The Role of Endothelial Progenitor Cells in the Aetiology and Pathogenesis of Coronary Artery Disease

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by

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Author's declaration

I declare that the work has been done and the thesis composed by myself, and that the books, papers, and websites cited were all consulted by me personally.

Advice on statistical methods was gratefully received from Ms Suzanne Stevens, after which I performed all statistical analyses.

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Publications

1. **Whittaker A**, Moore JS, Vasa-Nicotera M, Stevens S, Samani NJ. Evidence for genetic regulation of endothelial progenitor cells and their role as biological markers of atherosclerotic susceptibility. *Eur Heart J.* 2008; **29**: 332-338.

2. Brouilette SW, **Whittaker A**, Stevens SE, van der Harst P, Goodall AH, Samani NJ. Telomere length is shorter in healthy offspring of subjects with coronary artery disease: support for the telomere hypothesis. *Heart*; 2008: 94: 422-425.

The role of endothelial progenitor cells in the aetiology and pathogenesis of coronary artery disease.

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Introduction: Endothelial progenitor cells (EPCs) are produced by the bone marrow and are capable of endothelial repair and neovascularisation. The number of circulating EPCs and their function have been shown to be impaired in subjects with cardiovascular risk factors or coronary artery disease (CAD).

Aims: The primary aims of the work undertaken in this thesis were to examine whether there is genetic regulation of EPC number and / or function and to determine if EPC dysfunction precedes the onset of CAD. In additional studies, the role that EPCs play in two relevant coronary pathologies - in-stent restenosis (ISR) and coronary collateralisation – was examined. Finally, an exploratory analysis was undertaken to determine if cellular senescence, assessed by telomere length, impacts on EPC function,

Methods: A total of 162 subjects were studied including 24 healthy parent-healthy offspring pairs and 27 CAD parent-healthy offspring pairs for the principle objectives. The relationships between EPCs and ISR and coronary collateralisation were studied in 21 and 39 subjects, respectively. In all subjects, the number of circulating $CD34^{+}VEGFR\text{-}2^{+}$ and $AC133^{+}VEGFR\text{-}2^{+}$ EPCs, the number of EPCs grown *in vitro*, and the migration capacity of cultured EPCs towards VEGF were determined. Leukocyte telomere length was determined using Southern blotting.

Results: There was significant correlation in the number of cultured EPCs between parents and their offspring (Healthy: $R=0.492$, $p=0.015$; CAD: $R=0.751$, $p<0.001$) Offspring of subjects with CAD had significantly higher numbers of circulating $CD34^{+}VEGFR\text{-}2^{+}$ and $AC133^{+}VEGFR\text{-}2^{+}$ cells than offspring of healthy subjects ($p=0.018$ and $p<0.001$, respectively). There was no striking relationship between EPC number or function and either ISR or degree of coronary collaterilsation. Telomeres were significantly shorter in offspring of subjects with CAD than offspring of healthy subjects (5.7 ± 0.2 kb vs 6.7 ± 0.7 kb, $p < 0.001$). There was no association between telomere length and EPC function.

Conclusions: EPC number is at least partly genetically regulated. Circulating EPC number may represent biological markers of occult vascular damage in offspring with hereditary risk of CAD.

Abbreviations

ACE	:angiotensin converting enzyme
ACh	:acetylcholine
ADP	:adenosine diphosphate
APC	:allophycocyanin
AT	:angiotensin
b-FGF	:basic-fibroblast growth factor
BFU-E	:blood forming unit, erythroid
BMI	:body mass index
BMS	:bare metal stent
BP	:blood pressure
CABG	:coronary artery bypass grafting
$CaCl_2$	:calcium chloride
CAD	:coronary artery disease
CFU-GM	:granulocyte/macrophage colony forming unit
CFU-Mk	:megakaryocyte colony-forming unit
cGMP	:cyclic guanosine monophosphate
COX	:cyclo-oxygenase
CTO	:chronic total occlusion
DES	:drug-eluting stent
DiL-ac-LDL	:1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL
DNA	:deoxyribonucleic acid
EDHF	:endothelium-derived hyperpolarizing factor
EDTA	:ethylenediamine tetra-acetic acid
ELISA	:enzyme-linked immunosorbent assay
eNOS	:endothelial nitric oxide synthase
EPO	:erythropoietin
ET-1	:endothelin-1
FACS	:fluorescent activated cell sorting
FITC	:fluorescein isothiocyanate
G-CSF	:granulocyte colony stimulating factor
GM-CSF	:granulocyte-macrophage colony stimulating factor
Gro-β	:growth regulated protein-β
HDL-c	:high-density lipoprotein cholesterol
HGF	:hepatocyte growth factor
HIF-1	:hypoxia-inducible factor-1
HMG-CoA	:3-hydroxy-3-methylglutaryl coenzyme A
HSC	:haematopoietic stem cell
ICAM-1	:intercellular adhesion molecule-1
Ig	:immunoglobulin
IGF	:insulin-like growth factor
IHD	:ischaemic heart disease
IL	:interleukin
ISR	:in-stent restenosis
KDR	:kinase domain receptor
LAD	:left anterior descending coronary artery
LCx	:left circumflex coronary artery
LDL-c	:low-density lipoprotein cholesterol
MAPC	:multipotent adult progenitor cell

MCP :monocyte chemotactic protein
$MgCl_2$:magnesium chloride
MIP-1α :macrophage inflammatory protein-1α
MMP :matrix metalloproteinase
MSC :mesenchymal stem cell
MT-MMP :membrane-type matrix metalloproteinases
NaCl :sodium chloride
NaOH :sodium hydroxide
NFκB :nuclear factor kappa B
NO :nitric oxide
NOS :nitric oxide synthase
NSAID :non-steroidal anti-inflammatory drug
NYHA :New York Heart Association
PBMC :peripheral blood mononuclear cells
PBS :phosphate buffered saline
PCI :percutaneous coronary intervention
PCR :polymerase chain reaction
PDGF :platelet derived growth factor
PE :phycoerythrin
PECAM-1 :platelet endothelial cell adhesion molecule-1
PGE_2 :prostaglandin E_2
PGH_2 :prostaglandin H_2
PGI_2 :prostacyclin
PI3k :phosphoinositol 3-kinase
PPARγ :peroxisome proliferator-activated receptor γ
PSGL-1 :P-selectin glycoprotein ligand-1
PTCA :percutaneous transluminal coronary angioplasty
Q-FISH :quantitative fluorescent in-situ hybridisation
RCA :right coronary artery
RNA :ribonucleic acid
SCF :stem cell factor (aka c-kit)
SDF-1α :stromal cell derived factor-1α
SMC :smooth muscle cell
SSC :sodium saturated citrate
SVF :stromal vascular fraction
TERT :telomerase reverse transcriptase
TERC :telomerase RNA component
TGF-α :transforming growth factor-α
TGF-β :transforming growth factor-β
TNF-α :tissue necrosis factor-α
TRF :telomere restriction fragment
TRF-2 :telomere repeat binding factor-2
TXA_2 :thromboxane
VCAM-1 :vascular cell adhesion molecule-1
VE-cadherin :vascular endothelium cadherin
VEGF :vascular endothelial growth factor
VEGFR-2 :vascular endothelial growth factor receptor-2
VSMC :vascular smooth muscle cell
vWF :von Willebrand's Factor

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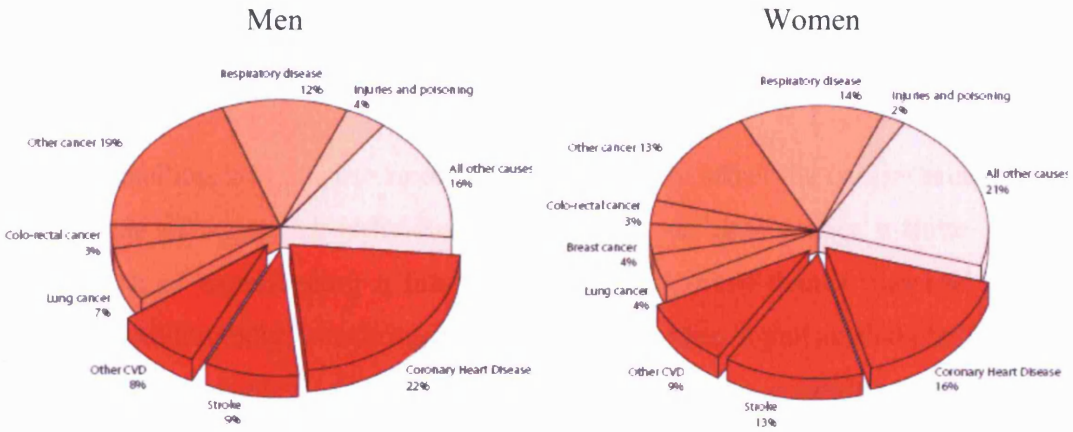
Chapter 1 Introduction

1.1 Ischaemic Heart Disease

1.1.1 Definition, Magnitude and Impact

Ischaemic heart disease (IHD) occurs as a result of atherosclerosis of the epicardial coronary arteries, which leads to an imbalance between coronary blood flow and myocardial requirement, and thus myocardial ischaemia. This can present clinically, as angina pectoris, myocardial infarction, heart failure, cardiac dysrhythmias and death. IHD remains the principal cause of morbidity and mortality in the Western world, despite continued advances in both its prevention and treatment. In the United Kingdom (UK) alone an estimated 2.6 million people (1.5 million men, 1.1 million women) have either had a myocardial infarction or have angina pectoris.[1] Data from the 2003 Health Survey for England & Wales suggests that the prevalence of IHD is 7.4% in men and 4.5% in women. Such prevalence rates increase with age, so that IHD affects around 1 in 4 men and 1 in 5 women over 75 years of age.[2]

Figure 1-1. Deaths by cause for men (left) and women (right) in the United Kingdom, 2003 (adapted from[1])



A total of 114,000 deaths were attributed to IHD in the UK in 2003 [Figure 1-1], and, despite falling mortality, in the 10 years from 1994-2003 the number of people in the UK adult population living with IHD has risen from 6% to 7.4% of men and 4.1% to 4.5% of women. Aside from the enormous health impact of the condition, IHD is estimated to cost the UK economy around £7,055 million per annum, higher than for any other disease.[1 2] Additionally, individuals with IHD are at considerable risk of developing atherosclerotic disease of the aorta and carotid, cerebral, renal and limb ateries, which may lead to aneurysm formation, stroke, renal failure and limb ischaemia.

1.1.2 Risk Factors for Ischaemic Heart Disease

The recognition that certain traits are associated with an increased risk of developing IHD began with prospective epidemiological studies in the USA and Europe in the late 1950's and early 1960's.[3-7] Consequently, factors have been identified that are strongly associated with an increased risk of developing IHD, which are traditionally considered as being either modifiable or non-modifiable. The main modifiable risk factors are hypertension, diabetes mellitus, dyslipidaemia, smoking, sedentary lifestyle, and obesity. Non-modifiable factors are male sex, age and genetic constitution. Despite increased public awareness and education, the increasing prevalence of some of these factors remains a major obstacle in reducing the burden of IHD.

A continuous relationship between increasing blood pressure (BP) and elevated cardiovascular disease risk is well established, and spans age, gender and racial categories.[8] Additionally, it has been reported that 'high-normal' BP levels (previously considered to be 'normal') are associated with poorer cardiovascular outcomes than lower than 'normal' BP.[9] An elevation in systolic BP of 20mmHg or diastolic BP of 10mmHg confers around a two-fold increase in IHD related mortality.[10] Numerous prospective studies have illustrated the beneficial effect of antihypertensive treatment on IHD risk, confirming the role of BP in IHD risk.[11]

Diabetes mellitus and glucose intolerance significantly affect the cardiovascular system in a multitude of ways. An individual with diabetes mellitus carries a three- to five-fold greater risk of experiencing a future cardiovascular event than a matched non-diabetic subject.[12] Additionally, clustering of risk factors is often found in diabetic patients, who frequently have dyslipidaemia, hypertension and obesity; a condition now referred to as metabolic syndrome.[13]

The incidence of IHD is strongly associated with increasing serum cholesterol levels in a continuous, graded manner, which is illustrated by a three-fold greater risk of IHD death in individuals in the highest quintile of the range compared with those in the lowest quintile.[14] The significance of hypercholesterolaemia has been borne out in the results of several prospective lipid-lowering trials, which have reported significant reductions in mortality and cardiovascular end-points.[15-18]

Tobacco smoking remains the most remediable of all the risk factors. The consequence of smoking is dysfunction of the vascular endothelium, leading to inflammatory cell recruitment and invasion, generation of reactive oxygen species and subsequent oxidation of low-density lipoproteins and, ultimately, accelerated formation of the atherosclerotic plaque. In addition, smoking exerts a detrimental effect on haemostatic factors, and leads to coronary vasoconstriction. The age-standardised mortality rate from IHD in men has been reported as 10.01 per 1000 men/year for current smokers compared to 6.19 per 1000 men/year for life-long non-smokers.[19]

Aside from a direct association with hypertension, dyslipidaemia and impaired glucose tolerance, obesity exerts an independent contribution to IHD risk and mortality.[20] Centripetal obesity and intrabdominal fat deposition are reported to confer the majority of the risk.

Physical exercise helps to maintain normal body weight, lower blood pressure and increase high-density lipoproteins and increase insulin sensitivity in peripheral tissues. Several studies have shown that the physical inactivity increases risk of cardiovascular disease similar to that seen in hypertension, dyslipidaemia and tobacco smoking.[21-23]

Compared to subjects in their fifties, the risk of dying from IHD increases three, ten and thirty fold for subjects in their sixties, seventies and eighties respectively. This effect is found regardless of blood pressure, which also increases with age.[10] Risk also varies with gender. Over a lifetime, men have approximately twice the risk of IHD related morbidity and mortality compared to women. However, this difference in risk narrows somewhat after the age of 45 years due to catch-up from women.[10 24] This gender-related effect is mainly seen in pre-menopausal women, with IHD risk increasing after the menopause, so that IHD incidence converges with that seen in men of matched age and risk factor profile. Oestrogen is purported to confer this protection and this may be related to the detrimental effects of androgens on high-density lipoprotein cholesterol (HDL-c) levels.[25]

Family history is now accepted as an independent risk factor for IHD.[26] In particular, first-degree relatives of an individual prematurely affected by IHD (< 55 years in men; < 65 years in women) posses a two to four-fold greater risk of developing IHD than those without such a history. The underlying cause of this genetic component in the aetiology of IHD remains the focus of intense research. Since the sequencing of the human genome,

and with rapidly improving technology, genome-wide scans are becoming more accessible and several genetic loci have been identified that may reveal some of answers in this field of research.[27-32]

Prospective studies of individuals from birth have revealed that low birth weight is associated with the development of diabetes mellitus and hypertension in later life. Additionally, such individuals have an increased risk of IHD above that expected from the presence of associated risk factors.[33]

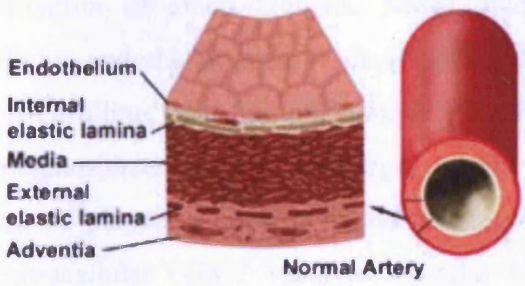
1.1.3 The pathogenesis of atherosclerosis

Derived from the Greek 'athero' (gruel) and 'sclerosis' (hardening), this condition was initially described as a degenerative process related to deposition of fats on the blood vessel luminal wall. Extensive research over recent years has revealed that in reality, atherosclerosis is a complex inflammatory process heavily influenced by cholesterol, which begins in childhood and generally leads to clinical manifestations in middle to late adulthood. This section describes the process by which atherosclerosis occurs starting with an introduction of a normal artery and its functions.

1.1.3.1 Normal artery anatomy

The structure of a normal, healthy artery is composed of the intima, media, and adventitia [Figure 1-2]. The luminal surface of the intima is covered by a monolayer of flat, specialised cells called endothelial cells, which lie lengthways in the direction of blood flow and are in regular contact with the circulating blood components. The endothelial cells are supported by a basement membrane composed of type IV collagen, laminin and fibronectin. In larger arteries, elastic fibres may amass to form a distinct internal elastic lamina. The media forms the elastic and muscular part of the artery. In this, elastic fibres are circumferentially distributed in layers around the artery, supported by extracellular matrix containing collagen fibrils, elastin, microfibrillar proteins and proteoglycans, and interspersed with vascular smooth muscle cells. This layer allows distension and recoil during changes in arterial pressure. The adventitia is the outermost layer of the muscular artery and contains nervous innervation, lymphatic drainage and blood supply via the vasa vasorum. It is comprised largely of collagen bundles, elastic fibres and fibroblasts, with scattered vascular smooth muscle cells.

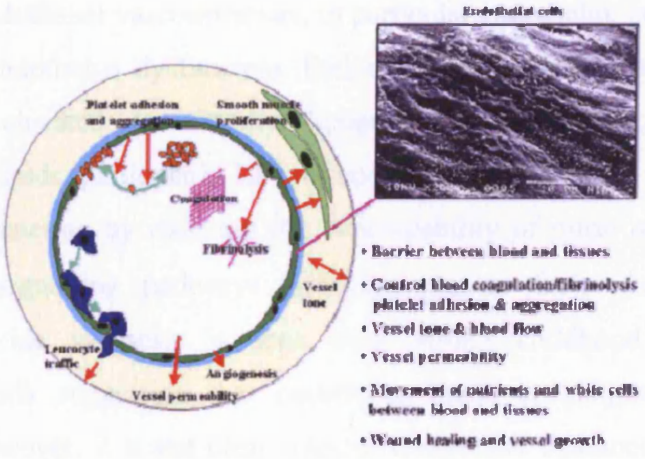
Figure 1-2. Normal artery anatomy (Adapted from www.cv-imagebank.com)



1.1.3.2 Vascular endothelial physiology

Endothelial cells form a continuous, overlapping, uninterrupted surface on the intimal aspect of all blood vessels. This represents the most extensive tissue in the adult body, covering approximately $7m^2$, playing a pivotal role in maintaining homeostasis between the artery wall and the circulating blood [Figure 1-3]. A low number of mature endothelial cells (1-3 per ml whole blood) are seen in circulating blood of healthy adult subjects,[34 35] which suggests that there is a low basal level of endothelial cell turnover.[36] Disruption to the endothelium, from such factors as oxidative stress, trauma, surgery or intravascular turbulence, increase endothelial cell turnover.[37]

Figure 1-3. Appearance and function of the vascular endothelial cell (Adapted from www.cv-imagebank.com)



When functioning normally, the vascular endothelium forms a selectively permeable membrane,[38] presents a non-thrombogenic surface,[39] and releases a variety of vasoactive factors including nitric oxide, prostacyclin and hyperpolarizing factor [Figure 1-3 & Table 1-1].[39] Normal endothelial function ensures the artery lies in a state of vasodilatation,

whereas denudation of the endothelium results in vasoconstriction and thrombosis. Nitric oxide (NO) is the main endothelial-signalling molecule, whose overall effects on the vessel inhibit the initiation of atherosclerosis. Nitric oxide is predominantly produced in endothelial cells via endothelial nitric oxide synthase (eNOS), which promotes oxidation of L-arginine to L-citrulline, releasing nitric oxide. This then exerts its effects via the cyclic guanosine monophosphate (cGMP) messenger system, leading to modification of arterial tone, inhibition of platelet aggregation and vascular smooth muscle cell proliferation. Increases in intracellular cGMP leads to vascular smooth muscle cell relaxation and vasodilatation. Nitric oxide, however, is also an active free radical and reacts with other, available free radicals and reactive oxygen species to generate toxic molecules such as peroxynitrite (ONOO-), which activate the endothelium causing dysfunction and initiation of the inflammatory response. This is discussed in more detail below. Endothelial nitric oxide synthase is regulated by a variety of signal pathways. Biomechanical forces on the endothelium and receptor-mediated signalling from agonists such as acetylcholine, bradykinin and oestrogen are the main mechanisms that trigger endothelial cells to initiate nitric oxide synthesis.

1.1.3.3 Endothelial dysfunction in atherosclerosis

One of the earliest demonstrable features of atherosclerosis is endothelial dysfunction, characterised by reduced bioactivity of nitric oxide (NO), which leads to activation of the endothelium and predisposition to inflammatory cell adhesion and invasion, and thrombosis. The endothelial vasconstrictors, in particular endothelin, counter the effects of NO and promote endothelial dysfunction. Endothelin levels are increased in response to oxidative stress, elevated low-density lipoprotein-cholesterol (LDL-c) levels and hypertension.[40 41] Lipids, particularly LDL-c, and oxidative stress are fundamental causes of endothelial dysfunction by reducing the bioavailability of nitric oxide and activating pro-inflammatory signalling pathways including nuclear factor kappa B (NFκB).[42] Reduced nitric oxide synthesis is seen even during childhood in subjects with hypercholesterolaemia suggesting that circulating lipids are important instigators of atherosclerosis. However, it is not clear whether endothelial dysfunction is the cause or effect of atherosclerosis, as fatty streaks are also present from a young age.[43]

Table 1-1. Properties of the vascular endothelium.

VASOMOTOR	**Vasodilators** Nitric Oxide (NO) Prostacyclin Endothelium-derived hyperpolarizing factor	**Vasoconstrictors** Endothelin Prostaglandin H2 Angiotensin II Platelet-derived growth factor Thromboxane A2 Endothelium-derived constricting factor
FIBRINOLTYIC	**Profibrinolytic** Tissue plasminogen activator Urokinase-type plasminogen activator	**Antifibrinoltyic** Plasminogen activator inhibitor-1
THROMBOSIS	**Anticoagulants** Thrombomodulin Heparan sulphate Dermantan sulphate **Platelet inhibitors** Nitric Oxide Prostacyclin Ecto-ADTPase	**Procoagulants** Tissue factor Fibronectin **Platelet activators** von Willebrand factor
CELL GROWTH	**Growth inhibition** Nitric Oxide Heparan sulphate Prostacyclin	**Growth promotion** Angiotensin II Platelet-derived growth factor Endothelin
INFLAMMATION	**Anti-inflammatory** Nitric Oxide	**Pro-inflammatory** E-selectin Intercellular adhesion molecule-1 Vascular cell adhesion molecule-1 Monocyte chemotactic protein Interleukin-8

Despite reduced bioactivity of nitric oxide, endothelial nitric oxide synthase levels seem to be preserved in endothelial dysfunction. Potential explanations for this include deficiency of factors necessary for endothelial nitric oxide synthase activation (such as L-arginine and tetrahydrobiopterin) or enhanced degradation of nitric oxide by reactive oxygen species. Endothelial dysfunction can be illustrated *in vivo* as abnormal nitric oxide-mediated vascular responses to pharmacological (acetylcholine) or haemodynamic (flow-mediated dilatation) stimuli. In addition to hypercholesterolaemia, endothelial dysfunction is seen in cigarette smoking, hypertension, heart failure and in macroscopically normal arteries of individuals with known atherosclerosis. High-density lipoprotein cholesterol (HDL-c), on

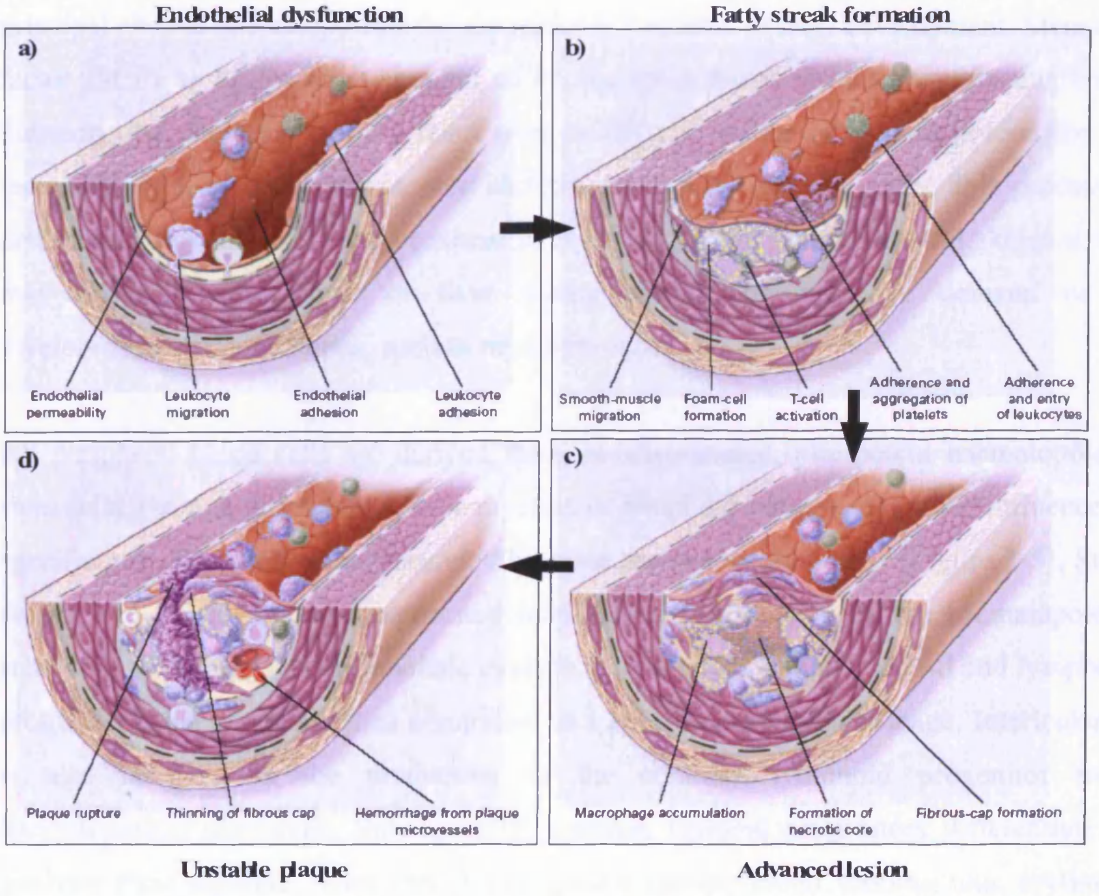
the other hand, prevents endothelial dysfunction and appears to be a protective factor for atherosclerotic disease.

1.1.3.4 The role of inflammation in atherosclerosis

In addition to nitric oxide-mediated dysfunction, when endothelial cells become activated they begin to express cell surface adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and P selectin, which leads to adherence of circulating inflammatory cells, including monocytes and T-lymphocytes. These cells then probe the endothelium for gaps and migrate into the sub-endothelium where they become activated. Once activated, the macrophages adopt a scavenger phenotype, with expression of modified LDL-c receptors, and synthesise a variety of pro-inflammatory cytokines and growth factors. The sub-endothelial macrophages ingest modified LDL-c particles and transform into foam cells. This process initially leads to the formation of the fatty streak [Figure 1-4]. These conditions cause the surrounding vascular smooth muscle cells to change from a contractile to a synthetic phenotype, enabling response to mitogens such as platelet derived growth factor (PDGF), and production of proteins capable of connective tissue matrix breakdown. This enables the vascular smooth muscle cells to migrate to the intima and proliferate, producing collagens and elastin in an attempt to 'repair' the damaged endothelium. However, this process leads to the generation of an atherosclerotic plaque, with a fibrous cap and a lipid rich, necrotic core. In response to these changes the artery undergoes compensatory dilatation to try to prevent the plaque from encroaching on the lumen and obstructing blood flow. As the plaque continues to grow, it gradually obliterates the lumen and impedes the normal flow of blood to the tissues. In the coronary vasculature, this leads to myocardial ischaemia, and clinical symptoms of chronic stable angina pectoris. A stable plaque can continue to develop, producing recurrent and progressive myocardial ischaemia, and eventually result in total occlusion of the lumen. In response to this, natural bridging vessels, termed coronary collaterals, develop through the myocardium in an attempt to restore perfusion to the ischaemic tissue.[44] This is discussed in greater detail in chapter 4. Not all plaques remain stable, and disruption of the surface of the plaque may encourage development of a thrombus and acute occlusion of the artery. Atherosclerotic plaques with thin caps and rich, necrotic lipid-laden cores are at higher risk of erosion and rupture than lesions with thick fibrous caps and smaller lipid pools. This is partly due to the extra protection from mechanical shear forces provided by a resilient cap. Pro-inflammatory cytokines and matrix metalloproteinases secreted by the migrated inflammatory cells and

activated vascular smooth muscle cells inhibit vascular smooth muscle cell proliferation and degrade collagen matrix, respectively.[45 46] Additionally, the recruited macrophages can directly induce vascular smooth muscle cell apoptosis through cell-cell communication.[47] In combination, these factors thin and weaken the fibrous cap, rendering it susceptible to erosion and rupture. Plaque rupture exposes the thrombogenic lipid-rich core to the circulating blood stimulating platelet aggregation, fibrin deposition and thrombus formation [Figure 1-4]. The thrombus generated fills the lumen and distal embolisation occurs.[45 46] If the intrinsic fibrinoytic system is not able to disperse the clot, complete vessel lumen occlusion results, with infarction of the surrounding tissue. In some circumstances significant extrusion of the lipid core occurs, itself causing sudden luminal occlusion. The clinical consequence of acute plaque rupture is severe tissue ischaemia and infarction, which in a coronary artery presents as an acute coronary syndrome or acute myocardial infarction.

Figure 1-4. The pathogenesis of atherosclerosis (Adapted from[48])



1.1.4 Management of stable ischaemic heart disease

The basis of the management of IHD involves lifestyle modification and pharmacological therapies. The benefits of the medications used in ischaemic heart disease are based on clinical trials designed to assess safety, efficacy and reduction in morbidity and mortality. This is an extensive subject, which will not be addressed in this thesis. However, it is important to mention that 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or 'statins' used as first-line therapy to improve dyslipidaemia, could have additional pleiotrophic effects that may influence telomere biology and endothelial progenitor cell function. In some patients percutaneous coronary intervention is performed to alleviate symptoms. This is discussed in greater detail in chapter 4. The main surgical treatment for CAD is coronary artery bypass grafting (CABG).

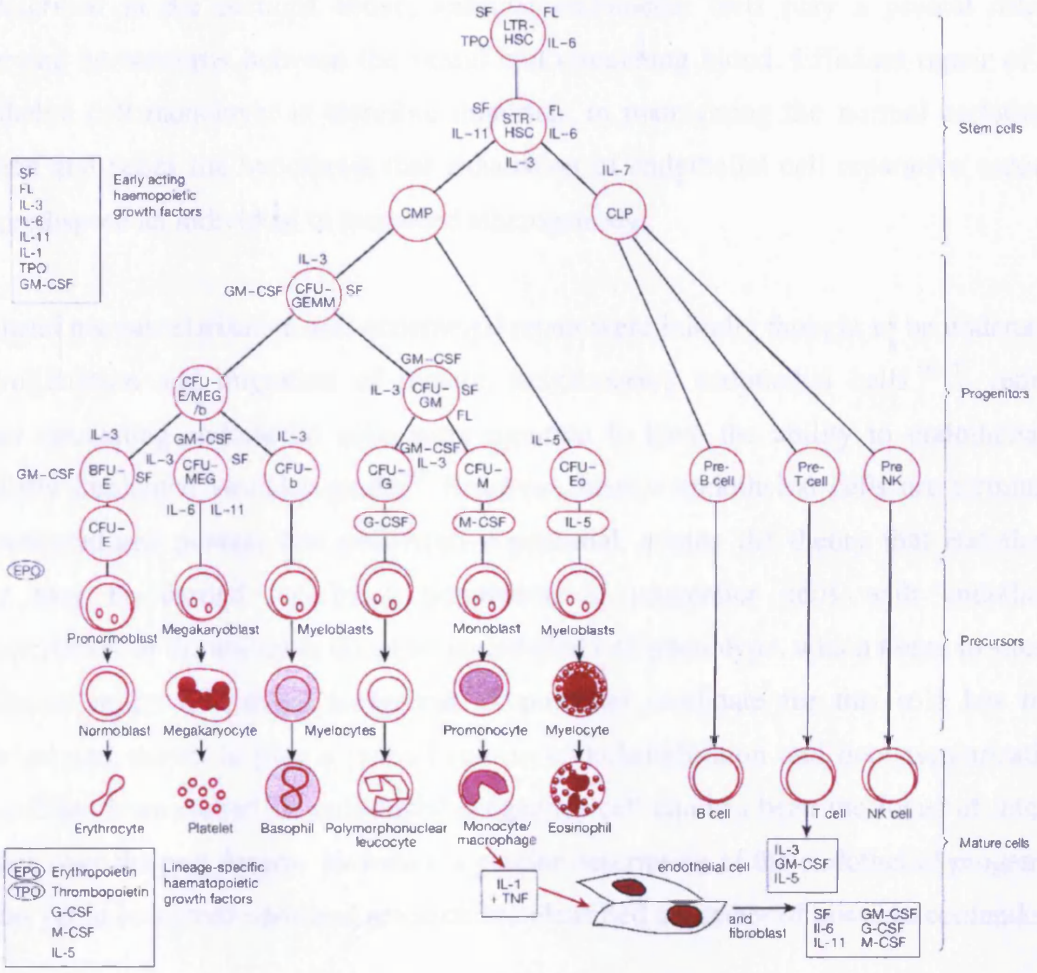
1.2 Haematopoiesis – blood cell formation

During human embryonic development primitive haematopoietic cells manufactured from blood islands within the yolk sac migrate to the liver and spleen, which become the principal sites of haematopoiesis for the initial 6-7 months of fetal development. Stem cell factor (SCF) or kit-ligand is thought to be the main factor influencing this migration. Subsequently, the bone marrow takes over as the sole source of haematopoiesis for the remainder of fetal development, into childhood and adult life. In adults, this process is predominantly confined to the proximal ends of long bones and the central skeleton, but may occur in the spleen or liver during periods of excessive demand or in myeloproliferative conditions, such as myelofibrosis.

All peripheral blood cells are derived from undifferentiated, pluripotent haematopoietic stem cells, through differentiation via myeloid or lymphoid lineages under the influence of specific growth factors mainly secreted by bone marrow stromal cells [Figure 1-5]. Steel factor, Flt3 ligand, thrombopoietin and interleukins-3, 6, and 11 act on haematopoietic stem cells early in the haematopoietic cascade to generate common myeloid and lymphoid progenitor cells, which are then committed to a specific phenotypic lineage. Interleukin-7 is also involved in the production of the common lymphoid progenitor from haematopoietic stem cells. Subsequently, common myeloid progenitors differentiate to generate three separate, more specific, progenitor groups; blood forming unit, erythroid (BFU-E), granulocyte/macrophage colony forming unit (CFU-GM), and megakaryocyte colony-forming unit (CFU-Mk). Mature, functional erythrocytes are generated from BFU-E under the influence of erythropoietin and platelet formation from the megakaryocyte is

driven by thrombopoietin. The leukocyte population is produced from both CFU-GM myeloid progenitors and the common lymphoid progenitor, induced by granulocyte-macrophage colony stimulating factor (GM-CSF) and lymphocyte derived growth factor, respectively. Mature monocytes and granulocytes are released from the bone marrow into the peripheral circulation.

Figure 1-5. Schematic representation of haematopoiesis (Adapted from[49])



A subset of the lymphocyte population, the T lymphocyte, undergoes further maturation in the thymus before taking up its role in immunity. Under normal circumstances few haematopoietic stem cells circulate in the peripheral blood, but numbers are increased during times of haematological or immunological stress, and can also be stimulated by injection of exogenous GM-CSF. Pluripotent haematopoietic stem cells are clonal and are capable of repopulating the entire bone marrow. It is this facility that is utilised following therapeutic bone marrow transplantation for haematological malignancy. Additionally, a

group of pluripotent stem cells have been identified that are capable of differentiation into other tissues, including endothelial cells, myocardium, skeletal muscle, liver, and neurones. These cells possess therapeutic potential for tissue regeneration and are the focus of concentrated research in several fields of medicine. These cells will be discussed in greater detail in subsequent sections.

1.3 Endothelial progenitor cells

As described in the sections above, vascular endothelial cells play a pivotal role in preserving homeostasis between the vessel and circulating blood. Efficient repair of the endothelial cell monolayer is therefore important in maintaining the normal endothelial function and raises the hypothesis that exhaustion of endothelial cell reparative capacity may predispose an individual to increased atherogenesis.

Post-natal neovascularisation and endothelial repair were initially thought to be undertaken by proliferation and migration of mature, neighbouring endothelial cells.[50] [51] Indeed, mature circulating endothelial cells were reported to have the ability to endothelialise surgically implanted vascular grafts.[52] However, mature endothelial cells are terminally differentiated and possess low proliferative potential, raising the theory that endothelial repair may be carried out by a population of progenitor cells with endothelial characteristics, or the ability to adopt an endothelial cell phenotype, which home to sites of endothelial injury and tissue ischaemia. A potential candidate for this role has been identified and shown to play a pivotal role in endothelialisation and neovascularisation. This cell has been named an endothelial progenitor cell and has been the focus of intense research over the past decade. However, a precise description of the endothelial progenitor cell has yet to be agreed upon and research has identified a number of possible contenders.

1.3.1 Definition, identification and source

1.3.1.1 Haematopoietic stem cell-derived endothelial progenitor cells

During embryogenesis, haematopoietic stem cells and endothelial cells develop contiguously suggestive of a shared derivation of these two cell lineages from the embryonic haemangioblast, which resides in the adult bone marrow.[53] In 1997, $CD34^+$ haematopoietic stem cells were isolated from peripheral blood and plated into culture dishes, which were either uncoated, or coated with human-fibronectin or collagen type I. The $CD34^+$ cells cultured on fibronectin became adherent and differentiated to an endothelial cell phenotype within 3 days, whereas only a minimal number of $CD34^+$ cells

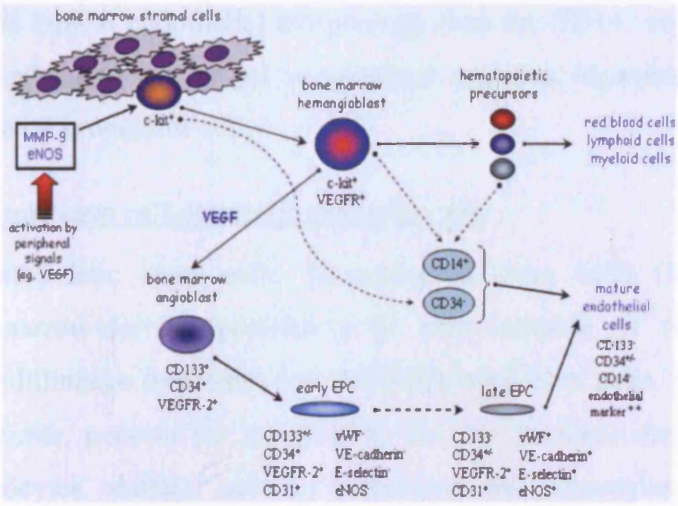
became adherent to the bare plates or collagen.[54] $CD34^-$ cells cultured in the same conditions failed to thrive. To confirm the endothelial cell phenotype, the $CD34^+$ cells were shown to co-express two other known markers of mature endothelial cells, namely endothelial nitric oxide synthase (eNOS) and vascular endothelial growth factor receptor 2 (VEGFR-2); also known as kinase domain receptor (KDR). In addition to surface marker expression, these cells displayed uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated LDL (DiL-ac-LDL) and binding of FITC-labeled *Ulex europaeus* agglutinin I (Lectin). Furthermore, these cells were shown to contribute significantly to *in vivo* angiogenesis in a rabbit model of hind-limb ischaemia.[54] Many subsequent *in vitro* and *in* vivo studies have confirmed the existence of circulating $CD34^+VEGFR\text{-}2^+$ precursor cells capable of angiogenesis and endothelialisation, supporting the early work of Asahara et al, and it was to this group of cells that the name endothelial progenitor cell was originally applied. It is now accepted that cells capable of both DiLacLDL uptake and Lectin binding *in vitro* represent endothelial cell phenotype. However, CD34 and VEGFR-2 are expressed on haematopoietic stem cells, endothelial progenitor cells, and mature endothelial cells and, therefore, may not represent a specific marker of progenitor cell type. A novel surface receptor, labelled AC133, which does not share homology with any other known haematopoietic cell surface antigen is co-expressed on circulating $CD34^+$ cells and appears to identify immature haematopoietic stem cells.[55 56] Both $CD34^+$ and $AC133^+$ cells have been shown to differentiate into endothelial cell phenotype in appropriate culture conditions *in vitro* and contribute to ischaemia-induced neovascularisation in animal models[54 55] AC133 is a marker of cellular immaturity, which is found on virtually all progenitor cells whilst in the bone marrow, is only found on a small number of peripheral blood mononuclear cells (0.02 – 0.1%),[57] and is absent on mature endothelial cells.[58] These studies of maturity-related surface marker expression have allowed the recognition of endothelial progenitor cells from mature endothelial cells and further distinction of early and late endothelial progenitor cells [Table 1-2 and Figure 1-6]. Early endothelial progenitor cells develop from adult peripheral blood mononuclear cells *in vitro* as quickly as four days, emerging as spindle-shaped cells, but by four weeks they gradually die out. Late endothelial progenitor cells are reported to appear between weeks 2-4 *in vitro* and expand rapidly to become a confluent monolayer of cells with cobblestone appearance capable of expansive serial population doubling. Late endothelial progenitor cells exhibit high expression of the endothelial surface markers VEGFR-2, eNOS, vWF, VE-cadherin, which is comparable to umbilical cord-blood derived cells and is higher than seen in early endothelial progenitor cells.[59]

Table 1-2 Identification of cell type by surface marker expression

	HSC	Early EPC	Late EPC	Mature EC
VEGFR-2	+	+	+	+
CD34	+	+	+/-	+/-
AC133	+	+	-	-
vWF	-	-	+	+
eNOS	-	+	+	+
VE-cadherin	-	-	+	+
CD31	+	+	+	+
E-selectin	-	-	+	+

VEGFR-2 - vascular endothelial growth factor receptor-2; vWF - von Willebrand Factor; eNOS - endothelial nitric oxide synthase; VE-cadherin - vascular endothelium cadherin; CD31 is also known as platelet endothelial cell adhesion molecule-1 (PECAM-1)

Figure 1-6. Schematic illustration of the source of endothelial progenitor cells (Adapted from[60])



See text for explanations of abbreviations

1.3.1.2 Haematopoietic stem cell-derived myeloid cells

Cells arising from the common myeloid progenitor that co-express surface markers of monocyte lineage (CD14) along with endothelial type markers (VEGFR-2) propose another source of endothelial progenitor cells. Such cells are capable of endothelial differentiation and formation of capillary-like structures *in vitro*, which integrate into neovascularisation in a murine model.[61] Romagnani et al illustrated that a population of circulating PBMCs which co-express CD14 and VEGFR-2, and also display low positivity

for CD34 ($CD34^{LOW}$), are multipotent and clonogenic as well as adopting endothelial cell phenotype in appropriate culture conditions.[57] Additionally, Zhao et al have illustrated that a subset of peripheral blood mononuclear cells expressing CD14, CD34 and CD45 acts as pluripotent stem cells in culture, differentiating into mature macrophages, endothelial cells, epithelial cells, T-lymphocytes, hepatocytes and neuronal cells when incubated in corresponding culture conditions and growth factors.[62] These cells are capable of improved neovascularisation in animal models despite showing a lower proliferation capacity than haematopoietic stem cell-dervied or cord blood-derived endothelial progenitor cells.[59 61] This may suggest that some of the angiogenic effect of monocyte-derived endothelial progenitor cells is through secretion of growth factors and/or recruitment of more active angiogenic cells. However, a separate study found that early endothelial progenitor cells and outgrowth endothelial cells develop from different starting sub-populations of peripheral blood mononuclear cells. Early endothelial progenitor cells were shown to differentiate primarily from $CD14^+$, whereas outgrowth endothelial cells arose solely from $CD14^-$ cells. Additionally, $CD14^-$ derived outgrowth endothelial cells exhibited a more pronounced endothelial phenotype with higher eNOS expression, greater proliferative capacity, and more typical endothelial morphology than the $CD14^+$ endothelial progenitor cells.[63] This has added to the as yet unanswered question regarding the identity of a functional endothelial progenitor cell.

1.3.1.3 Mesenchymal stem cell-derived endothelial cells

Alongside haematopoietic stem cells, mesenchymal stem cells (MSCs) represent a separate, bone marrow-derived population of cells capable of self-renewal, clonal expansion and multilineage differentiation. Mesenchymal stem cells, when isolated from bone marrow aspirate, possess the potential to develop multiple mesenchymal lineages including chondrocytes, skeletal muscle, adipocytes and osteocytes when cultured in appropriate conditions.[64] Additionally, mesenchymal stem cells are capable of *in vivo* differentiation into functional endothelial cells,[64] and release a combination of neoangiogenic growth factors.[65] Mesenchymal stem cells however, were traditionally thought to be fixed within the bone marrow structure and permanently resident therein. However, in 2001 mesenchymal stem cells were isolated from peripheral blood, so raising the possibility that this group of cells may represent an endothelial progenitor cell population.[66] In 2002, Reyes et al identified an additional bone marrow stem cell that co-purifies with mesenchymal stem cells, which was capable of producing endothelial cells *in vitro*.[67] This cell was noted to be distinct from the haematopoietic stem cell and was named

a multipotent adult progenitor cell (MAPC). Multipotent adult progenitor cells can not be identified by unique surface receptors, and in fact are CD34 and VE-cadherin negative, but do express low levels of AC133 and VEGFR-2 identifying them as potential endothelial progenitors. Indeed, they are able to differentiate *in vitro* into cells of mature endothelial cell phenotype that are capable of integrating into neovascularisation of wound healing and tumour development.[67]

1.3.1.4 Umbilical cord blood-derived endothelial progenitor cells

$AC133^+$ and $CD34^+$ cells are found in higher numbers in umbilical cord blood than adult peripheral blood and are capable of differentiation into mature endothelial cells.[68] [69] Umbilical cord blood-derived endothelial progenitor cells have been shown to differ from their adult blood-derived counterparts by their larger size, increased capacity to form more and bigger colonies, expression of higher levels of telomerase, and capacity to serially expand single-cells into secondary and tertiary colonies.[68] Both the cord and adult blood-derived endothelial progenitor cells expressed typical endothelial surface markers (including CD31, vWF, VEGFR-2) but neither population expressed CD45 (leukocyte common antigen; a haematopoietic stem cell marker) or CD14.

1.3.1.5 Adipose tissue-derived endothelial progenitor cells

Expansion of adipose tissue is undeniably possible throughout life and occurs as result of existing adipocyte hypertrophy and differentiation of multipotent progenitor cells located in the stromal vascular fraction (SVF). A rich blood supply is vital for adipose proliferation, and is utilised by mature adipose tissue to exert its endocrine effects. Cells of adipose lineage are capable of promoting angiogenesis through secretion of pro-angiogenic factors including vascular endothelial growth factor and leptin.[70] [71] Mesodermal stem cells in the stromal vascular fraction are mulitpotent, capable of producing chondrocytes, osteocytes, myocytes and neuronal cells.[72] Recent research has shown that these cells are also capable of differentiating into endothelial cell phenotype *in vitro* and augmenting neovascularisation in mice *in vivo*.[73] The cells successfully cultured from the stromal vascular fraction had high expression of CD34 and CD13 but did not express CD45 or CD14 suggesting that they are not from either haematopoietic stem cell or monocyte lineage. In a separate study, $CD34^+CD31^-$ cells isolated from the stromal vascular fraction of human adipose tissue differentiated *in vitro* into endothelial cells capable of vascular restoration when implanted into a murine ischaemic hind limb model.[74]

1.3.1.6 Summary

An authoritative consensus regarding the true identification and source of functional endothelial progenitor cells has yet to be determined. However, a current working guideline for the identification of an endothelial progenitor cell has been suggested as either:

(1) a cell that co-expresses haematopoietic stem cell (CD34 or CD133) and endothelial cell (VEGFR-2) surface antigens, or

(2) a non-endothelial cell that has stemness characteristics, clonal expansion capacity, and can give rise to an endothelial cell.[75]

1.3.2 Mobilisation and homing of endothelial progenitor cells

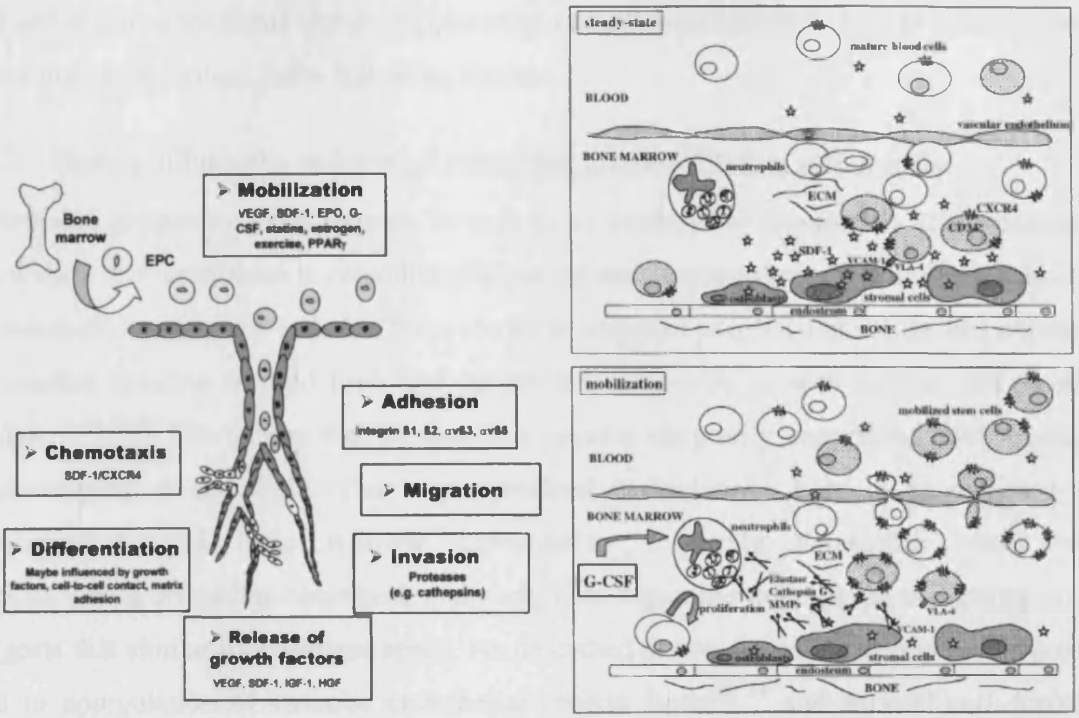
There is strong evidence to show that the endothelial repair process is substantially contributed to by endothelial progenitor cells, which are mobilised from the bone marrow and differentiate into endothelial cells at the site of injury.[76-78] Endothelial progenitor cells are attracted to sites of post-natal neovascularisation,[58 61 79] ischaemia,[79 80] and endothelial denudation.[76] Furthermore, endothelial progenitor cells integrate into developing collateral vessels in both animal and human models of ischaemia, augmenting neo-vascularisation.[81] In addition to the pathological circumstances described above, several physiological conditions affect circulating EPC numbers and migratory capacity. This section will concentrate on these factors and the elements involved in the mobilisation and homing of endothelial progenitor cells.

1.3.2.1 Molecular mechanisms of progenitor cell mobilisation

A complex relationship between resident haematopoietic stem cells, bone marrow stromal cells and the bone marrow extracellular matrix governs the differentiation and mobilisation of haematopoietic stem cells. During steady basal conditions both immature progenitor cells, and mature differentiated cells are released from the bone marrow into the peripheral circulation, regulated by interplay between the resident bone marrow cell niches, cytokines, chemokines, proteases and cell adhesion molecules. Stem cell mobilisation has been shown to be induced through the actions of several cytokines (G-CSF, GM-CSF, IL-7, IL-3, IL-12, stem cell factor), chemokines (IL-8, MIP-1α, Groβ, SDF-1α) and the use of some chemotherapeutic agents (cyclophosphamide, paclitaxel).[82] The primary step in the mobilisation of haematopoietic stem / progenitor cells is said to be the transendothelial migration of c-Kit$^+$ haematopoietic stem cells from the bone marrow stromal niche to the vascular zone, allowing access to the peripheral circulation. Cell-cell adhesive bonds

between the stromal and haematopoietic stem cells, formed by integrins, are disrupted by a selection of proteinases including cathepsin G, elastase and matrix metalloproteinase-9 (MMP-9) [Figure 1-7]. MMP-9 has been reported to play a pivotal role in the initial haematopoietic stem cell movement by promoting the transformation of membrane bound Kit-ligand to soluble Kit-ligand (also known as stem cell factor), which activates cKit+ cells.[83] A number of cytokines and chemokines exert their effect on the progenitor cells in an MMP-9 dependent manner, such as VEGF, G-CSF, IL-8, Groβ and SDF-1α.[53 82 84] Such mechanisms require sufficient numbers of functionally normal neutrophils to exert their effect. Furthermore, $CD34^+$ progenitor cells have been shown to secrete matrix metalloproteinases-9 and -2. Vascular endothelial growth factor and stromal cell derived factor-1α are upregulated during ischaemic conditions[84] and have been shown to play a role in the maturation and proliferation of haematopoietic stem cell-derived endothelial progenitor cells, through binding at the vascular endothelial growth factor receptor-2.[83] Gene transfection experiments producing upregulation of vascular endothelial growth factor-165 resulted in enhancement of endothelial progenitor cell mobilisation in humans.[85] Stromal cell derived factor-1α, interleukin-8, monocyte chemotactic protein-1, macrophage inflammatory protein-1α and -1β, and Groβ provoke rapid mobilisation of haematopoietic stem cells into the peripheral circulation 30 mins to a few hours after administration. Stromal cell derived factor-1α (SDF-1α) is the most potent chemoattractant for haematopoietic stem and progenitor cells and its receptor, CXCR4 is co-expressed on $CD34^+$ progenitor cells.[86 87] The activity of SDF-1α and its receptor, CXCR4, appears to be essential in normal haematopoiesis[88 89] and is pivotal in the cell mobilisation process.[82] Inhibition of CXCR4 and / or down-regulation of SDF-1α within the bone marrow, generating a bone marrow - peripheral blood gradient, appears to be vital for the granulocyte colony stimulating factor-induced mobilisation of haematopoietic stem / progenitor cells from the marrow niche.[90] Granulocyte colony stimulating factor has been utilised as a method of mobilising $CD34^+$ haematopoietic stem cells from the bone marrow into the peripheral circulation for many years for the purpose of bone marrow transplantation in the treatment of haematological malignancies.

Figure 1-7. Diagramatic representations illustrating the processes involved in G-CSF mediated progenitor cell mobilisation and homing from the bone marrow (Adapted from[53 82])



MMP-9 – matrix metalloproteinase-9; VEGF - vascular endothelial growth factor; VEGFR – vascular endothelial growth factor receptor; vWF – von Willebrand's Factor; VE-cadherin – vascular endothelium cadherin; eNOS - endothelial nitric oxide synthase; SDF-1α - Stromal cell derived factor-1α; CXCR4 – C-X-C receptor 4; IGF-1 – insulin-like growth factor-1; HGF – hepatocyte growth factor ; EPO – erythropoietin; PPARγ - peroxisome proliferator-activated receptor-γ ; G-CSF- granulocyte colony stimulating factor; VCAM-1 – vascular cell adhesion molecule-1

Granulocyte colony stimulating factor produces a much slower mobilisation than other cytokines, producing a peak at around 5 – 6 days after exogenous administration. Mature neutrophils activated by granulocyte colony stimulating factor release proteinases, including matrix metalloproteinase-9, cathepsin G, neutrophil elastase, and proteinase 3 from cytoplasmic granules. These substances disrupt cell-cell and cell-matrix adhesion through inactivation or cleavage of the leukocyte adhesive molecule including VCAM-1, c-kit, CXCR4 and SDF-1α, allowing mobilisation of haematopoietic stem cells. GM-CSF has been shown to heighten circulating endothelial progenitor cell numbers.[79] Interleukin-8, a chemokine with potent neutrophil chemo-attractant and activating activity, rapidly induces haematopoietic stem cell mobilisation, and involves activation of matrix metalloproteinase-9 and the $\alpha L\beta_2$-integrin.[82] The amplitude of endothelial progenitor cell mobilisation in response to interleukin-8 is, however, smaller and of shorter duration than granulocyte colony stimulating factor.[91] A close relationship between these two factors is

likely, given that the number of circulating $CD34^+$ cells following granulocyte colony stimulating factor administration positively correlates with the serum level of interleukin-8.[92] Once released into the circulation, the progenitor cells must localise to areas where they are required for tissue repair, regeneration or neovascularisation. This process, known as homing, is described in the following section.

1.3.2.2 Factors influencing endothelial progenitor cell mobilisation and homing

Endothelial progenitor cells migrate to regions of endothelial denudation and ischaemia whereupon they contribute to re-endothelialisation and neovascularisation. Indeed, infusion of endothelial progenitor cells has been shown to augment neovascularisation and improve myocardial function in hind limb and myocardial ischaemia in both murine and human models.[54 82 93-96] The factors that regulate this process are poorly understood, and research is developing in this field. The steps involved in leukocyte homing in response to inflammation, and bone marrow repopulation following therapeutic stem cell transplantation are better described, and early data regarding endothelial progenitor cells suggests that similar mechanisms apply. As described above, tissue ischaemia and hypoxia lead to upregulation of vascular endothelial growth factor[80 84] and stromal cell derived factor-1α, which mobilises EPCs. Additonally, both VEGF and SDF-1α are potent chemoattractants centrally involved in EPC homing. Genetic manipulation or exogenous administration of VEGF[85] or SDF-1α[97 98] increases EPC homing and integration into neovascularisation. The regulatory mechanisms determining tissue expression of such chemoattractants is not well described, however inflammatory factors such as tumour necrosis factor-α (TNF-α), some interleukins, and hypoxia-inducible factor-1 (HIF-1) all have the capacity to manipulate cell movement and may promote the increased production of such factors. TNF-α is a potent pro-inflammatory cytokine with increased expression in ischaemic and infarcted tissue. It is produced by a number of cells including monocytes/macrophages, degranulating mast cells, cardiac fibroblasts and cardiomyocytes, and is a chemoattractant to stem cells *in vitro*.[91] However, in patients with heart failure, elevated serum levels of TNF-α inversely correlate with circulating $CD34^+$ bone marrow-derived progenitor cells.[99] HIF-1 is fundamental in the regulation of cellular response to hypoxia, and regulates over 60 genes that promote cell survival during unfavourable conditions. Recently HIF-1 has specifically been shown to strongly control the production of SDF-1α and expression of its receptor CXCR4 in hypoxic tissue.[100] Additionally, HIF-1-induced SDF-1α production results in increased homing, adhesion and migration of $CXCR4^+$ EPCs in mice.[100] Endothelial platelet adhesion also promotes EPC mobilisation

and homing, which is mediated, in some part, by SDF-1α released from the activated platelets.[101] In addition, EPCs have the capacity to perpetuate further cell recruitment in a paracrine manner through secretion of VEGF and IL-8.[59 102] Significant amplification in the production of interleukin-8 / Gro-α mRNA is found in mouse cardiac endothelial cells following acute ischaemia, coupled with elevated serum levels. This is associated with chemotactic recruitment of $CD34^+CD117^{BRIGHT}$ bone marrow-derived progenitor cells, which co-express CXCR1, CXCR2, and CXCR4, to sites of myocardial ischaemia with augmentation of vasculogenesis and apoptotic resistance in cardiomyocytes.[103] MCP-1 is involved in monocyte recruitment to sites of inflammation and is found in high levels in atherosclerosis. It has been shown to promote progenitor cell migration *in vitro* and endothelial cell migration, re-endothelialisation, and integration into angiogenesis in a mouse model.[77] It may play a role in homing of EPCs, particularly $CD14^+$ cells, to sites of endothelial injury and angiogenesis. During physiological conditions, erythropoietin is predominantly released from the kidneys in response to hypoxia, thus encouraging increased erythrogenesis in the bone marrow. Additionally, activated macrophages have been shown to express erythropoietin mRNA, erythropoietin receptors are found on endothelial cells, and functionally active circulating EPC numbers are augmented following exposure to erythropoietin.[91] Taken together this suggests a role of erythropoietin in cell trafficking. Surface expression of integrins is important in cell adhesion and will be discussed in greater detail in the following section. Recent research has identified a potential role of $\alpha_4\beta_1$-integrin (very late antigen-4 [VLA-4]) in homing of circulating endothelial progenitor cells. In a study of tumour angiogenesis in mice, $CD34^+$ progenitor cells expressing $\alpha_4\beta_1$-integrin localised specifically to sites of active angiogenesis, which were shown to express VCAM-1 and cellular fibronectin, the ligands for $\alpha_4\beta_1$-integrin.[104] Furthermore, specific blockade of $\alpha_4\beta_1$-integrin prevented the endothelial adhesion, angiogenic homing, and differentiation of the $CD34^+$ cells *in vitro* and *in vivo*.[104]

Information regarding the number of circulating EPCs and *in vitro* ability to form EPCs from PBMCs in healthy adult humans is limited. Early EPCs, identified as $AC133^+$ $CD34^+VEGFR\text{-}2^+$ cells, have been reported to form approximately 0.002% of the circulating PBMC pool.[56] In a separate study, the number of circulating $CD34^+$ cells, as measured by anti-CD34 antibody-coated magnetic microbeads, was reported to be 645 cells per millilitre of blood.[105] A third study reported the number of circulating $CD34^+CD45^+$, $AC133^+$, and $CD34^+VEGFR\text{-}2^+$ cells in healthy control subjects to be 0.056

± 0.007%, 0.087 ± 0.01%, and 0.032 ± 0.005% of peripheral lymphocytes, respectively.[106] This same group found the number of PBMCs that differentiate to endothelial cell phenotype *in vitro* to be 57.2 ± 6.5 cells per high power field in the same healthy subjects (Vasa et al, unpublished data). Increasing age inversely correlates with endothelial function and circulating EPC number.[107] Aerobic exercise, both in healthy adults and subjects with IHD, increases the number of circulating EPCs.[108-114] Furthermore, both oestrogen[115] and exposure to high altitudes[116] have been associated with increases in circulating EPC number in humans.

The number of, and migratory capacity of, circulating EPCs is reduced by approximately 40% and 48%, respectively, in patients who have traditional risk factors for cardiovascular disease.[106 117] A family history of CAD and smoking are each associated with a significant reduction in circulating $CD34^+VEGFR\text{-}2^+$ cells, whereas hypertension correlates with impairment in EPC migration.[106] Increasing age and hypercholesterolaemia inversely correlate with the number of circulating $CD34^+VEGFR\text{-}2^+$ cells.[106] EPCs from individuals with either type I or type II diabetes mellitus display dysfunctional features including impaired adhesion, reduced proliferative capacity, impaired homing, and diminished angiogenic integration.[118 119] Hyperhomocysteinaemia is thought to promote CAD through endothelial injury and dysfunction. Recent work supporting this hypothesis shows a detrimental relationship between homocysteine and EPCs, characterised by increased cell senescence and reduction in cell numbers and function.[120 121] Studies of treatment naïve, asymptomatic individuals with evidence of preclinical atherosclerosis of large arteries, has illustrated an inverse correlation between greater extent of atherosclerosis and fewer numbers of circulating $CD34^+VEGFR\text{-}2^+$ cells.[122] Furthermore, the number of circulating EPCs is reduced by up to 60% in patients with angiographic CAD[123 124] suggesting an impairment in the endothelial repair mechanism in such patients. However, from all the data available thus far, it is unclear whether the reduction in EPCs precedes and promotes the development of CAD, or is a consequence of it. Myocardial infarction[80] and vascular injury[125] generate an acute elevation in the number of circulating $CD34^+AC133^+VEGFR^+$ cells, whilst heart failure shows a dichotomous effect on EPCs. Subjects with symptoms within New York Heart Association* (NYHA) class I or II (i.e. mild symptoms) exhibit elevated numbers of circulating $CD34^+AC133^+VEGFR^+$ cells and increased colony-

* New York Heart Association classification of heart failure
Class I: patients with no limitations of activities; they suffer no symptoms with ordinary activities
Class II: patients with slight, mild limitation of activity; they are comfortable at rest or on mild exertion
Class III: patients with marked limitation of activity; they are only comfortable at rest
Class IV: patients who should be at complete rest, confined to bed or chair; any physical activity brings on discomfort and symptoms occur at rest.

forming ability, whereas individuals with moderate to severe heart failure (NYHA class III or IV) demonstrate reduction in both circulating EPC numbers and colony formation.[99] Ethnic origin is also important. Healthy, young South Asian subjects in the UK have been shown to have fewer circulating EPCs and exhibit impaired endothelial function compared to matched UK Caucasian subjects.[126]

1.3.2.3 Mechanism of endothelial progenitor cell adherence and diapedesis

Upon reaching their destination, EPCs must adhere to the vessel wall and either begin differentiation into mature endothelial cells or undertake transendothelial migration. VEGF and its surface receptors are prime candidates for this process as both are vital in embryonic angiogenesis,[127] [128] and VEGF is frequently used *in vitro* to encourage endothelial differentiation. Additionally, a homeobox gene and Pim-1 gene have been identified which regulate haemangioblast and endothelial cell differentiation.[129] [130] Platelets at sites of endothelial injury support initial adhesion of circulating EPCs through binding of platelet P-selectin with its ligand, P-selectin glycoprotein ligand-1 (PSGL-1), present on $CD34^+$ progenitor cells.[131] Rolling of EPCs along the endothelium activates surface integrins including members of the β1-integrin (e.g. $\alpha_4\beta_1$-integrin) and β2-integrin (e.g. αLβ2, αMβ2, and αXβ2) families. Once activated, these bind to their counter-ligands (e.g. VCAM-1, ICAM-1 and surface associated fibrinogen) present on the endothelial surface, ensuring firm adhesion.[53] [132] β2-integrin mediated firm adhesion appears to be key for EPC adhesion at sites of neovascularisation in ischaemia tissue. αMβ2 also mediates cellular binding to fibrinogen, which is deposited at sites of tissue and vessel damage.[132] Alternatively, vitronectin receptors (e.g. $\alpha_v\beta3$- and $\alpha_v\beta5$-integrins) and possibly some of the β1-integrins are thought to mediate endothelial cell adhesion to extracellular matrix components at sites of endothelial denudation.[53] This may suggest varying roles and regulation of integrin expression dependent on whether the mobilised EPCs are required for re-endothelialisation, neovascularisation, or both. Coupling of MCP-1 to its receptor CCR2 produces a conformational change in β1-integrin and promotes firm adhesion of monocytes to injured endothelium. This has been shown to enhance functional re-endothelialisation of murine carotid arteries by $CD34^-CD14^+$ bone marrow derived monocyte lineage cells following balloon denudation.[77] Additionally, MCP-1 and IL-8 promote monocyte adhesion onto endothelial monolayers expressing E-selectin.[133] There is a strong possibility that this interaction also exists in EPCs, given that some reports suggest that EPCs, or a sub-population of EPCs, are of monocyte lineage, and that MCP-1 and IL-8 play and active role in EPC migration and homing. MCP-1 and VEGF are dependent on

β2-integrins to invoke transendothelial migration of human peripheral blood-derived EPCs *in vitro*.[132]

In order to augment neovascularisation, EPCs must migrate into the ischaemic tissue. Angiogenesis begins with nitric-oxide mediated vasodilatation and moderated vascular permeability under the actions of VEGF (increases permeability),[134] angiopoietin-1 (reduces permeability),[135] and platelet cell adhesion molecule-1 (cell stimulation).[136] $\alpha_v\beta3$-integrin expression is regulated by endothelium-derived nitric oxide and is central in the process of cell diapedesis, survival and angiogenesis.[137] The migrated, adherent cells must tunnel between endothelial cells and across the basement membrane. Surface markers including CXCR4,[138] PECAM-1, CD18 are involved in regulation of this stage.[139] Basement membrane degradation requires the action of MMP-2, a type IV collagenase. Subsequent burrowing through the extracellular matrix is reliant on several matrix metalloproteinases (including MMP-1, -8, -9 and -13) and non-MMP proteinases including cathepsin S and L.[140] Matrix metalloproteinases are secreted as an inactive precursor, or zymogen, by macrophages and endothelial cells, which are enzymatically cleaved into an active form. This activation step is performed by plasmin, membrane-type matrix metalloproteinases (MT-MMP) and enzymes produced by mast cells (chymase or tryptase). Urokinase-type plasminogen activator (uPA), also released from endothelial cells, liberates plasmin and has been shown to be vital in revascularisation post-myocardial infarction.[141] Recently, late outgrowth EPCs were shown to express higher levels of uPA and its receptor (uPAR) compared to human umbilical vein endothelial cells (as a mature endothelial cell control), which was associated with increased pro-MMP-2 secretion.[142] Furthermore, anti-uPA binding antibodies significantly inhibited the EPC proliferation, migration and ability to form capillary-like structures *in vitro*.[142] EPCs may augment neovascularisation through integration into angiogenesis, arteriogenesis and vasculogenesis, or through paracrine mechanisms through secretion of angiogenic growth factors promoting endothelial cell proliferation, or both.

1.3.3 The effect of pharmacological agents on endothelial progenitor cells

Some drugs commonly used in the treatment of cardiovascular disease exert significant influence on EPCs. The most widely studied of these are the lipid-lowering drugs, 3-hydroxy-3-methyl-gutaryl Co-enzyme A (HMG-CoA) reductase inhibitors, often referred to as 'statins'. Since their discovery, they have become the principal drug treatment for hypercholesterolaemia, and the cornerstone of primary and secondary prevention of

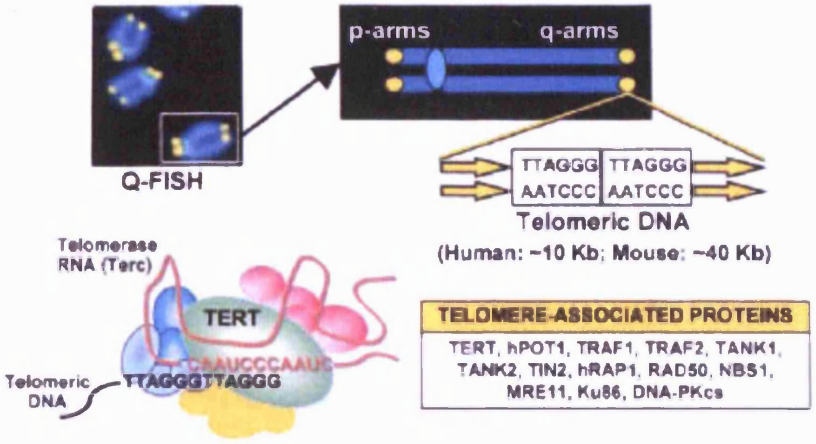
coronary artery disease. Aside from their lipid-lowering effects, it is thought that statins exert a number of pleiotropic effects including reduction of vascular inflammation and platelet aggregation, and increased endothelial nitric oxide synthesis.[143] Thus, the effect of statins on endothelial progenitor cells has gained considerable research interest. Recent data has shown that statins enhance the number, differentiation, and migratory capacity of endothelial progenitor cells *in vitro* and in animal models.[144] These effects are similar in extent to those seen following administration of vascular endothelial growth factor, and are mediated through the same, PI3/Akt pathway.[143] In addition, statins promote up-regulation of telomere repeat-binding factor-2 (TRF2), which delays replicative senescence in endothelial cells, prevents premature senescence in EPCs, and enhances *in vitro* migratory capacity.[145] Statin administration increases the number of circulating EPCs in humans with CAD[146] and accelerates re-endothelialisation following balloon denudation in rats.[147] Aspirin, widely used in cardiovascular disease for its antiplatelet effect, has been less extensively studied. Data from one, small *ex vivo* study suggests that aspirin reduces EPC adhesion, differentiation, proliferation, migratory capacity, vasculogenic integration, and inducible nitric oxide synthase levels.[148] Angiotensin II is a potent vasoactive substance at the heart of the renin-angiotensin system, important in cardiovascular disease, especially hypertension. Angiotensin II also impairs telomerase activity, accelerating the rate of senescence in cultured EPCs, and thereby reducing their proliferation.[149] Ramipril, an angiotensin converting enzyme inhibitor, reduces production of angiotensin II and also has positive effects on EPC number and function in humans with stable CAD.[150] A newer group of similar drugs are the angiotensin receptor blockers, which prevent angiotensin II binding with its receptor. A member of this group of drugs has been shown to increase the number of circulating EPCs and reduce the rate of senescence induced by angiotensin II in individuals with type II diabetes mellitus.[151] Conversely, subjects with angiographic CAD taking angiotensin receptor blockers have been reported as having lower circulating numbers of $CD34^{+}VEGFR\text{-}2^{+}$ cells.[112] Rapamycin (aka sirolimus) is used as an anti-proliferative agent on drug eluting stents to reduce in-stent restenosis. *In vitro* study of the effects of rapamycin on EPCs has shown that it reduces their number and function,[152 153] as well as impairing endothelial nitric oxide synthase production and inducing premature senescence through inactivation of telomerase.[152 154]

1.4 Telomeres

1.4.1 Telomere structure and function

Telomeres are found at the extreme tips of all eukaryotic chromosomes and are vital in sustaining cell proliferation capacity and preventing genome instability and mutation. The telomere complex is composed of a segment of G-rich, non-coding, double-stranded, repeat sequence of DNA, the enzyme telomerase, and specialised proteins that regulate synthesis and maintenance of telomeric length [Figure 1-8].[155] In humans the telomere repeat DNA sequence is TTAGGG, and this can be repeated several hundreds of times before ending in a 3' single strand overhang. The average human telomere length at birth is approximately 15-20 kilobases.[156] Human telomerase consists of telomerase reverse transcriptase (TERT) and a telomerase RNA component (TERC), which acts as a template for future telomere DNA production. Telomeric function is regulated through interaction of all three components of the telomere complex. Cells can compensate to a degree for impairment of one of these components, provided that the remaining factors are structurally and functionally intact (e.g. reduced telomerase activity with long telomeres). However, if more than one component is diminished (i.e. reduced telomerase activity plus short telomeres) then the affected cell(s) will become unable to proliferate (replicative senescence). The importance of telomerase reverse transcriptase in maintaining cell replication has been highlighted in gene transfer experiments, which result in prolonged lifespan and reduction in cellular senescence. Telomerase expression is highest during embryonic development, and in germ and tumour cells, and falls drastically after birth. Adult somatic cells possess low / absent telomerase activity, explaining their progressive telomere attrition with replication. Without all functional components, somatic cell telomeric DNA progressively shortens with each mitotic division until the telomere reaches a critical length, after which further cellular replication is unsustainable and the cell enters replicative senescence.

Figure 1-8. Human telomere structure (Adapted from[155])



Q-FISH: Quantitative Fluorescent in situ hybridisation; TERT – telomerase reverse transcriptase

1.4.2 Telomeres in aging, atherosclerosis and cardiovascular disease

Telomere attrition is accelerated in syndromes of premature human aging (e.g. Werner's Syndrome, ataxia telangiectasia, dyskeratosis congenita) and in conditions linked to human aging including hypertension and atherosclerosis. It is thought that the mechanism behind this, at least in part, is cumulative cellular damage from recurrent oxidative stress producing down-regulation of telomerase activity, and reduction in both TERT activity and TERT inhibition of apoptosis.[157] Additionally, variability in telomerase expression and telomere attrition rates is seen between different human tissues, with good maintenance of telomere length in the cerebral cortex and attrition rates of up to 60 base pairs per year in the liver and kidneys.[155] Interestingly, leukocyte telomere length is reduced in subjects with type I but not type II diabetes mellitus, which may reflect basic pathophysiological differences in the aetiology of these similar conditions. As discussed in earlier sections, atherosclerosis is a chronic inflammatory condition that begins with endothelial dysfunction, and is mediated by circulating leukocytes. Telomere attrition appears to be accelerated in endothelial cells exposed to greater shear stress with increased cell turnover,[158] and has been found to contribute to age-dependent endothelial dysfunction.[159] Examination of human atherosclerotic plaques has illustrated the presence of cells in replicative senescence.[160] Additionally, mean telomere length in circulating leukocytes of subjects with CAD is significantly shorter than in age and sex matched healthy controls[161] [162] and shortened leukocyte telomere length confers increased risk of premature myocardial infarction [Figures 1-9 and 1-10].[163] Although this may suggest that short telomeres or accelerated telomere loss is a predisposing factor for the development of atherosclerosis, it

is also plausible that the high rate of cell turnover associated with the chronic inflammatory process central to atherosclerosis results in accelerated telomere attrition. Pre-menopausal women exhibit higher levels of telomerase and reduced telomere attrition than age-matched males.[164] Research into this subject has revealed that endothelial telomerase is induced by oestrogen through activation of the phosphoinositol 3-kinase (PI3K/Akt)[165] pathway and nitric oxide signalling,[166] thus providing a potential explanation of the mechanism behind the gender variability. However, this may not be the whole answer as recent work suggests that non-specific Akt activation results in senescence-like arrest in endothelial cells.[167]

Figure 1-9. Mean telomere restriction fragment length in subjects with premature myocardial infarction (solid line) compared to age and sex-matched healthy controls (broken line) (Adapted from[163])

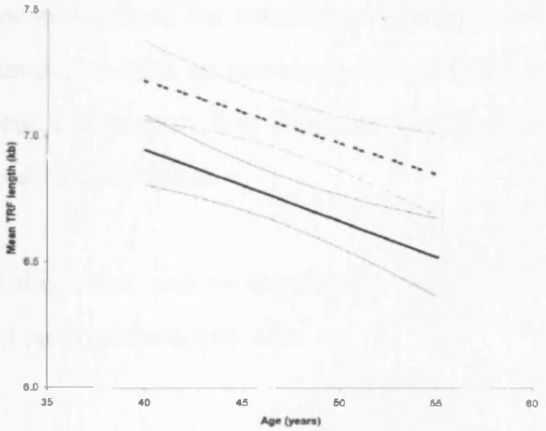
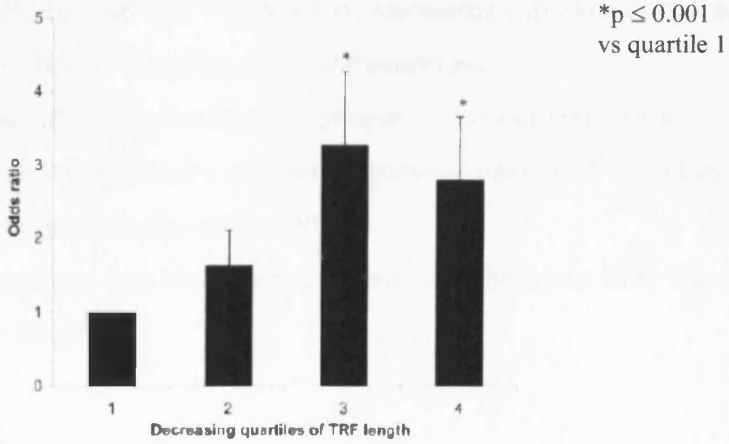


Figure 1-10. Relationship between mean leukocyte telomere restriction fragment (TRF) length and risk of premature myocardial infarction (Adapted from[163])



1.5 Aims of the work presented in this thesis

There is established evidence that atherosclerosis, and in particular CAD, occurs as a result of endothelial injury and is possibly exacerbated by impaired vascular reparative mechanisms. Percutaneous coronary angioplasty and stenting in CAD disrupts the vascular endothelium and relies on efficient re-endothelialisation for optimal results. Prolonged myocardial ischemia promotes neovascularisation through a combination of angiogenesis, arteriogenesis and vasculogeneis in an attempt to restore sufficient tissue perfusion. It has become clear that mature endothelial cells are not solely capable of completing these tasks, and evidence is mounting that circulating progenitor cells of endothelial lineage appear to play a major part in effective vascular repair and regeneration.

CAD has been associated with reduced numbers of EPC, however it is not yet clear whether this is a cause or an effect of the condition. A family history of premature CAD in a parent or sibling is associated with an increased risk of CAD in an offspring or sibling, signifying a strong genetic component. It is also now known that a family history of CAD is associated with reduced EPC numbers.

The overall purpose of this work was to develop an appreciation of the roles that EPCs play in the aetiology and pathogenesis of CAD.

In particular, the aims of this work were to:

- Examine whether EPC number and / or function is a heritable trait.
- Investigate whether EPC number and / or function represents a marker of risk of future development of atherosclerosis based on parental phenotype.
- Investigate the role that EPCs play in the pathogenesis of in-stent restenosis
- Explore the hypothesis that coronary collateralisation is improved in subjects with higher numbers or more functionally active EPCs
- Perform exploratory analysis into the possible relationship between EPC number and function, and telomere length.

Chapter 2
Materials and Methods

2.1 Overview of the project

A detailed explanation of the purpose of this work can be found in section 1.5 of the 1st chapter. In brief, the aim of was to develop an understanding of the role of endothelial progenitor cells (EPCs) in various aspects of coronary artery disease (CAD), including heritability, coronary in-stent restenosis, and coronary collateralisation. A total of 162 subjects were included in the project, categorised in 3 groups:

- 24 'healthy parent-healthy offspring' pairs and 27 'CAD parent-healthy offspring' pairs for the hereditary work

- 10 patients with, and 11 patients without, coronary in-stent restenosis.

- 39 subjects with chronic total occlusion of one main coronary artery and varying degrees of coronary collateralisation.

Further details of the recruitment process are provided in the relevant chapters. Aspects of the protocol that were common to all subjects are described in this chapter.

2.2 Ethical approval

This work was performed in accordance with the Declaration of Helsinki. A favourable opinion for the work was obtained from the Leicestershire Research Ethics Committee prior to any subject recruitment (Reference Nº 05/Q2501/63). With regard to the study participants recruited from the GRAPHIC study (see chapter 3 for details), the Leicestershire Research Ethics Committee had previously granted full ethical approval for the GRAPHIC study (Reference Nº 6463). This included approval to use DNA that had been previously collected in other studies investigating cardiovascular disease (with each participant's consent). Additional ethical approval was granted allowing us to re-contact GRAPHIC study participants, who had previously provided their consent for this, regarding future cardiovascular research projects (Reference Nº 7195).

2.3 Subject identification and recruitment

The identification and recruitment of suitable study participants was entirely performed by myself at the Glenfield Hospital, Leicester. Necessary information regarding coronary artery stenting procedures and cases of coronary in-stent restenosis was obtained from the Cardiorespiratory Audit Office at the Glenfield Hospital, Leicester, and participant's notes.

Potential study participants were provided with an approved invitation letter and patient information sheet, which provided details about the study and a contact telephone number. Candidates for the offspring study were also provided with a reply slip and stamped return addressed envelope. If potential subjects had not responded after one week, telephone contact was made with subjects (except offspring) to check the information had been received, answer any questions, and ascertain whether they would be interested in participation. Willing, suitable participants were seen at the Glenfield Hospital, Leicester and recruited. Written informed consent was obtained from all study participants prior to phenotypic data and sample collection. At the time of the study recruitment, the following information was obtained and recorded in hard copy:

- Date of birth and gender
- Height (metres) and weight (kilograms) for calculation of body mass index (BMI = weight (kg) ÷ [height (m)]^2)
- Ethnic origin
- Cardiovascular disease history including cerebrovascular disease, atrial fibrillation, peripheral arterial disease, prior percutaneous coronary intervention, prior coronary artery bypass grafting surgery
- List of traditional cardiovascular risk factors
- Family history of premature coronary artery disease in first-degree relatives
- Full list of medication
- Date of coronary angiogram(s) and angioplasty
- Degree of coronary artery disease and left ventricular systolic function
- Degree of coronary collateralisation (if appropriate)
- Details of stent (if appropriate)

Subjects were classed as having hypertension based on previous diagnosis by a physician or if they were taking anti-hypertensive medication specifically for elevated blood pressure. Smoking history and past medical history of hypercholesterolaemia were obtained from subjects self reporting.

2.3.1 Exclusion criteria

In all three investigative groups, subjects with diabetes mellitus, concomitant inflammatory disease (including asthma), malignancy, or renal impairment (serum creatinine >150μmol/L) were excluded from the study as it was felt that these conditions may significantly affect serum cytokine levels, EPC number, and / or EPC function. The

exclusion of subjects with known diabetes mellitus was done in view of the reported effects of type II diabetes mellitus on EPC number and function[119] in an attempt to minimise biasing factors. In addition, for the heritability study it was felt that, as type II diabetes mellitus exhibits a degree of heritability, non-diabetic offspring subjects of diabetic parents may carry genetic traits related to type II diabetes mellitus that could affect EPC number and / or function. Therefore, inclusion of only non-diabetic subjects would aim to remove the potential bias of this factor. The issues surrounding this decision are discussed in chapter 6. In the in-stent restenosis work, subjects with diabetes mellitus and obstructive CAD often have more severe, diffuse CAD which may require multivessel stenting, making subject matching difficult. In addtion, many diabetic subjects with CAD are now preferentially treated with drug-eluting stents rather than bare metal stents, which have different effects on re-endotheliasation and may affect EPCs. This is discussed further in chapter 4.

2.4 Phlebotomy and sample collection

Approximately 59 mL of peripheral venous blood was taken following clean puncture in the antecubital fossa using a Becton-Dickinson Vacutainer® System with 21 gauge hypodermic butterfly needle as follows:

- 27 mL in EDTA tubes for mononuclear cell isolation and culture
- 9 mL in EDTA tube for DNA extraction
- 10 mL in Lithium Heparin tube for plasma separation
- 10 mL in Plain tube for serum separation
- 3mL in EDTA tube for whole blood flow cytometry

2.4.1 Plasma and serum separation

Samples were processed within 30 minutes of collection. Plasma and serum were separated from cellular components by centrifugation at 2,500 rpm for 15 minutes at 20°C. 1 mL aliquots of each were then pipetted into labelled 1.8 mL NUNC cryotubes with blue caps (plasma) or red caps (serum). Samples were then "snap" frozen in liquid nitrogen before being placed in cryoboxes for storage at -80°C.

2.5 Flow cytometric quantification of circulating peripheral blood endothelial progenitor cells using fluorescent-activated cell sorting (FACS)

The number of circulating endothelial progenitor cells in the peripheral blood of each subject was determined by flow cytometry. Two types of EPC, characterised by different

surface marker expression (see below) were measured using duplicate flow cytometric analyses per subject.

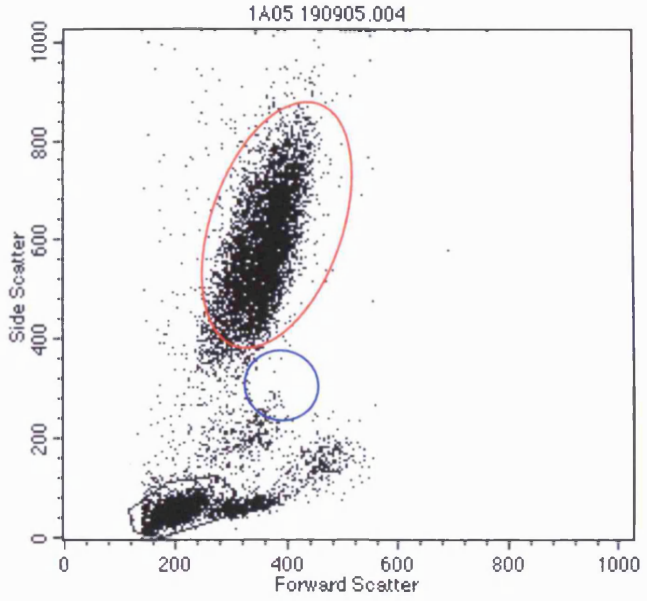
2.5.1 Explanation of flow cytometry

Flow cytometry is a method of counting and sorting cells that enables multiple characteristics of cells to be analysed as they file past electronic and optical detectors. The various cellular components of blood and subpopulations of leukocytes (i.e. granulocytes, monocytes, lymphocytes) can be identified due to their different sizes (forward scatter, FSC) and intracellular granularity (side scatter, SSC). More specific identification of cell subtype can be determined by fluorescent labelling, which uses antibodies to surface marker receptors or intracellular targets, conjugated with one of a variety of fluorescent markers known as fluorochromes. There are several different fluorochromes, each with their own specific wavelength range at which they are excited by laser light and emit a fluorescent signal.

Cells that are to be analysed by flow cytometry are labelled using appropriate antibodies (where required) and often fixed in formaldehyde. When analysing white blood cells from whole blood, the red blood cells must be lysed and, in some assays removed from the cells of interest prior to analysis on the flow cytometer. There are several methods and commercially available kits that can be used in conjunction with specific flow cytometers. Once the cells are prepared and suspended in the appropriate solution, they are introduced into the flow cytometer in single file using a method of hydrodynamic focusing. This ensures that a stream of single cells is passed through the beam of one, or a series of, laser(s) that either scatter the light or excite the fluorochromes. This can then be detected on one of a series of photomultiplier tubes. With regard to light scatter, one detector is situated directly in line with where the stream passes through the laser beam to measure cell size (forward scatter, FSC), and other detectors are located at ninety degrees to the laser beam to detect cell granularity (side scatter, SSC). This function alone enables separation of leukocyte sub-populations [Figure 2-1]. A number of fluorescent detectors are also present, which distinguish varying frequencies of light emission [Figure 2-2]. The signals detected on the photomultiplier tubes are transformed into digital signals, which can then be analysed using specific acquisition software on a linked computer. Data can be illustrated on two dimensional dot-plot (scattergrams) or histograms. A Becton Dickinson FACSCalibur™ flow cytometer coupled to a dedicated Apple Power Macintosh G5 computer with CellSystems® software was used for every sample in this study. The

FACSCalibur machine had a standard 488nm argon laser and an additional 633nm diode laser with four fluorescent detectors, allowing up to 4-colour immunophenotyping. Examples of commonly used fluorochromes are shown below [Figure 2-3].

Figure 2-1. Leukocyte separation by flow cytometry as determined by size (forward scatter) and granularity (side scatter).



Red loop – granulocytes; Blue loop – Monocytes; Black loop – lymphocytes.

Figure 2-2. Schematic representation of a Becton Dickinson flow cytometer and commonly used fluorochromes (http://facs.scripps.edu/images/facscolors.jpg)

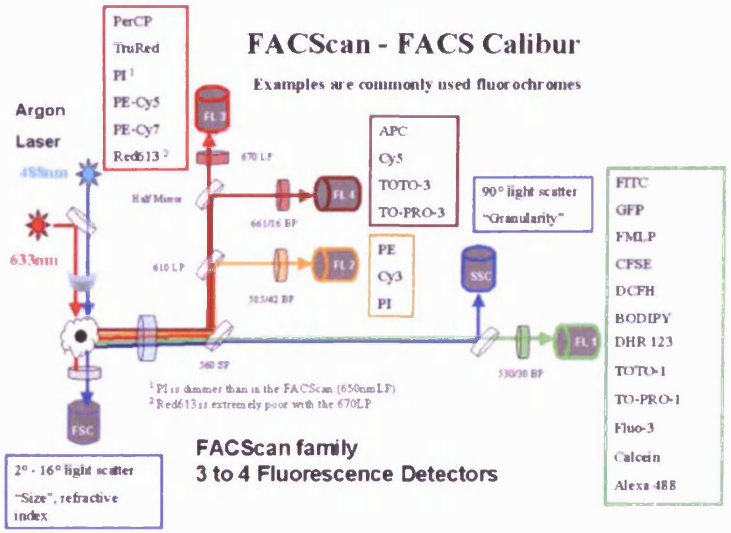
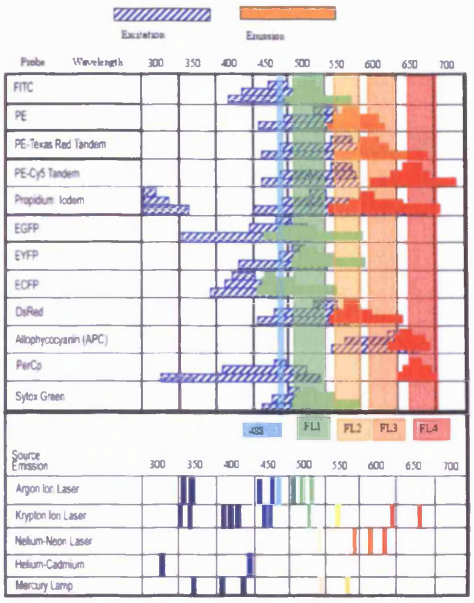


Figure 2-3. Fluorochromes and light sources commonly used in flow cytometry, highlighting excitation and emission wavelengths

(http://biology.berkeley.edu/crl/flow_cytometry_basic.html)

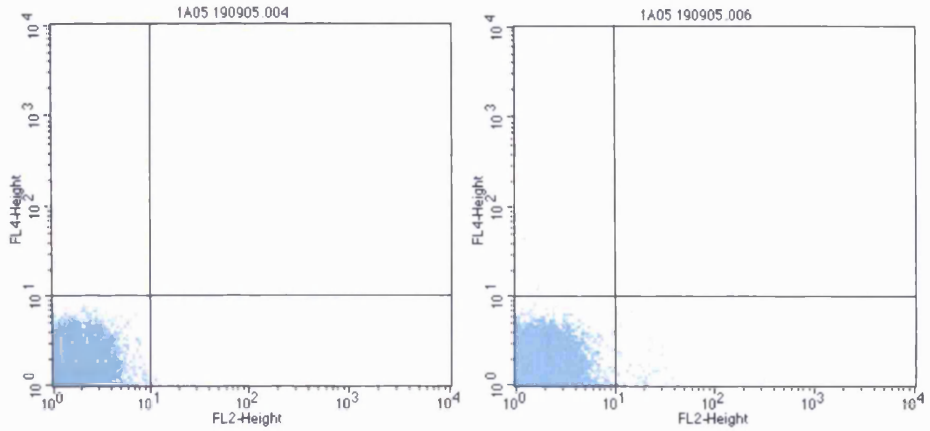


2.5.2 Gating, setting quandrants, and isotype controls

It is possible to focus on a particular subtype of leukocyte when performing flow cytometry. A "gate" is drawn around the cells of interest on a scattergram and a second histogram can be used to quantify a pre-decided number of events occurring within this gated cell population. For example, using a fluorochrome conjugated anti-human CD34 antibody and gating on the lymphocyte population can identify $CD34^+$ lymphocytes. This principle was the basis of my flow cytometric quantification of circulating endothelial progenitor cells. Isotype controls are monoclonal antibodies that are used to approximate the non-specific Fc-receptor background binding that occurs with the target primary antibody. Each isotype control should be of the same immunoglobulin subclass as the primary antibody (e.g. IgG1, IgG2a, IgG2b), and be used at the same concentration. Isotype controls can therefore be used to set the quadrants on the flow cytometer. A sample of the cells to be studied are labelled with the appropriate isotype control antobodies and passed through the flow cytometer, with the events recorded on a dot histogram split into four quadrants – lower left (LL), lower right (LR), upper left (UL) and upper right (UR). Whilst the isotype control sample is running the parameters are adjusted so that the majority of the cells are illustrated in the lower left quadrant. Then when the true sample is run, with the target antibodies, all background binding cells are in the lower left quadrant

and cells specifically binding the conjugated target antibodies are shown in either the upper left (e.g. single FL1 fluorescence), lower right (e.g. single FL2 fluorescence), or upper right quadrant (e.g. both FL1 and FL2 fluorescence) [Figure 2-4].

Figure 2-4. Examples of 4-quadrant dot histograms used to identify lymphocytes carrying fluorochromes detectable on FL2 (x-axis) or FL4 (y-axis)



Left image – isotype control; Right image – cells labelled with 2 target antibodies.

2.5.3 Anti-CD34 / anti-VEGFR-2 antibody assay

Circulating lymphocytes co-expressing the surface receptors CD34 and vascular endothelial growth factor receptor-2 (VEGFR-2) were used to identify more mature circulating EPCs, as previously reported.[60, 106] FITC-conjugated mouse $IgG_1\kappa$ (BD Pharmingen) and PE-conjugated mouse IgG_1 (Beckman Coulter) monoclonal antibodies were used for the isotype control. FITC-conjugated mouse anti-human CD34 monoclonal antibody (BD Pharmingen) and PE-conjugated mouse anti-human VEGFR-2 (R&D Systems) monoclonal antibodies were used to label $CD34^+VEGFR\text{-}2^+$ cells. All blood samples were stored at 2-4°C and were processed within 4 hours of collection.

Step 1: 100μL of whole peripheral blood in EDTA was pipetted into each of three tubes (1 isotype control plus 2 duplicate samples).

Step 2: 4μL of each appropriate FITC-conjugated antibody / isotype was added to each tube, gently agitated, and incubated for 15 minutes at room temperature in the dark.

Step 3: 4μL of each appropriate PE-conjugated antibody / isotype was added to each tube, gently agitated, and incubated for a further 15 minutes at room temperature in the dark.

Step 4: The red cells were lysed by 10 minutes incubation with 750 μL of 1X dilution BD FACS Lysing (BD Pharmingen) solution.

Step 5: The cells were spun at 1500rpm in a bench centrifuge (Heraeus Multifuge 3 $_{L-R}$) for 3 minutes to produce a pellet.

Step 6: The supernatant was discarded and the cells resuspended in 1 mL D-PBS w/o $CaCl_2$, $MgCl_2$ to wash.

Step 7: A second spin at 1500 rpm for 3 minutes was performed before the cells were finally resuspended in 500 µL D-PBS w/o $CaCl_2$, $MgCl_2$ for analysis on the flow cytometer.

The lymphocytes were identified by their light scatter characteristics, and were gated for dual colour analysis. 100,000 total events were counted per tube. A histogram with FITC fluorescence (FL1) on the 'y' axis and PE fluorescence (FL2) on the 'x' axis was used to quantify the target cells. Cells co-expressing $CD34^+$ and VEGFR-2^+ were displayed in the upper right quadrant. $CD34^+$VEGFR-2^+ cells were recorded as a percentage of the total lymphocyte gated population.

2.5.4 Anti-AC133 / anti-VEGFR-2 antibody assay

Circulating lymphocytes co-expressing the surface receptors AC133 and VEGFR-2 were used to identify a more immature type of EPCs, as previously reported.[60] [106] APC-conjugated mouse $IgG_1\kappa$ (BD Pharmingen) and PE-conjugated mouse IgG_1 (Beckman Coulter) monoclonal antibodies were used for the isotype control. APC-conjugated mouse anti-human AC133 monoclonal antibody (Miltenyi Biotec) and PE-conjugated mouse anti-human VEGFR-2 (R&D Systems) monoclonal antibodies were used to label $AC133^+$VEGFR-2^+ cells. The protocol was identical to that described in section 2.5.3 except for the use of different antibodies. As immature endothelial progenitor cells are reported to be less frequent, 200,000 total events were counted. $AC133^+$VEGFR-2^+ cells were recorded as a percentage of the total lymphocyte gated population.

2.6 Mononuclear cell isolation

A flow diagram of this procedure is shown in figure 2-5. Mononuclear cells were isolated from whole blood using Ficoll density gradient centrifugation. For each study participant, 27 mL peripheral whole blood in EDTA was divided into three separate 50 mL Falcon tubes (i.e. 9 mL per tube) and mixed with equal quantities of Dulbecco's phosphate buffered saline without calcium and magnesium chloride (D-PBS w/o $CaCl_2$, $MgCl_2$, Gibco / Invitrogen, UK) to produce a 1:1 dilution. Each 18 mL volume of diluted blood was pipetted slowly onto 15 mL Biocoll 1.077 ATS (Autogen Bioclear, UK), taking care

not to mix the solutions. The tubes were then placed in a bench centrifuge (Heraeus Multifuge 3 $_{L-R}$) at 2,000 rpm (800 x *g*) for 20 minutes with no brake. This ensured that the mononuclear cells (interphase) were separated from erythrocytes and granulocytes (pellet) and platelets (upper serum phase). Mononuclear cells were removed from the three tubes using serological pipettes and transferred into one clean 50 mL Falcon tube. The cells were made up to 50 mL with D-PBS w/o $CaCl_2$, $MgCl_2$ to wash, and centrifuged at 2,000 rpm (800 x *g*) for 10 minutes with full brake. At least two further cycles of D-PBS w/o $CaCl_2$, $MgCl_2$ resuspension and centrifugation were performed (until the supernatant became clear) to wash the cells. Finally, the pellet was resuspended in 20 mL D-PBS w/o $CaCl_2$, $MgCl_2$ and the number of mononuclear cells isolated was determined by manual haemacytometer count.

2.7 Ability of mononuclear cells to form endothelial progenitor cells *in vitro*

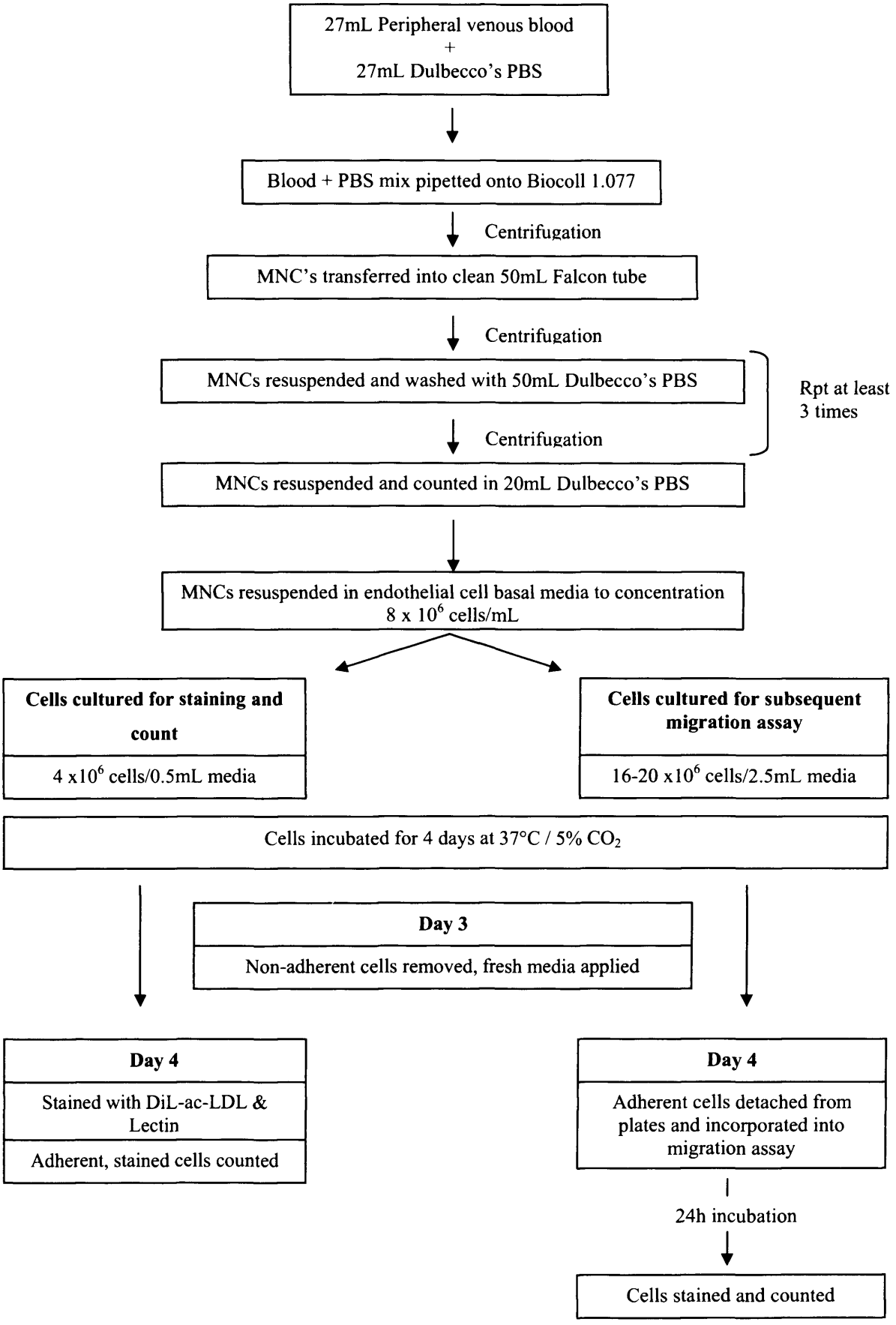
This was performed as previously reported.[106] This procedure is illustrated in figure 2-5. Separated mononuclear cells were resuspended in Endothelial Cell Basal Media supplemented with Endothelial Growth Media™ SingleQuots®[†] (Cambrex BioWhittaker, UK) and 20% Fetal Calf Serum (Cambrex BioWhittaker, UK) to a cell concentration of 8 x 10^6 cells/mL. They were then pipetted into plastic culture plates coated with human fibronectin (10μg/ml (Sigma-Aldrich, UK)) at a density of approximately 2.1 x 10^6 cells/cm^2:

- 24-well plate – 4 x 10^6 cells in 500μl media per well
- 6-well plate – 16-20 x 10^6 cells in 2.5mL media per well

For each subject, 4 x 10^6 cells per well were plated into two wells of a 24-well plate for subsequent staining and counting. The remainder of the mononuclear cells were plated into 6-well plates for subsequent use in the cell migration assay. Cells were incubated at 37°C / 5% CO_2 for a total of four days. Non-adherent cells were removed from all wells on day three by removal of the supernatant and gentle washing with D-PBS w/o $CaCl_2$, $MgCl_2$. Fresh media was applied to the adherent cells. Our methods resulted in approximately 0.5 – 1% of the initially incubated mononuclear cells differentiating into adherent endothelial progenitor cells.

[†] EGM™ SingleQuots® consist of 0.5 mL human endothelial growth factor, 0.5 mL hydrocortisone, 0.5 mL Gentamicin / Amphotericin B, and 2.0 mL bovine brain extract.

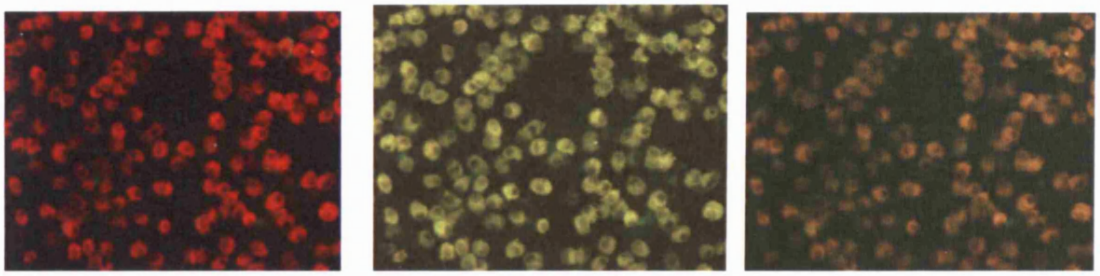
Figure 2-5. Flow diagram of mononuclear cell isolation, and culture and migration protocol



2.7.1 Fluorescent labelling of cultured endothelial progenitor cells

One accepted method to distinguish endothelial progenitor cells *in vitro* is through their capacity to take up acetylated low-density lipoprotein and bind *Ulex europaeus* agglutinin I.[60 106 117] On day four of cell culture, non-adherent cells were discarded by removal of the supernatant and gentle washing with D-PBS w/o $CaCl_2$, $MgCl_2$. The adherent cell fraction were supplied with fresh media and incubated for one hour. Adherent cells were then incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine-labeled acetylated low-density lipoprotein (DiL-ac-LDL, 2.4μg/mL, (BiogenesisLtd, UK)) at 37°C / 5% CO_2 for 1 hour. Culture media and DiL-ac-LDL was then removed by pipette and the adherent cells gently rinsed with D-PBS w/o $CaCl_2$, $MgCl_2$ before being fixed with 2% formaldehyde for 10 minutes in the dark at room temperature. The formaldehyde was removed and the cells were again washed with D-PBS w/o $CaCl_2$, $MgCl_2$ before being incubated with Fluorescein isothiocyanate (FITC)-labelled *Ulex europaeus* agglutinin I (lectin, 10 μg/mL, (Sigma-Aldrich, UK)) at room temperature in the dark for one hour. The cells were inspected using a Zeiss Axiovert 135 inverted fluorescent microscope at 20x magnification [Figure 2-6]. Counting was performed on a computer using a ProgRes C14 digital camera and utilising Jasc Paintshop Pro 8 software. Adherent, dual staining cells were judged to represent endothelial progenitor cells, and were counted in three randomly selected high-power fields per well (total of 6 high-power fields across two wells).

Figure 2-6. Examples of fluorescent labelling of EPCs in culture using DiL-ac-LDL (left), FITC-*Ulex europaeus* agglutinin I (centre), and dual staining (right) [x32 magnification].

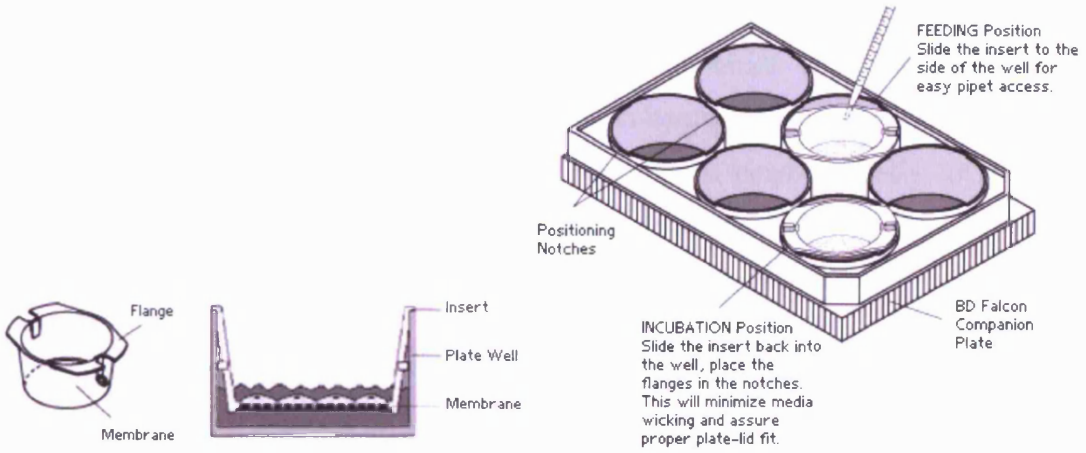


2.8 Propensity of cultured endothelial progenitor cells to migrate towards vascular endothelial growth factor

The functional capacity of cultured endothelial progenitor cells *in vitro*, was measured by their propensity to migrate towards human recombinant vascular endothelial growth factor. The number of cells that migrated across a porous membrane in a type of Boyden chamber

were counted, as previously reported[106] [Figure 2-7]. On day four of culture, non-adherent cells from the 6-well culture plates were removed by gentle washing with D-PBS w/o $CaCl_2$, $MgCl_2$. The remaining adherent cells were then incubated with 1 mM ethylenediaminetetraacetic acid (EDTA) pH 7.4 at 37°C / 5% CO_2 for 20 minutes to detach them from the human fibronectin. The plates were gently agitated to encourage cell detachment. The cells were removed from the plates, transferred into 15 mL Falcon tubes, and washed using 2 cycles of centrifugation at 2,000 rpm (800 x *g*) and resuspension in D-PBS w/o $CaCl_2$, $MgCl_2$. The harvested cells were resuspended in 2-3mL of D-PBS w/o $CaCl_2$, $MgCl_2$ and counted on a haemacytometer. The cells were then resuspended in endothelial cell basal medium supplemented with EGM™ SingleQuots® (maximum 2 x 10^5 cells/mL).

Figure 2-7. Illustration of Becton Dickinson Falcon cell culture insert used for endothelial progenitor cell migration assay (Adapted from www.bdbiosciences.com)



Two, 8µm pore, Falcon cell culture transwell inserts per study subject were placed in 24-well companion plates (Becton Dickinson, UK) with 750µL media in the lower chamber. Recombinant human vascular endothelial growth factor (50ng/mL (Sigma, UK)) was added to the lower chamber of one well only. The second well received no vascular endothelial growth factor and thus acted as a control. An equal number of endothelial progenitor cells (maximum 100,000) suspended in 500µL of media were placed into each upper chamber of the two transwell inserts. The apparatus was incubated for 24 hours at 37°C / 5% CO_2. After 24 hours, 2.4µg/mL DiL-ac-LDL (Biogenesis, UK) was added to the lower chamber and incubated for 1 hour at 37°C / 5% CO_2. Following this, the media and cells were removed from the upper and lower chambers, and the lower aspect of the transwell membranes were gently washed with D-PBS w/o $CaCl_2$, $MgCl_2$. Cells in the

migration apparatus were fixed with 2% formaldehyde for 10 minutes at room temperature in the dark. Non-migrated cells were then manually removed from the upper membrane of the transwell using a clean cotton bud. The number of cells on the lower aspect of the transwell membrane was counted by inverted fluorescent microscopy (as before) in five high-power fields per transwell.

2.9 Quantitative enzyme-linked immunosorbent assays (ELISA)

As described earlier serum and plasma samples were separated from whole blood by centrifugation and stored at -80°C until enzyme-linked immunosorbent assays (ELISA) were performed. Samples were thawed as described in the section for each ELISA. Each sample was thawed only once. Quantitative sandwich ELISA's were performed using commercially available kits as described for each cytokine below. All kits utilised the same protocol and theoretical basis. The studied cytokine binds to a solid-phase monoclonal antibody, and unbound antibody is removed by washing. The sample or standard is added and the cytokine binds to the solid-phase monoclonal antibody. An enzyme-linked polyclonal detection antibody is then added, which binds to the captured cytokine. Following this, a detection reagent (streptavidin-conjugated horseradish peroxidise) is used, and then a coloured substrate reagent is added to the sample. Finally, an acid solution is added to stop the reaction, and colour intensity is read on an optical density plate reader, whereby increasing colour intensity is proportional to cytokine levels.

2.9.1 Vascular endothelial growth factor and stromal cell derived factor-1α assays

Reagents were reconstituted, prepared and stored prior to use according to the kit protocols provided (R&D Systems, UK). Flat bottomed, 96-well plates (CoStar, UK) were coated with 100μL of monoclonal capture antibody at a concentration of 1.0μg/mL, and incubated at room temperature overnight. The following morning, the capture antibody was removed by three automated washes (Denley Wellwash 4) with 0.05% Tween 20 in filtered phosphate buffered saline (PBS). Excess fluid was removed by inverting the plate and blotting against absorbent paper. 300μL of filtered 1X reagent diluent was added to each well as a blocking agent and incubated at room temperature for a minimum of 1 hour. The triplicate wash step was repeated. Subject's plasma samples were thawed no more than 30 minutes prior to use. Sample standards were produced as per protocol, and seven, 2-fold serial dilutions, plus a zero standard of diluent only, were generated in duplicate to allow formation of an 8-point standard curve. 100μL of sample / standard was added to each well and incubated for 2 hours at room temperature, followed by a triplicate wash step. 100μL

of appropriate detection antibody was then added to each well and incubated for 2 hours at room temperature, again followed by three washes. Next, 100µL of Streptavidin-conjugated horseradish peroxidase was added to each well, away from direct light, and incubated in the dark for 20 minutes at room temperature. The 3-wash step was repeated. 100µL of substrate solution (1:1 mix of hydrogen peroxide and tetramethylbenzidine) was added to each well and incubated in the dark for 20 minutes at room temperature. Finally, 50µL of stop solution (2N sulphuric acid) was added to each well and the plate was immediately analysed on a plate reader (BIO-TEK ELx-800) set to 450nm, using KC Junior software. Regression coefficients for standard curves were 0.998 for VEGF and 0.996 for SDF-1α.

2.9.2 von Willebrand Factor (vWF) Activity Assay

Reagents were reconstituted, prepared and stored prior to use according to the kit protocols provided (Axis-Shield Diagnostics, UK). Ready to use, 96-well plates, pre-coated with capture antibody were provided within the kit. Plasma samples were thawed in a 37°C water bath for 15 minutes prior to use, and then diluted 20-fold in diluent. A calibration standard was provided in the kit, which had a maximum activity of 160%. Seven 2-fold serial dilutions of this maximum standard using diluent, plus a zero containing diluent only, were used to form an 8-point standard curve. Two control samples were provided within the kit; control 1 had an activity of 92%, control 2 had an activity of 15%, to represent normal and abnormal vWF activity. 100µL of each calibration standard, control, and subject sample were pipetted in duplicate into appropriate wells, and incubated for 1 hour at room temperature. Well contents were then decanted from the wells by rapid inversion of the plate, and the plate was blotted onto dry paper. Each well was then manually washed five times with 200µL of wash buffer, and blotted to dryness between each wash. 100µL of vWF conjugate (horseradish peroxidise-labelled murine monoclonal antibody to human vWF) was added to each well and incubated for 15 minutes at room temperature. The conjugate was removed by inverting the plate and blotting on paper. The 5-wash stage was repeated. 100µL of tetramethylbenzidine/hydrogen peroxide substrate solution (tetramethylbenzidine + hydrogen peroxide) was added to each well and incubated for 15 minutes at room temperature. Finally, 100µL of stop solution (2N sulphuric acid) was added to each well and the plate was immediately analysed on a plate reader (BIO-TEK ELx-800) set to 450nm, using KC Junior software. Regression coefficient for standard curves was 0.997.

2.10 DNA extraction

DNA was extracted from 9mL whole blood mixed with EDTA using a commercially available kit (FlexiGene DNA kit, QIAGEN Ltd, UK). Frozen samples were thawed in a 37°C water bath for 30 minutes, and then placed on ice before being added to a 50mL Falcon tube containing 25mL cell lysis solution. The samples were mixed by inversion. Centrifugation in a bench centrifuge (Heraeus Multifuge 3_{L-R}) was performed at 2000x*g* for 5 minutes to produce a pellet of mitochondria and white cells. The supernatant was discarded into a waste bottle containing disinfectant (Presept, Johnson & Johnson). The pellet was then resuspended by vortexing in a mixture of denaturation buffer (5mL per sample) and protease solution (50µL per sample) and incubated for 10 minutes in a 65°C water bath. This releases the white cell contents and efficiently removes contaminants. The DNA was precipitated using 100% isopropanol (5mL per sample) and pelleted by further centrifugation (2000x*g* for 3 minutes). Each sample was then suspended in 5mL 70% ethanol, pelleted by centrifugation (2000x*g* for 3 minutes), air-dried for a maximum of 5 minutes, and finally resuspended in hydration buffer (10mM Tris-Cl, pH 8.5).

2.11 DNA quantification

All samples were checked for yield and quality in duplicate by spectrophotometry. Nucleic acids have a peak absorbance in the ultraviolet range, at approximately 260nm. A solution containing 50µg/mL of double stranded DNA has an absorbance of 1 at 260nm. When the spectrophotometer has a path length of 1cm, absorbance (A_{260}) = optical density (OD), where OD = extinction coefficient (E) x concentration. Extinction coefficients vary with the type of nucleic acid: double stranded DNA has an extinction coefficient of 20g-1cm-1L. Thus,

$$DNA\ concentration = \frac{A260}{20 \times dilution\ factor}$$

The 260/280 ratio was assessed to provide an indication of sample purity. Pure DNA has an A260/280 ratio of 1.8-1.9. Lower ratios indicate protein contamination, whilst a higher ratio generally indicates RNA contamination. DNA samples were then stored in labelled cryotubes at -80 °C. Masterplates of DNA, containing 500uL of DNA at a concentration of 150 - 200ng/µl, were prepared and used for measurement of leukocyte telomere length.

2.12 Determination of mean telomere length by terminal restriction fragment analysis.

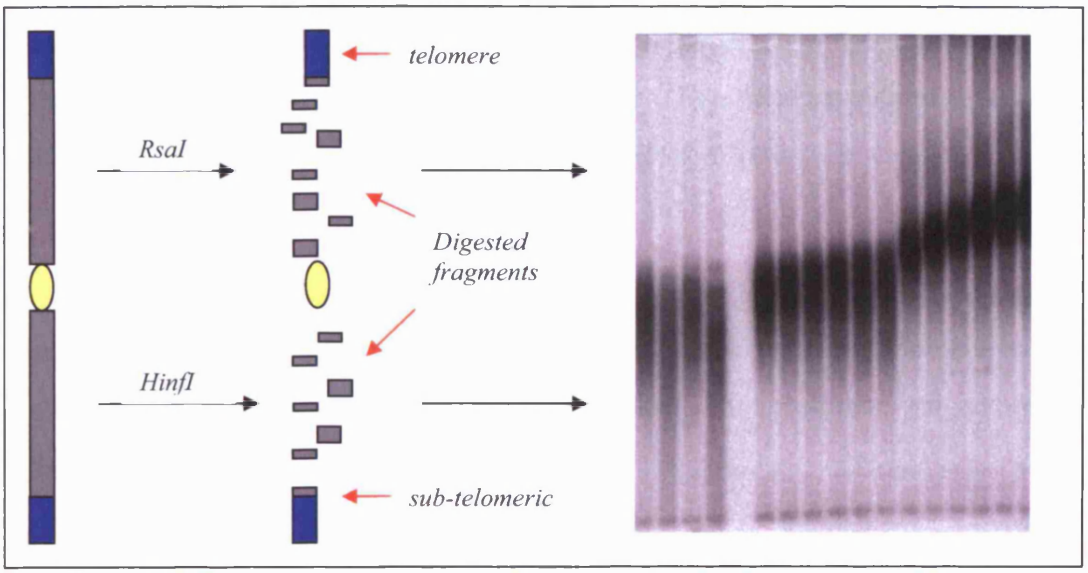
Mean terminal restriction fragment length (TRF) is a measure of average telomere size. It can be measured following incubation of genomic DNA with frequently cutting restriction enzymes (*RsaI*, 5'...G$^{\blacktriangledown}$ANTC...3', and *HinfI*, 5'...GT$^{\blacktriangledown}$AC...3'), which digest the DNA leaving the telomeric DNA intact as there are no recognition sites for the enzymes. The digested DNA is separated out by gel electrophoresis in order that small, digested fragments run off the end of the gel, whereas the larger telomeric DNA separates more slowly, forming a 12-15cm smear that can be used for analysis. The DNA was transferred to Hybond-N membrane by Southern blotting, labelled with a radio-isotope, and the resulting telomeric smear was measured using IMAGEQUANT® software (Molecular Dynamics Inc. USA) [Figure 2-8]. The smear is due to a combination of inter- and intra-chromosomal, and inter- and intra-cellular variability in telomere size. If this variability did not exist, a discrete band would be visualised on the gel electrophoresis. It is also important to note that a small, inconsistent amount of sub-telomeric DNA is not digested and is encapsulated in the telomeric smear.

2.12.1 Enzyme restriction digestion

This was performed as previously reported.[206] Briefly, 2μg of genomic DNA per subject was digested with 20U of each of *RsaI* and *HinfI* (Invitrogen, UK) at 37°C for 4-6 hours in a final solution of 1x One Phor All buffer (Amersham Pharmacia) made to correct volumes with distilled water. Samples were then resolved by electrophoresis on a 0.5% (w/v) agarose gel, containing 0.05μl/mL of 10mg/mL ethidium bromide to ease visualisation of the smears, in a horizontal submarine gel tank at 150V for the first 60 minutes followed by 50V for 18-20 hours in 1X TAE (40nM Tris-acetate, 1nM EDTA) at 2-8°C. 2μg samples were mixed with 4μL loading buffer and loaded onto the gel. Size standards were run on the outermost wells of each gel (500ng KB ladder, Invitrogen, and 700ng Hyperladders I and VI, Bioline). A specific control DNA sample, with known mean telomeric length, was also included on every gel to adjust for inter-gel variability. The gels were examined using an ultraviolet transilluminator (Alpha Innotech Corporations) to ensure the telomeric smear had been sufficiently run out. Each gel was then immersed in a depurination buffer of 0.25M hydrochloric acid for 20 mins with gentle shaking. Each gel was then rinsed with distilled water, soaked in a denaturation buffer (1.5mol/L NaCl, 0.5mol/L NaOH) for 30 minutes with gentle shaking, and rinsed again in distilled water. Finally, each gel was immersed in a neutralisation buffer (1.0mol/L Tris-HCl, 1.5mol/L NaCl, pH 7.2) for 30

minutes with gentle shaking before being rinsed in distilled water. DNA restriction fragment products were transferred to Hybond-N membrane (Amersham Pharmacia) by standard Southern blotting technique described below.

Figure 2-8. Schematic representation of genomic DNA enzyme digestion and separation by gel electrophoresis to visualise the telomeric terminal restriction fragments.



2.12.2 Southern Blotting

One tray per gel was filled with 10X sodium saturated citrate (SSC). A glass plate was placed across the top of the tray, not in contact with the liquid below. Sheets of 3MM paper and Hybond-N per gel were cut to the same width as the gel to be analyzed. The 3MM paper was then immersed in 10X SSC and carefully, but quickly, placed over the glass plate so that the top and bottom ends were resting in the 10X SSC in the tray below, allowing the 3MM to act as a wick. All air bubbles were removed by rolling a clean 25mL pipette over the 3MM paper. The gel was then carefully placed on top of the 3MM paper wick. Again, air bubbles were removed by rolling a 25mL pipette over the gel. The sheet of Hybond-N was then briefly immersed in 10X SSC and carefully placed onto the gel. Once the gel had touched the Hybond-N no further movements were made. Again, air bubbles were removed by rolling a 25mL pipette over the gel. Any gel that was extending beyond the Hybond-N was carefully trimmed. Dry sheets of QuickDraw™ Blotting paper (Sigma, UK) were piled on top of the gel, a second glass plate was placed on these sheets, and a 1kg weight was rested on top. To ensure that neither the Hybond-N nor the QuickDraw™ Blotting paper was in contact with the 3MM wick, strips of plastic were

positioned abutting the gel on all 4 sides. The transfer of DNA was allowed to proceed via capillary action overnight. After 10 minutes, and again after 1 hour, the apparatus was carefully dismantled, the lowest (originally dry) sheets of QuickDraw™ Blotting paper were replaced, and the apparatus re-assembled. Before leaving for the day, the QuickDraw™ Blotting paper was again replaced and additional clean paper towels were added to the apparatus, and an extra 1kg weight (total 2kg) was placed on top. The following morning the apparatus was carefully dismantled. The DNA was covalently cross-linked to Hybond-N membrane by exposure to 7 x 10^4 J/cm^2 UV light for 60 seconds using a UVP® CL-1000 Ultraviolet Crosslinker. The fixed membranes were stored, wrapped in tin foil at 2-4°C until hybridisation was performed.

2.12.3 Hybridisation

Pre-hybridisation and hybridisation were performed at 42°C in a hybridisation oven (Hybaid). Membranes were rolled up and placed into a hybridisation tube with 12ml RapidHyb™ Buffer solution (Amersham Biosciences). The membrane was pre-hybridised at 42°C for a minimum of 1 hour.

2.12.4 Terminal deoxynucleotidyl transferase (Tdt) labelling of T_1 probe

Tdt labelling was performed using a commercially available kit (Amersham Pharmacia). 20pmol of T_1 telomere probe $(AATCCC)_3$, 1X Tdt buffer (100 mM sodium cacodylate, pH 7.2, 0.2 mM 2-mercaptoethanol, 2 mM $CoCl_2$) 7.5μCi ^{32}P dCTP, 10mM $MgCl_2$ and 50 units Tdt enzyme. The reaction was allowed to proceed for 1 hour at 37°C, followed by 100°C for 8 minutes.

2.12.5 Radprime labelling of ladders

RadPrimer labelling was performed using a commercially available kit (Invitrogen). A combination of KB DNA ladder (30ng) and Hyperladders I and VI (45ng) in a total volume of 21μl were incubated at 100°C for 10 minutes, and then immediately placed on ice. The reaction was made up to 50μl with 1x Radprime buffer (50mM Tris-HCl (pH 6.8), 5mM magnesium chloride, 10mM 2-mercaptoethanol, 150μg/ml oligodeoxyribonucleotide primers (random octamers)), 30μM dATP/dGTP/dTTP mix, 12.5μCi ^{32}P dCTP and 40 units Klenow fragment. The reaction was allowed to proceed for 15 minutes at 37°C, followed by 100°C for 10 minutes. At this point approximately 2ml of the pre-hybridisation solution was poured into a 15ml sterilin tube and both the labelled ladders and the T1 probe added directly. This mix was then poured back into the hybridisation

tube, given a gentle shake to mix, and hybridised at 42°C for 2-4 hours. Washing in 5xSSC/0.1% SDS was carried out at 42°C for 10-15minutes; if the counts were still high and indicating high background, an additional and more stringent wash in 3xSSC/0.1% SDS was performed. The membrane was then allowed to dry for approximately 15 minutes before being wrapped in Saran-wrap and exposed to autoradiographic film within an intensifying screen at –80°C. Film was developed in a Kodak photo processor. Membranes were routinely exposed overnight and if the signal was strong enough to produce even a faint image, the membrane was then transferred to a phosphorimager plate and exposed for a further 24 hours. The plate was then scanned using a Molecular Dynamics Storm Phosphorimager to provide a digitised image of the hybridised membrane. Molecular Dynamics ImageQuant™ version 3.3 software was used to analyse the images.

2.12.6 Re-hybridising membranes

Occasionally it was necessary to re-hybridise a particular membrane. A container of water (500-1000ml) was heated to 100°C and the radio-labelled membrane was completely immersed. The container was then placed on an orbital shaker, and the water allowed to cool to room temperature, resulting in removal of the probe. The membrane was either re-hybridised immediately, as detailed previously, or stored at 4°C.

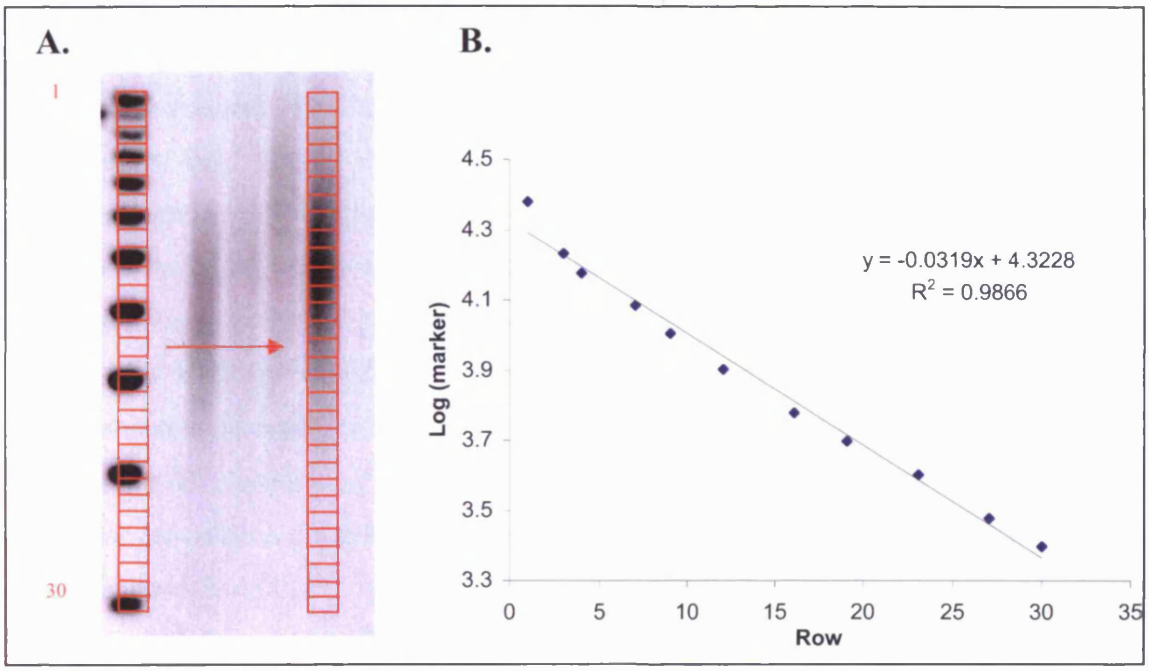
2.12.7 Estimation of mean terminal restriction fragment (TRF)

Once the gel had been exposed to the phosphorimager, the digitised image was analysed using Molecular Dynamics ImageQuant 3.3 software. Background signal was calculated in a rectangular object drawn in the space between the ladder lane, and the first sample lane. Therefore allowing determination of the total above background for each region of the gel image under analysis. The methods employed in construction of the standard curve are illustrated in figure 2-9. This was used to estimate the average molecular weight for each of the 30 regions of the grid. The grid was copied and placed over each sample lane. A rectangle was drawn in a region away from the lanes and defined as 'background', and the image was then quantitated by integrating the volume in each row. The section of output used for downstream analysis was the 'total above background'. The following equation was then used to estimate the size of the mean telomere restriction fragment (TRF):

$$TRF = \frac{\Sigma ODi}{\Sigma(ODi/MWti)}$$

Where MWt_i is the molecular weight (or size, in base pairs), and OD_i is the optical density (total above background) at that weight. This formula takes into account the fact that longer telomeres bind more labelled probe and consequently appear darker on the x-ray film and Phosphor Imager image.

Figure 2-9. Method of estimating mean telomere restriction fragment length.



A. Using IMAGEQUANT® software a grid (1 column x 30 rows) was drawn over the lane containing the KB DNA marker. It was important to try and draw the grid such that each of the bands in the marker lane lies within a row, and the number (1-30) into which each band falls recorded. Where a band lay between 2 rows, it was assigned to that row in which the majority of the band fell. **B**. A graph of log (molecular weight) against row number was plotted and used to interpolate values for each of the rows not containing a band. Thus, an average size (bp) was estimated for each of the 30 regions in the grid

2.13 Statistics

2.13.1 Demographic data

Continuous variables with a normal distribution were analysed using parametric statistical methods. Means comparison was performed using unpaired students t test or ANOVA test for continuous variables. Mann Whitney U test compared the medians for non-parametric variables. Dichotomous variables were analysed using χ^2 or Fisher's Exact Test as appropriate. Continuous variables that were not normally distributed were either analysed using the appropriate non-parametric test or transformed to fit a normal distribution where possible.

2.13.2 Laboratory derived data

Variables were tested for normal distribution using Kolmogorov-Smirnov and Shapiro-Wilk tests. Normally distributed continuous variables were compared using unpaired students t test, ANOVA, and analysis of co-variance (ANCOVA) when adjusting for significant variables. Mann-Whitney U test was used to compare the medians in non-normally distributed variables. Dichotomous variables and EPC categorical data were compared using χ^2 test or Fisher's Exact test. Correlation was determined using Pearson's coefficient for normally distributed data and Spearman's rank correlation for non-normally distributed data. Linear regression analysis was used to investigate the relationship between cultured EPC numbers, accounting for potential factors that may affect EPC numbers including age, gender, BMI and smoking status. Binary or multinomial regression analyses were performed for categorical variables, as appropriate. Two-sided tests were used with statistical significance deemed present at a level of $p < 0.05$. No formal adjustments were made for multiple testing but all P-values are interpreted with caution. P values are not provided for between group comparisons for the 'parent' subjects as it was felt that providing a given p value may be misleading in view of the marked demographic differences between the 'parent' groups. All statistical analysis was performed using Statistical Package for the Social Sciences (SPSS) v14. Graphs, charts and histograms were created using either SPSS v14 or GraphPad PRISM v3.02 for Windows (GraphPad Software Inc., USA). The number of circulating EPCs is always given as the percentage of EPCs in the lymphocyte fraction. The numbers of circulating $AC133^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+$ EPCs were markedly positively skewed, and could not be normalised by any transformation. As circulating EPCs were undetectable in a number of subjects (especially healthy subjects), they were categorised as undetectable, low level (< median) and high level (> median) where appropriate for certain analysis purposes.

Chapter

3

EPC number and function in subjects with and without premature CAD, and their healthy adult offspring

3.1 Introduction

There is an established hereditary component to the aetiology of CAD although the precise mechanism behind this has yet to be fully elucidated. Many of the tradional cardiovascular risk factors, such as hypertension, diabetes mellitus, hypercholesterolaemia, are also at least partly determined by hereditary factors. Over recent years, research has identified a number of candidate genes that show an association with the development of CAD. In addition to this, EPC number and function have been shown to be reduced both in individuals with risk factors for CAD and those with overt CAD, compared with healthy controls.[53 60 106 117 123 144] This generated the hypothesis that the number and / or function of EPCs may be genetically regulated and could contribute to the mechanisms by which CAD is inherited. This present work was undertaken to investigate the potential genetic regulation of EPCs by examining EPC number and function in parents and their offspring. This section described the studies performed to investigate whether EPC number and / or function differ between healthy adult offspring of subjects with CAD, and those whose parents are healthy. To try to ascertain whether circulating cytokines, which have been shown to influence EPC mobilisation and homing, could explain any potential variability in EPC number and function, the concentration of VEGF and SDF-1α were also measured.

3.2 Parent and offspring recruitment

Subjects aged 45 – 65 years with severe, premature coronary artery disease (>50% stenosis in three vessels) confirmed on coronary angiography with one of their healthy adult offspring (aged $\geq$ 18 years) were approached.[‡] Willing subjects and their offspring formed the CAD subject and CAD offspring cohorts. For the healthy cohort, healthy adult subjects with neither symptoms, nor prior history, of coronary artery disease, and one of their healthy adult offspring who had previously participated in the GRAPHIC Study[§] (see below) were invited to participate.[168]

85 subjects with premature CAD on angiography, all aged 45-65 years, were identified as potential participants following elective coronary angiography at Glenfield Hospital, Leicester. Letters of invitation were sent to these potential subjects. 14 subjects were subsequently unable to participate in the study as they either had no suitable offspring, or offspring not willing to participate. Although not recorded in the case notes, a further 4

[‡] All coronary angiography performed as per routine clinical care at the Glenfield Hospital, Leicester
[§] GRAPHIC Study – Genetic Regulation of Arterial Pressure in Humans in the Community.

subjects subsequently revealed a formal diagnosis of diabetes mellitus, 2 subjects had asthma, and 1 subject had inflammatory bowel disease. 34 subjects either did not respond to the study invitation or were not interested in participating when contacted. 30 subjects with premature CAD agreed to participate in the study and were recruited. Offspring from 3 of these subjects failed to attend despite agreeing to participate; therefore data from the 3 'parent' subjects was not included in any analysis.

The GRAPHIC study was designed to investigate the gene-environment interaction in the aetiology of blood pressure in at least 500 (unselected) nuclear families consisting of both parents (40-60 years) and two adult children (20-40 years) recruited from primary care practices in Leicestershire. As part of the GRAPHIC study, comprehensive demographic data and phenotypic measurements were obtained. This information allowed identification of potential healthy adult subjects plus one of their healthy adult offspring who (a) met our inclusion criteria, and (b) agreed to be re-contacted with information regarding future research projects. Subjects recruited from the GRAPHIC study were used to represent a normal, healthy United Kingdom population. A total of 76 families who matched the present study criteria were identified from the GRAPHIC study population. None of the family members had recently participated in any other research project. Invitation letters were sent to all 76 families. 29 responses were received in total. Of these, 26 pairings of parent and offspring, agreed to participate. 24 pairings were subsequently recruited, whilst 2 cancelled their study appointments on more than one occasion. 3 pairings were willing to participate, but could not arrange to attend a study appointment during the recruitment period due to offspring overseas work commitments.

3.3 Demographic details of parent and offspring subjects

3.3.1 Parent subjects

Information from 27 subjects with severe premature CAD and 24 subjects from the GRAPHIC study was available for analysis. Reflecting the different sources used for subject identification, there were significant differences in demographic and phenotypic parameters between the CAD subjects and the healthy adult subjects recruited from the GRAPHIC study [Table 3-1] Most of the parents with CAD were male, whilst nearly all of the healthy subjects were female. 2 parent subjects were of South Asian / Indian origin, whilst the remaining 25 were UK Caucasians. The subjects with CAD were significantly older and had a higher body mass index (BMI) than healthy subjects. Additionally, more

CAD subjects had hypertension, hypercholesterolaemia, and had smoked or were still smoking cigarettes [Table 3-1].

Table 3-1. Demographic details of parent and offspring subjects

Variable	Parent CAD subjects (n=27)	Parent healthy subjects (n=24)	Offspring of CAD subjects (n=27)	Offspring of healthy subjects (n=24)	P value (between offspring)
Age, years	59 ± 6	53 ± 4	31 ± 7	29 ± 6	0.200
Male Gender (%)	23 (85)	1 (4)	16 (59)	8 (33)	0.064
BMI, kg/m^2	28.8 ± 4.4	24.9 ± 2.8	26.0 ± 4.3	23.9 ± 3.5	0.069
Total cholesterol (mmol/L)	-	5.5 ± 1.0	-	4.7 ± 0.9	-
HDL-cholesterol (mmol/L)	-	1.7 ± 0.4	-	1.5 ± 0.3	-
Current Smoker (%)	5 (19)	1 (4)	6 (22)	6 (25)	0.815
Hypertension (%)	16 (59)	1 (4)	0 (0)	0 (0)	-
Hypercholesterolaemia (%)	22 (81)	5 (21)	1 (4)	0 (0)	1.000
FH of CAD in 1st degree relative (%)	11 (37)	7 (29)	27 (100)	0 (0)	< 0.001
Statin (%)	27 (100)	1 (4)	0 (0)	0 (0)	-
ACE-Inhibitor (%)	12 (44)	0 (0)	0 (0)	0 (0)	-

Continuous variables expressed as mean ± standard deviation. BMI – body mass index; HDL – high density cholesterol; FH – family history; CAD – coronary artery disease; ACE-I – angiotensin converting enzyme inhibitor. P values shown represent differences between each healthy group and CAD group.

The total cholesterol level of the CAD subjects was significantly lower than the healthy group (4.8 ± 1.1 vs 5.5 ± 1.0 mmol/l, p=0.016), which likely reflects the uniform use of lipid-lowering agents (mainly HMG Co-A reductase inhibitors; 'statins') in CAD subjects. High-density lipoprotein cholesterol levels were significantly lower in CAD subjects compared to healthy subjects (1.08 ± 0.32 vs 1.70 ± 0.41 mmol/l, p<0.001), however this data was only available in 41% of the CAD subjects [Table 3-1].

3.3.2 Offspring subjects

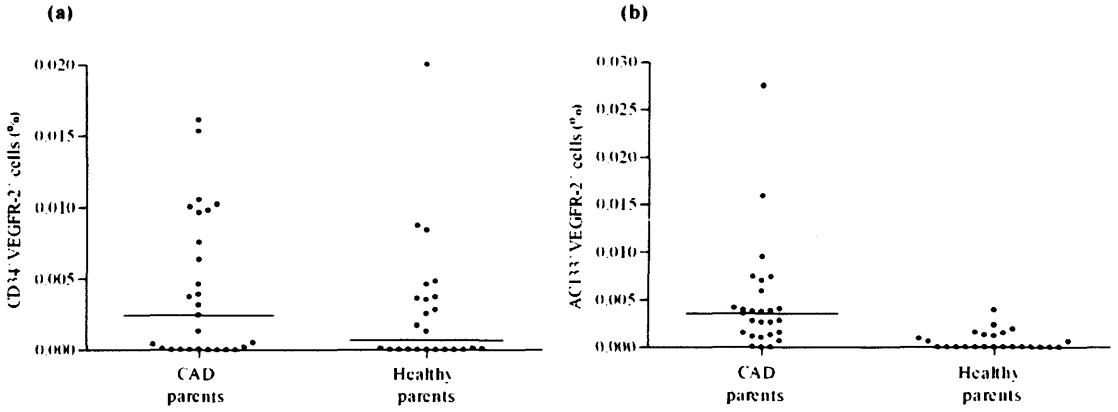
There was an expected significant difference in history of parental CAD between the offspring groups due to the study design [Figure 3-1]. 2 offspring subjects were of South

Asian / Indian origin, whilst the remaining 25 were UK Caucasians. In the offspring of healthy subjects, neither the recruited parent nor the other parent had a history of CAD. There was a non-significant treand toward more males, and higher BMI in the offspring of subjects with CAD. There was no difference in the prevalence of CAD in the grandparents between offspring subject groups.

3.4 Endothelial progenitor cell numbers in parents and offspring

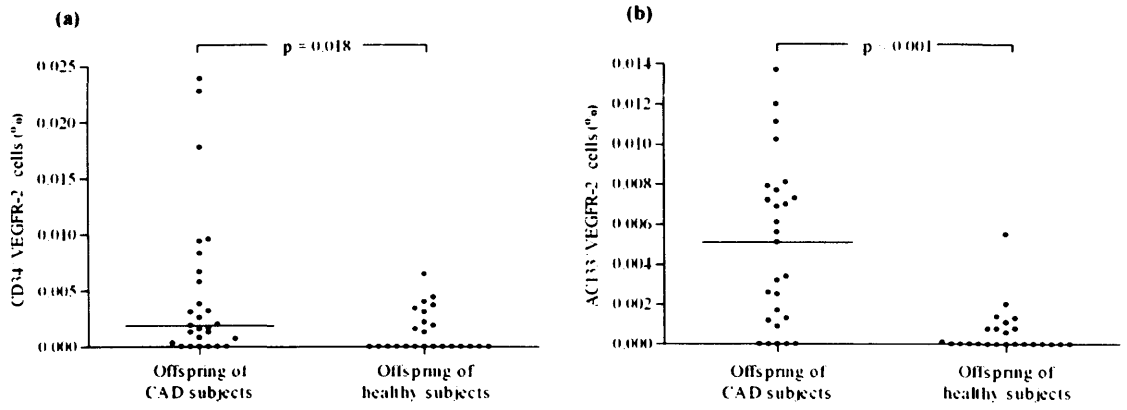
There was wide variation in the numbers of circulating and cultured EPCs in each of the parent and offspring subject populations studied [Figures 3-1 to 3-3]. There was marked positive skew in the distribution of the circulating EPCs, with several subjects having undetectable levels of circulating EPCs, therefore for certain statistical analysis, categories were generated as described in section 2.13.2.

Figure 3-1. Distribution of number of circulating EPCs in parent subjects.



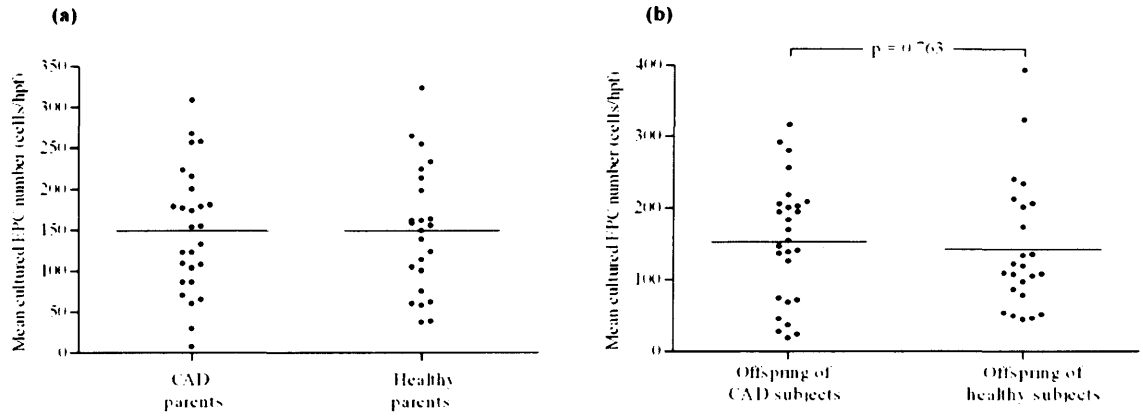
Bars represent median number of circulating EPCs. Where bar is not seen, the median is 0.000% therefore the median bar is obscured by the x-axis.

Figure 3-2. Distribution of number of circulating EPCs in offspring subjects.



Bars represent median number of circulating EPCs. Where bar is not seen, the median is 0.000% therefore the median bar is obscured by the x-axis.

Figure 3-3. Distribution of number of cultured EPCs in parents and offspring.



Bars represent mean cultured EPC number; hpf – high-power field

Table 3-2. Categories of circulating EPCs in offspring subjects.

		$CD34^+VEGFR\text{-}2^+$ cells	No. Subjects (%)	$AC133^+VEGFR\text{-}2^+$ cells	No. Subjects (%)
Category	Undetectable	0.0000	20 (39)	0.0000	19 (37)
	Low level	0.0001 – 0.0031	16 (31)	0.0001 – 0.0032	16 (31.5)
	High level	0.0032 – 0.0239	15 (30)	0.0033 – 0.0137	16 (31.5)

Number of $CD34^+VEGFR\text{-}2^+$ and $AC133^+VEGFR\text{-}2^+$ cells represent percentage of circulating lymphocytes.

3.5 The effect of demographic variables on circulating EPC numbers in parent subjects

In subjects with premature CAD, there was a borderline significant positive correlation between increasing age and circulating $CD34^+VEGFR\text{-}2^+$ cells only (R = 0.369, p = 0.058). There was no correlation between circulating EPCs and BMI. There was no difference in circulating EPC level when subjects were separated by gender, FH of CAD in a 1st degree relative, smoking, history of hypertension or hypercholesterolaemia.

In healthy subjects, there was a borderline significant positive correlation between increasing age and circulating $CD34^+VEGFR\text{-}2^+$ cells only (R = 0.386, p = 0.062). There was no correlation between circulating EPCs and BMI. Subjects with no history of hypercholesterolaemia were more likely to have undetectable levels of $AC133^+VEGFR\text{-}2^+$ cells (p = 0.042). There was no difference in circulating EPC level when subjects were separated by gender, FH of CAD in a 1st degree relative, or smoking.

3.6 The effect of demographic variables on circulating EPC numbers in offspring subjects

There were no associations between age, gender, BMI, smoking, or FH of CAD and circulating EPCs in either of the offspring subject groups.

3.7 Correlation in circulating EPCs between parents and offspring subjects

There was a borderline positive correlation in the number of circulating $AC133^+VEGFR\text{-}2^+$ cells between parents with premature CAD and their offspring (R = 0.390, p = 0.044). However, there was a significant inverse correlation in $AC133^+VEGFR\text{-}2^+$ cells between healthy parents and their offspring (R = -0.449, p = 0.028). There were no significant correlations in circulating $CD34^+VEGFR\text{-}2^+$ cells between parents and offspring in either of the groups.

3.8 Comparison of circulating EPC numbers between offspring subjects

The offspring of subjects with CAD had a significantly higher number of both $CD34^+VEGFR\text{-}2^+$ and $AC133^+VEGFR\text{-}2^+$ cells than offspring of the healthy subjects (p = 0.018 and p <0.001, respectively) [Figure 3-2]. On adjusted logistic regression analysis, with adjustment for age, gender, smoking, and BMI, $CD34^+VEGFR\text{-}2^+$ cells ≥ 0.0001% and $AC133^+VEGFR\text{-}2^+$ cells ≥ 0.0001% were the only significant predictors of CAD or

healthy status between the CAD offspring and healthy offspring [Table 3-3]. BMI showed borderline significance in predicting CAD/healthy status.

Table 3-3. Detectable levels of circulating EPCs are significant predictors of CAD offspring or healthy offspring status

	Odds ratio	95% confidence interval	P value
$CD34^+VEGFR\text{-}2^+$ cells ≥ 0.0001%	4.8	1.2 – 19.6	0.027
$AC133^+VEGFR\text{-}2^+$ cells ≥ 0.0001%	6.1	1.5 – 25.2	0.012
BMI, kg/m^2	1.2	1.0 – 1.4	0.056

3.9 The effect of demographic variables on cultured EPC number and migratory function in parent subjects

There were no significant correlations between cultured EPC number or migration ratio towards VEGF and either age or BMI in either of the parent cohorts. Moreover, there were no significant differences in the mean number of cultured EPCs or migration ratio when subjects were separated by gender, hypertension, hypercholesteroalemia, smoking, FH of CAD in a 1^{st} degree relative, or statin use.

3.10 The effect of demographic variables on cultured EPC number and migratory function in offspring subjects

There were no significant associations between any demographic variable and either cultured EPC number or migration ratio towards VEGF in either of the offspring groups.

3.11 Correlation in cultured EPCs between parents and offspring subjects

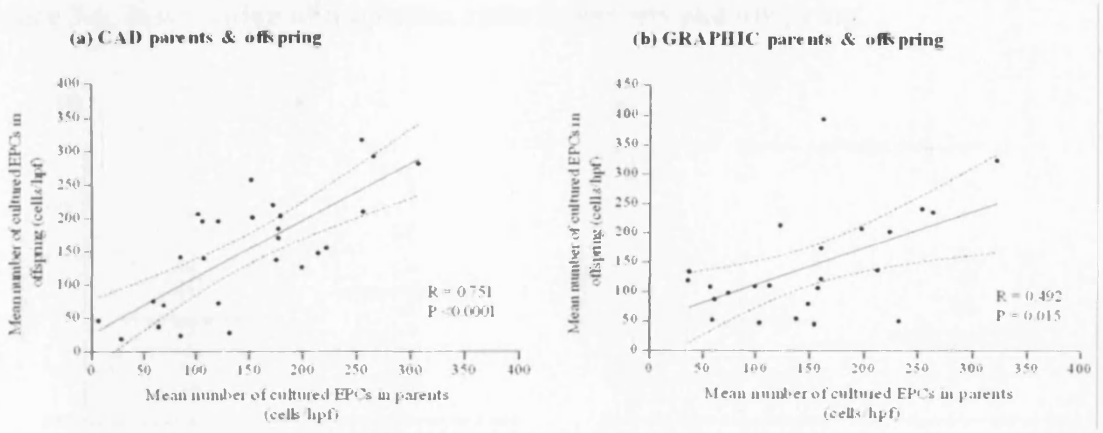
There was wide variation in the number of cultured EPCs in both parent and offspring subjects (9-fold in healthy parent and healthy offspring subjects, 17-fold in CAD offspring, and 44-fold in CAD parents) [Figure 3-3]. There was a strong positive correlation in the number of cultured EPCs between parents with premature CAD and their offspring ($R = 0.751$, $p < 0.001$) and between healthy parents and their offspring ($R = 0.492$, $p = 0.015$) [Figure 3-4]. For both healthy GRAPHIC parents and their offspring, and CAD parents and their offspring, the relationship in EPC numbers remained significant after adjustment for age, gender, BMI and smoking status in both generations ($p = 0.003$ and $p < 0.001$, respectively). In CAD subjects, for every one-unit increase in EPC number in parents, the EPC number in offspring increases by 0.93 (SE 0.16). Similarly for healthy subjects, for

every one-unit increase in EPC number in parents, the EPC number in offspring increases by 0.90 (SE 0.24). There was no significant correlation between unrelated subjects [Table 3-4].

3.12 Comparison of cultured EPC numbers between offspring groups

There was no difference in the number of cultured EPCs between offspring of subjects with premature CAD and offspring of healthy subjects (156 ± 85 vs 143 ± 90 cells/hpf, p = 0.763) [Figure 3-3].

Figure 3-4. Correlation in cultured EPC number between parents and offspring.



(a) Parents with CAD and their offspring; (b) Healthy GRAPHIC subjects and their offspring; Hpf – high-power field

Table 3-4. Correlation in mean cultured EPC number between parents and offspring

	CAD parents	Healthy parents	CAD offspring	Healthy offspring
CAD parents	1.000 (-)	0.043 (0.840)	**0.751** **(< 0.001)**	-0.117 (0.585)
Healthy parents	0.043 (0.840)	1.000 (-)	0.210 (0.324)	**0.492** **(0.015)**
CAD offspring	**0.751** **(< 0.001)**	0.210 (0.324)	1.000 (-)	0.224 (0.292)
Healthy offspring	-0.117 (0.585)	**0.492** **(0.015)**	0.224 (0.292)	1.000 (-)

Upper number represents correlation coefficient, lower number represents p value.

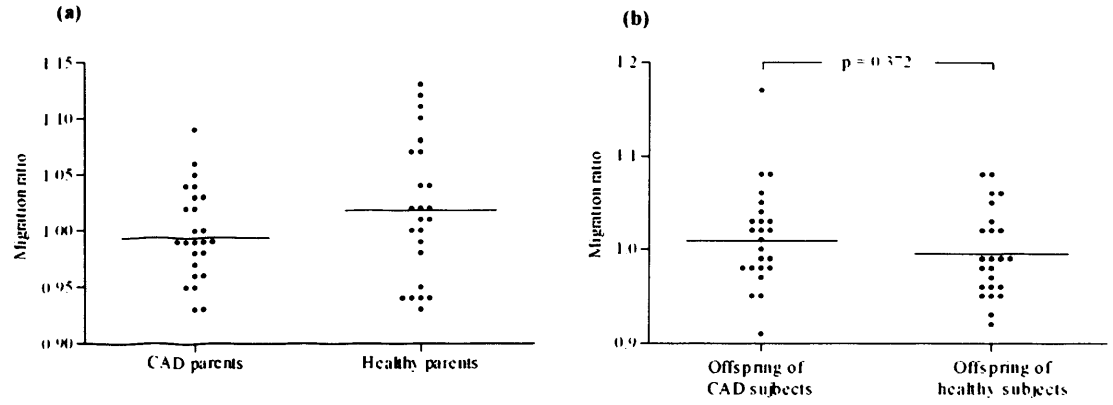
3.13 Correlation in EPC migration ratio between parents and offspring

There was no correlation in EPC migration towards VEGF between parents and offspring in any of the cohorts studied.

3.14 Comparison of EPC migration ratio in offspring

The distribution of EPC migration ratio towards VEGF is shown in figure 3-5. There was no significant difference in migration ratio between offspring of subjects with CAD and offspring of healthy subjects (1.01 ± 0.06 vs 1.00 ± 0.05, p = 0.372).

Figure 3-5. Distribution of migration ratio in parents and offspring.



In graph (a) bars represent mediam migration ratio; in graph (b) bars represent mean migration ratio; hpf – high-power field

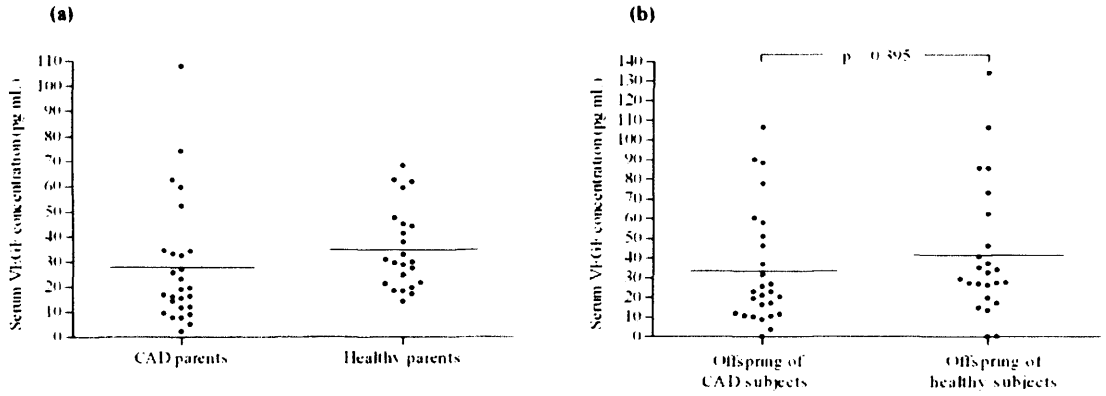
3.15 Correlation between circulating and cultured EPCs

In parent subjects with premature CAD, there was no significant correlation between circulating $AC133^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+$ cells, or between either type of circulating EPCs and cultured EPCs. In healthy parent subjects, a significant positive correlation was seen between circulating $AC133^+VEGFR\text{-}2^+$ cells and cultured EPC number ($R = 0.623$, $p = 0.001$). No significant correlation was seen between circulating $AC133^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+$ cells, or between $CD34^+VEGFR\text{-}2^+$ cells and cultured EPCs. In the offspring subjects, there were no correlations between circulating $AC133^+VEGFR\text{-}2^+$ and $CD34^+VEGFR\text{-}2^+$ cells, or between either type of circulating cell and cultured EPCs, in either offspring cohort.

3.16 Serum VEGF and SDF-1α concentrations in parents and offspring

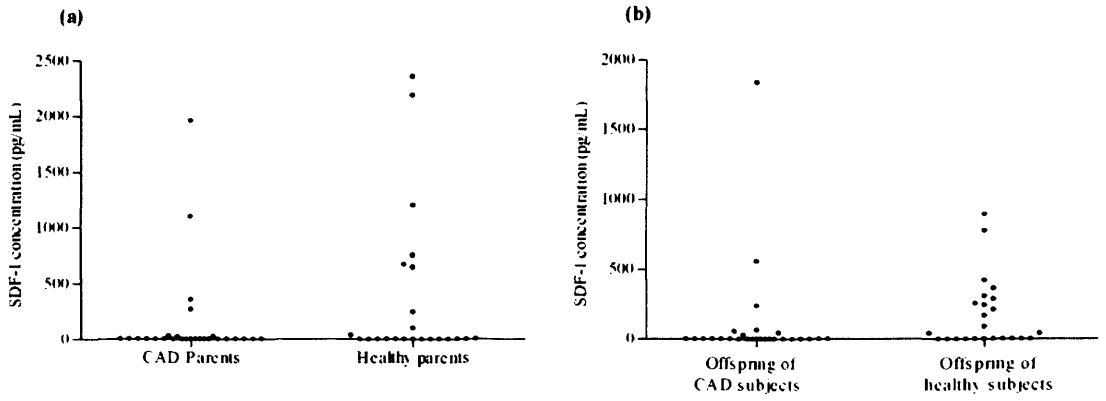
The distributions of serum VEGF and SDF-1α concentrations in parent and offspring subjects are shown in Figures 3-6 and 3-7. In the parent cohorts, serum SDF-1α was undetectable in 23 (85%) CAD subjects and 14 (61%) healthy subjects. In the offspring cohorts, serum SDF-1α was undetectable in 20 (74%) offspring of CAD subjects and 11 (46%) offspring of healthy subjects.

Figure 3-6. Distribution of serum VEGF concentrations in parents and offspring



Bars represent mean serum VEGF concentrations; VEGF - vascular endothelial growth factor

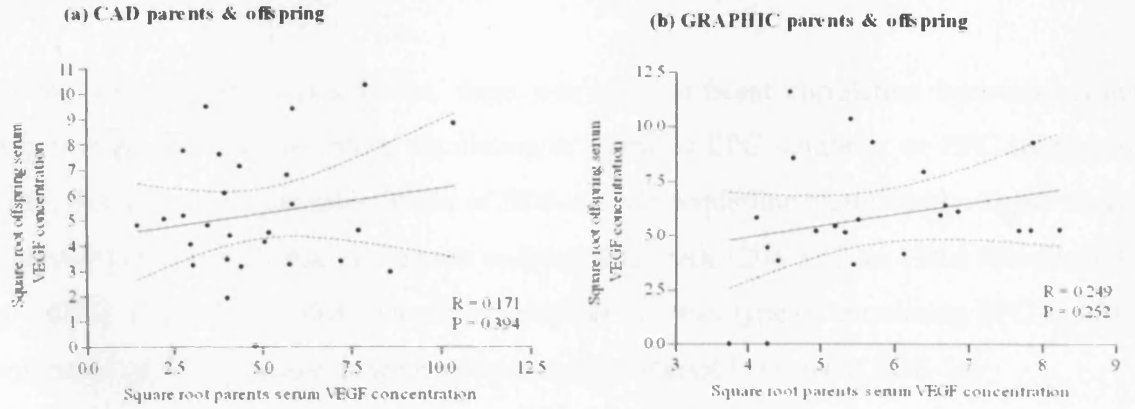
Figure 3-7. Distribution of serum SDF-1α concentrations in parents and offspring



3.17 Correlation in serum VEGF concentration between parents and offspring

No significant correlation in serum VEGF concentration was seen between subjects with CAD and their offspring, or between healthy subjects and their offspring [Figure 3-8].

Figure 3-8. Correlation in serum VEGF concentration between parents and offspring.



Graph (a) – CAD parents and their offspring; Graph (b) – healthy GRAPHIC subjects and their offspring; VEGF - vascular endothelial growth factor

3.18 Comparison of serum VEGF and SDF-1α concentrations in offspring subjects

There were no differences in mean serum VEGF concentrations between the two offspring groups, either when analysed unadjusted or after adjustment for age [Figure 3-6]. Offspring of healthy subjects were more likely to have detectable levels of serum SDF-1α levels than offspring of subjects with CAD (13 (54%) vs 7 (26%), p = 0.049) [Figure 3-7], although this showed only borderline significance.

3.19 Correlations between serum VEGF, serum SDF-1α, and EPCs

In parents with CAD, there was a significant inverse correlation between serum VEGF concentration and cultured EPC number (R = -0.433, p = 0.033). There was no correlation between serum VEGF and either circulating EPC numbers or EPC migration ratio. CAD subjects with undetectable levels of SDF-1α had significantly higher mean EPC migration ratio (1.00 ± 0.04 vs 0.94 ± 0.04, p = 0.009). There was no difference in the number of either type of circulating EPC or cultured EPCs when subjects were categorised by detectable levels of SDF-1α.

In healthy parent subjects, there was no correlation between serum VEGF concentration and either circulating or cultured EPC number, or EPC migration ratio. Subjects with detectable levels of SDF-1α had non-significantly higher mean EPC migration ratio than those with undetectable levels (1.04 ± 0.06 vs 1.00 ± 0.06, p = 0.09). There was no

difference in the number of either type of circulating EPC or cultured EPCs when subjects were categorised by detectable levels of SDF-1α.

In the offspring of CAD subjects, there was no significant correlation between serum VEGF concentration and either circulating or cultured EPC numbers, or EPC migration ratio. Subjects with detectable levels of SDF-1α had borderline significantly higher mean cultured EPC numbers than those with undetectable levels (204 ± 52 vs 134 ± 88 cells/hpf, $p = 0.06$). There was no difference in the number of either type of circulating EPC or EPC migration ratio when subjects were categorised by detectable levels of SDF-1α.

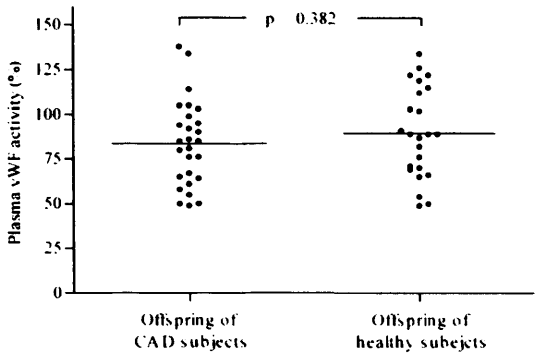
In the offspring of healthy subjects, there was no correlation between serum VEGF concentration and either circulating or cultured EPC number, or EPC migration ratio. Furthermore, there was no difference in the number of either type of circulating EPCs, cultured EPCs, or EPC migration ratio when subjects were categorised by detectable levels of SDF-1α.

3.20 von Willebrand Factor Activity and EPC number in offspring subjects

After demonstrating a difference in the number of circulating EPCs between offspring of subjects with CAD and those of healthy subjects, an exploratory analysis of extent of endothelial injury was undertaken in the offspring subjects. This was performed by measurement of plasma von Willebrand Factor (vWF) by ELISA (see section 2.9.2). vWF represents a surrogate marker of endothelial activation and if elevated could indicate an increase turnover of the vascular endothelium.

The distribution of plasma vWF activity in the offspring subjects is illustrated in figure 3-9. There were no significant associations between plasma vWF activity and any demographic variable. There were no significant differences in plasma vWF activity between the two offspring groups [Figure 3-9]. There were no significant correlations between plasma vWF activity and circulating EPC numbers, cultured EPC numbers, or migration ratio.

Figure 3-9. Comparison of plasma von Willebrand Factor activity in offspring subjects



Bars represent mean plasma vWF activity

3.21 Chapter discussion

The primary object of this part of the work was to ascertain whether endothelial progenitor cell number and / or function display heritability, both in healthy subjects and those with premature cardiovascular disease. Novel evidence of a striking correlation in cultured EPC number between parents and their healthy adult offspring in both subjects with premature CAD and healthy subjects was presented. In addition, parent cultured EPC number was an independent predictor of offspring EPC number in both the CAD and the healthy subject cohorts. The significant positive correlation between parents with premature CAD and their offspring in the level of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells is also novel. Together, these results suggest that EPC numbers are, at least partially, genetically regulated.

The inverse correlation seen in the level of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells between healthy GRAPHIC parents and offspring is more difficult to decipher as this result was the opposite of that seen between subjects with premature CAD and their offspring. In the healthy GRAPHIC cohort, 14 out of 24 (58%) of the parents and 14 out of 24 (58%) of the offspring had undetectable levels of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells. In the remaining 8 instances, when the parents' circulating $AC133^{+}VEGFR\text{-}2^{+}$ cell level was zero, their offspring had detectable levels, and vice versa, so producing an inverse relationship. The presence of so many undetectable levels raises the question of reliability of the Spearman's rank correlation used for the analysis. Unfortunately, as the data could not be transformed into a normal distribution this was felt to be the most appropriate statistical test. If all parent and offspring subjects with undetectable levels of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells were removed from the analysis then no significant correlation between parents and

offspring was seen. In view of this potential technical complexity it is very difficult to draw any firm conclusions regarding the heritability of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells. No significant correlation was seen in circulating $CD34^{+}VEGFR\text{-}2^{+}$ cells between either group of parents and their offspring, suggesting no genetic contribution to their levels. In summary, there was a statistically strong correlation in the numbers of cultured EPCs but not in the numbers of either type of circulating EPCs between parents and offspring of both subjects with premature CAD and healthy subjects. This may be explained by the possibility that the two separate assays measure different cell types. This is discussed in greater detail below.

The findings of a familial correlation could be explained by either a genetic aetiology, or the effect of shared environment. There is no published data on the effect that a shared environment exerts on EPC number and / or function. In the present study the effect of shared environment is unlikely to be significant as most of the offspring subjects were living separately from their parents at the time of participation. Furthermore, factors that are known to induce vascular endothelial damage, which could be present in a shared environment, such as cigarette smoking, showed no association with EPC number in our offspring subjects. Ideally further studies including either twins, or genetically unrelated subjects who share an environment (i.e. spouses or adopted offspring), could potentially be used to further clarify this issue.

The second interesting finding in this part of the work was that the offspring of subjects with CAD had significantly higher levels of circulating EPCs than offspring of healthy subjects. Although at first this may appear paradoxical, as CAD has been associated with reduced levels of EPCs,[106 117 123] it might suggest that although the offspring of children with CAD had no clinically apparent coronary disease, they could have occult vascular damage, and the raised EPCs reflect a heightened repair response. If this is the case, then our results suggest that elevated EPCs, particularly of the immature $AC133^{+}VEGFR\text{-}2^{+}$ type, could represent a biological marker of future risk of CAD in healthy young adults. vWF activity, which was measured in this work as a surrogate marker of endothelial activation, showed no difference between the offspring groups, but by itself cannot be taken as a conclusive measure of endothelial injury that could be stimulating an EPC response. To establish whether there is a heightened repair response replication studies involving greater numbers of people should be performed. These should also include additional methods of measuring endothelial activity/dysfunction. Previous studies of EPC

numbers in asymptomatic subjects have shown that circulating levels of $CD34^+VEGFR\text{-}2^+$ EPCs inversely correlate with degree of sub-clinical atherosclerosis as measured by carotid artery intima media thickness (IMT).[122 169] However, in both of these studies, the subjects were middle-aged (compared with our younger offspring cohort) and had a number of traditional cardiovascular risk factors. Recently it has also been shown that healthy young South Asian men with no traditional cardiovascular risk factors have fewer circulating $CD34^+AC133^+VEGFR\text{-}2^+$ EPCs and reduced endothelial vasomotor function compared to their Caucasian counterparts, and that impaired endothelial function correlates with reduced EPC number.[126] It is not clear how many of these subjects had healthy parents, and in how many one or both parents had premature CAD. In view of my findings, this factor would have important implications on how these results are interpreted. It is established that the incidence of CAD is greater in individuals of South Asian origin who reside in westernised countries compared to Caucasians, a difference which is unexplained by traditional risk factor profiling.[170-172] This disparity could be due to a genetic variation that is more prevalent in South Asians, and such a genetic difference could potentially also explain any difference seen in EPC number between South Asian and UK Caucasian subjects.

The lack of difference between the offspring groups in the number of cultured EPCs, suggets that either the inherent ability of the offspring's cells to adhere and differentiate is not impaired by a family history of premature CAD, or that a different cell type is measured by the *in vitro* culture method. Interestingly, there was no correlation between the number of EPCs grown *in vitro* and the level of either type of circulating EPCs. It has been accepted that EPCs can be reliably identified by two distinct methods: (1) *in vitro* culture of MNCs in endothelial conditions followed by dual staining with DiL-ac-LDL and Lectin.[51 60 106 117] which has been thought to represent the circulating EPC population,[55 173] or (2) identification of cells taken from the circulating blood by co-expression of progenitor and endothelial lineage surface markers by dual or triple antibody guided-labelling.[56 58 117] This present work would suggest that these two methods may not consistently identify the same cell or group of cells, and are therefore not interchangeable. Antibody guided detection of cells by surface marker expression is a specific method of cell identification, whereas *in vitro* culture of any precursor cell, exhibiting plasticity, under endothelial conditions could potentially result in an endothelial phenotype. Additionally, there is an argument that more mature endothelial cells, perhaps shed from the vascular endothelium,[174] or cells of monocyte/macrophage lineage[175] could also adhere

to fibronectin, survive during culture conditions and take up Dil-ac-LDL and Lectin, so resembling an endothelial progenitor cell. Therefore when employing an *in vitro* assay alone, the true origin and exact identity of the cultured cells may not be known. In a small study of healthy volunteers examining the expression of surface markers on MNCs cultured in endothelial conditions, only a small fraction of cultured cells were found to co-express $CD34^+VEGFR\text{-}2^+$ ($0.30 \pm 0.24\%$) or $AC133^+VEGFR\text{-}2^+$ ($0.03 \pm 0.01\%$) by day 4.[176] In a second study, comparing *in vitro* culture methods with FACS identification of EPCs, no correlation was seen between the number of adherent cultured cells and either $CD34^+VEGFR\text{-}2^+$ or $CD34^+AC133^+VEGFR\text{-}2^+$ circulating cells.[173] Both of these reports are consistent with my data, suggesting that the *in vitro* culture assay may measure the adherence and differentiation capacity of individual's cells, rather than represent the circulating EPC population. Since findings from either approach are currently being widely used as an index of EPC numbers, care needs to be taken in interpretation of data using the two methods.

VEGF and SDF-1 are important regulators of EPC mobilisation and homing.[53 82 85] The present work examined whether potentially heritable differences in these factors could explain the parent-offspring correlation in cultured EPC numbers. No relationship was seen between VEGF concentrations and cultured EPC numbers. SDF-1 levels could only be classified as detectable or not and there was no difference between the cohorts. The migratory capacity of cultured EPCs towards VEGF shows no correlation between parents and offspring, suggesting that this mechanism of homing of EPCs is not genetically determined. The measurement of chemokines in the systemic circulation may not be an accurate reflection of the mechanisms that control EPC mobilisation and homing. A better approach may be to measure chemokine concentration or gradients locally at sites of endothelial dysfunction or atherosclerosis.

An important issue to discuss is the significant difference in demographic variables between the parent subject groups. The CAD and healthy GRAPHIC parent groups are substantially different in the majority of characteristics, reflecting the recruitment strategy (see Materials & Methods). Overall, more men were found to have premature CAD, which is consistent with well-established epidemiological data. Therefore, it was easier to identify more potential male subjects who met my study criteria and were willing to participate. When suitable female subjects were identified strong attempts were made to recruit these individuals as they were infrequently identified. However, overall the end result was a

preponderance of male CAD subjects. When identifying potential healthy subjects from the GRAPHIC study, in virtually all cases the 'mother' of the family responded to the invitation letter and acted as point of contact for the potential family. The 'mothers' were more willing and logistically able to attend and participate in the study. The 'fathers' were often unable or unwilling to attend with many citing employment commitments. Although ideally, all parent subjects should have been at least gender matched it was important to ensure that enough parent/offspring combinations were recruited to allow the hereditary, and between-offspring, comparisons to be performed. Despite these drawbacks in the parental group recruitment, the offspring subjects were much more closely matched allowing for reliable between-group comparisons. Importantly, in the healthy GRAPHIC offspring group, neither the parent who participated in the study, nor the other parent gave any history of CAD.

The parental cohort were also not well matched for use of cardiovascular medications such as HMG-CoA reductase inhibitors ('statins') and ACE-inhibitors, which have been reported to affect the number of EPCs in both *in vitro* and *in vivo* studies.[143-147 150 177] It has been shown that statins influence EPC number and function through activation of eNOS via the PI3-Akt pathway.[143 144] ACE-inhibitors may also mediate EPC number and function through upregulation of eNOS, although this is not as well defined as for the statins. In a study of 20 subjects with CAD and 3 healthy volunteers, administration of ramipril 5mg od led to *in vitro* improvements in EPC number, migration towards VEGF, tubule formation, and proliferative capacity with independent of BP reduction.[150] The authors suggested that the effect of ramipril may be due to increased bradykinin binding with the B2-receptor leading to eNOS activation. Indeed, in these subjects serum NO concentrations were increased following 4 weeks' of ramipril therapy.[150] Since the primary objective of the study was to investigate parent-offspring correlations of EPC numbers and to examine EPC number and function in healthy subjects with contrasting familial risk of CAD, this does not affect the interpretation of my principle findings. However, because of the differences in demographics and the inability to account for variables such as treatment effect in the parental data, these results may not be an accurate representation of the wider population. This may explain the paradox between my results and previously published data comparing subjects with and without CAD.

Despite the differences between the methods of EPC identification, there does appear to be a genetic contribution to the number of EPCs and / or the differentiation capacity of a sub-

population of MNCs to endothelial lineage cells, between parents and offspring both with premature CAD and in healthy subjects. Further work is needed to determine the true identity of functionally active EPCs, which are capable of re-endothelialisation and neovascularisation shown in animal studies.[54-56] This is of paramount importance for the future of EPC research, and for studies examining the therapeutic use of EPCs in human randomised controlled trials. Once this is more clearly understood, the genetic contribution to EPC biology can be further delineated. In conclusion, this work suggests that a degree of the heritability seen in CAD may be explained through the genetic regulation of EPCs. Additionally, circulating EPCs may represent a biological marker of sub-clinical atherosclerosis in healthy young adults. Prospective studies are required to determine whether measurement of EPC numbers could be useful in identifying individuals at higher risk.

Chapter

4

EPC number and function in coronary in-stent restenosis and collateralisation

4.1 Introduction

This chapter concentrates on the role of EPCs in the pathogenesis of two different aspects of CAD, one of which is a reaction to coronary angioplasty and stenting (coronary in-stent restenosis [ISR]) and one that is a feature of long-standing CAD (coronary collateralisation). The first few sections of this chapter will provide a background to these two areas, before moving on to discuss the methods employed in the identification, recruitment, and investigation of subjects with either ISR or coronary collateralisation.

4.1.1 Cardiac catheterisation and coronary arteriography

The cardiac chambers, cardiac valves, coronary arteries, and great vessels can be assessed by introducing a catheter through a medium to large, peripheral artery, which is advanced under radiographic screening into the desired location. This procedure was first performed in humans in the late 1920's[178] and is now widespread in the field of cardiology. The femoral arteries, brachial arteries and radial arteries are the most common peripheral access sites. During cardiac catheterisation, measurements of pressure and oxygen saturation can be taken from varying areas of the cardiac chambers and great vessels, and can be used to determine circulatory abnormalities. Injection of contrast into the ventricles is used to assess chamber size and systolic function. Coronary arteriography involves selective catheterisation of the right and left coronary artery systems followed by injection of contrast and radiographic imaging. This enables the operator to visualise the anatomical route of the coronary arteries and assess the extent of luminal stenoses.

4.1.2 Percutaneous coronary intervention

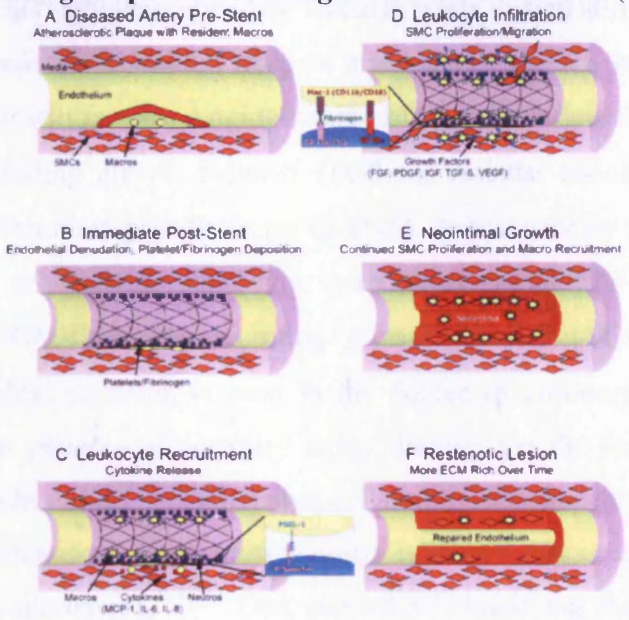
Following visualisation of a coronary artery stenosis on arteriography it may be appropriate, and technically feasible, to attempt to relieve a luminal obstruction by angioplasty and stenting. Initially pioneered by Andreas Gruntzig in 1979,[179] this now relatively common procedure is initially performed using the same technique as coronary arteriography, and is advanced by passing a guidewire down the lumen of the coronary artery, through the stenosis. Once the guidewire is in a suitable position a (deflated) cylindrical balloon is advanced over the guidewire and positioned, under fluoroscopic screening across the luminal stenosis. The balloon is then inflated for a short period of time (usually < 1 minute) before being deflated, and removed. This procedure is called percutanoeous transluminal coronary angioplasty (PTCA). It reduces the coronary stenosis through a combination of plaque disruption and intimal dissection, plaque compression, and dilatation of the healthy vessel wall. As a consequence there is denudation of the

endothelium, which gradually heals by re-endothelisation. The initial success rate of PTCA is around 90% in single lesions,[180] [181] however abrupt vessel closure secondary to dissection (80%), thrombus (20%) and coronary artery spasm occurs in 4-8% of cases.[182] [183] Restenosis of at least 50% angiographic luminal diameter occurs by 6 months in 30-50% cases, with up to 30% requiring repeat intervention.[184] In view of these complications coronary artery stents have been engineered and are widely used alongside PTCA in the majority of cases.[185] [186] Stents are cylindrical meshworks of metal, which act as a scaffolding within the vessel to maintain the maximal possible luminal diameter. They are delivered to the coronary artery in the same way as balloon angioplasty, but remain permanently, incorporated into the vessel wall. Although stents reduce abrupt vessel closure, they are still troubled by restenosis, which may occur in up to 30% of selected cases[187] and 59% of high risk cases.[188] Advancing knowledge and technology has enabled a rapid evolution of stents with the aim of abolition of restenosis. Drug-eluting stents loaded with anti-cell-proliferation agents, such as paclitaxel and rapamycin, have significantly reduced in-stent restenosis.[189-196]

4.1.3 Pathology of coronary in-stent restenosis

Plaque disruption and vessel injury induced by PTCA and stenting stimulates a number of processes, which result in in-stent restenosis. Denudation of the endothelium exposes the thrombogenic sub-endothelium leading to platelet aggregation, deposition of fibrin and development of thrombus. Activated platelets secrete an assortment of cell mitogenic factors and chemoattractants [Figure 4-1], stimulating local vascular smooth muscle cell proliferation and recruitment of circulating monocytes.[197] Subsequent migration and overproliferation of vascular smooth muscle cells, synthesizing an abundance of extracellular matrix, and a lack of endothelial cells results in neointimal hyperplasia, the hallmark of in-stent restenosis.[198-200] This process is halted by successful re-endothelialisation,[201] highlighting the vital role of the endothelial cells, and thus explains why restenosis risk is highest in the first 3 months after stenting.[202] Risk of in-stent restenosis is higher in cases involving longer stenoses, smaller vessel diameter (<3.0mm),[203] multiple stents,[204] and diabetes mellitus.[201] [205]

Figure 4-1. The pathological process leading to in-stent restenosis (Adapted from[197])



SMC - smooth muscle cells; MCP-1: monocyte chemotactic protein-1; FGF: Fibroblast growth factor; PDGF: platelet derived growth factor; IGF: insulin-like growth factor; TGF-β: transforming growth factor-β; VEGF: vascular endothelial growth factor; IL-6: interleukin-6; IL-8: interleukin-8.

4.1.4 Coronary artery chronic total occlusion and coronary collateralisation

As discussed in chapter 1, in coronary artery disease gradual plaque enlargement will eventually overcome a vessels ability to remodel and will encroach on the vascular lumen. A stable plaque can continue to develop, producing recurrent and progressive myocardial ischaemia, and eventually result in total occlusion of the lumen. In response to this, natural bridging vessels, termed coronary collaterals, develop through the myocardium in an attempt to restore perfusion to the ischaemic tissue.[44] Repeated short intervals of myocardial ischaemia (15 – 120 seconds) have been shown to stimulate coronary collateral generation.[206] Coronary collaterals may arise from the proximal part of the same vessel with forward filling (antegrade) or from other coronary arteries, back-filling the occluded vessel (retrograde).[44 207] It is thought that collaterals develop from tiny (<200μm diameter), anastomotic capillaries, which are present from birth but open in response to arterial occlusion and transform through a combination of vasculogenesis[**], angiogenesis[††], and

[**] *Vasculogenesis*: initial events in vascular growth in which endothelial cell precursors migrate to discrete location, differentiate *in situ* and assemble into endocardial tubes.
[††] *Angiogenesis*: development of new capillaries from post-capillary venules, which expand into a complex, mature vascular network.

arteriogenesis[‡‡] into larger resistance vessels capable of carrying arterial blood. [136] [208] Collateral growth is not only determined by tissue hypoxia, which stimulates angiogenesis, but also through shear stress, which triggers arteriogenesis. Each of these processes is driven by specific growth factors. Angiogenesis results from endothelial cell proliferation provoked by transforming growth factor-α (TGF-α), vascular endothelial growth factor (VEGF) and basic-fibroblast growth factor (b-FGF). Arteriogenesis is aided by vascular smooth muscle cell proliferation following secretion of granulocyte-macrophage colony stimulating factor (GM-CSF), transforming growth factor-β (TGF-β) and b-FGF.[209] Marked inter-individual variation is seen in the degree of coronary collateralisation in subjects with similar patterns of coronary artery disease, yet the reason underlying this difference is not understood.[50] [210] Extensive collateralisation appears to be important in protecting viable myocardium against future episodes of ischaemia and reduces the amount of angina pectoris experienced.[211-213] One method of classifying the extent of coronary collateralisation is the Rentrop scoring system, which allocates a grade to collateralisation seen at coronary angiography [Table 4-1]. [214]

Table 4-1. The Rentrop coronary collateralisation scoring system[214]

Rentrop Collateralisation Score	Angiographic features
0	No visible coronary collaterals
1	Filling of side branches of occluded artery without visualisation of epicardial segment
2	Partial filling of the epicardial segment of the occluded artery via collaterals
3	Complete filling of the epicardial segment of the occluded artery via collaterals

‡‡ *Arteriogenesis*: the transformation of pre-existing collateral arterioles into functional muscular collateral arteries

4.2 Coronary in-stent restenosis study cohort

4.2.1 Subject identification and recruitment

Potential participants were identified from data held within the Cardiorespiratory Audit database at the Glenfield Hospital, Leicester from January 2003 – December 2005. A full and detailed record of all cardiological procedures is held within this database, thus subjects who met the study criteria could be identified. Suitable participants had previously undergone single vessel coronary angioplasty and stenting for obstructive coronary artery disease. Subjects who subsequently developed coronary in-stent restenosis (ISR) as identified on repeat coronary angiography were regarded as ISR cases. The presence or absence of ISR was determined by the operating physician at the time of angiography. Control subjects, those without ISR, had undergone single vessel coronary angioplasty and stenting for obstructive coronary artery disease and had not developed coronary in-stent restenosis, as determined either by repeat coronary angiography or by complete alleviation of anginal symptoms following the initial angioplasty. As far as practicable, case and control subjects were matched for date of initial stenting procedure, stent type, stent diameter, total stent length, and vessel treated, in addition to age, sex and major coronary risk factors. Over the 3 year time period, data was available on 3304 non-diabetic subjects who had undergone percutaneous coronary angioplasty ± stenting. Of these, 189 subjects (6%) had been diagnosed with coronary in-stent restenosis. After initial matching of subjects based on age, sex, time of initial procedure, stent type and stent dimensions, medical notes were requested on 98 subjects. On review of the notes, 43 subjects had received drug-eluting stents and were therefore excluded. Medical notes could not be located for 3 subjects. 22 subjects were found to be unsuitable for recruitment: 9 subjects had more than one vessel stented or multiple areas of stenting in one vessel, 7 subjects had diabetes (not recorded in audit database), 3 subjects met other exclusion criteria, and 3 subjects had experienced acute stent thrombosis rather than ISR. Study invitation letters were sent to the remaining 30 suitable subjects. In addition, 23 potential subjects who had previously received a bare metal stent, and had undergone repeat angiography, were identified from the daily elective coronary angiography lists at the Glenfield Hospital. Of these, 5 subjects were unsuitable as they had undergone stenting to more than one vessel or had multiple areas of stenting within one vessel. Study invitation letters were sent to the remaining 18 subjects. A total of 21 subjects (10 ISR and 11 no ISR) agreed to participate in the study and therefore were finally recruited. The demographic details of the subjects

are shown in table 4-2. 1 subject in the ISR group and 2 subjects in the no-ISR group were of South Asian / Indian origin, whilst the remaining 18 subjects were UK Caucasians.

Table 4-2. Demographic details of coronary ISR subjects.

	In-stent restenosis (n=10)	No in-stent restenosis (n=11)	p value
Male gender (%)	7 (70)	10 (91)	0.311
Age, years	62 ± 8	58 ± 10	0.258
BMI, kg/m^2	28.8 ± 4.9	29.2 ± 3.7	0.842
Total cholesterol, mmol/l	4.4 ± 0.7	4.2 ± 1.1	0.602
HDL-cholesterol, mmol/l	1.2 ± 0.4 (n=7)	1.5 ± 0.6 (n=7)	0.202
Hypertension (%)	6 (60)	6 (55)	1.000
Cigarette smoking			
Ever smoker (%)	7 (70)	6 (55)	1.000
Current smoker (%)	2 (20)	2 (18)	1.000
FH CAD 1st degree relative (%)	7 (70)	4 (36)	0.198
Aspirin (%)	10 (100)	10 (91)	1.000
Statin (%)	10 (100)	11 (100)	1.000
ACE-Inhibitor (%)	7 (70)	4 (36)	0.198
Bare metal stent (%)	10 (100)	11 (100)	1.000
Vessel stented			
Left anterior descending artery(%)	8 (80)	8 (73)	1.000
Right coronary artery (%)	2 (20)	3 (27)	1.000
Stent length (mm)	17 ± 6	15 ± 2	0.403
Stent diameter (mm)	3 ± 0.2	3 ± 0.3	0.343

4.2.2 Distribution of EPCs in coronary ISR subjects

There was wide variation in the numbers of circulating and cultured EPCs in both the in-stent restenosis (ISR) and non-ISR groups [Figures 4-2 & 4-3]. There was positive skew in the distribution of both the circulating and cultured EPCs. $CD34^+VEGFR\text{-}2^+$ cells and cultured EPCs were square root transformed into a normal distribution for statistical analysis. However, $AC133^+VEGFR\text{-}2^+$ cells could not be normalised by any mathematical transformation therefore non-parametric tests were used. There were no correlations between the number of circulating $CD34^+VEGFR\text{-}2^+$ cells and $AC133^+VEGFR\text{-}2^+$ cells, or between the number of either type of circulating EPC and the number of cultured EPCs.

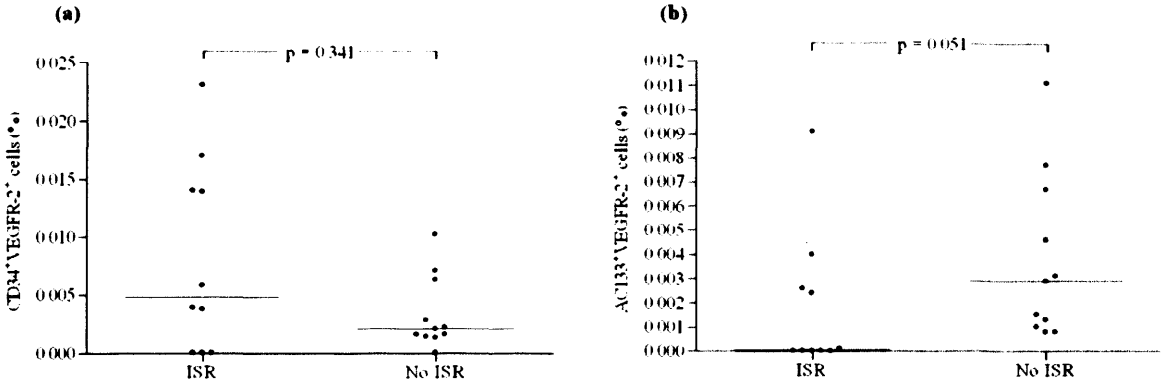
4.2.3 The effect of demographic variables on EPC number and migratory function

Subjects with a history of hypertension had a significantly lower mean number of cultured EPCs than those without (72 ± 59 vs 142 ± 79 cells/hpf, p = 0.032), yet showed a trend for greater migration capacity towards VEGF (1.01 ± 0.05 vs 0.97 ± 0.03, p = 0.067). There were no other significant associations between any other demographic variable and either type of circulating EPC, cultured EPC number or EPC migration ratio.

4.2.4 Case-control comparison of EPC numbers and EPC migratory function

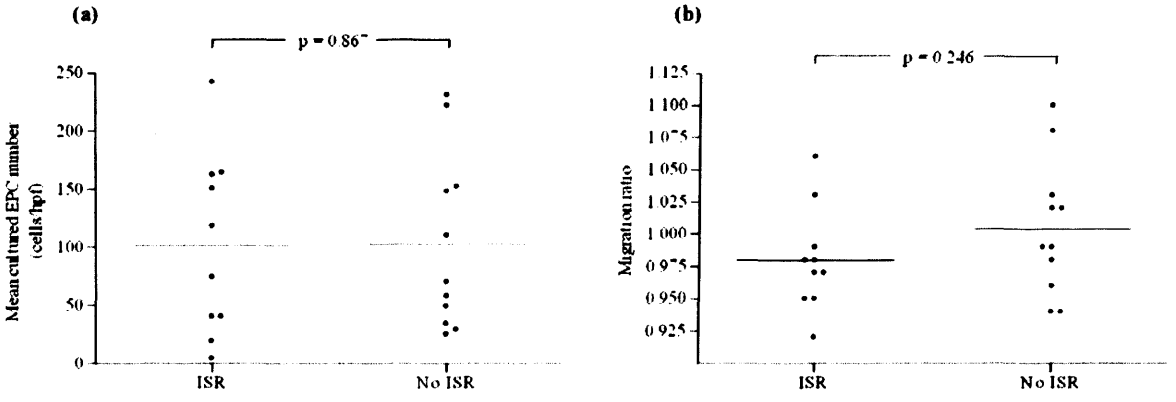
Subjects without ISR had borderline significantly higher circulating median $AC133^{+}VEGFR\text{-}2^{+}$ cells than subjects with ISR (p = 0.051) [Figure 4-2]. There were no significant differences in circulating $CD34^{+}VEGFR\text{-}2^{+}$ cell number, mean cultured EPC number, or EPC migration ratio between subjects with and without ISR [Figures 4-2 & 4-3].

Figure 4-2. Circulating EPC numbers in ISR cohort



Bars represent median number of circulating EPCs. ISR – in stent restenosis

Figure 4-3. Cultured EPC numbers and EPC migration ratio in ISR cohort



Bars represent mean number of cultured EPCs, graph (a), and mean migration ratio, graph (b)

4.2.5 Serum VEGF and SDF-1α concentrations, and EPC number and function

There was no significant difference in the mean serum VEGF concentration between subjects with and without ISR (33 ± 38 vs 44 ± 67pmol/L, respectively; p = 0.675). There were no significant associations between serum VEGF concentration and either $CD34^{+}VEGFR\text{-}2^{+}$ cells, $AC133^{+}VEGFR\text{-}2^{+}$ cells, cultured EPCs, or EPC migration ratio for subjects with or without ISR. Serum SDF-1α was undetectable in 80% of subjects with ISR and 83% of subjects with no ISR, showing no significant difference.

4.3 Coronary collateralisation cohort

4.3.1 Subject identification and recruitment

The initial plan was to identify potential study group participants from the Glenfield Hospital Cardiovascular Audit Database from Jan 2004 to Dec 2005. From this search, 77 subjects from 2004 and 72 subjects from 2005 were identified as having a chronic total occlusion (CTO) of a major epicardial coronary artery, and did not have diabetes. However, the database could not differentiate which subjects had subsequently undergone percutaneous intervention (PCI) or coronary artery bypass grafting (CABG) for their CTO. Review of angiograms and notes of 25 of these subjects revealed that the majority had undergone attempted or successful PCI or CABG, thus it was felt that these were not then suitable for recruitment, due to difficulties in subject matching. This method of subject identification was then abandoned and potential subjects were prospectively identified from the daily elective coronary angiography lists performed at the Glenfield Hospital. Subjects who had developed a chronic total occlusion (CTO) of one major epicardial coronary artery (i.e. left anterior descending artery, circumflex artery, or right coronary artery) were considered. Extent of coronary collateralisation was determined by review of the angiogram pictures and graded using the validated Rentrop Scoring System [Table 4-1].[214] Using this method 66 potentially suitable subjects were identified. 2 of these subjects subsequently were discounted as they met an exclusion criterion (1 with diabetes mellitus, 1 with malignancy). Invitation letters and study details were sent to the remaining 64 individuals. A total of 39 subjects were willing to participate and were therefore recruited. The demographic details of the subjects are shown in table 4-3. Due to the small participant numbers, subjects with collateral Rentrop score 0 and 1 were grouped together. Within this cohort, 4 subjects were of South Asian / Indian origin (0 Rentrop score 0-1, 2 in each of Rentrop groups 2 and 3), whilst the remaining 35 were UK Caucasians. Details

of the medication taken by one subject from the collateralisation study group are missing due to the subject not providing the information.

Table 4-3. Demographic details of coronary collateralisation subjects.

	Rentrop score 0-1 (n=11)	Rentrop score 2 (n=18)	Rentrop score 3 (n=10)	p value
Male gender (%)	10 (91)	16 (89)	9 (90)	1.000
Age, years	66 ± 6	65 ± 10	68 ± 8	0.680
BMI, kg/m^2	28.6 ± 2.8	27.3 ± 2.2	29.3 ± 3.6	0.174
Total cholesterol,	4.6 ± 1.2	4.7 ± 1.3	4.0 ± 0.7	0.292
mmol/l	(n=10)	(n=18)	(n=10)	
Hypertension (%)	5 (46)	7 (39)	8 (80)	0.113
Hypercholesterolaemia (%)	7 (64)	13 (72)	8 (80)	0.817
Cigarette smoking				
Ever smoker (%)	4 (36)	7 (39)	5 (50)	0.838
Current smoker (%)	3 (27)	3 (17)	0 (0)	0.286
FH CAD 1st degree relative (%)	1 (9)	12 (67)	4 (40)	0.008
Aspirin (%)	10 (100)	16 (89)	9 (90)	0.787
Statin (%)	10 (100)	17 (94)	10 (100)	1.000
ACE-Inhibitor (%)	3 (33)	7 (39)	4 (40)	1.000
CTO vessel (%)				
Right coronary artery (%)	3 (27)	13 (72)	6 (60)	
Left anterior descending artery (%)	6 (55)	5 (28)	3 (30)	0.092
Left circumflex artery (%)	2 (18)	0 (0)	1 (10)	

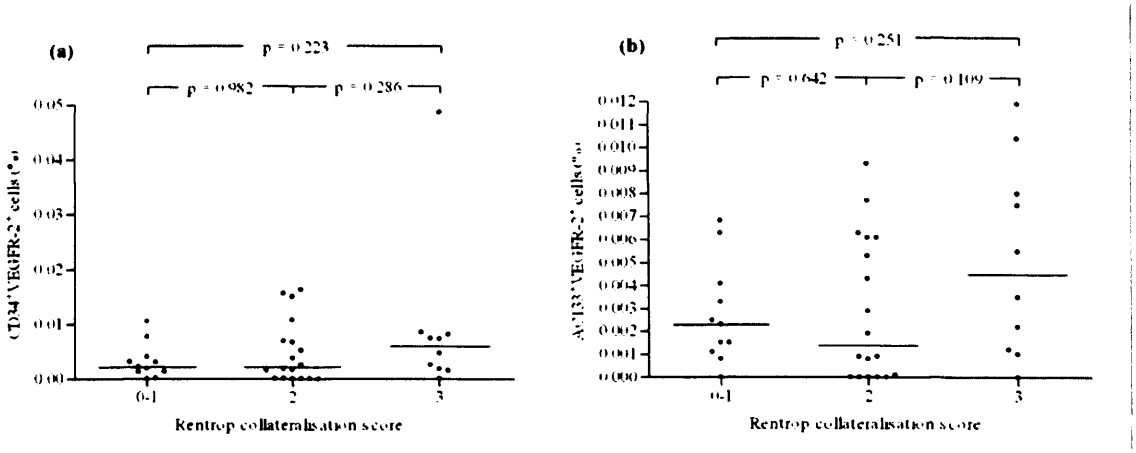
BMI – body mass index; HDL – high-density lipoprotein; FH – family history; CAD – coronary artery disease; CTO – chronic total occlusion

4.3.2 Distribution of EPCs in coronary collateralisation subjects

There was wide variation in the numbers of circulating and cultured EPCs in the subjects' comprising the coronary collateralisation group [Figures 4-4 & 4-5]. There was a significant positive skew in the distribution of both types of circulating EPC, which could not be overcome by mathematical transformation therefore non-parametric tests were employed. There were no correlations between the number of circulating CD34$^+$VEGFR-

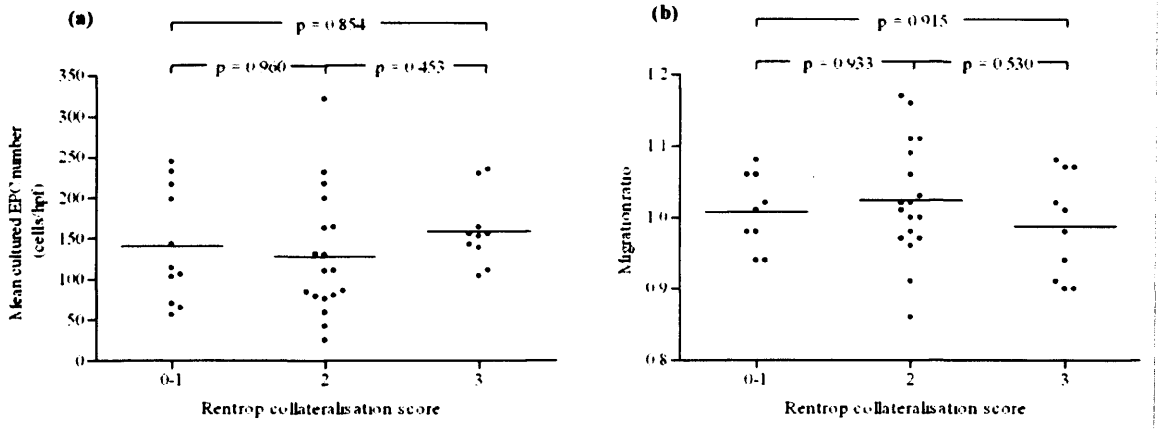
2^+ cells and AC133$^+$VEGFR-2^+ cells, or between the number of either type of circulating EPC and the number of cultured EPCs.

Figure 4-4. Distribution of circulating EPCs by collateralisation score



Bars represent median number of circulating EPCs

Figure 4-5. Distribution of cultured EPCs and EPC migration ratio by collateralisation score



Bars represent mean number of cultured EPCs, graph (a), and mean migration ratio, graph (b)

4.3.3 The effect of demographic variables on EPC number and migratory function

There were no significant associations between either type of circulating EPC and any demographic variable. Subjects with a family history of CAD in a first-degree relative had significantly lower mean number of cultured EPCs than those without (111 ± 55 vs 162 ± 68 cells/hpf, p = 0.017).

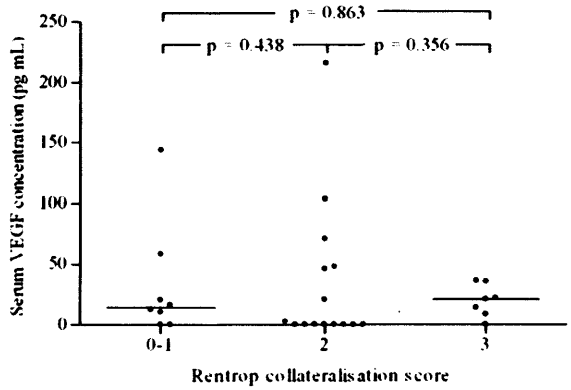
4.3.4 Comparison of EPC numbers and EPC migration ratio by collateralisation group

There was no significant difference in the median number of either $CD34^+VEGFR\text{-}2^+$ or $AC133^+VEGFR\text{-}2^+$ circulating cells when subjects were separated by collateralisation score [Figure 4-4]. In addition, there was no significant difference in the mean number of cultured EPCs, or EPC migration ratio towards VEGF when subjects were separated by collateralisation score [Figure 4-5].

4.3.5 Serum VEGF and SDF-1α concentrations and EPC number and function

Serum VEGF was undetectable in 18 (46%) of subjects in total. When separated by collateralisation score, VEGF was undetectable in 36% of subjects with Rentrop grade 0-1 collaterals, 61% of subjects with Rentrop grade 2 collaterals, and 30% of subjects with Rentrop grade 3 collaterals ($p = 0.248$). Serum SDF-1α was undetectable in 25 (64%) of subjects in total. When separated by collateralisation score, SDF-1α was undetectable in 73% of subjects with Rentrop grade 0-1 collaterals, 61% of subjects with Rentrop grade 2 collaterals, and 60% of subjects with Rentrop grade 3 collaterals ($p = 0.831$). There was no significant difference in median serum VEGF concentration when subjects were separated by Rentrop collateralisation grade [Figure 4-6]. Median comparison could not be performed for SDF-1α as the median concentration was 0 pg/mL in all three groups. There were no correlations between serum VEGF or SDF-1α concentrations and the number of either type of circulating EPC, cultured EPC number, or EPC migration ratio.

Figure 4-6. Distribution of serum VEGF concentration by collateralisation score



Bars represent median VEGF concentration. Where bar is not seen, median concentration is 0 and is therefore obscured by x-axis.

4.4 Chapter discussion

Subjects recruited into the coronary ISR and collateralisation study groups overall were well matched both for demographic variables and the use of cardiovascular medications, allowing direct statistical comparisons to be made. The most interesting result from this section of the work was that subjects who had not developed coronary ISR following bare metal stenting had higher numbers of circulating $AC133^{+}VEGFR\text{-}2^{+}$ cells than those with ISR. Although this showed only marginal statistical significance ($p=0.051$), this may be due to the small number of subjects in this cohort. Unfortunately, there is no published data regarding circulating EPCs in ISR, as identified by flow cytometry, available for comparison. Until this work is replicated the reliability of my results cannot be verified. Histopathological examination of coronary atherectomy specimens has revealed that $CD34^{+}$ and $AC133^{+}$ cells form a small percentage of the hypercellular matrix found in ISR lesions, and this proportion is higher than that seen in primary atherosclerotic lesions.[215] This suggests that the recruitment and integration of such cells is increased in response to coronary stenting, however does not clarify whether EPCs enhance endothelial healing or contribute to the neointimal formation archetypal of ISR. Another point to consider is that, in my study, EPC numbers and function were studied several weeks after the subjects had undergone coronary angioplasty and stenting. As the experiments were performed at a time distant from the procedure, it is likely that each individual's intrinsic EPC number and function was measured, rather than their response to angioplasty and stenting. This is supported by work on $CD34^{+}VEGFR\text{-}2^{+}$ cells, which have been shown to rise acutely following coronary angioplasty, with a peak on day 7, before returning to baseline.[216] In addition, elevation in circulating EPC numbers following vascular trauma induced by CABG or a burn injury is marked but transient, with levels returning to baseline within 72 hours.[125] This could be further clarified in a larger, prospective study with measurement of EPCs both prior to stenting, and then at 3- and 7-days and 1-month (and possibly longer) post-stenting in each subject.

The lack of difference between the groups with regard to $CD34^{+}VEGFR\text{-}2^{+}$ EPCs could suggest that each specific cell type performs a different role in the pathophysiology of endothelial repair following coronary stenting. For example, one type may be important in the initiation of endothelialisation of the stent, whereas the other may be vital to maintain this process. By measuring the circulating EPCs several weeks after angioplasty any early difference in EPCs between the study groups could have been missed, which may explain the lack of difference in $CD34^{+}VEGFR\text{-}2^{+}$ EPCs. Indeed previous work has shown that

acute change in the number of circulating $CD34^{+}VEGFR\text{-}2^{+}$ EPCs after angioplasty is higher in subjects who develop ISR than those who do not.[216] The lack of difference in the number of cultured EPCs in the present work is in line with the some of the previous studies examining the role of EPCs in ISR that have been published to date. There are only a few such studies published, which have reported mixed results on the number and function of EPCs and propensity to ISR.[216-218] In one comparititve study of 16 subjects with ISR and 11 subjects without, no difference in the number of EPCs was seen between the groups (as determined by culture methods).[217] However, when comparing subjects with either focal or diffuse ISR, a difference was seen.[217] In this particular study, by George *et al*, although similar culture and staining conditions were employed different antibodies were used to identify the cells on flow cytometry.[217] Again this raises the issue of EPC identification methods discussed in section 3.21. In a separate study, the colony-forming ability of EPCs *in vitro* was reduced in 16 subjects with ISR compared to 30 subjects without, showing borderline significance.[218] It would also appear from my work that a difference in tendency to develop coronary ISR is not due to impairment of VEGF-mediated EPC mobilisation. Again, no similar studies are available for comparison. The only other study of EPC function has suggested that EPC adherence to fibronectin is impaired in subjects with ISR, whereas adherence to an endothelial monolayer is not, which may reflect variations in integrin expression or function.[217] This is potentially important as the endothelial monolayer is denuded by coronary angioplasty and stenting, and the extracellular matrix (ECM) provides the surface for re-endothelialisation. An extension of my work with characterisation of the integrins involved in EPC adherence would be very interesting.

It is important to note that in the subjects classified as non-ISR on the basis of complete alleviation of anginal symptoms following angioplasty and stenting, ISR that is not causing significant obstruction to coronary blood flow may not cause recurrent angina. Therefore, some subjects may have a scientifically important degree of ISR (possibly up to 50-60% of luminal diameter) that is asymptomatic, and therefore such subjects would be incorrectly classified as non-ISR. Angiographic evidence of ISR, which was available for a proportion but not all of the study subjects, represents a more robust measure of the presence or absence of ISR independent of symptoms. This may explain the lack of difference seen between the ISR and non-ISR subjects in this work.

An interesting finding from this section is that subjects with a history of hypertension had significantly fewer cultured EPCs than those without, however demonstrated a trend

towards better migratory capacity. This could suggest that hypertension leads to an up-regulation in one or more of the VEGF mediated mechanisms involved in EPC mobilisation or homing, whilst also leading to an increase in endothelial turnover at sites of turbulent blood flow and abnormal shear stress. In this situation, EPCs would be able to mobilise to the area of endothelial injury appropriately and in doing so, may deplete the bone marrow capacity due to the increased demand. An association between hypertension and a reduction in EPC number has been demonstrated in both rat and human studies.[156 219] However, contrary to this hypothesis, one study has reported that EPCs from subjects with hypertension demonstrate impaired migration towards VEGF *in vitro*.[106] Overall data on this subject is relatively scarce and warrants further evaluation in larger numbers of hypertensive subjects.

A comprehensive understanding of the pathophysiology of ISR, including the cellular mechanisms of efficient re-endothelialisation is essential if we are to develop screening tests or improved methods for reducing coronary ISR. If subjects with higher levels of circulating $AC133^{+}VEGFR\text{-}2^{+}$ EPCs truly have a reduced propensity of developing ISR, as my results suggest, then pre-procedural measurement of $AC133^{+}VEGFR\text{-}2^{+}$ cell numbers could assist in prediction of ISR, and thus aid in the choice for preferred management of obstructive CAD.

Subjects in the collateralisation cohort were well matched for demographic variables. The results of this work show no evidence to support my hypothesis that either circulating EPCs, cultured EPCs, or EPC migration towards VEGF influences the degree of inter-individual coronary collateralisation. This may in some part be due to relatively small number of subjects in each group. In addition, although the scoring of coronary collateralisation on coronary angiography images is an accepted method,[214 220] it is somewhat subjective, and improvements in non-invasive imaging[221 222-224]and invasive measurement of collateral fractional flow reserve,[124 225-227] used individually or in combination, are likely to represent more objective and potentially more sensitive techniques. Indeed, Lambiase et al reported a strong positive correlation between coronary flow index (CFI) and numbers of circulating $CD34^{+}AC133^{+}$ cells, and a significantly reduced number of EPCs in those subjects with inadequate CFI.[124] This is an important issue as the majority of my subjects were scored as Rentrop grade 2 collaterals. It may be that some of these subjects would have been more accurately classified if a more

sophisticated collateral assessment method were employed. This is a vital consideration for any future study of EPCs in coronary collateralisation.

Coronary collateralisation is reported to be stimulated by recurrent or ongoing myocardial ischaemia.[44 208 228] As both VEGF and SDF-1α are mediated by an ischaemic stimulus, one may expect to see higher serum concentrations of these cytokines in subjects who had poor coronary collateralisation (i.e. greater ischaemia) compared to those subjects with good collateralisation (i.e. less ischaemia). However, this was not the case in this work, and is in support of a previous study of coronary collateralisation.[124] The lack of any correlation between the systemic concentrations of serum VEGF or SDF-1α, and EPC number or function is surprising given that previous reports indicate that each of these cytokines play a central role in EPC mobilisation and homing. This may suggest that either the ischaemic stimulus for coronary collateralisation is not mediated by systemic concentrations of VEGF or SDF-1α, but rather is due to local gradients of angiogenic cytokines, or that myocardial neovascularisation is more strongly mediated by alternative cytokines/factors.

The number of subjects successfully recruited for this part of the work was smaller than anticipated. In the in-stent restenosis cohort, this can be partially explained by the following factors. Firstly, coronary in-stent restenosis (ISR) occurs more frequently in individuals with diabetes[205] who were excluded from this study. Secondly, the use of drug-eluting stents (DES) has grown considerably over the time from when the study protocol was described (BCIS Audit data; www.bcis.org.uk), and DES are associated with a reduced incidence of ISR.[189-196 229-232] In addition, rapamycin, which is one of the pharmacological agents used on DES has been shown to affect EPC number and function.[152] Because of these factors it would not be appropriate to pool together, or compare, subjects who had received a DES with those who had received a bare metal stent (BMS), therefore only subjects with BMS were recruited into the study. Thirdly, it has become increasingly common practice to perform multiple stenting in 1 or more coronary arteries during the same procedure, often using a mixture of DES and BMS. Consequently, subjects may develop ISR in one stent in one vessel, but not in a second stent, sometimes even in the same vessel, making it extremely difficult to match subjects. Despite this limitation, this work on the role of EPCs in coronary ISR and collateralisation has raised some interesting points, which cannot be conclusively answered by the existing published data, suggesting that further research in this field would be interesting and worthwhile.

Chapter 5

Leukocyte telomere length, CAD risk, and EPCs: an exploratory analysis

5.1 Introduction

Ageing correlates strongly with cardiovascular risk. Although, traditionally age is measured chronologically, individuals who have clustering of cardiovascular risk factors, often seem older than their years and develop age-related diseases prematurely. This observation led to the concept of biological aging, whereby the tissues of the body 'age' sooner that might be expected for the number of years. Research has shown that cells of individuals who have premature age-related disease contain shorter telomeres and have a reduced replicative capacity than healthy controls, which could indicate that the body is less able to repair itself. Telomere length can therefore be used as a marker of biological aging in humans. Telomere length shortens with each mitotic division and this is a vital characteristic, as beyond a specific telomere length a cell cannot maintain its ability to undergo cellular replication, at which time it enters replicative senescence and may undergo apoptosis.[155 156 164] Previous work has suggested that an individual's telomere length is genetically determined[233] and is equal in all somatic cells within each individual.[156] Telomere length in leukocytes inversely correlates with the grade of atherosclerosis in human arteries, especially at site of high shear stress,[158 159 234] where endothelial cell turnover is increased, and repair is thought to be performed by EPCs. EPC number and function are said to be adversely affected by increasing age, which could affect reparative capacity.[106 235] Indeed, in younger patients with stable CAD who undergo CABG, the number of circulating EPCs increases, whereas the opposite is seen in older subjects.[235] This could suggest that older individuals cannot mount an effective reparative response due to impaired cellular replicative capacity. The propensity to generate sufficient numbers of active EPCs into the circulation during times of increased need could also be impaired if an individuals telomere length is inherently short. This hypothesis forms the basis of the present exploratory analysis into the relationship between telomere length, and EPC number and function, the results of which are described in this chapter. As EPCs are reported to arise from a leukocyte precursor cell, it seemed reasonable to work on the basis that the telomere length in circulating leukocytes would be representative of the telomere length in EPCs. This theory is reviewed in greater detail during the discussion at the end of this chapter. The methods that were used to determine leukocyte telomere length in this work are provided in chapter 2. Telomere length was normally distributed in all of the parent-offspring subjects, but was non-normally distributed in the coronary ISR and collateralisation groups. 10% of the telomere assays were repeated to form an internal

control measure of reproducibility. This showed strong positive correlation (R = 0.713, p = 0.001).

5.2 Telomere length and EPCs in parent and offspring subjects

5.2.1 Mean leukocyte telomere length and demographic variables

Distribution of mean leukocyte telomere length is shown in figure 5-1. There was a significant inverse correlation between telomere length and age (R = -0.590, p = 0.003) in subjects with premature CAD. In healthy parent subjects a significant positive correlation was seen between BMI and telomere length (R = 0.479, p = 0.021).

In the offspring subjects, there was a significant inverse correlation between telomere length and age when all offspring subjects were analysed together (R = -0.302, p = 0.041) [Figure 5-2]. Separately, there was a trend towards an inverse correlation between age and telomere length in healthy GRAPHIC offspring (R = -0.390, p = 0.066), but no correlation in offspring of subjects with CAD (R = 0.003, p = 0.991). There was no correlation between telomere length and BMI in the offspring. There was no difference in the telomere length, in any of the offspring cohorts, when subjects were separated by gender or smoking.

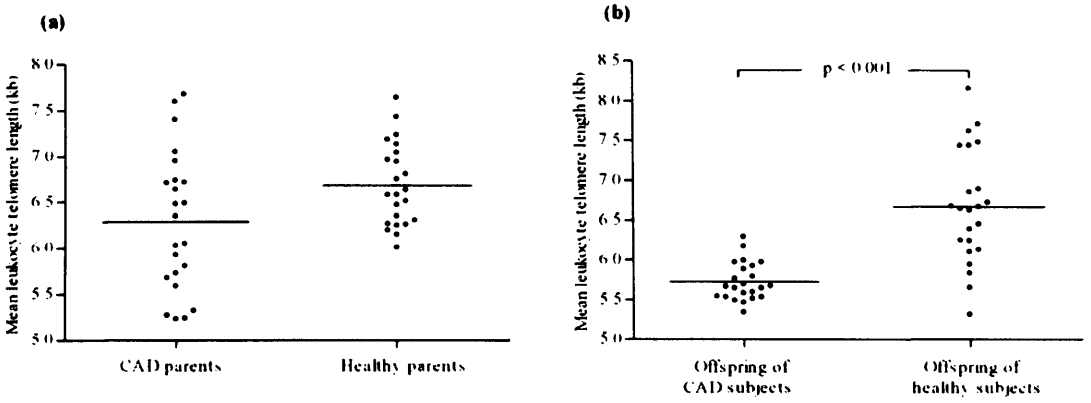
5.2.2 Correlation in mean leukocyte telomere length between parents and offspring

There was significant correlation in telomere length between healthy subjects and their offspring (R = 0.524, p = 0.01) [Figure 5-3]. On linear regression analysis including offspring age, BMI, gender, and smoking, parental telomere length was the only predictor of offspring telomere length (p = 0.01) in the healthy cohort. There was no correlation between parents with premature CAD and their offspring (R = -0.126, p = 0.567). There were no correlations between unrelated subject groups.

5.2.3 Comparison of mean leukocyte telomere length between offspring subjects

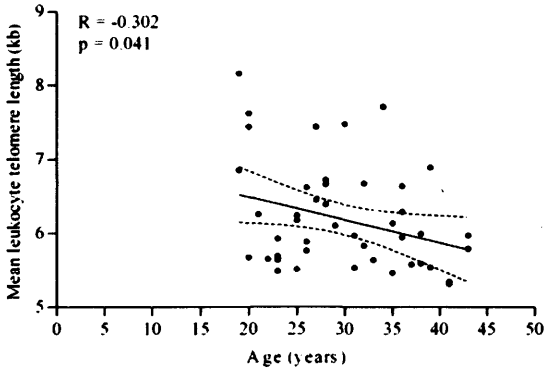
Distribution of mean leukocyte telomere length is shown in figure 5-1. Offspring of healthy subjects had significantly greater telomere length than offspring of subjects with premature CAD, both on unadjusted analysis, and after adjustment for age, BMI, gender, and smoking (6.7 ± 0.7 kb vs 5.7 ± 0.2 kb, $p < 0.001$).

Figure 5-1. Distribution of mean leukocyte telomere length in parent and offspring subjects



Graph (a) shows data for parent subjects; Graph (b) shows data for offspring subjects. Bars represent mean values; p-values shown refer to adjusted analyses. Kb - kilobases

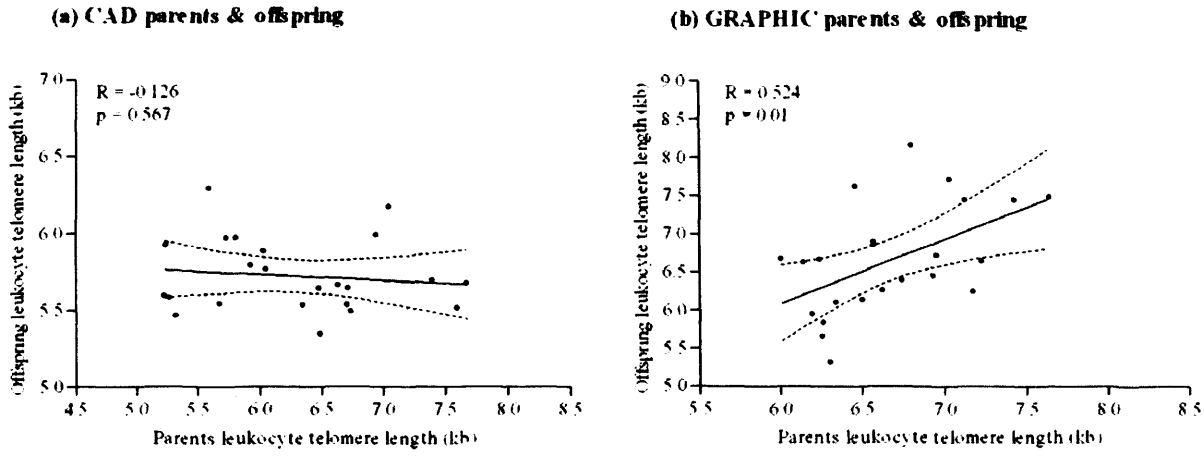
Figure 5-2. Inverse correlation between age and mean leukocyte telomere length in offspring subjects



5.2.4 Correlation between leukocyte telomere length and EPC number and function

There was a borderline significant inverse correlation between telomere length and circulating $CD34^+VEGFR\text{-}2^+$ cell number in parents with premature CAD (R = -0.413, p = 0.05). There was a significant inverse correlation between telomere length and cultured EPC number in the offspring of healthy subjects (R = -0.518, p = 0.011). Aside from this, there were no other significant correlations between telomere length and circulating $CD34^+VEGFR\text{-}2^+$ or $AC133^+VEGFR\text{-}2^+$ cell numbers, cultured EPC numbers, or EPC migration ratio.

Figure 5-3. Correlation in mean leukocyte telomere length between parents and their offspring.



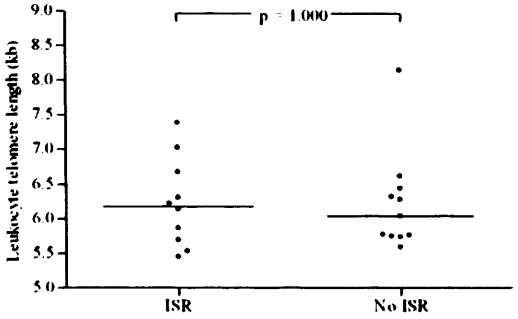
Panel (a) shows data for subjects with CAD and their offspring, and (b) healthy subjects and offspring, Kb - kilobases

5.3 Telomere length and EPCs, in coronary in-stent restenosis and collateralisation

There was no difference in mean telomere length between subjects with ISR and those without (6.2 ± 0.6 vs 6.0 ± 0.7, p = 1.000) [Figure 5-4]. There was a trend towards an inverse correlation between telomere length and circulating $CD34^+VEGFR\text{-}2^+$ cell number, which did not reach statistical significance (R = -0.429, p = 0.052). Aside from this, there were no other significant correlations between telomere length and circulating $CD34^+VEGFR\text{-}2^+$ or $AC133^+VEGFR\text{-}2^+$ cell numbers, cultured EPC numbers, or EPC migration ratio.

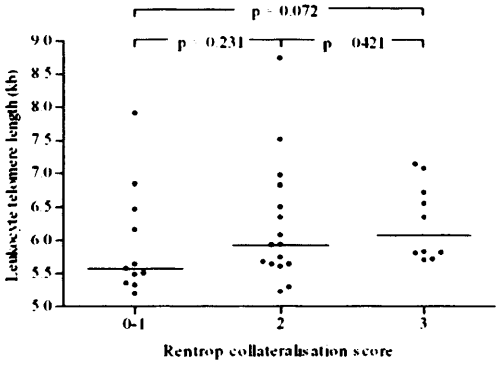
There was suggestion that mean leukocyte telomere length correlated with extent of collateralisation [Figure 5-5], with a trend towards subjects with Rentrop grade 3 coronary collateralisation having longer telomeres than subjects with Rentrop grade 1 collateralisation (6.1 ± 0.6 vs 5.6 ± 0.8, p = 0.072). There were no significant correlations between telomere length and circulating $CD34^+VEGFR\text{-}2^+$ or $AC133^+VEGFR\text{-}2^+$ cell numbers, cultured EPC numbers, or EPC migration ratio.

Figure 5-4. Comparison of mean leukocyte telomere length in ISR subjects



Bars represent median values. ISR – in stent restenosis

Figure 5-5. Mean leukocyte telomere length by collateralisation score



Bars represent median values

5.4 Chapter discussion

The age-related attrition in telomere length seen in both the parental and offspring cohorts is consistent with previously published data[158 163 164 233 236 237] and validates my experimental methods. In the healthy GRAPHIC parent group alone, this inverse relationship is not seen, and this could be explained by the fact that this cohort predominantly consists of women. It has been reported that oestrogens enhance telomerase activity, thus affording a slower rate of telomere attrition with ageing.[236 238 239]

The association between shorter telomeres and premature CAD has been shown in previous well-designed case-control studies.[161-163] In addition, there is evidence that individuals with CAD have reduced numbers of EPCs.[60 106 117 123] Given that EPCs are thought to arise from the common haemangioblast, the same precursor cell as circulating leukocytes, it was thought that any correlation between telomere length and EPCs could be shown by measuring telomere length in all leukocytes. No robust correlation between telomere length and EPCs were seen in this work. It is possible that because EPCs form

such a very small proportion of the circulating leukocyte population, the method employed to identify a correlation between EPCs and telomere length was too crude. Ideally, it would seem to be more appropriate for telomere length to be determined in EPCs directly. This is technically difficult when using a Southern blotting technique to measure telomere length, as it would require a large volume of blood from each subject in order to isolate enough EPCs to obtain sufficient quantities of DNA. One potential approach to enable specific identification and harvesting of a sufficient number of EPCs would be to isolate EPCs from the circulating blood pool by apharesis following cell-surface receptor antibody labelling. Other options would be to employ alternative assays for measurement of telomere length such as fluorescent in-situ hybridisation or real-time PCR techniques, which require significantly less DNA and can therefore be performed on much smaller numbers of cells.[240]

An interesting finding from this exploratory analysis was that of significantly shorter telomeres in the offspring of subjects with CAD compared to their healthy counterparts. This presents exciting and important implications, with at least 2 possible explanations. Firstly, this finding may indicate that each person has a genetically predetermined telomere length in all somatic cells. Thus, individuals with shorter telomeres are more susceptible to age-related conditions including CAD, and pass on this trait to their offspring, so rendering them also susceptible to age-related conditions.[233 241] Secondly, it may be that offspring subjects with a strong parental history of CAD have an increased predisposition to endothelial dysfunction and premature atherosclerosis, which is associated with increased endothelial and white blood cell turnover and therefore a higher rate of telomere attrition than subjects who have no heritable tendency towards early atherosclerosis.[158 159 234] The increased levels of circulating EPCs in offspring of subjects with premature CAD (chapter 3) supports this theory of endothelial activation and accelerated cell turnover in my cohort and this has been discussed in detail in chapter 3. This exploratory work by itself cannot answer whether this is a cause or effect association. In either case, measurement of telomere length may represent a biological marker of cardiovascular risk that can be easily and reliably determined, and could be applied to identify healthy young adults who could be targeted for aggressive lifestyle and risk factor modification as primary prevention of future atherosclerotic disease.

The finding of the longest telomeres in both the healthy GRAPHIC parents and their offspring, and the positive correlation in telomere length between these parents and

offspring supports a genetic contribution to telomere length, which is consistent with previously published data in twins.[233 241] The absence of correlation between parents with CAD and their offspring is interesting, and could be explained by the proposed X-linked mode of telomere inheritance.[239] Nawrot *et al* found no correlation in telomere length between fathers and sons, yet strong correlations between mothers and their sons and daughters, and between fathers and daughters.[239] In my study, the premature CAD cohort comprised 14 father-son pairings, 9 father-daughter pairings, and 2 each of mother-son and mother-daughter pairings. When analysed separately there was no correlation between fathers and sons ($R = 0.306$, $p = 0.390$) or fathers and daughters ($R = -0.254$, $p = 0.510$). There were insufficient numbers to generate meaningful correlations in the mother-offspring pairings. Whereas, in the healthy GRAPHIC cohort, there were 0 father-son pairings, 1 father-daughter pairing, 15 mother-daughter pairings, and 8 mother-son pairings, with positive correlations between mothers and daughters ($R = 0.512$, $p = 0.051$) and mothers and sons ($R = 0.758$, $p = 0.048$). No correlations were seen between any unrelated subjects. These results are in support of the previously proposed X-linked inheritance pattern of telomere length.

The influence that telomere length exerts in the development of coronary in-stent restenosis (ISR) and collateralisation cannot be determined from this work. As described in chapter 4, vascular smooth muscle cells (VSMC's) play a pivotal role in the neointimal hyperplasia that is characteristic of ISR[199 200] as well as in the development and maturation of functional collaterals.[242] Previous studies have shown that enhanced telomerase activity, which is generally associated with maintenance of telomere length and prevention of cellular senescence, promotes VSMC proliferation.[243 244] Although, telomerase activity was not measured in this work there was a suggestion that subjects with more extensive collateralisation had longer telomeres, which could potentially be due to better VSMC proliferative capacity. Overall, these results cannot be taken as conclusive evidence, especially as this work was performed on a relatively small cohort. However, with regard to ISR and coronary collateralisation, the results are novel and only a small amount of information currently exists in this area suggesting that further work is required.

Telomere biology has expanded over recent years and currently attracts enormous research interest across all fields of human (patho)physiology. Differences in telomere size and abnormalities of telomere function hold great promise in explaining age-related human conditions including CAD. As telomere biology and its interaction with such conditions

becomes better understood, therapeutic options in this area become a realistic possibility, which could significantly alter, and potentially delay, the development of age-related conditions in humans.

Chapter 6 Discussion

6.1 Limitations of this work

This work was diligently performed and attempts were made to accurately replicate the methods for each subject. This included the time at which subjects attended for their appointments, the way in which venepuncture was performed and the laboratory methods were carried out. There are however, limitations to this work that should be considered. The amount of exercise taken by each subject was not obtained during the recruitment interview. This could be important, as exercise has been reported to influence EPC number in both healthy subjects and those with established CAD.[108-114]

The exclusion of subjects with diabetes mellitus was done to try to minimise the patient factors that are known to affect EPC number and function, to allow for an assessment of the factors under investigation e.g. heritability. Moreover, it would not have been possible to recruit a healthy parental group of subjects for the heritability work, if subjects with diabetes were included. Inclusion of CAD parental subjects with type II diabetes mellitus could have implications on EPC number and function in the offspring generation, given the known complex heritability of type II diabetes.[245-247] However, it is important to note that although a formal clinical diagnosis of diabetes mellitus is made on set criteria, impaired glucose tolerance and insulin resistance are a continuous spectrum and can be abnormal before diabetes becomes overt. From this work the degree of glucose intolerance and insulin resistance was not quantified in the study subjects. Both of these factors have been shown to affect endothelial function,[248-250] and therefore potentially could affect EPC number and / or function. Future studies should include quantification of fasting serum glucose and insulin resistance in all subjects.

A second factor to consider is the effect of shared environment. This was addressed in the chapter 3 discussion. This work has demonstrated a correlation in EPC number between parents and their offspring, which suggests a genetic contribution. As almost all of the offspring did not share their current environment with their parents this was not felt to be a likely confounding factor. However, the effect of prior shared environment, whilst the offspring were growing up could potentially be important. As suggested in chapter 3, further studies involving either twins, or unrelated subjects who share an environment (e.g. spouses) could shed more light on this issue.

An additional point that should be considered when undertaking a genetic study, is that of survivor bias. Whilst all of the parent subjects recruited for this work had evidence of severe premature CAD, all had survived to middle age. Therefore, there may be a group of subjects who carry genetic traits associated with more severe phenotype of CAD, which leads to premature death. In that situation, such subjects may be under-represented in this study population, and their inclusion could have influenced the findings. To address this, a study of families with severe CAD, with affected parent subjects taken from representative age-groups e.g. 21-30 years, 31-40 years, 41-50 years etc may help to answer this issue.

The association between EPC number and function and endothelial function could not be fully addressed from this work. The decision to measure plasma vWF activity was taken after the significant difference in EPC number was seen between the offspring groups. Recalling subjects to perform additional measurements of endothelial function, such as flow-mediated dilatation, was not feasible but may have facilitated a clearer understanding of the relationship between EPCs and endothelial function in this work. Another blood derived marker that could be measured as a marker of endothelial damage would be endothelial microparticles, which are released into the circulation following cell membrane activation processes and apoptosis.

The measurement of systemic levels of circulating VEGF and SDF-1α as an indicator of the degree of chemical signalling associated with the homing and / or mobilisation of EPC is unlikely to accurately represent the actual mechanisms behind this process. With recent research developments leading to a more intricate understanding of EPC mobilisation and homing, measurement of chemokines local to the area of endothelial injury, may represent a superior method for future studies. A second consideration would be as to whether ELISA is a sensitive enough method to detect minor changes in concentrations of chemokines. In many subjects in this work, SDF-1α was undetectable despite using a sensitive ELISA kit, and in those whom it was detectable, the range in levels was very large. Other methods such as radiolabeling may be considered for future studies.

Finally, whilst the use of the Rentrop classification to grade the extent of coronary collateralisation is an acceptable method and clinically useful, it is based on subjective assessment and quite simple. There are other techniques that may allow a more sensitive measure of collateralisation, which should be considered for any similar studies in the future.

6.2 Future work and clinical application of endothelial progenitor cells

If it can be proven that paucity / exhaustion of EPC numbers and / or impairment of their function predispose an individual to atherosclerosis, EPCs may in the future be used as a reliable tool for risk assessment and diagnosis of CAD. The discovery of a relationship between EPCs and premature CAD within first-degree relatives would suggest a genetic control of EPC capabilities, may provide an explanation for the hereditary component of CAD, and could be utilised as a surrogate marker of atherosclerosis. Furthermore, if it were a hereditary trait this would allow for the identification of family members at risk of future development of CAD and thus allow modification of their risk factors and preventative therapy.

Data has emerged regarding the use of EPCs as prognostic markers in patients with angiographically proven CAD. This has highlighted that subjects with reduced numbers, and impaired colony-forming ability, of peripheral blood EPCs are at greater risk of death from cardiovascular causes, revascularisation, or hospitalisation for a cardiovascular event.[112] Interestingly, no specific association was found between reduced EPC numbers and myocardial infarction.[112] Additionally, circulating EPCs have been shown to rise following acute ischaemia, and therefore the finding of elevated levels in combination with other clinical features could be used to identify subjects at risk of significant myocardial ischaemia.[251] Further studies, with strict end-point measures and involving large numbers of subjects would help to clarify the potential role of EPCs in this area.

Once it had been shown that EPCs were integral in re-endothelialisation and neovascularisation, and could be isolated and expanded from either the bone marrow or peripheral blood, progression to assess whether these cells could be utilised in a therapeutic role was inevitable. Studies to date have evaluated whether EPCs can be utilised to facilitate rapid re-endothelialisation or improve LV function. Methods studied so far have included use of growth factors to mobilise EPCs from the bone marrow, and direct methods whereby EPCs are isolated from patients, expanded *in vitro* and re-injected into the same patient.

Systemic administration of G-CSF as a method of mobilising EPCs was shown to be effective in patients with CAD,[252-254] however there appeared to be an increased risk of MI in one study,[253] and in some cases was not associated with improvements in ventricular function following ST elevation myocardial infarction.[252 255] One trial comparing

intracoronary injection of bone marrow cells with placebo (BOOST trial),[256] and a second comparing intracoronary injection of bone marrow cells with circulating progenitor cells (TOPCARE-AMI)[93] in acute myocardial infarction have shown varying results. Data up to four months follow-up of subjects in TOPCARE-AMI reflect a benefit in post-myocardial remodelling and ventricular function in both the bone marrow and progenitor cell groups. However, although initial rapid improvements were seen in ventricular remodelling with endothelial progenitor cells in the BOOST trial up to six months, this was not sustained at eighteen months of follow-up. Importantly, both trials concluded that intracoronary delivery of bone marrow or progenitor cells in the setting of acute myocardial infarction is safe and feasible. This should pave the way for larger, blinded, randomised trials. A small study of autologous transplantation of bone marrow-derived mononuclear cells in patients with occlusive peripheral arterial disease yielded safe and significant improvements in limb ischaemia.[252] This was thought to be attributable to the presence of $CD34^+$ cells within the mononuclear fraction, however may also be the result of pro-angiogenic cytokines secreted from the transplanted cells. A potential problem with the use of autologous transplantation of EPCs in individuals with significant CAD is that the proliferative capacity and reparative function of these cells are likely to be impaired. In theory, EPCs isolated from younger, healthy subjects would be more likely to be effective when introduced into patients with CAD or LV dysfunction. This theory raises both ethical issues and scientific questions as to whether one individual's cells will function normally when injected into a new host.

Reports of unprompted seeding of EPCs onto the surface of vascular prostheses, including ventricular assist devices, has led to the development of biologically engineered vascular prostheses. Coronary artery stents have been designed that present anti-$CD34^+$ antibodies to the circulating blood, thus capturing circulating progenitor cells as they pass through the stent, and potentially accelerating re-endothelialisation. Again, early trials suggest that these stents are effective with no safety concerns.[257]

6.3 Conclusion

Over the past 10-15 years incredible progress has been made in the understanding of the pathophysiology of atherosclerosis and CAD. In addition, the role of telomeres in biological aging and their function in the development of age-related conditions has become increasingly understood. However, it remains extremely unlikely that any one factor will completely explain an individual's susceptibility to premature cardiovascular

disease. Rather, it is almost certain that complex interaction between inherent (genetic) predispositions, effects of metabolic disturbances (e.g. diabetes mellitus, dyslipidaemia), and exposure to environmental factors (e.g. tobacco smoke) all contribute to the risk in each person. The emergence of the inflammatory theory of atherosclerosis, and the extensive research undertaken to decipher the molecular elements in this process have been paramount in the scientific progress, and have led to the identification of endothelial progenitor cells (EPCs) which appear to have vascular reparative capacity. EPCs have become a focus of intense research interest as they present the potential to be utilised both as diagnostic markers of endothelial dysfunction / atherosclerosis, and hold the promise of a regenerative biological therapy for cardiovascular diseases. Once a comprehensive understanding of EPC biology is obtained, we will hopefully see great innovation in the diagnostic and therapeutic application of these interesting cells.

This present work provides novel data to suggest a strong genetic component to EPC biology in both healthy subjects and those with premature cardiovascular disease (Chapter 3). In addition, this work suggests that predisposition to atherosclerosis, and premature CAD, can be detected in healthy, asymptomatic young adults by measurement of circulating EPC numbers (Chapter 3). This work adds to the wealth of existing knowledge, and emerging genetic studies to improve our understanding of the mechanisms involved in the heritability of atherosclerotic diseases. Equally as important is the finding that *in vitro* culture and flow cytometric methods that are often used independently to quantify EPCs do not appear to be interchangeable, and likely measure subtly different aspects of EPC biology (Chapter 3). It is imperative that consensus on EPCs identification is reached soon to ensure that future work, especially studies involving use of EPCs as therapeutic tools, is performed appropriately.

The role that EPCs play in the development of coronary ISR and collateralisation remains a valid and interesting research question. Results from this work suggest that subjects with higher circulating levels of a more immature type of circulating EPC ($AC133^{+}VEGFR\text{-}2^{+}$) may be less likely to develop coronary ISR following bare metal stenting. This interesting result, if proved to be true through replication studies, could help in patient selection for PCI treatment of CAD and warrants further investigation in larger, well-described cohorts (Chapter 4).

Although the signalling pathways involved in EPC trafficking have been well described *ex vivo*, serum VEGF and SDF-1α levels do not show correlation with the number and / or migratory function of EPCs grown in culture or measured in the peripheral circulation (Chapters 3 and 4). This suggests a more complex interaction between circulating cytokines and EPC activity.

The observation of shorter telomeres in healthy offspring of subjects with premature CAD compared to offspring of healthy subjects is a novel finding from this exploratory work, and provides supporting evidence for the telomere hypothesis of age-related diseases. The positive correlation in telomere length between healthy parents and their offspring suggests telomere length is genetically determined and is in keeping with previous studies. This work failed to identify a link between leukocyte telomere length and circulating EPC number, which may have provided further insight into the heritability of premature CAD. The logical step for future work to investigate this association further would be to determine telomere length in circulating EPCs. Whilst these results were only an exploratory analysis, they suggest that a proportion of risk conferred by a family history of CAD may be attributable to the inheritance of shorter telomeres, and thus impaired cellular reparative capacity.

Although there are some limitations to this work, the interesting results presented in this thesis, some of which are novel, will help to guide future research into EPC and telomere biology in general and in the field of CAD specifically.

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