coronary endothelial outgrowth cells do not incorporate into murine vessels *in vivo*.

Immunohistochemistry of subcutaneously implanted sponges embedded with (A-C) coronary endothelial outgrowth (CEO) cells, (D-F) late outgrowth endothelial cells (EOCs) or (G-I) human umbilical vein endothelial cells (HUVECs). Sections were stained with antibodies cross-reactive to both mouse and human antigens (red) or specific to human antigen (green) with 4',6-diamidino-2-phenylindole (DAPI) as a counterstain. Auto-fluorescent erythrocytes are present in vessel lumen demonstrating that vessels are contiguous with the host circulation. Scale bar 50 μm. (J) In contrast to EOCs and HUVECs (arrows in E and H), CEO cells did not incorporate into murine vessels. Kruskal-Wallis analysis of variance (ANOVA). Error bars are standard deviation. P-values for Dunn’s post-test: *P<0.05.
5.7 DISCUSSION

Endothelial dysfunction is central to the pathogenesis of coronary artery disease (CAD). However, our understanding of the role of the coronary endothelium is based largely on the study of endothelial cells derived from more readily available sources. In this study, I report the first successful expansion of coronary endothelial cells from coronary atherothrombotic specimens obtained from patients undergoing treatment for acute myocardial infarction. I systemically compared the phenotype and function of these cells with endothelial outgrowth cells from the peripheral circulation and commercially acquired human coronary artery and umbilical vein endothelial cells. This demonstrated that coronary endothelial outgrowth (CEO) cells are phenotypically similar to control endothelial cells but have marked functional differences by measures of growth kinetics in vitro and angiogenesis in vivo suggesting that atherothrombotic specimens may be a viable and important source of endothelial cells to study the role of endothelial dysfunction in the pathogenesis of CAD.

Atherothrombotic specimens were isolated from the left anterior descending artery, the circumflex artery and the right coronary artery. Specimens were heterogeneous and rich in erythrocytes, leucocytes, fibrin and cholesterol clefts as previously reported\textsuperscript{[170, 171]}. Endothelial cells (CD146 cells with large circular nuclei) were found in histological sections but the number of CD146$^+$ and CD31$^+$ cells in digested specimens was low (<1%).

When atherothrombotic specimens were manually dissected with a surgical scalpel and plated on collagen-1, outgrowth of cells was reliably obtained (73%). Outgrowth occurred most frequently from specimens retrieved from the right coronary artery than the left anterior descending (LAD) artery, possibly due to the anatomical positioning of right coronary artery that accommodates aspiration by the thrombectomy catheter leading to larger atherothrombotic specimens for cell outgrowth.

As cell outgrowth from atherothrombotic specimens occurred without chemical digestion and cell-sorting techniques, I carefully characterised outgrowth cells to determine their identity and to rule out the inclusion of other cell types from atherothrombotic specimens. I identified endothelial cells using
a triad of tests: morphology, function, and antigen expression. Outgrowth cells had homogenous cobblestone morphology typical of endothelial cells, with no evidence of multipolar or elongated shapes typical of VSMCs, fibroblasts and pericytes. Outgrowth cells also incorporated acetylated LDL and formed tubule structures on Matrigel™ typical of endothelial cells. To fully confirm an endothelial cell identity of outgrowth cells, an array of commonly used mature (CD146, CD31, CD105, CD54, vWF and eNOS) and endothelial progenitor cell (CD34, KDR) antigens were used. The mature markers are essential for the identification of endothelial cells. In accordance with previous studies, outgrowth cells expressed all the mature antigens associated with endothelial cells during short- and long-term culture122. A proportion of cells expressed endothelial progenitor cell antigens characteristic of the endothelial cell hierarchy in culture125. Moreover, the proportion of outgrowth cells that expressed the pan-leucocyte marker CD45 was low (<4%) suggesting minimal contamination from blood mononuclear cells such as angiogenic monocytes119. Thus, outgrowth cells from atherothrombotic specimens were confirmed as endothelial and designated coronary endothelial outgrowth (CEO) cells.

To the best of my knowledge, this is the first report to isolate, expand and characterise endothelial cells from atherothrombotic specimens. One previous study obtained endothelial cells from coronary guidewires with CD146 immunomagnetic beads but limited characterisation was performed172. Other methods involving wire biopsies of the superficial veins and arteries of the forearm have been more successful in the isolation and expansion of endothelial cells in culture39, 173, but these beds are largely protected from atherosclerosis. As such, I suggest that CEO cells obtained from atherothrombotic specimens may be the most appropriate and relevant endothelial cells to study the pathogenesis of atherosclerosis.

As endothelial cell dysfunction is the quintessential pathophysiological features of CAD, I undertook a comprehensive phenotypic and functional analysis of CEO cells from atherothrombotic specimens and compared them to patient derived endothelial outgrowth cells (EOCs) as well as commercially available healthy controls- human coronary artery endothelial cells (HCAECs) and human umbilical vein endothelial cells (HUVECs).
Endothelial cell dysfunction is characterised by low nitric oxide production, the up-regulation of adhesion molecules for leucocyte adherence, and increases in endothelial cell permeability. Phenotypic analysis demonstrated that CEO cells were similar to control endothelial cells from other sources, as the levels of antigens expressed by patient derived EOCs and commercially acquired HUVECs were consistent with previous reports\textsuperscript{122, 125, 174} and identical to the levels expressed by CEO cells. I found no differences in the levels of eNOS expression which is responsible for the generation of nitric oxide in the vascular endothelium following increases in shear stress\textsuperscript{175}. And I found no differences in the expression of cell surface adhesion molecules such as ICAM (CD54) VCAM (CD106) which traffic leucocytes into the vessel wall during endothelial cell activation and dysfunction leading to atherogenesis\textsuperscript{176}. Nor did I find any differences in CD31 (Platelet endothelial cell adhesion molecule [PECAM]-1) expression, which contributes to barrier function and increases in endothelial cell permeability. It is unclear why the levels of hematopoietic CD133 (Prominin1) in CEO cells was higher compared to HCAECs, but the higher level of CD45 by EOCs was most likely due to carry over of mononuclear cells from the derivation of EOCs in early passages.

When endothelial cell proliferation is reduced it may not be possible to form a confluent monolayer following injury, which could lead to the development of atherothrombosis. Functional analysis \textit{in vitro} demonstrated that CEO cells had reduced growth kinetics compared to endothelial cells from other sources. The growth kinetics of endothelial cells were calculated by the PDT and CPDL for 9 passages in culture as previously described\textsuperscript{125}. While, commercially acquired controls cells had PDTs and CPDL similar to those of recent reports\textsuperscript{125, 177}, the potential of CEO cells to proliferate was reduced compared to endothelial cells isolated from the umbilicus vein. Interestingly, the proliferative potential of EOCs derived from the peripheral circulation of patients with CAD was similar to the proliferative potential of CEO cells. As atherosclerosis is a systematic disease, the reduced proliferative potential may have been a consequence of endothelial dysfunction. Indeed, previous studies have characterised the proliferative potential of EOCs from patients with CAD reporting similar PDTs and CPDL to those reported in this study\textsuperscript{10}. As these differences persisted in CEO cells following multiple passages and expansion in optimal culture conditions, this suggests there is an
inherent difference in the proliferation of endothelial cells derived from coronary atherothrombotic specimens.

This is the first study to successfully expand endothelial cells from atherothrombotic specimens. Previous studies have been unable to expand endothelial cells from the site of plaque rupture\textsuperscript{172}. While the authors speculate that they were unable to expand cells due to endothelial dysfunction, it is likely that the expansion failed due to the very low yield of cells and the inadequate environment for endothelial cell growth (DMEM with 20% FBS)\textsuperscript{172}. For proliferation of CEO cells, this study provided a collagen-I basement membrane and a powerful growth factor supplemented medium (EGM-2 with 20% Hyclone) facilitating cell expansion. The reduced proliferative potential of CEO cells compared to control endothelial cells could indicate a bias towards endothelial cell senescence which plays a key role in the attenuated angiogenic and regenerative capacity of endothelial cells\textsuperscript{178}. In addition, since CEO cells were isolated from ruptured plaques during an acute myocardial infarction, it is likely that they have experienced oxidative stress and ischemic injury prior to harvesting which is likely to further affect their function. Given this outcome, it would be worthwhile to measure the nitric oxide release from CEO cells by measure of nitrite accumulation following stimulation with acetylcholine. Reduced NO expression can result in reduced proliferation of endothelial cells and alterations in NO release can affect the proliferation/migration of cells in the vasculature contributing to atherogenesis.

When CEO cells was compared to commercially obtained healthy endothelial cells and endothelial cells from the peripheral circulation of patients with CAD by measures of cell attachment and wound closure \textit{in vitro}, CEO cells had equal potential to control endothelial cells. As previously reported\textsuperscript{40}, endothelial cells had a high potential to attach to a collagen substrate (>80%). This was similar to the level of attachment by CEO cells and, perhaps, not unexpected given the similar levels of expression of cell adhesion molecules (eg, VE Cadherin) from phenotypic profiling. CEO cells also had a similar potential for wound migration (30%) following serum starvation and the infliction of a wound across the endothelial monolayer. The wound closure of EOCs was similar to a recent reports (30%)\textsuperscript{40}, and
the robust potential of HUVECs to migrate (>80%) was also similar\textsuperscript{177, 179}. Of note, HCAECs failed to migrate which was inconsistent with previously reports\textsuperscript{180, 181}. This may have occurred due to donor variability demonstrating the challenges of using human endothelial cells of uncertain provenance from commercial sources.

Attachment and wound closure are important for repair of the endothelium following atherothrombosis. Impaired attachment and impaired response to chemoattractant is a characteristic of dysfunctional endothelial cells, which leads to impaired healing following PCI. It is interesting that CEO cells had a similar potential for attachment and migration compared to control cells as it has been shown that vessel wall endothelial cells from patients with CAD, have reduced microRNA expression with broad dysregulation of endothelial transcriptional networks which control cell function, such as attachment and migration\textsuperscript{40}.

To ascertain if CEO cells had a similar potential to participate in angiogenesis compared to control cells \textit{in vitro}, the matrigel angiogenesis was employed. Using this assay, I demonstrated that CEO cells formed tubule structures on a Matrigel-coated substrate but I did not demonstrate a quantifiable difference compared to endothelial cells from other sources. Other studies have reported similar findings when comparing endothelial cells, suggesting this assay, may not recapitulate the complexity of the real life situation. To this end, endothelial cells were implanted in murine sponges for 28 days to assess the potential for angiogenesis \textit{in vivo}. For this study, immuno-compromised male NOD-SCID mice lacking lymphocytes were selected as the recipient for xenogeneic endothelial cells. The study design was powered based on previous studies employing this model\textsuperscript{126} and the angiogenic potential was measured using the Chalkley count, which has previously been used as a prognostic measure of angiogenesis to assess metastasis of tumour cells\textsuperscript{169}. I found that CEO cells had a reduced angiogenic capacity \textit{in vivo} compared to HUVECs. This may be related to the low proliferative potential of CEO cells \textit{in vitro}, but requires further experimentation. I also found that CEO cells were unable to incorporate into new vascular beds in our experimental model of angiogenesis. While incorporation of HUVECs and EOCs is well documented in the literature\textsuperscript{122, 126, 136}, it was interesting that CEO cells were unable to incorporate as this could indicate an attenuated angiogenic and
regenerative capacity. It has previously been demonstrated that endothelial cells from patients with CAD have reduced release of tissue plasminogen activator with impaired functions such as vasodilatation and fibrinolysis \textit{in vivo}\textsuperscript{24, 164} so it is likely that other functions are affected also. Unfortunately, I could not relate the phenotype of CEO cells to it’s reduced \textit{in vivo} function as HUVECs and EOCs displayed a similar phenotype by flow cytometric profiling of surface and cytoplasmic markers. However, future studies assessing CEO cell phenotype after the addition of growth factors or following survival in ischemic conditions may be able to tease out differences compared to endothelial cells from other sources.

\section*{5.8 STUDY LIMITATIONS}

There are some limitations that merit consideration. First, I was unable to isolate coronary endothelial outgrowth from all patients, therefore in the CEO lines there may be an inherent selection bias whereby outgrowth of endothelial cells only arose from those patients with better vascular function. Although, it must be noted that I found no major differences in the clinical characteristics of those patients from whom CEO cells were and were not isolated. Second, as with all \textit{in vitro} studies the assays of endothelial cell function are artificial and therefore they may not adequately mimic endothelial function or dysfunction \textit{in vivo}. Third, the murine model of angiogenesis may not have recapitulated function or dysfunction of endothelial cells in CAD, as CEO cells were tested under non-stressed physiological conditions (the mice were healthy and immuno-compromised) rather than pathological conditions which can influence angiogenesis. However, it must be noted that despite this CEO cells were unable to incorporate into new vessels. Fourth, while it is very likely that endothelial cells from coronary atherosclerotic plaques possess an inherent impairment, it was not possible to obtain CEO from age- and sex-matched controls given the invasive nature of the thrombectomy procedure. Therefore, I cannot definitively conclude that the lower function displayed by CEO cells is a consequence of dysfunction due to atherosclerosis, method of isolation or inherent differences between cell types. Finally, the small sample size for experiments must be taken into account when interpreting these results. For most assays, functional differences observed between CEO and control
populations were marked, and therefore the statistical power was adequate even with a small number of replicates for the primary comparison. However, I cannot discount the possibility that some experiments were underpowered to detect more subtle differences in function between control cell lines.

5.9 CONCLUSION

Coronary endothelial cells can be reliably isolated and cultured from atherothrombotic specimens isolated from patients receiving thrombectomy for acute myocardial infarction. These cells have a lower capacity to proliferate compared to control endothelial cells in vitro and they do not incorporate into new vessels in vivo. These cells may be representative of the dysfunctional coronary endothelium in patients with coronary artery disease and they may help identify novel therapeutic targets to enhance endothelial function for the prevention of acute myocardial infarction.
CHAPTER 4

POLYMER SUBSTRATES FOR ENDOTHELIALISATION OF CORONARY STENTS
8.1 ABSTRACT

**Rationale:** Synthetic coatings that promote endothelial cell attachment, expansion and retention *in vitro* may promote endothelialisation of stents *in situ*, which may improve the clinical outcome for patients following angioplasty and stenting for coronary artery disease.

**Objective:** I sought to identify novel polymer substrates that promote endothelial cell attachment, expansion and retention *in vitro* and to assess the effect of these substrates on thrombus formation and platelet and leucocyte attachment *ex vivo* with the Badimon chamber.

**Methods:** Polyacrylates (PAs) and polyurethanes (PUs) (n=337) with high attachment (>500 nuclei/mm²) of coronary endothelial outgrowth (CEO) cells and late outgrowth endothelial cells (EOCs) but low attachment (<200 nuclei/mm²) of neutrophils and monocytes were identified by high-throughput polymer microarrays. These polymers were assessed by CEO cell and EOC attachment in steady-state flow, proliferation, migration, and cell retention at increasing levels of shear stress (0-85 dynes/cm²) in the IBIDI chamber and were compared with collagen (positive control), 8G7 (polymer standard control) and PA108 (polymer negative control). Polymers were further tested in a Badimon chamber with non-coagulated flowing blood, where thrombus formation on polymer substrates was compared to endothelium-denuded porcine aorta (control), and platelet and leucocyte attachment to polymer-coated prototype stents was compared to cobalt chromium bare metal stents (BMS).

**Results:** Polymer microarrays identified PA309 and PA318 (amine-functionalised methacrylate-based co-polymers) for high endothelial cell attachment (~1,000 nuclei/mm²) and low inflammatory cell (<200 nuclei /mm²) attachment. PA309 and PA318 supported endothelial cell attachment, migration and retention to the same extent as collagen or 8G7 (P>0.05), while PA108 did not. PA309, but not PA319, supported CEO cell and EOC proliferation compared to collagen (CEO cells 3.5±1.3 *versus* 4.5±0.2 cumulative population doubling level [CPDL]; EOCs 3.3±0.7 *versus* 3.7±0.4 CPDL). In *ex vivo* studies, PA309 (2,242±2,200μm²) and PA318 (3,043±4,054μm²) reduced thrombus formation compared to control (11,851±8,118μm²) (P<0.01 and P<0.001 respectively). Attachment of platelets and leucocytes was equivalent to conventional BMS in clinical use.
Conclusion: Polymer PA309 promotes endothelial cell attachment, expansion and retention similar to collagen or 8G7 in vitro. Furthermore, PA309 inhibits thrombus formation and has low attachment of platelets and leucocytes in an ex vivo model of thrombosis. As a synthetic coating for intravascular coronary stents, PA309 may promote endothelialisation without increasing the risk of stent thrombosis following implantation.
8.2 INTRODUCTION

Whilst percutaneous coronary intervention (PCI) improves myocardial perfusion and relieves the symptoms of myocardial ischaemia, the treated vessel endures significant mechanical trauma. The high-pressure balloon inflations of coronary angioplasty and the forceful apposition of rigid stent struts denude endothelial cells leading to segments of vessel wall with no endothelium. Re-endothelialisation occurs by the attachment and expansion of endothelial progenitor cells (EPCs) and neighbouring/local endothelial cells. But extensive endothelial cell denudation can disrupt this process initiating a cascade of inflammatory events that culminate in restenosis\textsuperscript{109,110,111} or stent thrombosis\textsuperscript{88}. Drug-eluting stents (DES) can also disrupt endothelialisation leading to an increased risk of late stent thrombosis as anti-proliferative drugs inhibit endothelial cell proliferation.

New therapeutic approaches that promote growth of the endothelium are required following PCI. Synthetic coatings that promote endothelial cell attachment, expansion and retention \textit{in vitro} may promote endothelialisation of intravascular stents \textit{in situ}, which may improve the clinical outcome for patients following angioplasty and stenting for coronary artery disease.
8.3 HYPOTHESIS

Defined polymer substrates will promote endothelial cell attachment, expansion and retention compared to controls collagen and 8G7 (a polymer previously characterised for high endothelial cell attachment), without increasing thrombosis or platelet and leucocyte attachment.

8.4 AIMS

4. Identify polymer substrates with high attachment of endothelial cells and low attachment of inflammatory cells using high-throughput polymer microarrays.

5. Identify polymer substrates that facilitate endothelial cell attachment, expansion and retention under dynamic flow conditions \textit{in vitro}.

6. Ensure polymer substrates do not aggravate thrombus formation or platelet and leucocyte attachment compared to bare metal stents in an \textit{ex vivo} model of thrombosis.
8.5 METHODS

Cell isolation

CEO cells were isolated by the explant culture of atherothrombotic specimens obtained from patients receiving PCI with thrombectomy for the treatment of STEMI (n=4). Peripheral blood was obtained from healthy volunteers to isolate EOCs, neutrophils and monocytes. All volunteers gave informed written consent and this study was approved by our Research Ethics Committee. EOCs were isolated by the in vitro expansion of PBMCs (n=4), neutrophils were isolated by dextran sedimentation of peripheral blood followed by buoyant density centrifugation over isotonic Percoll (n=3), and monocytes were isolated from PBMCs by MACS with CD14 microbeads (n=3).

High-throughput identification of polymer substrates

Polyacrylate (PA) (monomers composed of acrylates) and polyurethane (PU) (monomers linked by urethane groups) libraries were chosen as candidate polymer for this study, as are widely used in prosthetic grafts, exhibit good biocompatibility and are amenable to functionalisation with drugs\textsuperscript{182-184}. Previously characterised\textsuperscript{185} stock libraries of PA and PUs were provided by Professor Mark Bradley (School of Chemistry, University of Edinburgh) and printed by Susan Gallogly. 337 polymers were printed in quadruplicate, and spotted onto cytophobic agarose-coated silane glass slides by contact printing with 32 Qu solid pins. Each microarray contained two blank regions (agarose-coated silane glass) and one vehicle control (solvent) region as negative controls. Slides were dried under vacuum and UV sterilised.

Aliquots of $5 \times 10^5$ CEO cells, EOCs, neutrophils and CD14\textsuperscript{+} monocytes were suspended in 4ml of their respective medium and seeded onto individual polymer microarrays as previously described\textsuperscript{136}. After 24 hours, cells were fixed with 4% PFA for 30 minutes at room temperature. Slides were washed with PBS, rinsed in tap water and mounted with ProLong\textsuperscript{®} Gold Antifade Reagent with DAPI. Coverslips were applied and slides were dried at room temperature in the dark for 1 hour prior
to image capture (x20 objective) and analysis. Cell attachment to individual polymer spots was
quantified by manual counts of DAPI positive cells and expressed as the number of nuclei per mm²
area assuming a spot diameter of 150μm. High level attachment was defined as cell binding >500
nuclei no./mm², low level attachment was defined as cell binding <200 nuclei no./mm², and
intermediate level attachment was defined as binding between 200-500 nuclei no./mm².

**Polymer synthesis**

Polymers PA309 and PA318 were synthesised by co-polymerisation of methyl methacrylate and
glycidyl methacrylate prior to the addition of Di-n-hexylamine and N-methylhexylamine, respectively.
8G7 was synthesised by co-polymerisation of ethyl methacrylate with 2-(diethylamino) ethyl acrylate.

**Phenotypic characterisation of endothelial cells on polymer substrates**

Polymers were dissolved in tetrahydrofuran (THF) (T425; Fisher chemicals, UK) (2% w/v). 220μl of
the polymer solution was placed onto the centre of a 19mm² coverslip (Cat AGL46R19-1; Agar
Scientific, UK) and 120μl was placed onto the centre of a 13mm² coverslip (Cat AGL46R13-1; Agar
Scientific, UK) before rapid spin coating for 10 seconds at 200rpm (P6708 spin coater, Speedlines
Technologies, USA). Polymers were dried under vacuum and UV sterilised. Monolayers of EOCs
and CEO cells were serially passaged on selected polymers and phenotyped for flow cytometric cell
surface expression of endothelial and leucocyte antigens at day 7 and day 28.

**Assessment of endothelial cells on polymer substrates in vitro**

**Cell attachment-static conditions**

Polymers were spin-coated onto coverslips, dried under vacuum and UV sterilised. For EOC colony
formation, peripheral blood mononuclear cells were seeded onto polymer-coated coverslips and
colony formation was assessed at day 21. Colonies of EOCs were phenotyped with flow cytometric
cell surface expression of endothelial and leucocyte antigens at first passage. For attachment under
static conditions, CEO cells and EOCs were seeded onto polymer-coated coverslips for 24 hours. Slides were then washed with PBS, rinsed in tap water and mounted with ProLong® Gold Antifade Reagent with DAPI. Cell attachment to individual polymer spots was quantified as the number of attached nuclei no./mm² area.

**Cell attachment-flow conditions**

For cell attachment under continuous steady-state laminar flow, 700μl of polymer solution was placed along the centre of a 25mm x 64mm coverslips (Cat. MIC3248; SLS, UK) before rapid spin coating. Coverslips were dried under vacuum (24 h at 45°C/200 mbar) and irradiated with UV light for 20 minutes prior to use.

25mm x 64mm polymer-coated coverslips were attached to Sticky μ-Slides (L0.4 Leur) (Cat. 80178; IBIDI, Germany) and connected to a Yellow/green perfusion set (Cat. 10964; IBIDI, Germany). The perfusion set was mounted on the IBIDI unit and 18ml of EGM™-2 medium was added to the perfusion reservoirs. The system was then placed in a 37°C/5% CO²/95% relative humidity incubator for 24 hours for degasification. The IBIDI unit was attached to pump and computer equipped with Pump control software version 1.5.2. The system was primed and 500,000 endothelial cells were added into each perfusion reservoir. The system was placed back into the incubator and continuous steady-state laminar flow (2.5ml/minute) was initiated and maintained and cells were circulated throughout the chamber for 60 minutes. Slides were then detached and gently washed to remove non-adherent cells. Attached cells were fixed with 4% PFA and a mosaic of digital image of the entire slide was captured. The number of attached cells was manually quantified as the number of attached cells per mm² area.
EGM™-2 medium and endothelial cells were placed in the reservoirs of the IBIDI chamber and the system was connected to the pump and computer equipped IBIDI pump control software. Steady state flow was initiated (2.5ml/min) and endothelial cell attachment to polymer-coated μ-Slides was quantified after 60 minutes of flow.

**Cell expansion**

For proliferation, endothelial cells were seeded onto polymer-coated coverslips for 28 days and the PDT and CPDL were calculated. For the wound closure migration assay, endothelial cells were cultured to confluence on polymer-coated coverslips (48 hours), starved in serum-free medium and a wound in the endothelial monolayer was created by a linear vertical stroke across the diameter of the well with a pipette tip (time zero). The width of the wound was imaged at time zero and at 24 hours and the percentage of wound closure was calculated after 24 hours.

**Cell retention**

25mm x 64mm polymer-coated coverslips were attached to Sticky μ-Slides (I0.2 Leur) (Cat. 80168; IBIDI, Germany) and 200,000 endothelial cells in 220μl EGM™-2 medium were seeded into the flow well of each slide. For collagen coated coverslips, 200,000 endothelial cells in 220μl EGM™-2 medium were seeded in the flow well of collagen μ-Slides (I0.2 Leur) (Cat. 80162; IBIDI, Germany).
Slides were incubated at 37°C/5% CO2/95% relative humidity for 48 hours and then attached to a Yellow/Green perfusion mounted on the IBIDI unit with 18ml of degased EGM™-2 medium at 37°C.

For live cell imaging, the IBIDI unit was placed in a temperature controlled Zeiss Axio Observer microscope maintained at 37°C with AxioVision 4.8 software. The IBIDI unit was attached to a pump and a computer equipped with Pump control software (version 1.5.2). Laminar flow was initiated and endothelial cells in the IBIDI chamber were exposed to 20 minute intervals of increasing shear stress (Table 4.1) calculated and controlled by the IBIDI pump control software. Before the completion of each interval a mosaic of digital images were captured across the entire flow slide using a x5 objective.

For quantification of results, Image J Software version 2.0.0 was used. Images were trimmed, stacked, sharpened and threshold was applied. Cell number within the entire flow slide was calculated by automatic cell counts at each increment in laminar flow. Cell coverage was calculated by expressing the number of cells at each increment in laminar flow as a percentage of the cell count at 0 dynes/cm² shear stress (100% cell coverage) (Figure 4.2).

Shear stress is related to the third power of the radius. The levels of shear stress corresponding to pathophysiological levels within the coronary artery for Figure 4.12 were calculated according to the equation $4uQ/\pi r^3$ where $u$ (plasma viscosity) remains constant and $Q$ (flow rate) remains constant up to 75% stenosis. Thus, the level of shear stress is related to the third power of the radius. Consequently, from physiological conditions of 16 dynes shear stress, an atherosclerotic plaque of 40% stenosis would result in a 5-fold increase of shear stress to 80 dynes.
Table 4.1 Increasing levels of shear stress imposed by increments in laminar flow in the IBIDI chamber.

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Figure 4.2 Quantification of endothelial cell retention to polymer substrates with increasing levels of shear stress *in vitro*.

(A) Endothelial cells were cultured on polymer substrates within the flow well of IBIDI slides for 48 hours. (B) Endothelial cells were then exposed to increasing levels of shear stress by increments in laminar flow. For quantification of cell coverage, images of the entire flow well were captured at each increment in laminar flow. The number of cells present at each stage was determined by automatic cells counts using image J software and cell coverage was calculated by expressing the number of cells at each stage as a percentage of cell number at 0 dynes/cm² shear stress (100% cell coverage).
Assessment of polymers in an *ex vivo* model of thrombosis

The Badimon chamber is an *ex vivo* model of deep arterial injury and thrombus formation. To overcome the ethical and procurement challenges concerning the use of human tissue, this model employs porcine artery as the function and histological anatomy is comparable to its human counterpart\(^\text{187}\) and each aorta is of sufficient size to generate all the required arterial strips for each Badimon study.

The chamber was prepared by placing strips of endothelium-denuded porcine aorta into the three extracorporeal chambers (one chamber to mimic low shear stress of mild stenotic arteries and two chambers to mimic high shear stress of moderate stenotic arteries) of the Badimon chamber and submerging the chambers into a 37°C water bath. Stents were deployed distal to the Badimon chamber in the extracorporeal circuit (polypropylene tubing) (*Figure 4.3*).

![Figure 4.3 Badimon chamber set-up.](image)

*Figure 4.3 Badimon chamber set-up.*

Strips of endothelium-denuded porcine aorta were placed into three extracorporeal chambers of the Badimon chamber and maintained at 37°C in a water bath. Stents were placed distal to the Badimon chamber in the extracorporeal circuit. Each were exposed to non-coagulated blood flow for 5 minutes at a rate of 10ml/minute.
**Ethical approval**

Local Research Ethics Committee approved our study protocol and all subjects provided written informed consent. Healthy volunteers attended the Clinical Research Facility at the Edinburgh Royal Infirmary on one occasion for a Badimon study.

**Study design**

This study design was based on previous power calculations. Healthy male volunteers (n=12) were recruited and attended the clinical research facility at the Royal Infirmary of Edinburgh. Baseline characteristics were recorded (Age, height, body mass index, heart rate, systolic and diastolic blood pressure) and total blood count and clotting profile were assessed by the biochemistry lab at the Royal Infirmary of Edinburgh. A 17-guage intravenous cannula was placed into the large antecubital fossa vein in the forearm and blood was drawn into the Badimon chamber by a peristaltic pump (Masterflex model 7013, Cole-Palmer Instruments, USA) maintained at a rate of 10mls/minute for 5 minutes, as previously described. Patients were cannulated by Dr Simon Wilson and help with the study design was received from Dr Jennifer Raftis from the University of Edinburgh.

Each participant received four 5 minute runs of the Badimon chamber assessing thrombus formation as well as platelet and leucocyte attachment to four test substrates: PA309-coated, PA318-coated, 8G7-coated and uncoated porcine aorta with PA309-coated, PA318-coated, 8G7-coated and uncoated cobalt chromium BMS respectively. The order of the substrates was randomised with a balanced block design and the operator was blinded to the condition being assessed.

**Procedure**

The endothelium in porcine aorta was removed to expose subendothelial components within the intima of the artery (Figure 4.4). Strips of endothelium-denuded porcine aorta were dip coated in polymer, dried at room temperature and placed in the extracorporeal chambers of the Badimon chamber (1 low shear stress and 2 high shear stress chambers; See Figure 4.3 for setup). Abbott Multi-link Vision L-605 cobalt chromium stents were dip coated in polymer, dried under vacuum, and
placed in the extracorporeal circuit distal to the Badimon chamber. A 17-gauge cannula was placed into the large antecubital fossa vein in the forearm of healthy volunteers and a peristaltic pump drew blood into the extracorporeal chambers and through the extracorporeal circuit. Blood was maintained at 37°C and the pump was maintained at a rate of 10mls/minute for 5 minutes. Before the completion of each study, blood was obtained from the effluent for flow cytometric analysis of antigens associated with platelet activation (platelet-monocyte aggregates, platelet neutrophil aggregates and P-selectin expression on platelets). At completion, the system was flushed and denuded-endothelium porcine aorta and stents were rinsed in saline for 1 minute. Porcine aorta and stents were then fixed in 4% PFA overnight, transferred to sterile PBS and stored at 4°C for 1-3 weeks prior sectioning.

Total area of thrombus formation on the coated and uncoated aortic strips was assessed after PFA fixation, paraffin sectioning (4μm), and histological staining with Massons trichrome. Platelet and CD45 leucocyte attachment was assessed on six regions of each stent (n=4 for each substrate) after PFA fixation and immunocytochemistry for cell surface antigens with human monoclonal antibodies and fluorochrome conjugated secondary antibodies.

![Figure 4.4 Preparation of endothelium-denuded porcine aorta strips for ex vivo studies.](image)

Scanning electron microscope images of porcine aorta as it is prepared for use in the Badimon chamber. (A) The cobblestone endothelial cell monolayer present in the intima of the artery (B) was removed to expose the underlying fibro-muscular layer of the vessel wall (denuded endothelium control). For studies with polymer, this layer was (C) dip-coated in polymer with uniform coverage across the surface of the strip. Scale bars 100 μm.

*Dip-coating of porcine aorta and stents*
Polymers were dissolved in THF (2% w/v). Cobalt chromium (L-605) (Abbot multilink, UK) stents were suspended from threads and dip coated into 2ml of polymer solution. While suspended, stents were dried under vacuum (12 h at 45°C/200 mbar) and sterilized by irradiation with UV light for 20 minutes. The endothelium of porcine aorta (Cat no. 59402; Pel-Freeze, USA) was removed and strips were dip coated into 5ml of polymer solution. Strips were air dried at room temperature for 2 hours and stored at 4°C for 24 hours before use.

**Scanning electron microscopy**

The surfaces of endothelium-denuded porcine aorta and stents were imaged using a scanning electron microscopy to verify uniform coverage by polymer. To this end, strips of endothelium-denuded porcine aorta and stents were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.3) for 2 hours. They were then washed in 3 x 10 minute changes of 0.1 M sodium cacodylate buffer. Samples were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 45 minutes. A further 3 x 10 minute washes were performed in 0.1 M sodium cacodylate buffer. Samples were then dehydrated in graded concentrations of ethanol (50%, 70%, 90%, and 3 x 100%) for 10 minutes each, followed by critical point drying with liquid carbon dioxide. After mounting on aluminium stubs with carbon tabs attached, BMS and denuded porcine aorta were sputter coated with 20 nm gold palladium and viewed with a Hitachi S-4700 scanning electron microscope.

**Histology of porcine aorta**

Porcine aortic strips were paraffin embedded and transverse sectioned to 4µm segments, by the histology department in SuRF at the University of Edinburgh. In brief, sections were deparaffinised, rehydrated and stained with Masson’s trichrome stain. Segments received a 10 minute incubation with Weigert's iron hematoxylin, a 15 minute incubation with Biebrich scarlet-acid fuchsin solution and a 10-15 minute incubation with phosphomolybdic-phosphotungstic acid solution until fibrin was stained red. Slides were then washed and incubated with aniline blue solution for 5 minutes and 1% acetic acid solution for 2 minutes. Total thrombus area was imaged and measured by an operator blinded to the coating applied to each strip. Results from six sections at equal intervals were averaged to determine thrombus area for each chamber (µm²). Total thrombus area was measured with help from
Mr Shea Connell with an Axio Scan automated slide scanning microscope (Zeiss, Germany) and image analysis system (Definiens Tissue Studio, Germany).

**Flow cytometry for platelet activation**

Fresh peripheral blood (baseline) and peripheral blood from the effluent of the extracorporeal circuit was placed in PPACK (Cat no. CAY15160; Cambridge Biosciences, UK) (100uM) and incubated for 20 minutes at room temp with monoclonal antibodies CD42a-FITC and CD62p-PE to identify P-selectin expression on platelets (*Table 2.3*) (*Figure 4.5*), CD42a-FITC and CD14-PE to identify platelet-monocyte aggregates and CD42a-FITC and CD16-APC/Cy7 to identify platelet-neutrophil aggregates (*Table 2.3*) (*Figure 4.6*). Tubes for P-selectin expression were washed with PBS (15 minutes 800g) and fixed with 1% PFA and tubes for platelet-leucocyte aggregates were washed with PBS (5 minutes 400g) and fixed with BD FACS™ Lysing Solution.

Samples were analysed immediately with a BD-Fortessa II flow cytometer. A minimum of 500,000 CD42a+ events were collected on the log scale to assess P-selectin expression and a minimum of 3,000 CD14+ events were collected to assess platelet-leucocyte aggregation. Data was analysed with FlowJo version 10.0.6 (TreeStar Inc., Switzerland).
Figure 4.5 Gating strategy for the identification of P-selectin expressing platelets in blood.

(A) Platelets were distinguished from other cells in blood by their characteristic light scatter signal for side scatter-area (SSA-A) against forward scatter-area (FSC-A) on the log scale. (B) Platelets were identified by positive displacement of CD42a- Fluorescein isothiocyanate (FITC) on the histogram scale. (C) Platelets positive for CD42a-FITC with co-expression of CD62-p (P-selectin)-Phycoerythrin (PE) (top right quadrant) were identified as activated.

Figure 4.6 Gating strategy for the identification of platelet-leucocyte aggregates in blood.

(A) Neutrophils (top sphere) were distinguished from other cells in blood by their characteristic light scatter signal for side scatter-height (SSC-H) against forward scatter-height (FSC-H). (B) Neutrophils were identified by positive displacement of CD16-Allophycocyanin (APC) Cy7 on the histogram scale, compared to unstained control. (C) Platelet-neutrophil aggregates were identified as CD16 APC Cy7 positive neutrophils with co-expression of CD42a-Fluorescein isothiocyanate (FITC) (top right quadrant). (D) Monocytes (bottom sphere) were selected based on their characteristic SCC-H against FSC-H and identified by (D) expression of CD14-APC on the histogram scale. (E) Platelet-monocyte aggregates were identified as CD14-APC positive monocytes with co-expression of CD42a-FITC (top right quadrant).
Platelet and leucocyte attachment to stents

Stents were incubated with 2% NGS in PBS for 30 minutes to block non-specific staining. Stents were then incubated overnight at 4°C with monoclonal antibodies: rabbit anti-human CD45 and mouse anti-human CD42a antibodies (Table 2.3) with isotype controls for mouse primary antibody (Cat no. 086599; Invitrogen, UK) and rabbit primary antibody (Cat no. 086199; Invitrogen, UK). Cells were washed and incubated with fluorescent labelled secondary antibodies for 30 minutes: Alexa Fluor® 568 goat anti-rabbit or Alexa Fluor® 488 goat anti-mouse to detect CD45 and CD42a respectively (Table 2.3). Stents were washed in PBS, incubated in 1mg/ml DAPI for one hour. They were then rinsed and visualised with a fluorescent Zeiss Axio Observer microscope (Zeiss, Germany). Using a x20 objective, six regions were chosen subjectively from each slide based on visual scanning for DAPI positive nuclei (blue). Composite multicolour images of each region were generated. Platelet attachment was quantified as fluorescent area (green) expressed as a percentage of stent surface area within the six regions. Leucocyte attachment was quantified as the number of attached cells per mm² area.
8.6 RESULTS

High-throughput identification of polymer substrates for high endothelial cell attachment and low inflammatory cell attachment

High-throughput screening of PA (n=302) and PU (n=35) libraries identified 45 polymers capable of supporting high endothelial cell attachment (>500 nuclei no./mm\(^2\)) for both CEO cells (Figure 4.7 A) and EOCs (Figure 4.7 B). Of this subset, 28 polymers had low neutrophil attachment (<200 nuclei no./mm\(^2\)) (Figure 4.7 C) and 17 of these had low monocyte attachment (<200 nuclei no./mm\(^2\)) (Figure 4.7 D).

PA309 and PA318 (amine-functionalised methacrylate-based co-polymers) (Figure 4.8) were chosen from this group as candidate polymers for stent coatings. This was because PA309 had very high attachment of CEO cells (1,043±1040 nuclei no./mm\(^2\)) and EOCs (1,390±785 nuclei no./mm\(^2\)) with very low attachment of neutrophils (164±194 nuclei no./mm\(^2\)) and monocytes (96±112 nuclei no./mm\(^2\)). Likewise, PA318 had very high attachment of CEO cells (1,202±1193 nuclei no./mm\(^2\)) and EOCs (1,490±1016 nuclei no./mm\(^2\)) with low attachment for neutrophils (93±94 nuclei no./mm\(^2\)) and monocytes (71±94 nuclei no./mm\(^2\)).

Each polymer microarray contained 8G7 (a polyacrylate previously characterised for high endothelial cell attachment\(^{136}\)) as an internal synthetic polymer standard control and each microarray contained two blank regions (agarose-coated silane glass) and one vehicle control (solvent) region as negative controls. Polymer 8G7 had high attachment of CEO cells (816±744 nuclei no./mm\(^2\)) and EOCs (661±520 nuclei no./mm\(^2\)) with intermediate attachment of neutrophils (250±263 nuclei no./mm\(^2\)) and monocytes (453±401 nuclei no./mm\(^2\)) (Figure 4.7).

The blank and solvent controls were not compromised during microarray studies with negligible endothelial cell and inflammatory cell attachment (<5 nuclei no./mm\(^2\) for all). In microarray studies, PA108 was selected as a negative polymer control for in vitro studies as it did not attach endothelial...
cells or inflammatory cells (1±1 nuclei no./mm² for all).
Figure 4.7 High-throughput identification of polymer substrates for intravascular stent coatings.

Images display cell attachment to spotted polymers quantified by nuclei staining with 4',6-diamidino-2-phenylindole (DAPI). Graphs display the top 45 endothelial cell-attaching polyurethanes (PUs) and polyacrylates (PAs).

Polymers are ranked in order of cell attachment by (A) coronary endothelial outgrowth (CEO) cells, (B) endothelial outgrowth cells (EOCs), (C) neutrophils, and (D) CD14+ monocytes. PA309 and PA318 (solid rectangular box) facilitated (A and B) high endothelial cell attachment with (C and D) low inflammatory cell attachment. Polymer 8G7 (broken rectangular box) had previously been characterised for high endothelial cell attachment and was included as an internal synthetic polymer standard control. The nuclei number/mm² was calculated based on a spot diameter of 150μm. Error bars are standard deviation.
Polyacrylate (PA)318 and PA309 are methyl methacrylate and glycidyl methacrylate co-polymers. PA309 has the amine functional group Di-n-hexylamine attached while PA318 has N-methylhexylamine attached. 8G7 is also a polyacrylate. It is a co-polymer of ethyl methacrylate and the amine containing 2-(diethylamino) ethyl acrylate.
Assessment of endothelial cells on polymer substrates *in vitro*

PA309 and PA318 were spin-coated onto coverslips for further analysis *in vitro*. Collagen (the gold standard substrate for endothelial cell culture) was used as a positive control and polymer 8G7 as an internal synthetic polymer control based on previous observations.\(^{136}\)

PA309 and PA318 cultured endothelial cells displayed typical endothelial cell cobblestone morphology comparable to control polymers collagen and 8G7 (*Figure 4.9*).

*Figure 4.9 Endothelial cell morphology on polymer substrates *in vitro*.*

Endothelial cells had a healthy circular cobblestone morphology with even spacing following culture on PA309, PA318, collagen and 8G7 for 24 hours. Scale bar 100μm.
Endothelial cell attachment to polymer substrates in vitro

Endothelial outgrowth colony formation

Peripheral blood mononuclear cells cultured on PA309 and PA318 supported generation of EOC colonies with similar propensities to collagen (2/3 donors gave EOC colonies for all). In contrast to previous findings, EOC colony formation was not observed on 8G7 (0/3 donors gave EOC colonies). Colonies formed on PA309 and PA318 had a similar phenotype to colonies formed on collagen with high CD31, CD146 and CD34 expression (> 89% for all) and low CD45 expression (<2.5%) after 28 days in culture (Table 4.2).

Table 4.2 Phenotypic characterisation of endothelial outgrowth colonies raised on candidate polymers at first passage.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Alternative name</th>
<th>PA309</th>
<th>PA318</th>
<th>Collagen</th>
</tr>
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<tbody>
<tr>
<td><strong>Cell surface</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>MCAM</td>
<td>98.7</td>
<td>95.3</td>
<td>94.7</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>95.8</td>
<td>99.5</td>
<td>88.2</td>
</tr>
<tr>
<td>CD144</td>
<td>VE-Cadherin</td>
<td>20.3</td>
<td>95.5</td>
<td>10.7</td>
</tr>
<tr>
<td>CD34</td>
<td>HPCA-1</td>
<td>93.4</td>
<td>86.3</td>
<td>99.4</td>
</tr>
<tr>
<td>CD309</td>
<td>KDR</td>
<td>67.5</td>
<td>14.0</td>
<td>70.0</td>
</tr>
<tr>
<td>CD133</td>
<td>Prominin-1</td>
<td>31.5</td>
<td>8.6</td>
<td>12.3</td>
</tr>
<tr>
<td><strong>Leucocyte</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD45</td>
<td>PTPRC or LCA</td>
<td>0.2</td>
<td>1.1</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Values are raw data for percentage antigen expression measured by flow cytometry (2 biological replica). KDR=kinase domain receptor; MCAM=melanoma cell adhesion molecule; LCA=leucocyte common antigen; PA=polyacrylate; PECAM=platelet endothelial cell adhesion molecule; VE=vascular endothelial.
Endothelial cell attachment to polymer substrates under static conditions

CEO cells attached to PA309 and PA318 with similar densities to controls collagen and 8G7 under static conditions (P>0.05). EOC attachment was similar compared to CEO cells (ANOVA=0.060) and neither CEO cells nor EOCs attached to the negative control polymer PA108 (Figure 4.10 A).

Endothelial cell attachment to polymer substrates in continuous steady-state laminar flow

When endothelial cells were circulated in continuous state steady laminar flow, cell attachment was ten-fold lower compared to static conditions. Nevertheless, CEO cells attached to PA309 and PA318 with similar densities to collagen and 8G7. Attachment of EOCs was greater than CEO cells while circulating (ANOVA=0.038). While EOCs attached to PA309 and PA318 with similar densities to collagen, EOC attachment to 8G7 was reduced (ANOVA=0.003). Neither CEO cells nor EOCs attached to PA108 (Figure 4.10 B).
Figure 4.10 Endothelial cell attachment to polymer substrates in dynamic flow conditions.

(A) Coronary endothelial outgrowth (CEO) cells and endothelial outgrowth cells (EOCs) attached to PA309 and PA318 with similar densities to controls (collagen and 8G7) under static conditions.

(B) When endothelial cells were circulated in continuous state steady laminar flow, EOC attachment to PA309 and PA318 was higher than CEO cells. While endothelial cells attached to candidate polymers with similar densities to collagen, EOC attachment to 8G7 was reduced. Endothelial cells did not attach to PA108. Two-way analysis of variance (ANOVA). Error bars are standard deviation. P-values for Bonferroni post-test: *P<0.05; **P<0.01 are cell attachment across substrates; †P<0.05; ††P<0.01 are differences in cell attachment to individual substrates.
Endothelial cell expansion on polymer substrates in vitro

Proliferation

Proliferation of CEO cells was high on PA309 with a similar PDT to collagen (2.8±0.5 versus 1.8±0.9 days) and CPDL to collagen (3.5±1.3 versus 4.5±0.2 level). Proliferation of CEO cells was low on PA318 with a reduced CPDL compared to collagen (P<0.01). Proliferation was also low on 8G7 with a similar CPDL to PA318 (1.3±0.4 and 1.8±0.5 respectively). EOCs had similar proliferative activity as CEO cells for PDT (ANOVA=0.546) and CPDL (ANOVA=0.183) on all substrates (Figure 4.11 A i and A ii).

Monolayers of endothelial cells serially passaged on PA309 had a similar phenotype to cells cultured on collagen and 8G7 during short-term culture (7 days) (Table 4.3) and long-term culture (28 days) (Table 4.4). Endothelial monolayers passaged on PA318 had higher levels of KDR antigen expression compared to cells cultured on collagen after short-term culture (P<0.05 for EOCs) and long-term culture (P<0.001 for CEO cells).

Wound closure migration assay

CEO cells and EOCs had a similar migratory capacity for wound closure on PA309 and PA318 as collagen and 8G7 (ANOVA=0.068) (Figure 4.11 B).
Unlike polyacrylate (PA)318, PA309 facilitated proliferation of coronary endothelial outgrowth (CEO) cells and endothelial outgrowth cells (EOCs) with similar (A i) population doubling times (PDTs) and a similar (A ii) cumulative population doubling level (CPDL) to collagen. (C) All substrates facilitated endothelial cell migration for wound closure compared to controls collagen and 8G7. Error bars are standard deviation. Two-way analysis of variance (ANOVA). P-values for Bonferroni post-test: *P<0.05; **P<0.01, ***P<0.001, ****P<0.0001 are cell proliferation across substrates.
Table 4.3 Phenotypic characterisation of coronary endothelial cells and endothelial outgrowth cells on candidate polymers after 7 days of *in vitro* culture.

| Antigen | CEO cells (n=3) | | | | EOCs (n=3) | | | |
|---------|----------------|--------|--------|--------|----------------|--------|--------|--------|--------|
|         | PA309          | PA318  | 8G7    | Collagen | P-Value | PA309  | PA318  | 8G7    | Collagen | P-Value |
| Cell surface |             |        |        |          |         |        |        |        |          |         |
| CD146   | 99.4 ± 0.9     | 99.9 ± 0.1 | 99.6 ± 0.4 | 99.6 ± 0.3 | 0.306  | 99.8 ± 0.1 | 99.8 ± 0.2 | 99.8 ± 0.2 | 99.9 ± 0.1 | 0.909 |
| CD31    | 93.4 ± 3.2     | 88.9 ± 2.4 | 93.7 ± 3.3 | 90.4 ± 4.9 | 0.227  | 79.3 ± 11.3 | 76.2 ± 16.0 | 81.1 ± 12.8 | 78.7 ± 15.9 | 0.706 |
| CD144   | 43.7 ± 6.3     | 30.0 ± 16.1 | 52.0 ± 3.7 | 34.9 ± 9.1 | 0.061  | 34.1 ± 4.7 | 25.3 ± 14.0 | 28.2 ± 4.6 | 29.9 ± 14.9 | 0.762 |
| CD34    | 47.7 ± 11.4    | 45.7 ± 13.8 | 43.5 ± 6.0 | 54.9 ± 7.4 | 0.820  | 41.5 ± 11.2 | 39.8 ± 21.7 | 50.9 ± 14.7 | 47.7 ± 24.3 | 0.920 |
| KDR     | 17.0 ± 16.6    | 33.4 ± 16.7 | 14.2 ± 2.1 | 13.9 ± 11.1 | 0.291  | 12.1 ± 4.3 | 19.6 ± 1.9 | 8.9 ± 4.3 | 8.3 ± 1.9 | 0.050 |
| CD133   | 0.0 ± 0.1      | 0.0 ± 0.1 | 0.1 ± 0.1 | 5.2 ± 7.6 | 0.266  | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.6 ± 0.9 | 2.8 ± 4.9 | 0.207 |
| Leucocyte |             |        |        |          |         |        |        |        |          |         |
| CD45    | 0.0 ± 0.1      | 0.3 ± 0.2 | 0.4 ± 0.4 | 0.2 ± 0.1 | 0.347  | 0.0 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.1 | 0.1 ± 0.3 | 0.987 |

Values are mean ± standard deviation for percentage antigen expression measured by flow cytometry. CEO=coronary endothelial outgrowth; EOC=endothelial outgrowth cell; PA=Polyacrylate. P-values are values for one-way analysis of variance (ANOVA). Kruskal-Wallis analysis of variance. P-values for Dunn’s post-test.
Table 4.4 Phenotypic characterisation of coronary endothelial cells and endothelial outgrowth cells on candidate polymers after 28 days of \textit{in vitro} culture.

| Antigen | CEO cells (n=3) | | | | EOCs (n=3) | | | |
|---------|-----------------|--------------|-----------------|---|----------------|---|-----------------|---|-----------------|---|-----------------|---|
|         | PA309 | PA318 | 8G7 | Collagen | P-Value | PA309 | PA318 | 8G7 | Collagen | P-Value |
| Cell surface | | | | | | | | | | | | |
| CD146   | 97.3 ± 0.5 | 99.0 ± 0.7 | 96.5 ± 3.8 | 99.0 ± 1.1 | 0.507 | 98.4 ± 2.2 | 99.2 ± 0.5 | 94.9 ± 6.0 | 99.0 ± 0.3 | 0.875 |
| CD31    | 98.4 ± 1.2 | 99.0 ± 1.0 | 99.0 ± 1.0 | 99.0 ± 0.7 | 0.306 | 99.1 ± 0.6 | 99.7 ± 0.2 | 98.1 ± 1.4 | 99.6 ± 0.2 | 0.364 |
| CD144   | 78.0 ± 6.3 | 84.6 ± 5.3 | 83.65 ± 4.2 | 88.3 ± 10.1 | 0.625 | 86.1 ± 4.9 | 92.2 ± 1.7 | 68.0 ± 29.7 | 82.8 ± 0.4 | 0.229 |
| CD34    | 93.9 ± 2.9 | 97.0 ± 0.5 | 95.0 ± 2.3 | 96.3 ± 3.4 | 0.397 | 93.9 ± 2.3 | 95.4 ± 1.5 | 91.9 ± 5.4 | 95.8 ± 1.3 | 0.556 |
| KDR     | 43.6 ± 1.2 | 94.5 ± 5.0 | 46.6 ± 1.4 | 43.6 ± 1.1 | \(<0.001\) | 49.6 ± 15.4 | 90.7 ± 8.6 | 47.3 ± 5.4 | 40.1 ± 4.4 | 0.056 |
| CD133   | 1.5 ± 1.4 | 1.3 ± 0.6 | 3.2 ± 4.5 | 1.7 ± 0.15 | 0.982 | 10.4 ± 9.7 | 10.8 ± 4.2 | 5.9 ± 2.6 | 3.1 ± 2.0 | 0.200 |
| Leucocyte | | | | | | | | | | | |
| CD45    | 0.2 ± 0.2 | 0.1 ± 0.1 | 0.3 ± 0.4 | 0.2 ± 0.1 | 0.779 | 0.3 ± 0.2 | 0.2 ± 0.1 | 0.5 ± 0.4 | 0.2 ± 0.3 | 0.265 |

Values are mean ± standard deviation for percentage antigen expression measured by flow cytometry. CEO=coronary endothelial outgrowth; EOC=endothelial outgrowth cell; PA=polyacrylate. Kruskal-Wallis analysis of variance. P-values for Dunn’s post-test.
Retention of endothelial cells on polymer substrates with increasing levels of shear stress *in vitro*

When CEO cells were exposed to increasing levels of shear stress by increments in laminar flow, CEO cells did not detach from PA309 and PA318 at 4, 8 and 16 dyne/cm² (physiological levels of shear stress). At 25 dyne/cm², CEO cells began to detach rapidly with a 70% loss by 35 dyne/cm² (pathophysiological level imposed by 20% stenosis), an 80% loss by 55 dyne/cm² (pathophysiological level imposed by 30% stenosis) and 100% loss by 85 dyne/cm² (pathophysiological level imposed by 42% stenosis) (ANOVA=0.037) (*Figure 4.12*).

CEO cell retention to PA309 and PA318 was similar to collagen at 16 dyne/cm² but reduced at 25, 35 and 45 dyne/cm². By 55 dyne/cm², retention of CEO cells was similar on PA309 (16±26%), PA318 (12±20%) and collagen (18±25%). PA309 and PA318 had similar retention of CEO cells compared to 8G7 at 25, 35 and 45 dyne/cm² shear stress (*Figure 4.12 A*).

In contrast to CEO cells, EOC retention to PA309 and PA318 decreased similarly to collagen and 8G7 for all levels of shear stress (ANOVA=0.486) (*Figure 4.12 B*).
Figure 4.12 Retention of endothelial cells to polymer substrates with increasing levels of shear stress in vitro.

(A) Coronary endothelial outgrowth (CEO) cell retention to PA309 and PA318 was similar to collagen at 16 dyne/cm² but reduced at 25, 35 and 45 dyne/cm². (B) Endothelial outgrowth cell (EOC) retention to PA309 and PA318 was similar to collagen for all levels of shear stress. Error bars are standard deviation. Two-way analysis of variance (ANOVA). P-values for Bonferroni post-test: *P<0.05 and **P<0.01 for PA309, PA318 and 8G7 versus collagen.
**Ex vivo model of thrombosis**

The Badimon chamber is an *ex vivo* model of deep arterial injury and thrombus formation. It was used to determine the effect of candidate polymer coatings on thrombus formation compared to endothelium-denuded porcine aorta as control. The chamber was modified to determine if polymer-coated prototype stents would have similar levels of platelet and leucocyte attachment compared to BMS in clinical use following exposure to non-coagulated flowing blood.

Participants recruited for Badimon studies were 36±14 years old with a healthy body mass index and blood pressure (*Table 4.5*). Total and differential cell counts and clotting profiles in blood were within normal range (data not shown). One participant did not complete all four studies with the Badimon chamber and was excluded from data analysis.

**Table 4.5** Baseline characteristics of participants in *ex vivo* studies.

<table>
<thead>
<tr>
<th>Total population (<em>n=11</em>)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>36 ± 14</td>
</tr>
<tr>
<td>Height (m)</td>
<td>178 ± 6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24 ± 2</td>
</tr>
<tr>
<td>Heart rate (beats per minute)</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>130 ± 23</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>85 ±14</td>
</tr>
</tbody>
</table>

Values are mean ± standard deviation.

PA309 (2,242±3,200μm²) and PA318 (3,043±4,054μm²) reduced total thrombus area under low shear stress compared to control strips of endothelium-denuded porcine aorta (11,851±8,118μm²) (*P*<0.01 and *P*<0.001 respectively). Likewise PA309 and PA318 reduced total thrombus area compared to control under high shear stress (*P*<0.01 and *P*<0.05 respectively) (*Figure 4.13*).

Platelet activation following contact with PA309, PA318, denuded endothelium and 8G7 was similar to baseline measures of activation. Furthermore, platelet activation was similar between all substrates when measured by percentage of platelet-monocyte aggregates (50.3±27.8%, 50.1±27.3%, 49.6±19.8%
and 43.2±24.8% respectively), platelet-neutrophil aggregates (25.4±13.6%, 20.4±11.1%, 28.2±12.8% and 23.7±9.9% respectively) and P-Selectin expression on platelets (28.8±22.7%, 25.1±18.8%, 27.4±18.8% and 31.6±23.8% respectively) (Figure 4.14).

Prototype stents coated with PA309 and PA318 had similar levels of platelet attachment compared to conventional cobalt chromium stents in clinical use as well as 8G7-coated stents (PA308: 19.1 ± 9.6%, PA318: 12.4 ± 3.7%, BMS: 13.5 ± 9.8%, 8G7: 18.2 ± 9.9% area coverage/mm²). Likewise, the number of attaching CD45 leucocytes was similar (PA309: 20 ± 14, PA318: 11 ± 5, BMS: 33 ± 15 cells, 8G7: 31 ± 14 cells respectively) (Figure 4.15).
Figure 4.13 Polymer substrates reduce thrombus formation in ex vivo studies.

(A) Scanning electron microscopy demonstrates uniform polymer coatings of endothelium-denuded porcine aorta strips for ex vivo studies. (B) Masson’s trichrome staining demonstrates thrombus formation on the porcine aorta with or without polymer coating following exposure to non-coagulated flowing blood in the Badimon chamber. (C) Total thrombus area was quantified with a semi-automated platform calibrated using colour to differentiate between background (light blue), thrombus (red) and aorta (green). For measurement of total thrombus area, six equidistant regions of porcine aorta was averaged for each participant. Polyacrylate PA309 and PA318 reduced thrombus formation under (D) low shear stress and (E) high shear stress compared to the denuded control. Polymer 8G7 also reduced thrombus formation under low and high shear stress. Error bars are standard deviation. One-way analysis of variance (ANOVA). P-values for Bonferroni post-test: *P<0.05; **P<0.01, ***P<0.001.
Figure 4.14 Platelet activation in *ex vivo* studies.

Blood was obtained before (baseline) and after (effluent) contact with polymer substrates in the Badimon chamber. Flow cytometric detection of platelet activation demonstrated similar levels of platelet activation following contact with substrates by measures of (A) platelet-monocyte aggregation, (B) platelet-neutrophil aggregation and (C) P-selectin expression on platelets. Error bars are standard deviation. Two-way analysis of variance (ANOVA).
Figure 4.15 Platelet and leucocyte attachment to polymer-coated stents in \textit{ex vivo} studies.

During \textit{ex vivo} studies, an exploratory study assessing platelet and leucocyte attachment to polymer-coated stents was performed in a small subset of patients (n=3). (A) Scanning electron microscopy demonstrates uniform polymer coatings of stents for \textit{ex vivo} studies. (B) The attachment of platelets and leucocytes to stent surfaces following exposure to non-coagulated blood was identified by CD42a cell surface staining (green) for platelets and CD45 cell surface staining (red) for leucocytes with 4',6-diamidino-2-phenylindole (DAPI) as a nuclei counterstain (blue). Candidate polymers PA309 and PA318 had similar attachment of platelets (C) and leucocytes (D) compared to conventional cobalt chromium stents in clinical use. Error bars are standard deviation. Kruskal-Wallis analysis of variance (ANOVA).
8.7 DISCUSSION

New therapeutic approaches that promote endothelialisation are required to improve the clinical outcomes in patients undergoing angioplasty and stenting for coronary artery disease. Stent coatings that promote endothelial cell attachment, expansion and retention \textit{in vitro} may promote endothelialisation of intravascular stents \textit{in situ}. Here I report that PA309 promotes endothelial cell attachment, expansion and retention compared to collagen or 8G7 \textit{in vitro}, with reduced thrombus formation and similar levels of platelet and leucocyte attachment compared to conventional bare metal stents in an \textit{ex vivo} model of thrombosis. As a synthetic coating for intravascular coronary stents, PA309 may promote endothelialisation following percutaneous coronary intervention without increasing the risk of stent thrombosis following implantation.

Polymers have widespread biomedical application and have been used for coatings of cardiovascular implants\textsuperscript{182, 184}, drug delivery devices\textsuperscript{182} and intraocular lens. While polyethyleneimines (such as poly[2-ethyl-2-oxazoline]) and polyoxazolines (such as poly[2-ethyl-2-oxazoline]) have demonstrated high endothelial cell attachment\textsuperscript{5}, I chose to assess polyacrylates (PAs) (monomers composed of acrylates) and polyurethanes (PUs) (monomers linked by urethane groups) as they had characteristics which could be advantageous for coronary stents such as their biocompatibility, mechanical resistance to abrasion, tissue-compatibility and amenity to functionalisation with drugs. Furthermore, I selected libraries of PAs and PUs whose chemical identify has previously been documented\textsuperscript{185}. As previous studies have suggested that growth of endothelium over denuded vessel segments and the abluminal surface of stents is believed to occur through the actions of local endothelial cells and circulating endothelial progenitor cells\textsuperscript{116}, I employed coronary endothelial outgrowth (CEO) cells and endothelial outgrowth cells (EOCs) as representative cells from these localities. For high-throughput screening of PA and PU libraries, I included polyacrylate 8G7 as an internal comparator control as this polymer had previously been characterized for high endothelial cell attachment in the absence of platelet attachment\textsuperscript{136}. My studies identified 45 polymers with high endothelial cell attachment. I selected polyacrylate PA309 and PA318 from this selection, as both polymers demonstrated exceptional endothelial cell attachment with low neutrophil and monocyte attachment indicating that
they might promote endothelial cell attachment without exacerbating the local inflammatory response. Indeed, PAs have previously been reported as efficient polymers for endothelial cell attachment\textsuperscript{136, 189}. Unexpectedly, PA309 and PA318 had higher levels of endothelial cell attachment compared to the synthetic control 8G7.

PA309 and PA318 are methyl methacrylate and glycidyl methacrylate co-polymers with the addition of amine functional groups Di-n-hexylamine and N-methylhexylamine, respectively. The amine groups present on these structures could have promoted endothelial cell attachment as they become protonated under biological conditions (pH 7.4) conferring a positive charge\textsuperscript{185}. As a consequence, structure modification by means of further amine functionalisation could improve the biological function of these polymers leading to increased levels of cell attachment. 8G7 is a co-polymer of ethyl methacrylate and 2-(diethylamino) ethyl acrylate and it also has amine groups incorporated similar to PA309 and PA318. However, the amines present on 8G7 are incorporated into the backbone of the polymer structure instead of on the peripheral side branches like PA309 and PA318. Thus, physico-chemical properties of 8G7 could have prevented cellular interactions with the amines resulting in lower attachment of endothelial cells.

While it is unclear why PA309 and PA318 would favour endothelial cell attachment over inflammatory cell attachment, it is possible that the positive charge conferred by the amine group promoted hydrophilicity (due to the hydrogen bonding between the functional groups and water). Hydrophilicity can exert anti-inflammatory effects discouraging the attachment of inflammatory monocytes whereas hydrophobic polymers are known to promote adhesion of inflammatory cells\textsuperscript{190}. Indeed, experiments assessing the wettability of polymers by the contact angle measurement of the water droplet on the surface of the polymer could shed light on the hydrophobic or hydrophilic nature of these polymer structures. Using this information, future experiments altering the ratio of the co-monomers could lead to increased hydrophilicity, which could improve the biological function of these polymers.

The \textit{in-situ} endothelialisation of coronary stents involves endothelial cell attachment, expansion and
retention to polymer substrates. As the underlying surface material can affect each of these functions, I carefully assessed each of these parameters in vitro comparing to collagen and the 8G7 synthetic polymer control. I compared to collagen as it is the gold standard substrate for in vitro culture of endothelial cells although it cannot be used as a stent coating as it attaches platelets making it unsuitable for clinical use.

To measure endothelial cell attachment to polymer substrates, I assessed EPC attachment by EOC colony formation and I also assessed CEO cell and EOC attachment. As with previous studies, the attachment of CEO cells and EOCs was examined by directly seeding cells onto surface materials in static conditions. I also examined cell attachment under continuous steady-state laminar flow, as this state is more representative of the vascular environment in situ. For this assay, I used a flow rate of 2.5ml/min which is representative of haemodynamic forces experience in venous flow (2 Dyn/cm²) rather than physiological coronary arterial flow (16 Dyn/cm²), but this is common when assessing cell attachment in vitro as cell do not attach quickly at higher speeds.

My preliminary study of 3 donors, found that candidate polymers PA309 and PA318 supported EOC colony formation with efficiencies similar to collagen. Unlike previous reports, EOC colony formation was not seen on 8G7 but further studies are necessary with a larger number of donors to confirm this finding. This may be a consequence of heterogeneity between donors, which is common and due to the low number of circulating endothelial progenitor cells that are able to form EOC colonies. It was also found that PA309 and PA318 promoted CEO cell and EOC attachment with densities similar to control substrate collagen in static conditions. Reassuringly, the levels of endothelial cell attachment to collagen was similar to previous studies. When endothelial cells were circulated with continuous steady-state laminar flow in the IBIDI system, PA309 and PA318 promoted CEO cell and EOC attachment with densities similar or better than control polymers, collagen or 8G7. Interestingly EOCs showed a higher potential to adhere to PA309 and PA318 compared to CEO cells. While the reasons for this are unclear, it is possible that EOCs are conditioned to attach to substrates under flow condition, as they circulate in blood and attach to existing endothelial or sub-endothelial space in the vasculature. As this was the first study to explore
endothelial cell attachment following circulation in the IBIDI chamber, further studies are warranted to investigate this result.

Previous studies have only assessed endothelial cell expansion on polymers up to 7 days\textsuperscript{5}, or at first passage\textsuperscript{6}. In this study I assessed endothelial cell expansion long-term in culture (28 days which is equivalent to 4 passages). I found that only PA309 was able to support long-term endothelial cell expansion, where PA318 and 8G7 did not. This is in keeping with the current literature whereby PAs with different monomer combinations and functional groups have shown different potential for endothelial cell growth\textsuperscript{5}. It has also been reported that while endothelial cells may attach and show good initial results for cell growth, longer-term culture can result in delamination of endothelial cells leaving behind a bare polymer surface\textsuperscript{5}.

The endothelial cells expanded on PA309 had a phenotype comparable to those cultured on collagen characterised by high CD31 expression. Reduced CD31 (Platelet endothelial cell adhesion molecule [PECAM]-1) expression has previously been reported following long term growth on poly(L-lactide) (PLLA) with reduced barrier function and endothelial dysfunction observed as a consequence\textsuperscript{194}. Interestingly, endothelial cells on PA318 had increased KDR expression (a receptor for vascular endothelial growth factor\textsuperscript{161, 195}), although the mechanism for this needs to be further investigated. Encouragingly, all substrates supported wound closure (>50\%) by migration of endothelial cells. Previous studies\textsuperscript{196} have demonstrated that polymer stiffness can influence the speed and direction of cell migration which can adversely affect wound healing capabilities.

To measure the capacity of polymer substrates to retain endothelial cells as they experience shear stress forces encountered in the diseased coronary circulation, cell attachment to PA309 and PA318 was compared to control substrates during increasing levels of shear stress imposed by increments in laminar flow in the IBIDI system. Normal physiological and elevated pathophysiological levels of shear stress were employed to determine cell retention in healthy and stenotic conditions, but laminar flow was employed rather than disturbed or oscillatory flows which are more representative of the
type of flow experienced \textit{in vivo}\textsuperscript{36}. This study demonstrated that PA309 and PA318 retained endothelial cells similar to 8G7 although collagen was better at retaining CEO cells at pathophysiological levels of shear stress (25, 35 and 45 a dynes/cm\textsuperscript{2}). In the clinical situation, the goal of PCI is to restore normal vessel patency and normalise coronary blood flow (defined as post-procedure thrombolysis in myocardial infarction [TIMI] flow grade 3\textsuperscript{197}). When this occurs, physiological levels of shear stress (16 dynes/cm\textsuperscript{2}) are restored. This level of shear stress would be most relevant for the clinical scenario at implantation. But at 6 month follow-up angiography, the frequency of target vessel revascularisation may be as high as 40\textsuperscript{197} with >50% stenosis in the treated segment resulting in higher pathophysiological levels of shear stress. Thus, higher levels of shear stress may be relevant for the clinical scenario at 6 months following implantation, and these conditions need to be studied in further experiments. It is important that polymer substrates retain endothelial cells with increasing levels of shear stress to avoid late endothelial cell denudation with the increased flow rates during arterial remodelling.

As surface-mediated thrombogenicity is a major cause of intravascular stent failure, I evaluated the thrombotic potential of selected polymer substrates \textit{ex vivo} before efficacy studies in animals. The Badimon chamber is a highly reproducible technique of \textit{ex vivo} thrombus formation\textsuperscript{188, 198} and it has previously been used to assess the thrombogenicity of different biological\textsuperscript{199} and synthetic materials\textsuperscript{200}. Using this chamber, I exposed polymer-coated and uncoated subendothelial components in the fibromuscular layer of porcine aorta to non-coagulated flowing blood (endothelium-denuded porcine aorta). While the pump rate is set at 10ml/ml, the rheological conditions within each extracorporeal chambers are altered by channel height leading to moderate changes in average blood flow rate exposing substrates to low shear stress (wall shear rate 106\textsuperscript{-1}) and high shear stress (wall shear rate 1690s\textsuperscript{-1}) representative of mild and moderate stenosis\textsuperscript{201}. Under low and high shear stress, PA309 and PA318 reduced thrombus formation compared to endothelium-denuded porcine aorta, as did 8G7. As thrombus formation on the denuded endothelial control in the low shear stress chamber and high shear stress chamber was in accordance with a recent report\textsuperscript{202}, this indicated that selected polymers were not prothrombotic. And whilst the integrity of the polymer was not assessed following Badimon
studies, it is unlikely that the polymer degraded within 5 minutes following contact with blood, given the results of the *in vitro* studies with the IBIDI chamber where polymers were exposed to flowing material for ~1 hour. Surprisingly, the thrombogenic difference between candidate polymers and endothelium-denuded porcine aorta was not reflected in the flow cytometric assessment of platelet activation (platelet-leucocyte aggregates and p-selectin expression) within circulated blood, nor was there an increased from baseline measure of platelet activation in the effluent. This phenomenon has previously been reported in Badimon studies\textsuperscript{203} as activated platelets in the chamber may have incorporate into the thrombus making them undetectable in the effluent from the extracorporeal circuit.

Inflammation mediated against the implanted biomaterial can increase the morbidity associated with stent implantation therefore studies commonly assess platelet aggregation on substrates *in vitro*\textsuperscript{136} by assays of indium-labelled platelet accumulation on substrates *ex vivo*\textsuperscript{204} and leucocyte attachment under constant rotation *in vitro*\textsuperscript{204}. In this study, I modified the Badimon chamber to include polymer-coated and conventional cobalt chromium stents to allow the assessment of platelet and leucocyte attachment *ex vivo* following exposure to non-coagulated flowing blood. These studies were exploratory and not based on any formal power calculation, but interestingly I observed that PA309- and PA318-coated prototype stents had similar attachment of platelets and leucocytes in comparison to cobalt chromium surface coating suggesting that PA309 and PA318 may not promote inflammatory cell attachment. These observations need to be confirmed in a larger set of experiments and *in vivo*.

The success and patency of a stent coating is related to its ability to integrate into its vascular environment and prevent adverse events following implantation. This study has identified two amine functionalised methacrylate based co-polymers as candidate polymers for stent coatings. The *in vitro* study results presented here have demonstrated that PA309 supports endothelial cell attachment, expansion and retention at similar levels to collagen or 8G7. While PA318 supported endothelial cell attachment and retention it did not support endothelial cell proliferation necessary for endothelial cell expansion on coronary stents. This indicates that PA309 is more suitable for clinical use despite the fact that both polymers reduced thrombus formation and had low levels of platelets and leucocyte attachment equivalent to conventional stents in clinical use. Thus, PA309 could be used as a surface
coating for coronary stents to promote the formation of a confluent endothelium and should be taken forward to in vivo experimental studies.

8.8 STUDY LIMITATIONS

A number of limitations need to be taken into consideration when interpreting this data. First, as with all in vitro studies the conditions are artificial and therefore they do not reflect the complexity of in vivo situation. Thus, assays of endothelial cell attachment, expansion and retention might not adequately mimic endothelialisation in situ. Second, the effect of polymer substrates on VSMC attachment and proliferation has not been assessed in this study. As VSMCs mediate neointimal hyperplasia, future studies are warranted to assess this risk. Third, the Badimon chamber was used to evaluate thrombosis on polymers. While it provided useful insight into the pro-thrombotic potential of polymer substrates, the short 5-minute cycles of blood flow may not recapitulate the thrombotic effect of polymer substrates as prolonged exposure may increase adsorption of vWF and soluble tissue factor from blood plasma which could increase the risk of thrombosis. As such, further studies are required to stratify each polymer’s risk for thrombosis in vivo, perhaps in a porcine model of coronary angioplasty. Lastly, the small sample size for in vitro experimentation must be acknowledged. Because of this, assumptions such as normal distribution and homogeneity of variance could not be determined due to the inability to assess the distribution shape when there are so few observations. This must be taken into account when interpreting these results.

8.9 CONCLUSIONS

New therapeutic approaches that promote endothelialisation are required to improve the clinical outcomes in patients undergoing angioplasty and stenting for coronary artery disease. I have demonstrated that PA309 promotes endothelial cell attachment, expansion and retention in vitro with reduced thrombus formation and low attachment of platelets and leucocytes in an ex vivo model of
thrombosis. As a coating for coronary stents, PA309 has major potential to promote endothelialisation and it may limit the risk of stent thrombosis following implantation.
CHAPTER 5

CYCLIN-DEPENDENT KINASE INHIBITORS
FOR THE RESOLUTION OF
NEUTROPHILIC INFLAMMATION
FOLLOWING
PERCUTANEOUS CORONARY
INTERVENTION
12.1 ABSTRACT

**Rationale:** Resolution of the acute neutrophil-mediated inflammatory response to stenting could improve the clinical outcome for patients following percutaneous coronary intervention. AT7519, a cyclin-dependent kinase (CDK) inhibitor, is effective at resolving neutrophilic inflammation but its effect on the coronary endothelium is unknown.

**Objective:** I aimed to test the effect of AT7519 on endothelial cell function and viability at concentrations that induce neutrophil apoptosis *in vitro*. I also aimed to test the effect of AT7519 on vascular smooth muscle cell (VSMC) proliferation and viability.

**Methods:** Neutrophils were incubated with AT7519 for 6 and 24 hours (Astex Pharmaceuticals, UK) and optimal concentrations to induce neutrophil apoptosis were determined by nuclei pyknosis and Annexin V binding measured by flow cytometry. The effect of these concentrations on coronary endothelial outgrowth (CEO) cell and late endothelial outgrowth cells (EOC) function was assessed by measures of proliferation, attachment, wound closure, and tubule formation *in vitro*. The effect on VSMC proliferation was also measured. For cell viability, endothelial cells and VSMCs were stimulated with vascular endothelial growth factor (VEGF165) or platelet-derived growth factor (PDGF)-BB, respectively, in the presence or absence of AT7519. Apoptosis was quantified by Annexin V binding and cell toxicity was quantified by lactate dehydrogenase (LDH) release by colorimetric assay.

**Results:** At a concentration of 0.1μmol/L, AT7519 induced neutrophil apoptosis compared to vehicle control at 6 hours (11±1 *versus* 1±1 nuclei pyknosis, *P*<0.05) and 24 hours (76±3% *versus* 45±7% Annexin V binding, *P*<0.01). This concentration did not impact endothelial cell or VSMC proliferation (*P*>0.05 for both) nor did it effect CEO cell attachment (32±23% *versus* 57±5% attachment) wound closure (94±8% *versus* 70±19%) or tubule formation (44±6 *versus* 58±11 tubule structures) (*P*<0.05 for all). Similarly AT7519 at a concentration of 0.1μmol/L did not have any impact on EOC function, nor did it induce endothelial cell or VSMC apoptosis or cytotoxicity in stimulated cells.
**Conclusion:** The CDK inhibitor AT7519 selectively induces neutrophil apoptosis with no adverse effect on endothelial cell function or viability at a defined concentration *in vitro*. Controlled elution or infusion of AT7519 could resolve the acute neutrophil-mediated inflammatory response to stenting without adversely affecting the function and integrity of the coronary endothelium.
12.2 INTRODUCTION

Compounds released by drug-eluting stents (DES) have reduced the incidence of neointimal hyperplasia but they have increased the incidence of late stent thrombosis as they adversely affect re-endothelialisation following percutaneous coronary intervention (PCI). Current compounds, such as M-inhibitor Paclitaxel and G₁-inhibitor Rapamycin (Sirolimus), inhibit vascular smooth muscle cell (VSMC) proliferation for the reduction of neointimal hyperplasia but they also inhibit endothelial cell proliferation leading to segments of vessel with no endothelial cell lining and an environment predisposed to thrombus formation⁹⁵. Re-endothelialisation by neighbouring/local endothelial cells and circulating endothelial progenitor cells is essential to prevent adverse outcomes following PCI. As such, next generation DES seek compounds that can inhibit neointimal hyperplasia without adversely affecting the coronary endothelium.

Cyclin-dependent kinase (CDK) inhibitors are small molecules that prevent formation of CDK/cyclin complexes at cell cycle checkpoints which can lead to cell arrest or apoptosis. The archetypal CDK inhibitor, R-roscovitine, has been shown to induce apoptosis in neutrophil promoting the resolution of inflammation in many diverse models¹⁵¹-¹⁵³. R-roscovitine induces broad-spectrum inhibition of CDKs, which reduces Mcl-1 in neutrophils leading to apoptosis and phagocytosis by monocytes. This induces the release of IL10 and TGF-β from monocytes with the resolution of neutrophilic inflammation in murine models of inflammation¹⁵¹-¹⁵³.

Neointimal hyperplasia is an inflammatory driven process²⁰⁶, ²⁰⁷. As such, CDK-inhibitors may reduce neointimal hyperplasia by preventing inflammation cell accumulation and thereby VSMC proliferation and migration. Neutrophils have been implicated in the development of restenosis with peri-strut accumulation (6 hours – 3 days) priming macrophages for prolonged accumulation in the neointimal²⁰⁸. Indeed, neutrophils usher monocytes to the site of inflammation by the expression of monocyte-attracting chemokines (MCP-1) and the secretion of cathelicidin²⁰⁹. They also polarise monocytes to their pro-inflammatory phenotype by the secretion of TNF-α, IFN, and IL-6²⁰⁶. Upon entry, monocytes secrete growth factors that modulate VSMC phenotype leading to neointimal
hyperplasia. The central role of neutrophils in the development of neointimal hyperplasia is well
established and it has been demonstrated that targeted inhibition (by Mac-1 β2 integrin blockage) in
the initial phase of inflammation reduces VSMC proliferation and neointimal hyperplasia following
balloon angioplasty210. Thus, the resolution of the neutrophil-mediated inflammation could inhibit
neointimal hyperplasia and improve the clinical outcome in patients following PCI.

AT7519 is a new generation of cyclin-dependent kinase (CDK) inhibitor discovered by Astex's
fragment-based medicinal chemistry approach using high-throughput X-ray crystallography211.
AT7519 is currently undergoing clinical development as an anti-cancer agent as it inhibits CDK 1 and
CDK 2 in tumour cells inducing cell cycle blockage8-11. Like its predecessor R-roscovitine, it has been
shown to induce neutrophil apoptosis by down regulation of Mcl-1 without impairing monocyte
phagocytosis of apoptotic neutrophils. It has also been shown to resolve inflammation in established
model of respiratory inflammation in vivo153. AT7519 is 100 times more potent at inducing neutrophil
apoptosis compared to first generation CDK inhibitors153. It is also more selective in its inhibition of
CDK families (1, 2, 4, 6 and 9)212. Indeed, patients have demonstrated good tolerability in phase II
clinical trials with a better toxicity profile compared to first generation CDK inhibitors149. Of
particular interest, our collaborator has demonstrated that AT7519 reduces atherosclerotic plaque size
in mice (unpublished) suggesting AT7519 may have a role in resolving the chronic inflammatory
response to coronary stenting. Currently, the effect of AT7519 on the coronary endothelium is
unknown.
12.3 HYPOTHESIS

The cyclin-dependent kinase inhibitor AT7519, will selectively induce neutrophil apoptosis without adversely affecting endothelial cell function or viability in vitro.

12.4 AIMS

1. Determine the concentrations of AT7519 that induce neutrophil apoptosis in vitro.

2. Assess the effect of these concentrations on endothelial cell proliferation, function and viability in the presence or absence of pro-inflammatory growth factor (vascular endothelial growth factor) in vitro.

3. Assess the effect of these concentrations on coronary artery vascular smooth muscle cell proliferation and viability in the presence or absence of pro-inflammatory growth factor (platelet-derived growth factor-BB) in vitro.
12.5 METHODS

Cell isolation

Coronary endothelial outgrowth (CEO) cells were isolated by the explant culture of atherothrombotic specimens obtained from patients receiving PCI with thrombectomy for the treatment of STEMI (n=4). Peripheral blood was obtained from healthy volunteers who provided informed written consent. All volunteers gave informed written consent and this study was approved by our local Research Ethical Committee. Endothelial outgrowth cells (EOCs) were isolated by the \textit{in vitro} expansion of PBMCs and neutrophils were isolated by dextran sedimentation of peripheral blood followed by buoyant density centrifugation over isotonic Percoll. Human coronary artery VSMCs were obtained from Lonza (UK.)

Pharmaceutical agent

AT7519 (N-[4-piperidinyl]-4-[2,6-dichlorobenzoyla-mino]-1H-pyrazole-3 carboxamide) (>98%) was synthesized by Astex Therapeutics, Astex, Cambridge, England, UK\textsuperscript{211} and PBS was used as the diluent. As previously described\textsuperscript{151}, cells were incubated with a range of concentrations (0, 0.001, 0.01, 0.1, 1, 10μmol/L) of AT7519 for 6, 24, or 48 hours in their respective medium at 37°C/5% CO\textsubscript{2}/95% relative humidity.

Apoptosis and cell cytotoxicity of arterial cells with AT7519

Morphology

Following incubation in the respective drug solutions, neutrophil apoptosis was assessed morphologically by cyto-centrifuging 150μl of 2 x 10\textsuperscript{6} cells per ml at 300rpm for 3 minutes (Shandon\textsuperscript{TM} Cytospin\textsuperscript{TM}, Thermo Fisher Scientific, UK). They were then fixed for 1 minute in 100% methanol, air-dried for 5 minutes and stained for one minute with eosin and haemotoxylin supplied in
the Diff-Quik™ kit (Cat no. CA53000-052; Dade Behring, Germany). Slides were then washed in tap water and air dried overnight before coverslips were applied. Cells were viewed under oil immersion light microscopy at x1000 magnification. 500 cells were counted over 5 fields of view to identify apoptotic cells with one or more darkly-stained pyknotic nuclei.

**Annexin V staining**

Apoptosis was assessed by flow cytometric measurement of externalised phosphatidylserine. Adherent cells were trypsinised from tissue culture plastics and aliquots of 5 x 10^4 cells were seeded into 1 well of a 24-well tissue culture plate overnight. The next day, cells were washed three times with PBS and medium was replenished. Adherent cells or freshly isolated polymorphonuclear leucocytes were incubated with AT7519 and growth factors for 6, 24 or 48 hours at 37°C/5% CO₂/95% relative humidity. After 6, 24 or 48 hours, adherent cells were trypsinised. Aliquots of 1 x 10^5 cells in 100μl medium were incubated with 200μl Annexin V-FITC for 10 minutes at 4°C (Cat. 11858777001; Roche, UK). The volume is each tube was then diluted 1/500 with binding buffer (GIBCO® Hank's Balanced Salt Solution with Ca²⁺ (Cat. H9394; Sigma Aldrich, UK). Samples were immediately analysed with a BD-Fortessa II flow cytometer. A minimum of 5,000 events in the relevant gate was collected for data analysis with FlowJo version 10.0.6 (TreeStar Inc., Switzerland) and measurements were made as percentages of total gated cells.

**Cell cytotoxicity**

Cell cytotoxicity was assessed by supernatant lactate dehydrogenase (LDH) release detected with a Cytotoxicity Detection Kit (Cat no. 11644793001; Roche, UK). Three sets of replicates were used for each condition: one high control, one test well and one culture medium control to serve as the blank, as previously reported.²¹ Three sets of replicates were used for each condition: one high control, one test well and one culture medium control to serve as the blank, as previously reported.²¹ Cells were trypsinised from tissue culture plastics and aliquots of 5 x 10^4 cells were resuspended in their respective medium and seeded into 1 well of a 24-well plate overnight. The next day, cells were washed three times with PBS and 400ul phenol red free-medium ([Cat no. CC-3129, Lonza, UK] supplemented with BulletKit™) was added to each well. AT7519 and growth factors were then added. After 48 hours, 2% Triton™ X-100 (Cat no. T8787; Sigma Aldrich, UK) was added to the high control wells for 10 minutes to disrupt cell membranes. The plates were then
centrifuged and 100μl of supernatant from the top of each well was transferred to a flat bottom 96-well plate. Cytotoxicity reagents were then prepared according to the manufacturer’s instructions and 100μl was added to wells in a flat bottom 96-well plate. The 96-well plate was then incubated at room temperature in the dark for 20 minutes prior to measurement with a reference wavelength of 490nM and an OPTIMA II plate reader (BMG Labtech, GmbH, Germany).

**In vitro functional assessments of endothelial cells with AT7519**

For proliferation, endothelial cells were incubated with AT7519 for 48 hours. Sets of digital images were taken at 0 hours and 48 hours and fold-increase was calculated as the number of cells at 48 hours divided by the number of cells at zero hours. For cell attachment, endothelial cells were incubated with AT7519 for 48 hours. Cell attachment was quantified by seeding these cells into a 6-well collagen I-coated plate for 30 minutes. Plates were washed and attachment within a defined region of each well was quantified and expressed as a percentage of seeded cell number. For the wound closure migration assay, confluent endothelial cells were starved in serum-free media with AT7519 for 24 hours. A wound was created by a linear vertical stroke across the diameter of the well with a pipette tip (time zero) and cells were incubated with AT7519 for a further 24 hours. The width of the wound was imaged at time zero and at 24 hours and the percentage of wound closure was calculated. For the tubule formation assay, endothelial cells were pre-incubated with AT7519 for 24 hours. They were then seeded into a 48-well plate pre-coated with Matrigel™ basement membrane matrix and incubated with AT7519 for a further 24 hours. Tubule structures were quantified at 24 hours.

**Cell stimulation with growth factors**

For apoptosis and cytotoxicity experiments requiring simulation, endothelial cells and VSMCs were incubated for one hour with growth factors prior to the addition of AT7519. For stimulation of endothelial cells, VEGF was omitted from the EGM-2 BulletKit™ and serum content was reduced to 5% (starvation media). PBS was used as the vehicle control.
Aliquots of $1 \times 10^4$ endothelial cells were seeded into 1 well of a 24-well plate overnight. Cells were washed three times with PBS, replenished in starvation media and incubated with a range of concentrations (0, 5, 10, 100ng) of recombinant human VEGF$_{165}$ (Cat no. 100-20; Peprotech, UK) for 48 hours. Sets of digital images were taken at 0 hours and 48 hours and the cell number was quantified by automatic counts using Image J Software (version 2.0.0) where a suitable concentration of VEGF$_{165}$ to stimulate cell proliferation was selected.

Aliquots of $5 \times 10^3$ VSMCs were seeded into 1 well of a 48-well plate overnight. Cells were washed three times with PBS, replenished in SmGM$^{TM}$-2, and incubated with a range of concentrations (0, 5, 10, 20, 100ng) of recombinant human platelet derived growth factor (PDGF)-BB (Cat. 100-14B; Peprotech, UK) for 48 hours. Sets of digital images were taken at 0 hours and 48 hours and the cell number was quantified by automatic counts using Image J Software (version 2.0.0) where a suitable concentration of PDGF-BB to stimulate cell proliferation was determined.
12.6 RESULTS

**AT7519 induces neutrophil apoptosis in vitro**

Morphological assessments of nuclei pyknosis indicated that AT7519 induced neutrophil apoptosis at concentrations of 0.1 μmol/L (11±1 cells), 1.0 μmol/L (18±9 cells) and 10 μmol/L (19±6 cells) compared to vehicle control (1±2 cells) after 6 hours of treatment (ANOVA<0.001) (**Figure 5.1 A**).

Flow cytometric detection of Annexin V indicated that AT7519 induced neutrophil apoptosis at concentrations of 0.1 μmol/L (76±3%) 1.0 μmol/L (81±5%) and 10 μmol/L (79±4%) compared to vehicle control (45±7%) after 24 hours of treatment (ANOVA<0.001) (**Figure 5.1 B**).
Figure 5.1 AT7519 induces neutrophil apoptosis in vitro.

(A) apoptosis was assessed by morphological detection of nuclei pyknosis (arrow heads) and (B) flow cytometric detection of phosphatidylserine expression identified by Annexin V binding after treatment with AT7519 for 6 (purple bars) or 24 hours (grey bars). Despite high baseline levels of apoptosis, typical of neutrophils in vitro, AT7519 induced apoptosis from concentrations of 0.1μmol/L after 6 and 24 hours. Error bars are standard deviation. One-way analysis of variance (ANOVA). P-values for Bonferroni post-test at 6 and 24 hours: *P<0.05; **P<0.01; ***P<0.001.
Functional assessments of cells treated with AT7519 \textit{in vitro}

\textit{Proliferation of endothelial cells and vascular smooth muscle cells with AT7519.}

AT7519 at a concentration of 0.1 μmol/L did not affect endothelial cell or VSMC proliferation with a similar fold-increase from baseline after 48 hours incubation for CEO cells (2.7±0.4 \textit{versus} 2.8±0.6), EOCs (2.8±0.3 \textit{versus} 1.9±0.4) and VSMCs (1.8±0.9 \textit{versus} 1.7±0.6). Higher concentrations of AT7519 (1 and 10μmol/L) inhibited endothelial cell and VSMC proliferation with reduced cell numbers compared to baseline (ANOVA<0.001) (\textbf{Figure 5.2}).

\textbf{Figure 5.2 Proliferation of endothelial cells and vascular smooth muscle cells with AT7519 \textit{in vitro}.}

Coronary endothelial outgrowth (CEO) cells, endothelial outgrowth cells (EOCs), and human coronary artery vascular smooth muscle cells (VSMCs) proliferated at similar levels to baseline when treated with 0.1μmol/L of AT7519 for 48 hours. Higher concentrations of AT7519 (1 and 10μmol/L) did not support endothelial cell or VSMC turnover with a reduced number of cells compared to baseline at 48 hours. AT7519 inhibited proliferation of endothelial cells and VSMCs at identical concentrations. Error bars are standard deviation. Two-way analysis of variance (ANOVA).
In vitro attachment, wound closure and tubule formation of endothelial cells with AT7519

At a concentration of 0.1 μmol/L AT7519, CEO cells retained functional capacity compared to baseline with a similar potential to attach to a collagen substrate (32±23% versus 57±5% attachment) (Figure 5.3 A), migrate for wound closure (94±8% versus 70±19% wound closure) (Figure 5.3 B), and form tubule structures on a Matrigel™ membrane matrix (44±6 versus 58±11 tubule structures) (P>0.05 for all) (Figure 5.3 C).

At a higher concentration of 1 μmol/L, there was a rapid decline in CEO cell and EOC attachment, wound closure and tubule formation (ANOVA<0.001 for all). AT7519 affected CEO cell and EOC function at identical concentrations (ANOVA=0.820).
Coronary endothelial outgrowth (CEO) cells and endothelial outgrowth cells (EOCs) retained their ability to (A) attach to a collagen substrate, (B) wound close following the infliction of a linear wound in the endothelial cell monolayer, and (C) form tubule structures on a Matrigel™ membrane matrix compared to the baseline after treatment with 0.1μmol/L AT7519. Endothelial cells lost functional capability following treatment with a higher concentration. AT7519 inhibited attachment, wound closure, and tubule formation of CEO cells at identical concentrations to EOCs. Error bars are standard deviation. Two-way analysis of variance (ANOVA). P-values for Bonferroni post-test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Apoptosis and cytotoxicity of endothelial cells and vascular smooth muscle cells with AT7519 in vitro

To determine if AT7519 would damage the integrity of the arterial wall while inducting neutrophil apoptosis, the apoptotic and cytotoxic effect of AT7519 on endothelial cells and VSMCs was assessed. To mimic pathological conditions in situ, endothelial cells were stimulated with VEGF165 and VSMCs were stimulated with PDGF-BB. The optimal concentration of growth factors to stimulate cells was determined by increased cell proliferation in the absence of AT7519 (Figure 5.4). VEGF165 at a concentration above 10ng stimulated endothelial cell proliferation (P<0.05 for CEO cells) and PDGF-BB at a concentration of 20ng stimulated VSMC proliferation (P<0.05 versus baseline). Thus, endothelial cells were stimulated with 10ng of VEGF165 and VSMCs were stimulated with 20ng of PDGF-BB to mimic pathological conditions in vivo.
Figure 5.4 Stimulation of endothelial cells and vascular smooth muscle cells in vitro.

(A) Coronary endothelial outgrowth (CEO) cells and (B) endothelial outgrowth cells (EOCs) were incubated with vascular endothelial growth factor (VEGF)165 while (C) human coronary artery vascular smooth muscle cells (VSMCs) were incubated with platelet-derived growth factor (PDGF)-BB to determine concentrations to stimulate cell proliferation. Proliferation of endothelial cells was stimulated with concentration of VEGF165 above 10ng while proliferation of VSMCs was stimulated with concentrations of PDGF-BB above 20ng. Error bars are standard deviation. Kruskal-Wallis analysis of variance. P-values for Dunn’s post-test: *P<0.05.
Apoptosis of endothelial cells and vascular smooth muscle cells with AT7519

The addition of VEGF165 did not impact the susceptibility of CEO cells to apoptosis (ANOVA=0.606) (Figure 5.5 A) but it desensitised EOCs to apoptosis (ANOVA=0.028) (Figure 5.5 B). The addition of PDGF-BB sensitised VSMCs to apoptosis (ANOVA=0.006) (Figure 5.5 C) with increased apoptosis following incubation with 10 µmol/L AT7519 (P<0.05 versus vehicle control).

In endothelial cells and VSMCs, with and without growth factors, 0.1 µmol/L of AT7519 did not induce apoptosis (<6% increase from baseline for all). There were low levels of apoptosis at 1 µmol/L of AT7519 but this did not reach statistical significance. At a concentration of 10 µmol/L, there was a significance increase of apoptosis in EOCs and VSMCs (ANOVA=0.002 and 0.057 respectively).

Cytotoxicity of endothelial cells and vascular smooth muscle cells with AT7519

The addition of growth factors did not impact the susceptibility of CEO cells, EOCs or VSMCs to cytotoxicity (Figure 5.6 A-C). In endothelial cells and VSMCs, in the presence or absence of growth factors, 0.1 µmol/L of AT7519 did not induce cell cytotoxicity. All cell types had low levels of cytotoxicity at 1 µmol/L and 10 µmol/L but this did not reach significance.
Figure 5.5 Apoptosis of endothelial cells and vascular smooth muscle cells with AT7519 in vitro.

(A) Coronary endothelial outgrowth (CEO) cell and (B) endothelial outgrowth (EOCs) were stimulated with vascular endothelial growth factor (VEGF)_{165} (C) Human coronary artery vascular smooth muscle cells (VSMCs) were stimulated with platelet-derived growth factor (PDGF)-BB. The addition of growth factors desensitised EOCs to apoptosis and sensitised VSMCs to apoptosis and in all cell types there were low levels of apoptosis following treatment with 1 and 10 μmol/L of AT7519.

Two-way analysis of variance (ANOVA). Error bars are standard deviation. P-values for Bonferroni post-test: *P<0.05; **P<0.01 are apoptosis for differing concentrations of AT7519; †P<0.05 is difference in apoptosis for vehicle and stimulated cells.
Figure 5.6 Cytotoxicity of endothelial cells and vascular smooth muscle cells with AT7519 \textit{in vitro}.

(A) Coronary endothelial outgrowth (CEO) cell and (B) endothelial outgrowth (EOCs) were stimulated with vascular endothelial growth factor (VEGF)$_{165}$ (C) Human coronary artery vascular smooth muscle cells (VSMCs) were stimulated with platelet-derived growth factor (PDGF)-BB. The addition of growth factors did not affect cell susceptibility to cytotoxicity and all cells had low levels of cytotoxicity with treatment of 1 and 10 μmol/L of AT7519. Error bars are standard deviation. Two-way analysis of variance (ANOVA).
Resolution of the acute neutrophil-mediated inflammatory response to stenting could improve the clinical outcome for patients following percutaneous coronary intervention (PCI). It has previously been reported that AT7519, a cyclin-dependent kinase (CDK) inhibitor, activates neutrophil apoptosis in vitro and promotes the resolution of neutrophilic inflammation in vivo\textsuperscript{151-153}. I add to this body of work, demonstrating that AT7519 selectively induces neutrophil apoptosis at concentrations that do not adversely affect endothelial cell function or viability at a defined concentration in vitro. These observations suggest that AT7519 could resolve the acute neutrophil-mediated inflammatory response to stenting without adversely affecting the function and integrity of the coronary endothelium.

Cell cycle progression is dependent on the formation of CDK/cyclin complexes at cell cycle checkpoints and failure of the formation of CDK/cyclin complexes can lead to cell cycle arrest or, in the case of neutrophils, apoptosis. Apoptosis leads to fragmentation into smaller bodies, ingestion by phagocytes without activation of the inflammatory system. To reliably detect the concentrations of AT7519 that induced neutrophil apoptosis, I assessed characteristic morphological changes (nuclei pyknosis) as well as characteristic cell surface changes (externalisation of phosphatidylserine) detectable with Annexin V staining by flow cytometry. I found that concentrations of AT7519 from 0.1, 1 and 10\(\mu\text{mol/L}\) induced neutrophil apoptosis after acute (6 hour) and chronic (24 hours) phase incubation. This was consistent with previous studies that have examined the concentrations of AT7519 that induce neutrophil apoptosis\textsuperscript{153} and the high levels of neutrophil apoptosis (60% pyknosis) observed in the absence of drug is typical for neutrophils ex vivo\textsuperscript{151, 214}.

The viability of endothelial cells is essential for re-endothelialisation following PCI. As such, I assessed the effect AT7519 would have on endothelial cells at concentrations that induce apoptosis of neutrophils. For this (and other assays), I only used active concentrations of AT7519 that induced neutrophil apoptosis. To model the coronary endothelium, I used coronary endothelial outgrowth (CEO) cells to represent local coronary endothelial cells and I used endothelial outgrowth cells (EOCs)
as the progeny of circulating endothelial progenitor cells, as both of these populations contribute to endothelialisation following PCI\textsuperscript{116}. I found that AT7519 at a concentration of 0.1μmol/L permitted CEO cell and EOC proliferation and I determined that this concentration had no adverse effects on endothelial cell function (attachment, wound closure and tubule formation) \textit{in vitro}. This indicated that 0.1μmol/L of AT7519 would not interrupt re-endothelialisation which is a critical to prevent stent thrombosis following PCI. All concentrations above this dose adversely affected endothelial cells with inhibition of cell function indicating that the optimal therapeutic window was narrow.

Determining the effective therapeutic range of AT7519 is important for vascular drug delivery as inhibition of endothelial cell function may lead to stent thrombosis in patients following PCI. Local delivery by a drug eluting stent is advantages over systematic delivery as it aderts the need for high systemic dosing to achieve adequate levels at the target site. It also leads to reduced systemic side effects and reduces the drug cost\textsuperscript{215}. However locally applied drugs must enter the tissue and diffuse through the target area down a concentration gradient, which can be influenced by the hydrophobic or hydrophilic nature of the drug. It has previously been shown that hydrophilic Rapamycin and hydrophobic Paclitaxel do not distribute uniformly through the media and adventitia, resulting in arterial drug concentration more than an order of magnitude above the applied levels\textsuperscript{69}. Given the narrow therapeutic window for hydrophilic AT7519 (0.1μmol/L), it may require systemic delivery as this could result in adequate levels at the target site leading to neutrophil apoptosis with no adverse effect on the function of the endothelium. Fortunately, the systemic pharmacokinetics of AT7519 are well described\textsuperscript{148} and minimal side effects have been reported in patients\textsuperscript{149}. Thus, systematic administration of AT7519 to patients may resolve the acute neutrophil-mediated inflammatory response to stenting with no adverse effect on the endothelium for the prevention of restenosis in following PCI.

VSMC proliferation is central to the pathogenesis of neointimal hyperplasia. Previous studies have employed CDK inhibitors (such as Flavopiridol\textsuperscript{216}) to prevent proliferation of VSMCs in neointimal hyperplasia as CDK inhibitors are negative regulators of the cell cycle. These studies demonstrated that CDK inhibitors were efficacious at inhibiting VSMC proliferation but the impact on endothelial
cells was not explored in detail. Unfortunately, these studies also demonstrated that Flavopiridol had an unfavourable toxicity profiles in patients with reports of diarrhoea, hypokalemia and hypotension which is typical of first generation CDK inhibitors. As AT7519 is a second generation CDK with a better toxicity profile, inhibition of VSMCs could add an extra dimension to AT7519 for the prevention of restenosis so long as endothelial cell function is not adversely impacted. Unfortunately, my study showed that VSMC proliferation was inhibited at identical concentrations to endothelial cells indicating that VSMC proliferation could only be suppressed at the cost of inhibiting endothelialisation. Concentrations that permitted endothelial cell proliferation (0.1μmol/L) also permitted VSMC proliferation, suggesting that AT7519 would not have any other benefits at this concentration other than inducing neutrophil apoptosis.

In my last experiment, I assessed the apoptotic and cytotoxic effect AT7519 would have on vessel wall cells while inducing neutrophil apoptosis, as cytotoxicity may lead to arterial necrosis with the release of cytotoxic contents and activation of the inflammatory system leading to an increased incidence of thrombosis. To mimic the injured vascular environment following PCI, I stimulated endothelial cells with vascular endothelial growth factor (VEGF) and I stimulated VSMCs with platelet-derived growth factor (PDGF)-BB, as these growth factors are abundant following vessel injury encouraging cell survival and cell proliferation. I demonstrated that there was no appreciable increase in apoptosis or cytotoxicity in endothelial cells or VSMCs up to relatively high concentrations of AT7519 while VSMCs had increased susceptibility to AT7519-induced apoptosis following stimulation with PFGF-BB. Also, while I did not measure the cytotoxic effect of AT7519 on neutrophils, previous studies have measured it by flow cytometric profiling of Annexin V+PI+ binding cells at 6 hours. These studies demonstrated limited cytotoxicity with no histotoxic release of contents into host tissues. Thus, the limited cytotoxic effect on endothelial cells and VSMCs was expected. The results from this study indicate that AT7519 is unlikely to induce cell death even in conditions of cell activation and abundant coronary artery disease.
12.8 STUDY LIMITATIONS

There are some limitations that merit consideration. As with all \textit{in vitro} studies, the conditions are artificial and therefore endothelial cell function and viability \textit{in vitro} may not adequately mimic these processes \textit{in vivo}. Also, this is the first investigation to target neutrophilic-mediated inflammation in the setting of angioplasty with stent implantation. Stent implantation is associated with chronic neutrophil influx with sustained recruitment of monocytes over days to weeks as the immune system mounts an attack on the newly implanted biomaterial. It is believed that chronic inflammation occurs due to a failure in the resolution of acute inflammation \cite{218} and it has been shown that preventing acute inflammation can reduce neointimal hyperplasia following balloon angioplasty \cite{210}, but there is limited information on the effect of resolving neutrophilic-mediated inflammation in coronary stent implantation. Thus, future studies are required to determine if inhibition of neutrophil-mediated inflammation will resolve the chronic inflammatory response to stenting and reduce neointimal hyperplasia. Lastly, the small sample size for experiments must be acknowledged, but the number of replicates is consistent with previous dose ranging studies using this compound \cite{151,153}.

12.9 CONCLUSIONS

Resolution of the acute neutrophil-mediated inflammatory response to stenting could improve the clinical outcome for patients following percutaneous coronary intervention. I have demonstrated that the cyclin-dependent kinase inhibitor AT7519, selectively induces neutrophil apoptosis at concentrations that do not have adverse effects on endothelial cell function or viability at a defined concentration \textit{in vitro}. These observations suggest that controlled elution or infusion of AT7519 could resolve the acute neutrophil-mediated inflammatory response to stenting without adversely affecting the function and integrity of the coronary endothelium.
CHAPTER 6

SUMMARY AND DISCUSSION
Percutaneous coronary intervention (PCI) can be life-saving in patients with acute myocardial infarction (MI), but adverse events related to vascular repair and remodelling continue to be problematic. Adverse events, such as neointimal hyperplasia and stent thrombosis, compromise vessel patency increasing both morbidity and mortality. These events arise due to the sequela of vascular trauma caused by the procedure. The high-pressure balloon inflations during coronary angioplasty and the forceful apposition of rigid stent struts denude the endothelium from the injured arterial segment initiating vascular smooth muscle cell (VSMC) proliferation and neointimal hyperplasia. Also, the implantation of a stent in the arterial wall further exacerbates endothelial cell injury and VSMC proliferation due to the persistent inflammatory response mediated against the implanted biomaterial. Drug eluting stents (DES) employ anti-proliferative drugs to prevent neointimal hyperplasia by inhibiting VSMC proliferation but these drugs also prevent endothelial cell proliferation. This prevents re-endothelialisation of the injured vessel segment as well as endothelialisation of the stent surface leading to the prolonged exposure of biomaterials for inflammatory cell attack. The lack of endothelium results in an environment poised for thrombus formation.

There is a substantial need for new therapeutic approaches that improve the clinical outcomes for patients following PCI. There is mounting evidence that promoting endothelialisation of injured vessel segments and promoting endothelialisation of the implanted biomaterial and can prevent adverse events by reducing neointimal hyperplasia and stent thrombosis. This occurs as the endothelium is anti-mitogenic to VSMCs and it possesses anticoagulant properties. There is also evidence that resolving the inflammatory response to stenting may prevent adverse events, as neointimal hyperplasia is an inflammatory driven process.

This thesis set out to assess new therapeutic approaches that promote repair of the vasculature following PCI. It sought to identify stent coatings that promote endothelialisation following balloon angioplasty and stenting as this could inhibit neointimal hyperplasia and reduce the risk of stent thrombosis. It also sought to uncover a novel compound to resolve inflammation without adversely
affecting the endothelium, as this too could prevent adverse events by reducing neointimal hyperplasia and the risk of restenosis.

In order to assess these therapeutic strategies, it was essential to re-create the endothelial cell environment for stent implantation. Therefore, the first result chapter (Chapter 3) was concerned with the development of a new in vitro model of the coronary endothelium, as this would better reflect the coronary environment for stent implantation in patients with coronary artery disease (CAD). Although more replicates of the experiments are required to confirm the findings presented in this chapter, the main observations were that coronary endothelial outgrowth (CEO) cells could be reliably isolated from atherothrombotic specimens following thrombectomy for acute myocardial infarction and that these cells had lower proliferative activity in vitro and did not incorporate into new host vessels in vivo compared to endothelial cells derived from other sources (endothelial outgrowth cells [EOCs] and human umbilical vein endothelial cells [HUVECs]). This indicated that coronary endothelial outgrowth (CEO) cells had impaired function and thus, may be a suitable model to study endothelial function in patients with myocardial infarction.

The requirement to isolate vascular bed specific endothelial cells has generated much controversy in vascular research. Gene profiling has demonstrated that endothelial cells perform different functions along the vascular tree depending on their location and the local tissue-specific requirements. Thus, subsets of endothelial cells vary slightly in their phenotype in situ. Despite this finding, vascular research often employs endothelial cells from other sources or distal locations. This is perhaps because endothelial cells show no great distinction of phenotype in culture. One study demonstrated differences in gene expression between endothelial cell lines revealed by global expression profiling. In this study endothelial cells from the coronary, pulmonary and iliac artery were compared to endothelial cells derived from the umbilical and saphenous vein. The up-regulation of Notch 4, CD44 Ephrin-B1 and C17 was reported for arterial cells, while the up-regulation of Ephrin Receptor B4 and Lefty-1 was reported for venous endothelial cells. In addition, the expression of Hey2 and C17
transcript was confirmed with real-time polymerase chain reaction and CD44 surface expression with flow cytometry. In support of these findings, it has been found that activation of the Notch signal pathway in the endothelium causes induction of Ephrin B2 expression and suppression of Ephrin receptor B4 expression leading to an arterial identity. But despite these findings, studies in our laboratory (unpublished) and studies from other laboratories have not been able to distinguish between venous and arterial endothelial cells in culture. This is reportedly due to the loss of ‘venous’ and ‘arterial’ related markers once cells are placed in cell culture conditions. Thus, while cell types are inherently different they display similar properties in culture due to the plasticity of endothelial cells and the uniformity of cell culture conditions where their physiological microenvironment is kept constant.

Chapter 3 demonstrated negligible differences in the in vitro phenotype or function of CEO cells isolated from atherothrombotic specimens compared to endothelial cells isolated from other sources, with the noted exception of the decreased growth potential for CEO cells compared with HUVECs. When CEO cells were implanted into mice for the assessment of function in vivo, they did not incorporate into new host vessels in vivo while the other cells did incorporate. This difference between CEO cells and other cells was only evident when cells were introduced into a biological environment. Indeed, in Chapter 4 no notable functional difference was observed between CEO cells and EOCs on polymer substrates in static culture conditions. But when cells were exposed to dynamic perfusion conditions using the IBIDI chapter, CEO cells showed a reduced potential to attach to a substrate while circulating in continuous steady state flow and they also showed a reduced potential to adhere to a polymer substrate when exposed to increasing levels of shear stress in vitro. With this in mind, future experiments comparing endothelial cells in vitro should attempt to mimic physiological conditions, rather than static monolayers of endothelial cell culture, to tease out functional differences between cell types.
In summary, Chapter 3 has provided a novel cell line for scientific investigations into endothelial cell function in coronary artery disease. Atherothrombotic specimens are a valuable reservoir of endothelial cells and they are routinely discarded following thrombectomy procedures. Given the simplicity of the method to isolate endothelial cells described in this thesis, it is anticipated that future groups will retain atherothrombotic specimens to isolate vascular bed specific endothelial cells for investigation into endothelial cell function.

Following the generation of an in vitro model in Chapter 3, this thesis aimed to uncover new therapeutic approaches to promote endothelialisation and improve the clinical outcomes for patients following PCI. Polymers have widespread application in cardiology, in particular during PCI as coatings for stent platforms and coating matrices for DES. It is well established that endothelialisation can reduce the degree of neointimal formation and it can reduce the impetuous for stent thrombosis following PCI. Given this, a polymer coating for stents that enables endothelialisation of its abluminal surface would be of great value in the clinical setting as it would suppress VSMC growth and it would generate a surface that is non-thrombotic and non-inflammatory. In Chapter 4, I identified synthetic polymer substrates to promote endothelialisation of coronary stents using EOCs and the in vitro model of the coronary endothelium established in Chapter 3.

For selection of a substrate in Chapter 4, high-throughput polymer microarrays and in vitro assays of endothelial cell attachment, expansion and retention were used to gauge endothelialisation in situ. Although more replicates of the in vitro experiments are required to confirm the findings presented in this chapter, the main observations were that PA309 supported endothelial cell attachment in dynamic flow conditions as well as endothelial cell expansion and retention when exposed to increasing levels of shear stress in vitro. The suitability of PA309 as a potential stent substrate was further supported by thrombogenic studies ex vivo where it inhibited thrombosis and had lower attachment of platelets and leucocytes equivalent to platelet/leucocyte attachment to conventional stents in clinical use. Given that the success and patency of a stent coating is related to its ability to integrate into its vascular
environment and prevent adverse events following implantation, I concluded that PA309 may be suitable for clinical use and it should be taken forward for future testing as a potential substrate to promote endothelialisation of coronary stents. Furthermore, the polymer (PA309) may have application in other fields as it is capable of long-term culture of endothelial cells. As an alternative to collagen and other synthetic extracellular matrices, chemically defined polymer substrates can be used for generation of good manufacturing practice (GMP) compliant clinical grade endothelial cells for cell therapy trials. Also, as this study demonstrated a phenotypic change in endothelial cells following culture on PA318, this substrate could be used to induce kinase domain receptor (KDR) expression on cells for vascular therapy. Ultimately, polymer PA309 may have clinical use in next generation intracoronary stents and if successful at encouraging endothelialisation in situ, PA309 may have future application in the field of tissue engineering for endothelialisation of vascular conduits in the peripheral circulation.

Chapter 4 detailed on a novel approach to test the suitability of synthetic substrates for stent coatings by parameters of endothelialisation in vitro and thrombogenicity ex vivo. Polymer microarrays are widely used for selection of biomaterials and they have been validated as an efficient and reproducible means to select substrates for biological application. However, to the best of my knowledge, the IBIDI chamber has never been used to assess endothelial cell attachment to stent coatings. Nor, to the best of my knowledge, has the Badimon chamber been used to assess leucocyte/platelet attachment to stent coatings or test the thrombogenicity of stent coatings. The IBIDI system is a pump system for the cultivation of cells under flow. It generates mechanical forces in vitro that are similar to those produced by physiological hemodynamic forces of blood vessels in situ. Most studies analysing the impact of a substrate on cell attachment use static conditions, or rarely, they use the parallel plate flow chamber, such as in studies by Busch et al., where material-dependent endothelialisation was demonstrated on poly(ethylene-co-vinylacetate) (PEVA) and poly(butyl methacrylate). The parallel plate flow chamber is similar to the IBIDI system in that endothelial cells are seeded onto the polymer surface and they are exposed to culture medium as it circulates through the chamber at an adjustable flow rate creating a defined laminar shear stress. However, the IBIDI chamber can be
manipulated to re-create various physiological conditions that endothelial cells on a stent might be exposed to (e.g. the turbulent flow of restenosis), while the parallel plate flow chamber cannot. This type of flow is of particular interest for studies of artificial devices where endothelial cell attachment as a strong continuous monolayer is essential. Similarly, the modification of the Badimon chamber to access the polymer substrate may also be of interest for future testing of stent biomaterials as this system mitigates the safety and logistical difficulties of using radiolabelled platelets. Thus, the techniques described in this thesis could enable future studies of biomaterials where endothelialisation and thrombogenicity are important criteria for selection.

In the final chapter of results (Chapter 5), I continued to explore new therapeutic approaches to promote endothelialisation and improve the clinical outcomes for patients following PCI. CDK inhibitors have gained attention as novel anti-inflammatory agents and their future application for the treatment of inflammation is a realistic prospect as their suitability for translation has been demonstrated as an anti-mitotic treatment for cancer. In Chapter 5, I evaluated the potential of AT7519 to resolve neutrophil-mediated inflammation and the effect this would have on the function and integrity of the endothelium. For this experiment, I used EOCs and I again employed the in vitro model of the coronary endothelium established in Chapter 3. In this study, I also evaluated the effect of AT7519 would have on vascular smooth muscle cell (VSMC) proliferation and viability. After identifying the concentrations of AT7519 that induced neutrophil apoptosis, I demonstrated that AT7519 impacted endothelial cells and VSMCs at identical concentrations, and determined that a concentration of 0.1 μmol/L could selectively induced neutrophil apoptosis with no adverse effect on endothelial cell function and viability or VSMC proliferation and viability. Thus, my findings indicated a therapeutic window for AT7519 to resolve neutrophil-mediated inflammatory and support endothelialisation, although AT7519 would not inhibit VSMC proliferation.

The compound for elution is one the main factor underlying the current deficiencies in DES. We have learned from DES that inhibiting endothelial cell proliferation leads to an injured endothelium and an
inflammatory environment poised for thrombus formation. But finding a drug that supports endothelialisation while suppressing VSMC proliferation is challenging. Non-steroidal anti-inflammatory drugs have been shown to inhibit VSMC proliferation and NF-κB inhibitors (such as Plumericin) have been shown to inhibit VSMC proliferation and therefore have been proposed as dual anti-inflammatory and anti-proliferative treatments to suppress neointimal hyperplasia following PCI. But these studies rarely assess the effect these compounds have on the endothelium. Often, studies employ an assay to show endothelial cell viability (such as the Resazurin conversion assay to demonstrate cell metabolism) but endothelial cell proliferation and function are almost never addressed. In the study where the CDK inhibitor Flavopiridol was assessed as a dual anti-inflammatory and anti-proliferative treatment for neointimal hyperplasia, the study demonstrated suppression of VSMC proliferation but only the cytotoxic effect of Flavopiridol on endothelial cells was assessed. Chapter 5 clearly demonstrated that the CDK inhibitor AT7519, could only suppress VSMC proliferation at the cost of inhibiting endothelial cell proliferation. Given this result, this thesis suggests assessing endothelial cell proliferation alongside assessing endothelial cell viability in future experiments where novel anti-stenotic compounds are being trialled. This is of the upmost importance for the next generation of compounds for DES as preservation or enhancement of endothelial cell function is essential for endothelialisation and the prevention of stent thrombosis.

The work performed in this thesis supports the use of PA309 to encourage endothelialisation following PCI and it supports the use of AT7519 to resolve neutrophilic inflammation without impairing endothelial cell function. If AT7519 can be infused at a concentration that reduces neutrophil-mediated macrophage infiltration and neointimal hyperplasia, then it may be possible to combine it with PA309 to promote endothelialisation. The elution of AT7519 from polymer PA309 would be possible as polyacrylates are amenable to functionalisation with drugs. However, given the narrow therapeutic window of AT7519 for endothelial cell function (0.1-1 μmol/L), a tight elution profile would be required. Further testing is required to assess the effect of drug attachment to PA309, as this can modify the surface structure of the polymer substrate which could in turn effect endothelial cell attachment, expansion and retention. If the release kinetics of PA309 cannot be tailored within the
defined therapeutic range, patients could receive oral administration of AT7519 following stent implantation to resolve neutrophilic inflammation. This would be feasible as oral administration of CDK inhibitors have previously been used to treat metastatic refractory solid tumors\textsuperscript{224}.

13.1 FUTURE DIRECTIONS

To summarise, the work performed in this thesis has established a novel model to study the coronary endothelium in patients with coronary heart disease. This thesis has identified PA309 as a potential coating for coronary stents to promote endothelialisation following coronary intervention. Furthermore, I have identified a potential new role for the novel CDK inhibitor AT7519 in preventing neointimal hyperplasia by inducing neutrophil apoptosis without impairing endothelialisation. Future studies are required to further characterise these cells and to develop these therapeutic strategies:

1. Additional assays of endothelial cell function (such as nitric oxide release \textit{in vitro} and angiogenesis \textit{in vivo} with the murine hindlimb ischaemia model) to delineate the mechanism of endothelial cell dysfunction in coronary endothelial outgrowth cells.

2. Genome microarray studies and mRNA studies to pinpoint novel therapeutic targets to restore endothelial function in coronary endothelial outgrowth cells.

3. Studies to determine whether PA309 promotes endothelialisation, limits inflammation and reduces neointimal hyperplasia in an experimental model of restenosis. This work would shed light on the long-term consequences of PA309 following implantation \textit{in vivo} and studies are planned using a mouse model of restenosis by Rodriguez-Menocal \textit{et al}, 2010 and Ali \textit{et al}, 2007\textsuperscript{227, 228}.

4. Systemic administration of AT7519 to resolve neutrophilic inflammation in a mouse model of restenosis\textsuperscript{227, 228} to determine the effects of this drug on neointimal hyperplasia and re-endothelialisation \textit{in vivo}. 

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CONCLUDING REMARKS

To conclude, coronary endothelial outgrowth cells can be reliably isolated and cultured from thrombectomy specimens and represent a novel and relevant model to study endothelial cell function. Polymer PA309 promotes coronary endothelial cell attachment and expansion in vitro with similar potential to collagen and inhibits thrombus formation ex vivo. Furthermore, the CDK inhibitor AT7519 selectively induces neutrophil apoptosis with no adverse effect on endothelial cells at a defined concentration in vitro.

This thesis has provided a novel cell line for investigations into endothelial cell function that may help identify novel therapeutic targets to enhance endothelial function for the prevention of acute myocardial infarction. It has provided a candidate synthetic coating for intravascular coronary stents to promote endothelialisation without increasing the risk of stent thrombosis following implantation, and it has determined that controlled elution or infusion of AT7519 could resolve the acute neutrophil-mediated inflammatory response to stenting without adversely affecting the function and integrity of the coronary endothelium. The techniques and strategies described in this thesis have major potential for future clinical application and they may help reduce the morbidly and mortality in patients undergoing angioplasty and stenting for coronary artery disease.
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