Molecular mechanisms and modulation of Endothelial Progenitor Cell function in South Asian men

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Submitted in accordance with the requirements for the degree of Doctor of Philosophy

The University of Leeds
School of Medicine
December 2012

The candidate confirms that the work submitted is his own and that appropriate credit has been given where reference has been made to the work of others.

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Acknowledgements

The research included in this thesis is my own, though where the assistance of other investigators has contributed in any manner this is explicitly indicated.

I would like to thank my supervisors, Professor Mark Kearney, Dr. Richard Cubbon and Professor Peter Grant for their insightful guidance, unfaltering support and encouragement throughout my studies. Furthermore, the expertise and assistance offered by Dr Hema Viswambharan and Dr Nadira Yuldasheva has been crucial to the running of my western blot and transfusion studies.

Finally, this project would not have been possible without the financial support of the British Heart Foundation who kindly sponsored my PhD studentship. Next, I would like to express my gratitude to all of the researchers and technicians who have in any way assisted me in developing and running this research project. Without their knowledge, skills and organisation the project would have required many more years to reach this stage! In particular I would like to thank (in no specific order): Dr Stephen Wheatcroft, Dr Piruthivi Sukumar, Dr Matthew Kahn, Mrs Jessica Smith, Dr Mark Rakobowchuk, Dr Helen Imrie, Dr Matthew Gage, Dr Sam Stephen, Dr Kate Gatenby, Dr Amir Aziz, Dr Romana Mughal and Mrs Stacey Galloway. I am also grateful to all of the volunteers who gave up their time in order to participate in the project.

Finally, I am extremely thankful to my family and my best friend Dr Ramachandra for their unfaltering support and guidance.
Abstract

South Asian ethnicity has long been considered a contributory factor in the development of atherosclerosis and cardiovascular disease. Endothelial dysfunction is considered to be the precursor of atherosclerosis, and South Asian ethnicity has been linked to endothelial progenitor cell dysfunction and impaired endothelium dependent vasodilatation. Endothelial progenitor cell (EPC) dysfunction may contribute to ineffective endogenous endothelial repair, although the exact mechanisms remain unclear. This project aimed to validate existing data, and identify signalling pathways and molecular mechanisms that may be involved in EPC dysfunction in healthy South Asian men when compared to matched white European controls. In addition, the study was designed to assess the impact of South Asian ethnicity on EPC function in vivo, and whether modifying the signalling pathways linked with EPC dysfunction improved EPC function.

Early outgrowth EPC numbers were significantly lower in South Asian men. EPC numbers were significantly increased in both South Asian and white European groups by treating EPCs with Simvastatin in vitro. Functional parameters such as migration to VEGF, adhesion to fibronectin and endothelial tubule formation induced by EPC conditioned medium were also impaired in South Asian EPCs. Some aspects of functional impairment were improved by Simvastatin. Late outgrowth EPCs derived from South Asian men demonstrated reduced proliferation and increased senescence. Western blot analysis showed reduced basal Akt and endothelial Nitric Oxide Synthase (eNOS) expression in South Asian late EPCs. Vascular repair of injured conduit vessel following transfusion of South Asian late
EPCs was significantly impaired - this was rescued by increasing Akt activity using Lentiviral delivery of constitutively active Akt. Abnormal phosphotidyl-inositol-3-kinase/Akt signalling may therefore contribute to EPC dysfunction in South Asian men. These data provide novel insights into mechanisms underlying EPC dysfunction which may contribute to increased atherosclerosis burden in people of South Asian origin. Future research avenues have been identified, which may guide development of novel strategies in preventing and treating cardiovascular disease.
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Publications arising from this project


Publications arising during this project


**Abstracts arising during this project**


2. Hema Viswambharan; Helen Imrie; Afroze Abbas; Matthew Gage; Matthew Kahn; Adil Rajwani; Nadira Yuldasheva; Shouhong Xuan; **Vivek Baliga**; Jessica Surr; Richard Cubbon; Stephen Wheatcroft; Mark Kearney Endothelial Cell Insulin Sensitivity and Nitric Oxide Bioavailability Are Regulated by Insulin-Like Growth Factor-1 and Insulin Receptor Levels. *American Heart Association* November 2010 122 (Meeting Abstract Supplement) A17875
Glossary

AcLDL – Acetylated LDL
ACh – Acetyl choline
ApoE – Apolipoprotein E
Akt – Protein kinase B
BH4 – Tetrahydrobiopterin
BMI – Body mass index
BP – Blood pressure
CD – Cluster differentiation marker
CFU – Colony forming unit
CRP – C-reactive protein
CV – Coefficient of variation
CVD – Cardiovascular disease
CXCR4 – Chemokine X receptor-4 (SDF receptor)
Dil - 1, 1’-dioctadecyl-3, 3’, 3’-tetramethylindocarbocyanine perchlorate
DMSO – Di-methyl sulphox ide
EBM – Endothelial basal medium
EDTA – Ethylenediaminetetraacetic acid
EGFP - Enhanced Green Fluorescent Protein
EGM – Endothelial growth medium
ELISA – Enzyme linked immuno-sorbent assay
EMP – Endothelial microparticle
eNOS – Endothelial nitric oxide synthase
EPC – Endothelial progenitor cell
FACS – Fluorescence activated cell sorting
FAD – Flavin adenine dinucleotide
FCS – Foetal calf serum
FGF – Fibroblast growth factor
FITC – Fluorescein isothiocyanate
Flk-1 – Fetal liver kinase-1
FMD – Flow mediated vasodilatation
FMN – Flavin mononucleotide
FOXO – Forkhead transcription factor 12
GTN – Glyceryl trinitrate
HbA1c – Glycosylated haemoglobin
HDL – High-density lipoprotein
HIV - Human Immunodeficiency Virus
HMG CoA – Hydroxy-methyl glutaryl Coenzyme A
HOMA-IR – Homeostasis assessment of insulin resistance
HUVEC – Human umbilical vein endothelial cell
IGF-1 – Insulin-like growth factor-1
IGT – Impaired glucose tolerance
IHD – Ischaemic Heart Disease
IR – Insulin resistance
IRS-1 – Insulin receptor substrate -1
KDR – Kinase domain receptor (or VEGF receptor-2)
LDL – Low-density lipoprotein
L-NMMA – Levo-N-monomethyl arginine
LTR - Long Terminal Repeats
MAP-kinase – Mitogen activated protein kinase
MMP – Matrix metalloproteinase
NADPH – Nicotine adenine dinucleotide phosphate
NE – Norepinephrine
NO – Nitric oxide
NOS – Nitric oxide synthase
NOX – Nicotinamide adenine dinucleotide phosphate oxidase
PBMC – Peripheral blood mononuclear cell
DPBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PI3K - Phosphatidy1 Inositol-3 kinase
ROS – Reactive oxygen species
SA – South Asian
SD – Standard deviation
SDF – Stromal cell-derived factor
SEM – Standard error of mean
SIN - Self Inactivated
SMR – Standardised mortality ratio
TNF – Tumour necrosis factor
UEA-1 - *Ulex Europeus* -1 agglutinin
VEGF – Vascular endothelial growth factor
WE – White European
Chapter One:
General
Introduction
1. General Introduction

Despite advances in medicine and a reduction in cardiovascular mortality in the past 5 years, variable outcomes have been noted between genders and ethnicities.\(^1\) South Asian populations in particular have been demonstrated to exhibit a higher incidence and prevalence of cardiovascular disease (CVD), with studies showing a 50-60% greater death rate from CVD compared to white European males.\(^2\) Given this group forms a significant portion of the population of the United Kingdom, it presents a significant burden on the healthcare system. Conventional risk factors such as smoking and hypertension may play an important role; however non-conventional risk factors may also play a significant part in the pathogenesis of cardiovascular disease in this group. A detailed discussion of this concept follows.

1.1 South Asian ethnicity and Cardiovascular risk

1.1.1 Defining ‘South Asian’ ethnicity

The Indian subcontinent represents one quarter of the developing world’s population, and so the South Asian ethnic group forms one of the largest ethnic groups in the world. Unlike race, whose definition relies heavily on physical attributes such as hair and skin colour, ethnicity is a complex concept that encompasses many components.\(^3\) Numerous factors play an important role in defining ethnicity, including geographic origin, religion, diet and other cultural practices. In short, clearly defining ethnicity is a difficult task, and most published studies define South Asian ethnicity based on geographic location alone i.e. those subjects hailing from India, Pakistan, Nepal, Bangladesh and Sri Lanka. This, however, does not include a homogenous group of individuals as geographic origin alone neglects numerous additional
aspects of ethnicity. Furthermore, within commonly defined ethnic groups exists many subgroups, each with distinct cultural practices. This has been observed in studies that have shown variable prevalence in disease between subgroups of subjects residing in Pakistan.\textsuperscript{2} When put into this perspective, South Asian population groups studied within the UK are likely to exhibit further diversity. Given the wide variability in the definitions of ethnicity in the relevant literature, for the purposes of this discussion, ‘South Asian’ is defined geographically as hailing from India, Pakistan, Nepal, Bangladesh and Sri Lanka unless stated otherwise.

1.1.2 Epidemiology

In 2004, the Health Survey for England observed a high prevalence of cardiovascular disease in South Asian populations, with the highest prevalence seen in Pakistani men (9.1\%) when compared to the general population (7.9\% IHD and stroke combined).\textsuperscript{1} Over a 5 year period, the prevalence had nearly doubled in both these groups, with an increase from 4.8\% to 9.1\% seen in Pakistani men, and 2.3\% to 4.2\% seen in Indian women.\textsuperscript{1} Age-standardised risk ratios demonstrated a similar pattern to these observed percentages. In particular, risk ratios for angina were high in South Asians, particularly Pakistani men (2.85) whose prevalence was significantly greater than the general population (1.0 by definition). Cardiovascular mortality has also been demonstrated to be significantly higher in subjects born in the Indian subcontinent, with values 15-60\% greater than the entire population of England and Wales.\textsuperscript{4,5} In a cross sectional survey assessing heterogeneity of various coronary heart disease risk factors within South Asian population groups, a number of potentially relevant differences were found in the risk factor profile between various groups.\textsuperscript{6} These differences between South Asians and white
Europeans aside, there were also differences noted in economic circumstances and anthropometric measurements between Bangladeshi, Pakistani and Indian groups. Smoking was more prevalent in Bangladeshis, who also had the highest triglyceride and fasting glucose concentrations. However, they were shorter than and had lower blood pressure than Indian and Pakistani men. This led the authors to the conclusion that risk factors between groups are not uniform, and could underlie varying prevalence of coronary artery disease within these groups.

1.1.3 South Asian ethnicity, socio-economic class and health care provision

The relationship between lower socio-economic class and poorer health outcomes has been extensively studied.\(^7\),\(^8\) In India, cardiovascular disease (CVD) was initially considered to primarily affect the affluent class, though this trend seems to have changed since the 1990’s.\(^9\) Over time, it has become apparent that CVD mostly affects the socioeconomically disadvantaged groups.\(^10\) Within the UK, a similar trend has been noticed in migrant South Asian populations in keeping with the changes in India. Recent work by Tillin \textit{et al} demonstrated an increased risk of CVD in South Asians within lower socioeconomic classes, defined by fewer years of education.\(^11\)

In a study conducted to identify issues related to lifestyle risk factors in a focus group of South Asians over the age of 40yrs in Leicester, UK, Farooqi \textit{et al} demonstrated that language barrier played a key role in accessing appropriate healthcare in a clinical setting.\(^12\)

The 1991 UK census data, whilst showing similar proportions of general and South Asians populations in specific social classes, revealed higher all cause mortality and
mortality due to ischemic heart disease in South Asians within comparable classes.\textsuperscript{13} This disproportionality was mostly driven by the higher disease rates in Bangladeshi men. Individual groups’ experiences in the UK health care system have been variable and conflicting in terms of investigations and management of cardiac disease. Earlier work has suggested that South Asians wait longer for referral \textsuperscript{14} and treatment \textsuperscript{15} of ischemic heart disease for reasons that are not very clear. However, the Whitehall II study showed a higher rate of coronary artery disease in South Asians despite appropriate utilisation of investigations and secondary prevention measures.\textsuperscript{16} Despite this conflict, South Asian populations have experienced similar improvements in outcome and a decline in mortality following myocardial infarction when compared to the white European population.\textsuperscript{17}

1.1.4 Pathogenesis

A number of factors may play a role in the higher prevalence of CVD in South Asian populations groups. These are discussed below.

1.1.4.1 South Asian ethnicity, Insulin resistance and Diabetes mellitus

In a study of anthropometric and biochemical data in 3,754 South Asian, Afro-Caribbean and European subjects between 40-69 years of age, McKeigue \textit{et al} found a high prevalence of type 2 diabetes mellitus in the South Asian population groups, with values approaching 20\% compared to 4\% in white European counterparts.\textsuperscript{18} In India, urbanisation is thought to have had a significant impact in the prevalence of type 2 diabetes, with an age-standardised prevalence of 11.2\% in urban population compared to 2.4\% in rural population.\textsuperscript{19} Within the UK, South Asian subjects develop diabetes 10 years earlier than white Europeans.\textsuperscript{20}
The pathogenesis of diabetes mellitus involves both pancreatic β-cell dysfunction and insulin resistance, though the actual prevalence of this may vary between ethnic groups. In South Asians, insulin resistance plays a key role in the pathogenesis of type 2 diabetes, while in Afro-Caribbeans pancreatic β-cell dysfunction may make a larger contribution.

Diabetes has been identified as an independent risk factor for the development of cardiovascular disease. However, numerous additional factors play a role, including smoking, obesity, hypertension, elevated plasma cholesterol and raised triglycerides, all of which are more prevalent in South Asians (see table 1).

Clustering of these risk factors was initially described by McKeigue et al, who identified a relationship between glucose intolerance, hyperinsulinemia and intra-abdominal fat distribution in South Asians. These risk factors put together form the ‘metabolic syndrome’, and previous studies have shown that subjects with metabolic syndrome face a greater risk of all cause and cardiovascular mortality when compared to those without the syndrome.

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<th>White Europeans</th>
<th>UK South asians</th>
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<tr>
<td></td>
<td></td>
<td>Punjabi Sikh</td>
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<tr>
<td>Cigarette Smoking (%)</td>
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<tr>
<td>Systolic BP (mmHg)</td>
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<tr>
<td>Total Cholesterol (mmol/L)</td>
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<td>6.1</td>
</tr>
<tr>
<td>Diabetes (%)</td>
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<td>20</td>
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<tr>
<td>2 hr insulin (mU/L)</td>
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<td>39</td>
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<tr>
<td>---------------------</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L)</td>
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**Table 1**: Prevalence of risk factors for coronary artery disease in South Asians vs. White Europeans

Insulin resistance describes a reduction in the signalling effects of insulin, which over time results in a phenotype of impaired glucoregulation. It manifests early in South Asians; Whincup et al have demonstrated that in children between ages 8-11 years, South Asians had a higher fasting and post-glucose insulin concentrations compared to their white European counterparts, despite lower body weight. Blood glucose concentrations were similar in the two groups. Asian Indians have significantly higher proportions of visceral and total abdominal fat, levels of which were found to be inversely related to glucose disposal rate and directly related to insulin resistance. Foetal under-nutrition is believed to play a part in these phenomena, with metabolic adaptations resulting in a ‘thrifty phenotype’ designed to preserve energy and promote brain growth *in utero* (see below).

South Asian diets consist predominantly of carbohydrates, consumption of which are higher in India than in migrant Indians in the UK. A high carbohydrate diet has been shown to induce hyperinsulinemia in South Asian subjects. Dietary fat intake is higher in migrant South Asians, and interestingly intake of saturated fat does not correlate with degree of hyperinsulinemia. Vegetarian diets did not confer additional protection when compared to non-vegetarian diets, with reports suggesting a higher prevalence of truncal obesity and higher BMI in vegetarians.
However, the impact of a vegetarian diet on insulin sensitivity is yet to be extensively investigated. Higher post glucose load hyperinsulinemia has been reported in South Asian vegetarians when compared to white European counterparts.\textsuperscript{33}

Physical inactivity has a direct relationship to obesity and onset of hyperglycaemia. South Asians in particular lead a sedentary lifestyle when compared to other ethnic groups, which may contribute to higher insulin levels.\textsuperscript{34} To some extent, this has been due to urbanisation and migration patterns. However, there is insufficient data available at present to causally link this with insulin resistance in South Asian groups.

The role of genetics in development of insulin resistance has been inadequately studied. In a study assessing the inter-relationship between truncal obesity and insulin resistance in Asian Indians, Chandalia \textit{et al} found Asian Indians to be more insulin resistant than Caucasians, independent of generalised or truncal adiposity.\textsuperscript{35} In a study assessing insulin resistance and cardiovascular risk in immigrant Asians, fasting insulin levels were measured followed by levels after 1 and 2 hours of an oral glucose load. Fasting insulin levels were significantly higher in British Asians and Indian Asians when compared to white European subjects. There was no difference between the two South Asian groups two hours after glucose load. This could suggest a genetic component to insulin resistance in this group.\textsuperscript{36} However, with respect to inheritance of coronary artery disease risk, recent genome wide association studies have failed to show material differences in effect size or allelic frequency of established susceptibility variants between European and South Asian cohorts.\textsuperscript{37}
1.1.4.3 South Asian ethnicity, dyslipidemia and obesity

Prospective studies have shown an increase in CAD amongst South Asians when compared with white Europeans, after accounting for insulin resistance, diabetes, metabolic syndrome and socio-economic status.\textsuperscript{2} Hyperinsulinemia and consequent insulin resistance is associated with reduced high density lipoprotein (HDL) cholesterol concentrations and hyper-triglyceridemia.\textsuperscript{38} In addition to this, South Asians also demonstrated elevated small-dense low density lipoprotein (LDL) concentrations.\textsuperscript{39} Small LDL particles are more atherogenic than normal sized particles and there is evidence that individuals with these particles are at a higher risk of developing coronary artery disease.\textsuperscript{40} Furthermore in South Asians, total HDL is usually low, and most HDL particles are small in size which renders them less protective.\textsuperscript{41} The abundance of HDL 2b, which is the most protective component of HDL, is significantly lower in Asian Indians when compared to non-Asian Indians.\textsuperscript{42}

Obesity, defined as a BMI $>$30 kg/m\textsuperscript{2}, is highly prevalent in migrant South Asian populations, and in urban populations in India.\textsuperscript{43} This pattern of obesity starts in childhood, and appears to increase with urbanisation and migration.\textsuperscript{44, 45} In addition, South Asians have a lower lean body mass and a relatively higher body fat, suggesting a BMI of $<$ 25 kg/m\textsuperscript{2} may not necessarily reflect ‘normal’ body composition.\textsuperscript{46} The International Diabetes Federation guidance in defining metabolic syndrome uses a lower waist circumference cut off to measure central obesity in South Asians.\textsuperscript{47} Abdominal and intra-abdominal fat mass is high in South Asians as well, with thicker truncal skin folds.\textsuperscript{48, 49} However, despite this, waist to hip ratio remains comparable to white Europeans, primarily due to small waist size in South Asians.\textsuperscript{50} This combination of increased abdominal and intra-abdominal fat and body
fat patterning may be important determinants of dyslipidemia and insulin resistance.\textsuperscript{48, 49}

1.1.4.2 The ‘thrifty phenotype’ hypothesis

The thrifty phenotype hypothesis, also termed ‘Foetal origin of Adult Disease (FOAD)’ proposes that foetal and infant under-nutrition induces permanent changes in metabolism, resulting in a predisposition to type 2 diabetes and cardiovascular disease in adulthood.\textsuperscript{51} These adaptations include a reduced capacity for insulin secretion and insulin resistance, which when combined with other risk factors such as obesity and physical inactivity, may be important determinants of the development of type 2 diabetes. The mean birth weight of South Asian babies is one of the lowest in the world at 2.6-2.8 kg, and remains so in UK South Asians.\textsuperscript{52} Low body weight in infancy is common, and adaptation of an urban lifestyle results in infants ‘catching up’, eventually leading to childhood and adult obesity and thus increasing their risk. This mismatch of ‘post natal plenty’ with ‘foetal thrift’ has been described as the ‘adaptation-dysadaptation’ phenomenon.\textsuperscript{52}

1.1.4.3 Other conventional risk factors

Smoking, hypertension and hypercholesterolemia are important risk factors in the development of CVD. The Health Survey for England 1999 provided useful insights into the prevalence of cardiovascular risk factors in ethnic minorities in the UK.\textsuperscript{53} Indian and Pakistani men tended to smoke less, though Bangladeshi men were 60% more likely to smoke. Women tended to smoke less than men, which probably reflect traditional practices. Systolic blood pressure was comparable between South Asian and white European counterparts. However, this does not dismiss them as important
risk factors, as migration and adoption of local practices can have a significant impact on inter-ethnic risk profile. Patel et al demonstrated migrant South Asian populations demonstrated a higher body mass index (BMI), systolic blood pressure, blood glucose and serum cholesterol when compared to their siblings in India.  

1.1.4.4 Non conventional risk factors

A west London study demonstrated a 1.5 fold excess of pathological Q waves on ECG (indicative of a previous myocardial infarction) amongst South Asians after adjusting for age, smoking, diabetes, cholesterol, waist-hip ratio and glucose intolerance. This implies that conventional risk factors alone may not explain the higher prevalence of CVD in South Asians. Ethnic variations in inflammation, homocysteine concentrations, lipoprotein profiles and diet have been proposed as possible risk factors.

Inflammation is a central feature of atherosclerosis. Inflammatory cytokines such as adipokines released from adipose tissue have been shown to play a role. They include TNF-alpha, C-reactive protein, leptin and resistin, and high concentrations of these markers have been found in healthy South Asians when compared to white Europeans. Alterations in adipokine production may therefore play a role in the increased cardiovascular risk observed in South Asians. In addition, high concentrations of Lipoprotein (a) and homocysteine have been noted in South Asians, and this are speculated to play a role in the higher incidence of CVD, but these associations are purely speculative.

1.2 The Endothelium
The endothelium is the cellular monolayer lining all blood vessels that forms an interface between the circulating blood and the perfused tissues. Once considered just a simple barrier, the endothelium has now been demonstrated to possess a number of properties that affect numerous physiological processes.

1.2.1 The Endothelium in health

The vascular endothelium serves not merely as a passive barrier between flowing blood and the vascular wall, but plays a pivotal role in the maintenance of vascular tone. It produces a number of substances that mediate vasoconstriction (Endothelin-1, Angiotensin-II and Thromboxane A-2) and dilatation (Nitric oxide, Prostacyclin, EDHF) in addition to modulating inflammatory cell extravasation, regulating thrombosis and fibrinolysis. The presence of an intact endothelium has been demonstrated to be essential for acetylcholine (ACh) induced vasodilatation of isolated arteries. Subsequent studies showed the substance mediating ACh induced vasodilatation to be nitric oxide (NO). NO is thought to be an important mediator of vasomotor endothelial function, and exerts numerous other effects including inhibition of platelet aggregation, impairing adhesion of leucocytes to endothelium and inhibition of vascular smooth muscle migration and proliferation, all of which may retard the progression of atherosclerosis.
Endothelium derived NO is synthesised by the enzymatic conversion of L-Arginine to L-Citrulline by endothelial Nitric Oxide Synthase (eNOS), using tetrahydrobiopterin (BH4), Flavin Adenine Dinucleotide (FAD) and Flavin Mononucleotide (FMN) as co-factors (see figure 1). eNOS is predominantly located in ‘caveolae’ – flask shaped invaginations in the cell membrane that forms around 30% of the cell surface in capillaries. The caveolar coat protein Caveolin-1 interacts with eNOS through calmodulin and inhibits its activity. However, an increase in intracellular calcium in response to shear stress or certain vasodilatory stimuli results in displacement of caveolin-1 from calmodulin, thus releasing eNOS. The NO subsequently generated diffuses to the vascular smooth muscle and increases cyclic guanosine monophosphate generation, resulting in smooth muscle relaxation. NO appears to be a critical effector in a number of endothelial signalling pathways, and this is discussed in detail below.

1.2.1.1 Endothelial intracellular signalling pathways

1. eNOS signalling and Nitric Oxide production
As a principle modulator of vasoreactivity, NO has a number of downstream effects. The degree to which it exerts these effects depends on a delicate balance between its production and degradation. As NO production is dependent on eNOS activity, adequate transcription and translation of eNOS is essential to maintain NO synthesis. eNOS transcription is augmented by a number of stimuli ranging from shear stress, insulin, statins, VEGF, exercise and low levels of oxidised LDL. This is in contrast to stimuli such as hypoxia and inflammatory cytokines.

The eNOS protein consists of a reductase and an oxygenase domain which are connected to each other by a flexible protein strand. NADPH catalyses dehydrogenation of the reductase region, resulting in the production of electrons that transfer across the flexible protein strand to the oxygenase region. These electrons are required for nitric oxide generation. In addition to this, the oxygenase region binds tetrahydrobiopterin (BH4), an important cofactor in synthesis of NO. Deficiency of BH4 or even L-arginine can result in uncoupling of eNOS, resulting in superoxide (O$_2^-$) and hydrogen peroxide radical production instead of NO. In conditions of oxidant stress, despite adequate production of NO, O$_2^-$ production can inactivate it rapidly. Interaction between NO and O$_2^-$ produces peroxynitrite, itself a reactive oxygen species, which can oxidise BH4 and deplete it.

The flexible protein strand of eNOS has a specific binding site, to which attaches calmodulin. Calmodulin is an essential activator of eNOS. When electrons essential for NO production are released from the reductase region of the eNOS protein, the transfer across to the oxygenase region is facilitated by binding of calcium to calmodulin. A deficiency of calmodulin can thus result in reduced NO production.
Agonists such as acetylcholine that increase intracellular calcium promote binding of calmodulin to eNOS, thus increasing its activity.

Besides the above mechanisms, eNOS is also regulated through other post-translational mechanisms which include phosphorylation at Serine 1177 (Ser1177). Phosphorylation of Ser 1177 is catalysed by Akt in addition to other kinases such as AMP-activated protein kinase (AMPK), and results in activation of eNOS by inhibiting calmodulin dissociation from eNOS. This interaction results in increased NO production; a similar effect is seen following phosphorylation of Ser 617 by Akt or PKA which renders eNOS more susceptible to activation by calmodulin.

2. The PI-3 kinase / Akt pathway and relationship to eNOS

The PI-3 kinase / Akt signalling pathway has been shown to play a pivotal role in the phosphorylation of eNOS at serine 1177, resulting in an increase in endothelial NO production. Ziche et al demonstrated that VEGF stimulated an eNOS-dependent angiogenic response in a model of corneal angiogenesis, and eNOS mediated NO production was demonstrated by Dimmeler et al to be dependent on Akt signalling. Signalling via this pathway confers increased cell survival, reduced apoptosis and promotes angiogenesis. Indeed, infection of rabbit arterial endothelial cells by a viral vector encoding a constitutively-active Akt demonstrated increased blood flow and local NO-mediated vasodilatation, a process which was blunted when a virus encoding a dominant negative Akt was used. Similar results have been shown in a study of penile erection in rats, where Akt dependent eNOS phosphorylation was demonstrated to play a key role in maintaining erection- a process that was reduced...
by wortmannin and LY294002 (inhibitors of PI3-kinase). Interestingly, amongst the Akt isoforms, Ackah et al demonstrated Akt-1 to be the crucial isoform in phosphorylation of eNOS and angiogenesis in mouse hind limb ischemia studies.

3. Reactive oxygen species

Reactive oxygen species (ROS) are products of normal cellular metabolism. They are usually produced as a result of reactions catalysed by cellular enzyme systems, which include NADPH oxidase, xanthine oxidase, mitochondrial enzyme complexes, cytochrome P450 and uncoupled eNOS. Classically, ROS were considered to be products of cellular metabolism that exerted detrimental effects on numerous cellular structures. However, more recently, they have also been identified as critical regulators of a number of intracellular signalling pathways. Enhanced ROS production has however been implicated in the pathogenesis of a number of cardiovascular risk factors including hypertension and diabetes mellitus. In particular, NADPH oxidases are a major source of ROS in the vasculature, with high levels of Nox2 and Nox 4 NADPH oxidases evident in atherosclerotic plaques in diseased coronary arteries. NO degradation is principally mediated by ROS, which react with NO and produce peroxynitrite, which in itself is a form of ROS. While NADPH oxidase plays a critical role in cell signalling processes, evidence suggests that excess NADPH oxidase activity is linked to worse cardiovascular outcomes. Despite the wealth of evidence supporting the detrimental effects of ROS, certain groups have shown activation of endothelial NADP(H) oxidase by angiogenic factors such as VEGF promotes endothelial cell migration and proliferation, which in turn may contribute to postnatal angiogenesis in vivo. In keeping with available evidence, it appears physiological concentrations of reactive oxygen species appear
essential to maintain normal endothelial function, and concentrations higher than this appear to be detrimental.

1.2.2 The Endothelium in disease

1.2.2.1 Atherosclerosis and endothelial dysfunction

Atherosclerosis has been described as a systemic inflammatory disease affecting the arterial wall. Atherosclerotic plaques mediate the mortality and morbidity of cardiovascular diseases, including angina, claudication, myocardial infarction and strokes. Cholesterol forms a major component of atherosclerotic plaques, and it is well established that hypercholesterolemia is an important risk factor in the development of atherosclerosis. However, atherogenesis is more than just lipid accumulation in the vessel wall, and the precursor event leading onto atherosclerosis is widely thought to be endothelial dysfunction, even in the absence of angiographically evident coronary disease.

The endothelium possesses innate antioxidant properties which are overwhelmed in the presence of high LDL. Oxidised LDL is a potent inducer of ROS generation that play a major role in the pathogenesis of atherosclerosis. Endothelial injury appears to be the triggering factor (‘response to injury’ hypothesis) and the resultant endothelial dysfunction results in a cascade of events that alter the homeostatic properties of the endothelium. Enhanced endothelial expression of luminal leukocyte surface adhesion molecules leads to increased adhesion of circulating monocytes. These release a number of pro-inflammatory cytokines, which promote smooth muscle cell proliferation in the tunica media, along with further monocyte recruitment. Monocytes cross the endothelium and differentiate into macrophages, which ingest
and oxidise free lipoproteins, resulting in the formation of ‘foam cells’. The accumulation of foam cells within the vessel wall forms fatty streaks which progressively grow and coalesce, with surrounding smooth muscle and fibrous tissue encapsulating the fatty streak to form more advanced plaques. Macrophages also release pro-inflammatory mediators which further stimulate atherogenesis. Eventually these plaques can obliterate the vessel lumen, causing a reduction in flow of blood flow and ischemia of target tissues, resulting in clinical syndromes such as angina and claudication. Ongoing inflammation destabilises the fibrous cap of the plaque predisposing to plaque rupture, which promotes overlying thrombosis and abrupt vessel occlusion, causing myocardial infarction and some forms of ischemic stroke (See figure 2).
Atherogenesis begins early in life, and the conventional cardiovascular disease risk factors have all been linked with endothelial dysfunction. Cigarette smoking is a potent inducer of endothelial dysfunction, with studies showing significant functional impairment in conjunction with hypercholesterolemia. Hypercholesterolemia is a well-recognised risk factor in the development of atherosclerosis, and certainly oxidised LDL seems to play a major role in the pathogenesis of the atherosclerotic
plaque, as previously discussed. Studies have demonstrated a significant risk reduction in the major cardiovascular events following the reduction in levels of LDL cholesterol.\textsuperscript{101} Furthermore, a reduction in LDL cholesterol by HMG-CoA reductase inhibitors is associated with an improvement in endothelial function.\textsuperscript{102} Central adiposity is also recognised as important risk factor, and certainly a strong link has been established with endothelial dysfunction, as evidenced by blunted FMD in obese subjects.\textsuperscript{103} Central obesity is associated with altered release of adipokines that modulate fatty acid and glucose catabolism. While adipocyte products such as adipocyte-derived relaxing factor\textsuperscript{104} and adiponectin\textsuperscript{105} confer protection against development of atherosclerosis, the overall effect of increasing obesity is increased production of non-esterified free fatty acids and numerous pro-inflammatory cytokines including TNF-\(\alpha\), plasminogen activator inhibitor-1 (PAI-1) and C-reactive protein (CRP) to name a few. The free fatty acids deposit in ectopic sites such as the liver and muscle, eventually leading to insulin resistance and diabetes mellitus. Clustering all the above risk factors, Reaven coined the term 'syndrome X’, establishing a link to insulin resistance.\textsuperscript{106} Over the years the definition has evolved and is termed ‘metabolic syndrome’, which clumps together abdominal obesity, atherogenic dyslipidemia (elevated triglycerides and low HDL), elevated blood pressure, insulin resistance and elevated pro-inflammatory and prothrombotic markers.\textsuperscript{107} It is now established that it is linked with increased risk of future cardiovascular events.\textsuperscript{108}

NO released from healthy endothelium plays an important role in retarding platelet aggregation and thrombus formation. In addition to this, dysfunctional endothelium secretes more plasminogen activator inhibitor -1, an inhibitor of fibrinolysis, which
contributes to an overall prothrombotic state. These form an important aspect in the pathogenesis of atherosclerosis.

1.2.2.2 Insulin resistance and endothelial dysfunction

It is well established that obesity, type II diabetes and insulin resistance are major risk factors in the development of atherosclerosis and subsequent cardiovascular disease. Insulin regulates glucose disposal at a number of sites in the body, including muscle and adipose tissue. It does so by attaching to its cell surface receptor thus activating a cascade of downstream signalling molecules mediating its metabolic effects. This is mediated by activation of the insulin receptor's tyrosine kinase domain, which via a number of intermediaries promotes activity of the PI3 kinase and MAP kinase pathways. The PI3 kinase molecule is essential for insulin stimulated GLUT-4 translocation, which is responsible for insulin-regulated glucose translocation into cells.

In addition to its metabolic effects, insulin also plays a role in vascular endothelial NO production, leading to enhanced blood flow, vasodilatation and augmented skeletal muscle glucose disposal. Studies have demonstrated the presence of insulin receptors on the surface of the endothelium. However, insulin signalling pathways that regulate endothelial NO production are similar to the insulin signalling pathways that regulate its metabolic effects in adipose tissue and skeletal muscle. The relative contributors of whole body insulin resistance and endothelium specific insulin resistance to endothelial dysfunction and atherosclerosis remain unclear, although recent data indicate endothelium-specific insulin resistance is atherogenic.
In the vascular endothelium, insulin receptor activation stimulates two major signalling cascades: the PI3 kinase pathway which is involved in NO production, and the MAP kinase pathway which is predominantly involved in cell growth / division. The activation of the PI3 kinase pathway is dependent on phosphorylation of insulin receptor substrate-1 (IRS -1), which appears to play a major role in mediating insulin stimulated endothelium-dependant vasorelaxation \(^1\) (see figure 3). Following phosphorylation by the activated insulin receptor, IRS -1 binds and activates PI3 kinase, which leads to generation of phosphoinositide (3,4,5) triphosphase which stimulates membrane localisation of phosphoinositide-dependent kinase 1 (PDK-1). PDK-1 is then able to phosphorylate and activate Akt \(^1\), which in turn phosphorylates eNOS at serine 1177, resulting in enhanced eNOS activity and NO production. However, phosphorylation is not the only mechanism involved in regulating eNOS activity, and other post translational modifications have also been described.

Insulin also stimulates the production of Endothelin-1 (ET-1), a potent vasoconstrictor. Work by Potenza et al \(^2\) demonstrated the role of MAP kinase signalling in endothelial ET-1 secretion. By studying bovine aortic endothelial cells, they showed that insulin treatment induced an increase in ET-1 levels in conditioned medium, a process blocked by PD-98059 (a MAP kinase inhibitor) and not by wortmannin (a PI3 kinase inhibitor). A similar effect was seen in cell lysates, implicating regulation of ET-1 secretion by MAP kinase signalling and not PI3 kinase signalling.
In insulin resistant states, signalling via the PI3 kinase pathway is downregulated, while the MAP kinase signalling is overactive, resulting in ‘pathway-specific insulin resistance’, a term coined by Quon et al, as a contributor to the pathophysiology of endothelial dysfunction associated with insulin resistance. At a molecular level, insulin resistance is accompanied by reduced insulin stimulated tyrosine phosphorylation of IRS-1, perhaps due to inhibitory phosphorylation of nearby serine residues. The MAP kinase pathway is activated normally by interaction between Grb-2/Sos and IRS-1 or Shc. However, in insulin resistant states, the former interaction is downregulated but the reduced phosphorylation of the IRS-1 receptor is sufficient enough to maintain a normal interaction between Grb-2/Sos and Shc, resulting in normal MAP kinase pathway activity. A number of factors seem to

**Figure 3:** Stimulation of insulin receptor and downstream effects on endothelium
play a role, including hyperglycemia and elevated free fatty acid levels. Hyperglycemia increases the production of ROS, which can activate transcription factors Nuclear Factor κB (NF-κB) and activator protein -1 (AP-1). These transcription factors can also be activated by lipopolysaccharides and free fatty acids. Once stimulated, they enhance transcription of proinflammatory cytokines (IL-1, TNF-α), which results in insulin resistance by affecting insulin signalling via paracrine effects.¹²⁵ Thus NF-κB plays a pivotal role in inflammation mediated pathway-specific insulin resistance, and a recent study has corroborated this.¹²⁶ Metabolic insulin resistance is accompanied by compensatory hyperinsulinemia in order to maintain a euglycemic state. Hyperinsulinemia results in over-activity of MAP kinase dependent pathways, resulting in an imbalance between PI3-kinase and MAP kinase dependent effects of insulin. By doing so, molecules whose secretion is regulated by MAP kinases such as ET – 1 are over-expressed and molecules dependent on PI3 kinase activity (such as NO) are reduced, which is characteristic of endothelial dysfunction.

By mimicking pathway specific insulin resistance via pharmacological inhibition of PI3 kinase pathway, Montagnani et al demonstrated enhanced mitogenic effects of insulin mediated via the MAP kinase pathway.¹²⁷ This also results in increased expression of leukocyte adhesion molecules such as ICAM-1, VCAM-1 and E-selectin due to greater availability of prenylated Rho and Ras proteins. These adhesion molecules are believed to play an important role in the initiation of atherogenesis.¹²⁸ Furthermore, increased circulating concentrations of soluble adhesion molecules correlate strongly with future cardiovascular events.¹²⁹,¹³⁰ Reduction in NO production blunts vasodilatation in response to insulin – an effect
that is mediated by the PI3 kinase pathway. Similar effects are seen in obese subjects and patients with type 2 diabetes mellitus.

It is evident from the above discussion that insulin signalling pathways involving the PI3-kinase regulate GLUT-4 translocation and subsequent glucose uptake in adipose tissue and skeletal muscle. The same pathway regulates eNOS activation and NO production in the endothelium. Thus, metabolic insulin resistance and endothelial dysfunction appear to be pathophysiologically related to selective downregulation of the PI3-kinase signalling.

Insulin resistance is particularly prevalent in South Asian population groups, and is associated with endothelial dysfunction. A non-invasive study of the endothelium-dependent conduit vessel flow mediated dilatation in SA males suggested significant blunting when compared with WE counterparts and this was associated with insulin resistance. Insulin resistant patients have elevated ET-1 levels, and patients with hyperinsulinemia associated with metabolic syndrome also demonstrate elevated ET-1 levels.

1.2.3 Endothelial cell senescence

In 1961, Hayflick and Moorhead first described senescence in human skin fibroblasts. The term 'replicative senescence' was coined; implying diploid cells had a limited capacity to divide in cell culture, eventually leading to an arrest in cell division. Numerous hypotheses were stated, including the belief that cellular senescence was an anticancer mechanism. At one point, senescence was believed to be the only marker of ageing – the loss of regenerative capacity of cells in vivo.
This meant that senescence had both beneficial and detrimental effects. Future studies conducted have shown that associated with these effects is a rather striking phenotypic change, which include apoptosis and altered gene expression. In 1995, Dimri et al demonstrated the histochemical marker beta-galactosidase detected senescent keratinocytes and fibroblasts in skin biopsies of elderly people.\textsuperscript{136} Experiments conducted by Kurz et al have demonstrated, through beta-galactosidase staining, deposition of senescent cells at site of denudation following balloon injury in rabbit carotid artery \textsuperscript{137}, confirming the occurrence of vascular senescence \textit{in vivo}.

A number of factors play a role in triggering cell senescence. The vasculature in humans is exposed to a circulating reactive oxygen species and other insults, including modified lipoproteins. These can result in rapid telomere damage and shortening, which can result in senescence.

\begin{figure}[h]
\centering
\includegraphics[width=0.6\textwidth]{figure4.png}
\caption{Mechanism of senescence and resultant phenotype}
\end{figure}
In essence, telomere length is inversely related to endothelial cell age \textit{in vivo} \textsuperscript{138}, and senescence is reached when the telomeres are shortened below critical length.\textsuperscript{139} Previous work has demonstrated the presence of senescent endothelial cells in human atherosclerotic lesions but not in non-atherosclerotic lesions.\textsuperscript{140} However, the role of diabetes and insulin resistance in endothelial cell senescence is poorly understood. Hyperglycaemia, a feature of both insulin resistance and diabetes, can lead to oxidative stress, which in turn can result in premature senescence of normal endothelial cells.\textsuperscript{141} Studies examining the relationship between coronary artery disease and telomere length found that telomeres from endothelial cells derived from diseased portions of coronaries were shorter than the non-diseased regions.\textsuperscript{142}

\textbf{1.3 Endothelial progenitor cells}

The first indication of the presence of endothelial progenitor cells dates back to the early 1930’s, when Heuper and Russell found capillary-like formations in leukocyte cultures\textsuperscript{143}. In the early 1950’s, human patients with cardiovascular disease were having diseased segments of large blood vessels replaced by artificial vascular grafts, which upon later excision were covered in a layer of endothelial cells.\textsuperscript{144} For a number of years, the established theory regarding postnatal new vessel growth invoked proliferation and migration of fully differentiated endothelial cells from pre-existing vessels – a process termed ‘angiogenesis’. However, in 1997, Asahara \textit{et al} published a landmark study suggesting the presence of circulating cells bearing the CD34 (or the KDR) surface marker that possessed some properties of endothelial cells when cultured \textit{in vitro} with angiogenic growth factors.\textsuperscript{145} Furthermore, these cells promoted \textit{in vivo} angiogenesis when transplanted into murine hind limb
ischemia models. These cells were termed ‘Endothelial cell Progenitors’ or ‘angioblasts’, and were believed to form blood vessels de novo – a process called ‘vasculogenesis’, initially thought to occur only in utero. However, these data have been extrapolated by many other investigators to suggest the in vivo presence of ‘endothelial progenitor cells’, defined by the co-expression of CD34 and KDR (though no cell culture modification). However, the in vivo role of such cells has never been proven, and this has resulted in controversy and confusion in the field of vascular repair. Notwithstanding these concerns, Asahara’s initial findings and subsequent studies have resulted in a paradigm shift in our understanding of endothelial injury and repair. A delicate balance between endothelial damage and repair is now thought to influence the development of atherosclerosis, and hence modulating repair mechanisms could represent a novel way retarding, or reversing atherogenesis.

1.3.1 Defining endothelial progenitor cells

To date, there is no clear consensus on the definition of an endothelial progenitor cell (EPC) and the term is still used interchangeably to describe circulating cells bearing CD34 with or without other markers, or various types of cell-culture derived populations. The former group is perhaps better defined as circulating progenitor cells (CPC), acknowledging our ongoing uncertainty as to their role in vivo. CPC will be used from now on in this thesis to describe circulating cells counted with flow cytometry, whereas EPC will be used to denote culture derived cells with typical functional properties. Phenotypic characterisation of CPC has been controversial, and attempts by numerous groups to successfully define these cells have been confounded by the presence of circulating endothelial cells (CEC). Furthermore,
CECs and CPCs form only 0.01% and 0.0001% respectively of cells in peripheral blood, making isolation and thus further characterisation of these cells difficult.\textsuperscript{147} Circulating endothelial cells are believed to be derived from mature vascular endothelium following mechanical disruption or apoptosis \textsuperscript{148}, though a small subset of these cells may be derived from bone-marrow derived progenitors.\textsuperscript{149} Their numbers are increased in various conditions that are associated with vascular injury, including septic shock \textsuperscript{150} and systemic lupus erythematosus.\textsuperscript{151} In order to ascertain the origin of CECs further, Lin et al conducted novel experiments on blood samples derived from recipients of gender-mismatched bone marrow transplants. They demonstrated that in vitro endothelial cell colonies appearing within nine days were of a recipient phenotype thus arising from CECs, while those arising later were of donor origin suggesting the possibility of bone marrow derived CECs or EPCs.\textsuperscript{152} Furthermore, CECs derived from the bone marrow are thought to form only a small fraction of the total CEC count.\textsuperscript{152}

Identifying the specific cellular origin of EPCs is difficult given the various cell types in the blood that can differentiate into cells with endothelial-like gene expression in vitro. These include hematopoietic stem cells, monocyte-macrophages and CECs.\textsuperscript{153} However, there are a variety of measures that can help assist in isolation and quantification of EPCs. These include identifying cell surface phenotype using flow cytometry with subsequent flow sorting and in vitro culture. Cells for analysis can be derived from whole blood or by density layered centrifugation.

Preliminary work conducted by Asahara et al\textsuperscript{145} identified that transfused culture-modified murine CD34\textsuperscript{+} or Flk1\textsuperscript{+} cells localised to areas of neoangiogenesis in vivo.
This led them to hypothesise that circulating CD34+ and Flk-1+ mononuclear cells may contribute to neoangiogenesis in adult mice. Following this, Peichev et al.\textsuperscript{154} attempted to differentiate circulating endothelial cells (sloughed mature endothelial cells) and CPCs using cell surface markers. Identifying that HSCs strongly expressing CD34 and CD133 cell surface markers are downregulated during HSC differentiation, they postulated that cells expressing these markers may represent immature progenitor cells. Furthermore, they postulated that cells expressing CD133 co-expressed KDR, and that this could be used to differentiate between mature endothelial cells and CPCs. Of all the samples analysed, they found that only 2% of mobilised peripheral blood CD34+ cells co-expressed CD133 and KDR. This was not seen in umbilical cord derived endothelial cells, which only expressed CD34+ and KDR+, but did not express CD133. Based on this information, it was concluded that circulating CD34+KDR+CD133+ cells may represent a putative immature CPC phenotype. Examination of luminal surfaces of left ventricular assist devices implanted in humans identified the presence of cells expressing all three surface markers, though they only constituted 3% of the total mononuclear cells on the surface.\textsuperscript{154} Based on all the above evidence, the authors concluded that CD34+ cells in the circulation that co-expressed CD133 and KDR could be defined as ‘circulating EPCs’ (or CPCs), and quantification of these cells could provide valuable information of their role in diseased states. In addition, we now understand that late outgrowth EPCs are not derived from CD133 cells.\textsuperscript{155}

In addition to identifying CPCs \textit{in vivo}, a number of groups have attempted to identify putative EPCs \textit{in vitro}. Initial work by Asahara \textit{et al} identified CD34+ expressing cells as putative EPCs \textit{in vitro}, after a period of culture modification whilst adherent to
fibronectin coated plates. These cells formed clusters of round cells surrounded by spindle shaped cells when co-cultured with CD34 depleted mononuclear cells, and expressed ‘endothelial’ surface marker proteins. Ito et al. expanded on this work and isolated human peripheral blood mononuclear cells and plated them on fibronectin. The non-adherent cells were removed after 24 hours in order to deplete the population of monocytes, macrophages and any circulating endothelial cells, and were re-plated onto fibronectin coated dishes. A number of similar clusters were identified as EPC colonies 7 days after plating.

The definition of an EPC remained unclear. Hur et al. recognised this lack of clarity and attempted to demonstrate the heterogeneity of EPCs based on culture properties. In a series of experiments using human peripheral blood, they identified two types of EPCs in culture. The spindle shaped cells that appears 3-5 days following plating were termed ‘early EPCs’, and were akin to the EPCs initially described by Asahara. Following an increase in their number over 2 weeks, a decline in their number was later observed with their gradual disappearance in 4 weeks. During the early EPC decline, another cell type with a similar appearance to endothelial cells was identified, with the capability to rapidly proliferate over numerous population doublings without senescence (see figure 5). These were called late-outgrowth EPCs and ingested acetylated LDL and bound to lectin in a similar fashion to early EPCs. Another interesting observation noted was the proliferation rate of late EPCs was greater than mature endothelial cells (gastro-epiploic artery endothelial cells – GEA EC), which could imply the origin of late EPCs was not simply via vessel wall detachment.
Figure 5 - Different growth curves of early and late EPC and mature endothelial cells (Modified from Hur et al\textsuperscript{157})

In 2003, Hill \textit{et al} demonstrated a technique of culturing EPCs purported to avoid subculture of circulating mature endothelial cells.\textsuperscript{158} PMNCs were placed on fibronectin, and after 24-48 hours non adherent cells were removed and re-plated. After 7 days in culture, colonies formed which were counted. These colonies had a central core of round cells with spindle shaped cells around the periphery. Work by Hur \textit{et al} has shown the central core to be composed of T-lymphocytes \textsuperscript{159}, while the spindle shaped cells in the periphery to be macrophages bearing some non-specific endothelial surface markers.

1.3.2 Identifying Endothelial Progenitor Cells in vitro

1.3.2.1 Morphology in vitro
It is now apparent that Asahara’s initial work described early outgrowth EPCs, sometimes referred to by other groups as circulating angiogenic cells. These cells when derived from peripheral blood mononuclear cells remain adherent to fibronectin 4-7 days following plating. They are spindle shaped cells with limited proliferative capacity, and rarely survive beyond 2-3 weeks in culture. When co-cultured with endothelial cells, they promote angiogenesis in a paracrine fashion, though they do not form vascular networks in isolation. This is achieved by a number of pro-angiogenic factors released by these cells, which include VEGF, MMP-9 and IGF-1, concentrations of which are significantly higher compared to the conditioned medium of late outgrowth EPCs. Work conducted by Rohde et al has shown these early EPCs to be culture derived monocytes, and the expression of endothelial surface markers has been demonstrated to be via phagocytosis of platelet derived microparticles generated from debris in the culture plate.

As outlined earlier, the other form of early outgrowth EPC is the colony-forming units (CFU-Hill). These colonies are derived by plating PBMCs in fibronectin coated plates with modified growth medium for 2 days, following which the non-adherent cells are extracted, re-plated and grown for a further 3 days. A typical CFU appears on day 5 and consists of a cluster of spherical cells surrounded by spindle shaped cells (see figure 6). The central core has been demonstrated to be strongly positive for the surface marker CD-3, indicating a T-lymphocyte-rich core. These T-lymphocytes release numerous angiogenic growth factors including VEGF, IL-8 and TNF-α, towards which the surrounding monocytes migrate. These surrounding monocytes are akin to early outgrowth EPCs described by Asahara, and express endothelial antigens.
Figure 6: Different subsets of endothelial progenitor cells derived from culture of PBMCs.

When PBMCs are cultured for prolonged periods, late outgrowth EPC colonies of cells appear after 7-21 days. These cells exhibit a cobblestone pattern in culture, have a high proliferative potential, being capable of serial passages, and express typical endothelial surface markers. When placed in matrigel plugs, these cells can form capillary-like networks in contrast to early outgrowth EPCs that are incapable of doing so.¹⁶⁰ Seminal work by Ingram et al has demonstrated that some individual late outgrowth cells can form secondary late outgrowth colonies that exhibit high proliferative capacity; this proliferative hierarchy is considered a feature of ‘true progenitor’ populations.¹⁶⁴ Hence, late outgrowth EPCs are considered by some to be the true EPC, and share similar gene expression profiles to HUVECs.¹⁵⁷, ¹⁶⁵
1.3.2.2 Uptake of Dil-Ac-LDL

In 1990, Voyta et al.\textsuperscript{166} incubated endothelial and smooth muscle cells with a fluorescently tagged acetylated LDL cholesterol molecule Dil-Ac-LDL, for 4 hours and visualised them with fluorescence microscopy. Endothelial cells were highly fluorescent, while the smooth muscle cells only demonstrated mild fluorescence when compared to the background, and so this method was adopted to identify endothelial cells, and later EPCs.\textsuperscript{167} We now understand that acetylated LDL is taken up by endothelial cells via the ‘scavenger cell pathway’ of metabolism of LDL.\textsuperscript{166} However, high uptake is also seen in monocytes and macrophages, and hence this assay in isolation is not specific for the endothelial phenotype.

1.3.2.3 Binding to UEA-1 lectin

Ulex Europeus 1 agglutinin (UEA-1) is a lectin that binds to certain glycol-compounds present in cells and can be visualised under fluorescence microscopy when conjugated with fluorescent molecules such as FITC. Studies have shown that it binds to endothelial cells lining blood vessels\textsuperscript{168}; however, more recent work has shown that UEA-1 also binds to other cell types\textsuperscript{169}, and so binding of UEA-1 alone is a non-specific assay of endothelial phenotype. Combined assessment of UEA-1 with that of Dil-Ac-LDL binding may improve specificity for endothelial phenotype\textsuperscript{167}, and so can be used in defining early EPCs. However we now understand these cells to be of monocyte origin, so highlighting the poor specificity of this assay.
1.3.3. EPCs, CPCs and cardiovascular outcomes

Despite the numerous methods to culture and identify EPCs in vitro, it is unclear whether these culture-derived cells exist in vivo, and if so whether they are reflected by circulating progenitor cells in vivo. This is primarily due to the lack of a unique identifier that is present within EPCs. This makes interpreting and correlating cardiovascular outcome data regarding EPCs and CPCs difficult. Previous work has demonstrated an inverse relationship between circulating progenitor cell number and cardiovascular risk factors.\(^{170}\) The number of circulating progenitor cells has also been shown to independently predict future major cardiovascular events in patients suspected of having coronary artery disease by Werner et al.\(^{171}\) Similar findings have been seen in patients with congestive cardiac failure.\(^{172}\) Cultured EPC-CFU numbers have been demonstrated to correlate with endothelial dysfunction and Framingham cardiovascular risk index.\(^ {158}\)

The relationship between CPCs linked to cardiovascular outcome by Werner et al.\(^ {171}\) and cell-culture derived EPC remain unclear. Recent research has shown that within CD34\(^+\)CD45\(^-\) CPCs are the cells that give rise to late outgrowth EPCs, although not all CD34\(^+\)CD45\(^-\) cells possess this potential.\(^ {155}\) This work has also showed that late outgrowth EPCs lack CD133, a primitive cell surface marker, and that CD133\(^+\) cells cannot form late outgrowth EPCs in vitro. Timmermans et al also demonstrated that CD34\(^+\)CD45\(^-\) cells which eventually generated late EPCs lacked CD133. In contrast to this, some groups have shown that purified CD133+ cells can generate endothelial cells.\(^ {173}\)
It is evident from the above discussion that a unique marker of an EPC is lacking, and whether or not results obtained from \textit{ex vivo} work with EPCs are relevant to \textit{in vivo} observations remains unclear.

1.3.4 Functional properties of EPCs

1.3.4.1 Endothelial Progenitor Cell mobilisation

The bone marrow is home to a stem cell niche which consists of fibroblasts, osteoblasts and endothelial cells, and provides an environment of self renewal and differentiation of stem and progenitor cells. Progenitor cells are mobilised from the bone marrow into the circulation in response to a number of cytokines and growth factors which include Vascular Endothelial Growth Factor (VEGF), Stromal-cell Derived Factor (SDF), angiopoetin-1 and erythropoietin.\textsuperscript{174} These cytokines can stimulate the cleavage of molecules mediating retention of progenitor cells in the bone marrow by activating proteases such as matrix metalloproteases (e.g. MMP-9) and cathepsins.\textsuperscript{175} Both VEGF and SDF-1 up-regulate bone marrow MMP-9 activity, which cleaves the progenitor cell membrane bound kit-ligand, resulting in progenitor mobilisation into the vascular zone of the bone marrow.\textsuperscript{174} Nos3\textsuperscript{-/-} (eNOS\textsuperscript{-/-}) mice have greatly reduced basal activity of pro-MMP-9 resulting in a reduction in sKitL release from mKitL. Consequently progenitors fail to mobilise in Nos3\textsuperscript{-/-} mice in response to physiological stimuli, a phenomenon that was rescued by infusion of sKitL. This establishes a mechanistic link between VEGF signalling and MMP-9 in bone marrow stroma.\textsuperscript{174} NO released by the bone marrow stromal cells as a result of VEGF signal transduction promotes nitrosylation of MMP-9, and is an absolute requirement for VEGF stimulated EPC mobilisation.\textsuperscript{176}
Tissue hypoxia appears to be a major stimulus for VEGF release, mostly due to increased VEGF messenger RNA levels in response to hypoxia inducible factor -1 (HIF-1). Animal studies have demonstrated increased mobilisation of circulating progenitors and EPCs in response to VEGF. Similar results have been seen in human studies, with numbers of EPCs increasing following burn injuries and myocardial infarction mirroring increased VEGF levels. Other cytokines that mobilise EPCs include granulocyte colony stimulating factor (G-CSF) and granulocyte monocyte colony stimulating factor (GM-CSF). However, recent evidence has shown these to be pro-inflammatory and possibly contributory towards plaque instability and has questioned their therapeutic use in patients with the sole aim to progenitor cell mobilisation. Erythropoietin is another cytokine that mobilises EPCs in vivo. Numerous other stimuli result in release of EPCs from bone marrow, and these have been listed in the figure below.

**Figure 7:** Factors affecting various stages in EPC mobilisation
Endothelial NO synthase is essential to maintain EPC mobilisation in response to various stimuli. More specifically, NO is a critical molecule in EPC mobilisation from the bone marrow. Mice deficient in eNOS show reduced EPC and stem cell mobilisation from the bone marrow. Numerous other stimuli such as exercise and statins have been demonstrated to mobilise EPC in a NO dependent mechanism. This would imply a reduction in NO production, as noted in patients with endothelial dysfunction, would lead to impaired mobilisation of CPCs.

Recent work conducted by our group assessing the effect of moderate intensity exercise on mobilisation of CPCs provided the first proof of a pivotal role for NO in mobilisation of CPCs in humans.

1.3.4.2 EPC adhesion and transmigration

In order for EPCs to participate in vascular repair, it is essential for them to adhere to the vasculature at the sites of ischemia or injury. Integrins have been demonstrated to mediate adhesion of leukocytes to extracellular matrix proteins and endothelial cells. In particular, β1 integrins are expressed on the surface of endothelial cells, while β2 integrins are expressed on hematopoietic stem cells. Integrins are also expressed on the surface of peripheral blood derived EPCs, and are required for EPC adhesion to endothelial cells and trans-endothelial migration in vitro. Furthermore, HSCs lacking β2 integrins demonstrate impaired homing and reduced capacity to improve post-ischemic neovascularisation. In denuded arteries, reendothelialisation may be mediated to an extent by β1 integrins’ role in adhering EPCs to extracellular matrix. However, studies have shown that the α₆β₃-
and αvβ3-integrins play a major role in mediated adhesion of EPCs to denuded vessels.189

1.3.4.3 EPC chemotaxis and migration

Given the small number of circulating progenitor cells released in response to tissue injury and ischemia, it is necessary to have a sufficient concentration of chemo-attractant factors to facilitate recruitment to the injured tissue. VEGF has been demonstrated to act as a chemo-attractant factor to EPCs190,191. In addition to this, SDF-1 has also been shown to be a potent chemotactic factor and stimulates recruitment of progenitor cells to ischemic tissues.192 IGF-1 has also been demonstrated to exert similar effects193, and is itself released from ischemic tissues.194

1.3.4.4 EPCs and angiogenesis

As previously discussed, early outgrowth EPCs release numerous mediators that can stimulate capillary growth, though they themselves do not directly or permanently contribute to vascular endothelial networks.195 These results have been noted in both in vitro and in vivo studies.196

In 2001, Kocher et al197 mobilised CD34+ cells from the bone marrow using G-CSF, purified them, labelled them with Dil-Ac-LDL and injected them into rats where the main coronary artery had been ligated (to cause myocardial infarction) 48 hours earlier. Significantly enhanced neoangiogenesis was noted at the site of the infarct, with 20-25% of the new vessels co-localising with labelled cells. Also noted was an improvement in left ventricular ejection fraction. This improvement was not seen with CD34− cells. Other groups have also shown similar results.198 Transplantation of ex
vivo expanded EPCs into a mouse model of hind limb ischemia demonstrated successful promotion of neovascularisation with a 50% reduction in limb necrosis and auto-amputation.\textsuperscript{199} This preliminary work has demonstrated the possible role of bone marrow derived progenitor cells in neoangiogenesis \textit{in vivo}, though cannot confirm the direct formation of endothelial cells from transplanted bone marrow cells.

Since then, work has demonstrated that neoangiogenesis may occur from interplay between EPCs and mature endothelium. In 2003, Rehman \textit{et al} demonstrated that endothelial progenitor cells secreted a number of angiogenic factors, including VEGF.\textsuperscript{195} Other groups have shown that CD14\textsuperscript{+} and CD34\textsuperscript{+} EPCs also release numerous other growth factors such as insulin – like growth factor-1 (IGF-1) and basic fibroblast growth factor (bFGF).\textsuperscript{200, 201} Work by Hur \textit{et al} demonstrated early outgrowth EPCs, when co-cultured \textit{in vitro} with HUVECs on Matrigel, were incorporated into tubular network structures, though not to the extent seen with late outgrowth EPCs.\textsuperscript{160} This indicated the involvement of early outgrowth EPCs \textit{in vitro} in the formation of new blood vessels, and is likely to be secondary to the numerous growth factors released which generate a pro-angiogenic environment. However, there is much scepticism regarding \textit{in vitro} assays of angiogenesis using Matrigel, as cells that have no angiogenic potential \textit{in vivo} may also induce tubulogenesis.\textsuperscript{202} In order to account for this problem, Sieveking \textit{et al}\textsuperscript{201} performed a tubulogenesis assay specific for endothelial cells using co-cultures of differentiated endothelial cells (which included HUVECs, coronary artery and microvascular ECs) or non-endothelial cells with monolayers of human fibroblasts. They showed via 3D immune-fluorescence microscopy that early EPCs do not directly participate in tubulogenesis, but in fact release mediators in a paracrine fashion that facilitate tube
formation by differentiated ECs. Further analysis of late outgrowth EPCs demonstrated direct participation in both *de novo* tubulogenesis and direct incorporation into pre-formed EC tubules, without exerting a paracrine effect. As previously discussed, this suggests that late outgrowth EPCs are true EC progenitors.

Clearly, more research is required to identify all the relevant paracrine mediators of tube formation. By isolating and delivering these paracrine mediators, potential future therapeutic strategies may be developed in patients suffering from CAD and vascular disease.

*1.3.5 Endothelial Progenitor Cell signalling pathways*

The functional properties of EPCs described above are dependent on a number of critical signalling pathways and molecules. The following section discusses these important molecules.

*1. Nitric oxide*

Endothelial regeneration may be dependent on the normal functioning of EPCs in response to ischemia or injury, requiring mobilisation, homing and eventually endothelial repair. These steps are all critically dependent on NO. NO is produced in EPCs by eNOS, via the same molecular signalling mechanisms as endothelial cells.\(^{203}\) Aicher et al demonstrated in a hind limb ischemia model in eNOS-deficient mice (*Nos3\(^{-/}\)*), infusion of wild type EPCs, but not *Nos3\(^{-/}\)* EPCs rescued the phenotype of defective neovascularisation, indicating the role of eNOS beyond simply mobilising EPCs.\(^{176}\) In human subjects with type 2 diabetes, early EPCs
demonstrate impaired migration *in vitro*, a phenomenon that was reversed by addition of a NO donor drug; further studies suggested a role for NO in EPC cytoskeletal plasticity and so migration properties. These findings indicate a strong and essential role for EPC-derived NO in many of its biological functions. Coupled with its requirement for progenitor mobilisation and endothelial homeostasis, NO is likely to be a critical mediator in vascular repair.

2. **PI3K/Akt signalling pathway**

The PI3K/Akt pathway augments the production of NO through stimulatory phosphorylation of eNOS at its serine residue 1177 in mature endothelial cells – a phenomenon that has also been demonstrated in EPCs. The pathway's activity is stimulated by a number of factors including VEGF, exercise and drugs such as statins. As NO plays a significant role in EPC mobilisation, the PI3K/Akt pathway may play an important role in this process, as discussed below.

Experiments conducted by Ackah *et al* have shown Akt 1 to play a pivotal role in progenitor mobilisation and EPC function. They demonstrated that infusion of Akt1−/− EPCs following hind-limb ischemia had no significant effect on tissue perfusion, which was in stark contrast with wild-type EPCs. A number of other functional properties, such as EPC homing and migration have also been linked to PI3 kinase Akt pathway. In particular, the γ subunit of the PI3K molecule is thought to play an important role in regulation of EPC function, with PI3Kγ−/− EPCs showing a defect in proliferation, survival, integration into endothelial networks, and migration toward SDF-1. Pharmacological agents such as statins have been demonstrated to enhance EPC mobilisation and migration, a phenomenon that is PI3K/Akt-pathway.
dependent.\textsuperscript{167} Similarly, homing of EPC toward chemotactic ligands such as VEGF and SDF-1 released in response to tissue hypoxia are PI3K/Akt dependent.\textsuperscript{88, 207}

Despite the overwhelming evidence in support of the PI3K/Akt pathway in regulating EPC function, recent work has demonstrated a possible detrimental role of Akt in EPC function and survival. Wang \textit{et al} have postulated that chronic upregulation of Akt may result in enhanced apoptosis and impaired angiogenesis.\textsuperscript{208} Furthermore, Nishi \textit{et al} observed impaired angiogenesis in VEGFR-1 haploinsufficient mice as a result of uninhibited VEGFR-2 signalling. This effect was mediated by excessive Akt activation.\textsuperscript{209} It thus appears that many of EPC functional properties are mediated via PI3K/Akt signalling, though hyper-stimulation of the pathway may be detrimental.

3. MAP kinases
Mitogen activated protein kinases (MAP kinases) are a family of serine/threonine kinases that comprise 3 majors subgroups – extracellular signal regulated kinases (ERK) which includes p42/44, p38 MAPKs and c-Jun N-terminal kinases (JNK). They co-ordinate and regulate cell proliferation and differentiation in response to stimuli such as TNF-\(\alpha\) in different cell types.\textsuperscript{210} The MAP kinase pathway broadly promotes cell proliferation. Pathway specific insulin resistance results in hyper stimulation of MAP kinase pathways, leading to cell apoptosis and senescence.\textsuperscript{122} Previous work has demonstrated the p38 MAP kinase signalling pathway to be hyper stimulated by high glucose concentrations\textsuperscript{211}, indicating a role in EPC dysfunction in diabetes. When compared to healthy subjects, patients with CAD and underlying EPC dysfunction have demonstrated profound upregulation in p38 phosphorylation. A similar effect has been noted when EPCs derived \textit{ex vivo} are cultured with TNF-\(\alpha\) or
glucose. Oxidised LDL has also been demonstrated to reduce EPC number and function by inducing p38 MAPK in a time and dose dependent manner. Interestingly, inhibition of p38 MAPK is associated with enhanced angiogenesis. Thus, by targeting p38 MAPK, potential therapies could be designed to halt premature senescence and thus promote vascular repair.

4. Reactive oxygen species and oxidative stress

For a long time now, ageing and disease has been partially attributed to endogenous reactive oxygen species. As previously discussed, ROS have also been implicated in the pathogenesis of numerous cardiovascular risk factors. Recent evidence has shown ROS to serve as a secondary intracellular messenger, playing an important role in cellular processes including differentiation, senescence and cell apoptosis. While detrimental effects are primarily seen at high concentrations of reactive oxygen species, lower concentrations have been shown to play a critical role in mobilisation and homing of EPCs, thus playing a key role in neovascularisation following ischemic injury. An alteration in ROS generation may thus alter EPC function and modulate cardiovascular risk.

Studies have demonstrated that defective endothelial repair is closely linked to oxidative stress in disease states. In subjects with type 2 diabetes, a reduction in EPC mobilisation and number has been attributed to uncoupling of eNOS, resulting in the production of superoxide anion (O$_2^-$) instead of NO. In addition, Sorrentino et al demonstrated enhanced NADP(H) oxidase catalysed superoxide anion production as a possible aetiological factor in the impaired in vivo re-endothelialisation capacity of EPCs derived from patients with diabetes. Similar
results have been seen in recent work by other groups, with high NADP(H) oxidase activity and superoxide levels seen in EPCs derived from diabetic subjects.\textsuperscript{221} Hyperglycaemia induced EPC dysfunction was rescued by superoxide dismutase and by restoration of BH4 levels.\textsuperscript{219, 222, 223} However, excess ROS in isolation cannot explain EPC dysfunction in diabetes, and mechanisms such as increased activation of p38 MAP kinase have also been postulated.\textsuperscript{212}

Despite enhanced ROS production in conditions of stress such as injury and ischemia, EPCs still manage to facilitate vascular repair, suggesting a possible resistance to ROS-induced toxicity. Work performed by Dernbach \textit{et al} demonstrated that EPCs derived from healthy donors were rich in antioxidants superoxide dismutase and glutathione peroxidise when compared to HUVECS.\textsuperscript{224} This could potentially augment their ability to repair vasculature, although whether these defences are diminished by insulin resistance or diabetes is unclear.

5. Inflammation

As previously discussed, atherosclerosis is a systemic inflammatory disease. Mediators released by inflammation such as C-reactive protein can have a detrimental effect on endothelial progenitor cell function, resulting in impaired survival and differentiation.\textsuperscript{225} However views supporting the contrary exist, and a study assessing circulating EPCs and EPC-CFUs in patients with anginal syndromes demonstrated a positive correlation between CRP levels and both CPC and EPC-CFU numbers, suggesting inflammation can promote CPC mobilisation.\textsuperscript{226} The authors did however acknowledge the small sample size. Recent evidence has shown that acute systemic inflammation alone is insufficient to mobilise EPCs, and
vessel wall injury is also required. The group showed that systemic inflammation induced by *Salmonella typhus* increased levels of VEGF, however failed to cause mobilisation of progenitor cells.\(^{227}\)

6. **Insulin resistance and EPCs**

A number of studies, both human and animal, have demonstrated abnormalities in EPC biology, ranging from reduced numbers, impaired mobilisation and impaired function in insulin resistant states.\(^{228,229}\) Studies have also shown subjects with type 2 diabetes to have reduced numbers of circulating EPCs.\(^{230,231}\) Furthermore, a 12 week course of Rosiglitazone increased EPC numbers and migratory function in newly diagnosed type 2 diabetic patients, independent of its glucoregulatory properties.\(^{232}\) Unpublished work by our group has already shown a negative correlation between homeostasis model assessment of insulin resistance (HOMA-IR) and number of circulating EPCs.

Studies linking EPC function and insulin resistance have mostly been conducted in subjects with diabetes. Teasing out the effect of insulin resistance alone on EPC function from all other facets in a diabetic phenotype is difficult. Recently our group performed a series of experiments on normoglycemic mice with whole body haplo-insufficiency of the insulin receptor (insulin receptor knockout IRKO), assessing the impact of whole body insulin resistance alone on progenitor function.\(^{233}\) CPCs derived from IRKO mice demonstrated impaired endothelial repair following vascular injury - in addition IRKO had absent VEGF induced CPC mobilisation and their EPCs showed impaired paracrine functionality. Transfusion of wild type CPCs into IRKO
mice significantly enhanced vascular repair, providing novel insights into the effect of insulin resistance on vascular repair, progenitor biology and EPC function.

Insulin resistance aside, impaired EPC function in individuals with diabetes can be partly explained by a direct glucotoxic effect on the cells. Hyperglycaemia is a feature of insulin resistance and diabetes, and plays an important role in the pathogenesis of vascular complications in patients with diabetes mellitus. EPCs derived from patients with type 2 diabetes demonstrate impaired adhesion, proliferation and incorporation into vascular structures.\textsuperscript{234} Chronic incubation of early and late outgrowth EPCs with high glucose dose-dependently reduces the proliferation, migration and colony forming numbers, effects not seen with the comparable osmotic control mannitol.\textsuperscript{235} In addition to this, Krankel et al demonstrated that hyperglycaemia resulted in the induction of protein phosphatase 2A (PP2A – an eNOS de-phosphorylator at serine\textsuperscript{1177}), which resulted in a decline of eNOS phosphorylation and NO production in EPCs. Impaired Akt activation was proposed as a possible additional mechanism, though PP2A inhibition did not restore Akt activity completely, indicating other possible hyperglycaemia-mediated mechanisms in EPC dysfunction.\textsuperscript{236}

1.3.6 Endothelial Progenitor Cells and vascular re-endothelialization

An important phenomenon in the pathogenesis of atherosclerosis, in-stent restenosis and vascular conduit failure is endothelial damage/dysfunction and neo-intimal proliferation. By accelerating healthy re-endothelialisation following endothelial insult by ischemia or injury, neo-intimal proliferation could be prevented thus resulting in improved vessel wall healing and reduced atherosclerosis.
Transfusion of EPCs derived from splenic mononuclear cells into splenectomised mice following arterial injury demonstrated enhanced re-endothelialisation and reduced neo-intima proliferation. In balloon denuded rabbit carotid arteries, transfusion of bone marrow derived rabbit MNCs has shown similar results. Hypercholesterolemia plays an important role in atherogenesis, and certainly spleen-derived EPCs transfused from wild type mice into high-fat diet fed splenectomised Apo E−/− mice restored endothelium dependent dilatation, though the effects were not as pronounced as spleen derived MNCs. Furthermore, chronic treatment of aged Apo E−/− mice with bone-marrow derived progenitor cells from young Apo E−/− mice or wild type mice prevented the development of atherosclerotic lesions. This effect is enhanced by statins, and certainly bone marrow derived progenitor cells harvested from C57/BL6 mice treated for 10 days with Rosuvastatin demonstrated increased reendothelialisation and decreased neointimal formation when compared to controls. Similar results have also been demonstrated by other groups.

In subjects with type 2 diabetes mellitus, Sorrentino et al demonstrated that in vivo reendothelialisation of injured carotid arteries in nude mice following transfusion of EPCs derived from these subjects is significantly impaired - a phenomenon that is improved by Rosiglitazone, implying the role of oxidant stress and NAD(P)H oxidases in the EPC dysfunction. Similar effects have been demonstrated in rats with type 1 diabetes mellitus, with insulin administration restoring NO-mediated inhibition of neointimal hyperplasia. Certainly, insulin has a vaso-protective effect, and improves re-endothelialization and decreased neointimal growth following arterial injury. Furthermore, a recent study by Kahn et al demonstrated impaired endogenous vascular repair in insulin receptor knock out (IRKO) mice when
compared to wild type (WT) mice. This effect was normalized when IRKO mice were transfused with EPCs derived from insulin-sensitive animals but not from insulin-resistant animals.\textsuperscript{233} Despite the available evidence, the effect of insulin resistance per se on \textit{in vivo} re-endothelialisation in humans requires further investigation. Furthermore, studies assessing the relevance of these observations in South Asian people are lacking.

1.3.7 \textit{South Asian ethnicity and endothelial progenitor cells}

Data confirming a direct relationship between endothelial progenitor cell dysfunction and South Asian ethnicity is limited. So far, I have discussed the increased propensity of South Asians to develop atherosclerosis, and have linked endothelial dysfunction to the pathogenesis of atherosclerosis. The work of Murphy \textit{et al} assessed endothelial function in South Asian men (n=24) in comparison with matched white European men (n=25).\textsuperscript{132} Ethnicity was defined as subjects, or both of their parents, having been born in India, Pakistan, Sri Lanka or Bangladesh. Brachial artery flow mediated dilatation (FMD) and fore-arm vascular response to intra-arterial injection of Levo-N-monomethyl arginine (LNMMA – a nitric oxide synthase antagonist) was used to assess endothelial dysfunction. South Asian men demonstrated significantly reduced FMD (6.9\% vs. 8.5\%; \textit{p}=0.003) and reduced response to LNMMA, implying impaired NO mediated endothelium-dependent vasodilatation at baseline and in response to shear stress. Furthermore, CPC numbers and EPC-CFU colony numbers were significantly reduced. However as limited functional assays were performed in a small subset of the study group, more
detailed studies in a larger cohort are required. Also, no data regarding the mechanisms of dysfunction were published.

Since then, our group has conducted further work assessing the effect of exercise on circulating progenitor cells in South Asian men. We assessed basal CPC numbers in 15 South Asian and 15 matched white European men. Basal CPC numbers were significantly lower in South Asian men. The study went on to assess the effects of exercise on CPC mobilisation, and demonstrated a significant increase in CPC numbers following exercise, though this was blunted in South Asian men in a NO dependent manner.

To date, this is the only available published evidence linking South Asian ethnicity and endothelial progenitor cell number and function.

1.4. Statins, the Endothelium and Progenitor cells

1.4.1 Introduction

In 1971, Akira Endo and Masao Kuroda commenced research into inhibitors of HMG-CoA reductase. Their work looked into the microbial metabolites that would inhibit this rate limiting enzyme in cholesterol synthesis. They subsequently discovered Mevastatin, which they derived from a strain of *Penicillium citrinum*, a subclass of fungi. Since then a number of statins have been developed with variable potency and pharmacokinetics. Statins are a class of drugs used clinically to lower serum cholesterol. They act by inhibiting the enzyme Hydroxy-methyl-glutaryl
Coenzyme A reductase (HMG CoA reductase), which catalyses the rate limiting step in hepatic cholesterol synthesis. In particular, this group of drugs reduce serum low density lipoprotein (LDL) concentrations, which studies have shown to be a major risk factor in the development of coronary artery disease.\textsuperscript{246} Meta-analyses studying the effects of statins on future coronary events and cardiovascular mortality have demonstrated a significant reduction in major coronary events independent of gender and the presence of hypertension and diabetes.\textsuperscript{247} Reductions in cardiovascular mortality persist in insulin resistant populations.\textsuperscript{248}

1.4.2 Statins and atherosclerosis

As has been discussed previously, inflammation plays a key role in the development of atherosclerosis. Besides their cholesterol lowering properties, statins possess a number of pleiotropic effects, which are speculated to contribute to their clinical efficacy. They have been demonstrated to exhibit anti-inflammatory properties by modulating immune cell activation and subsequently reducing the number of inflammatory cells in the atherosclerotic plaque.\textsuperscript{249} In addition to this, they retard the formation of oxidised LDL, which plays a major role in plaque formation. A number of mechanisms have been postulated, including preservation of superoxide dismutase (an endogenous antioxidant)\textsuperscript{250} and alterations in macrophage handling of oxidised LDL.\textsuperscript{251} Statins also decrease the expression of the endothelial cell leukocyte adhesion molecules VCAM-1 and E-selectin, which are important mediators in the initiation of atherosclerosis.\textsuperscript{252}

The pleiotropic effects of statins include an effect on endothelial dysfunction, with restoration of vasomotor function seen at early stages of treatment.\textsuperscript{102,253} A number
of mechanisms have been postulated, ranging from increased NO production\textsuperscript{254} to complex signalling\textsuperscript{255}.

1.4.3 Statins and endothelial cell signalling pathways

1.4.3.1 Enhanced eNOS expression

NO is a labile-freely diffusing molecule that not only exerts its effects on the cell it is synthesised within, but also on the surrounding cells and molecules\textsuperscript{256}. Reduced bioavailability of NO is a hallmark of endothelial dysfunction. One of the primary mechanisms through which NO production is reduced in endothelial dysfunction is through reduced expression and activation of eNOS. A study on the effects of statins on cerebral ischemia in mice demonstrated that statins up-regulated eNOS expression and activity\textsuperscript{257}. Studies conducted in dogs\textsuperscript{258} and primates\textsuperscript{259} have yielded similar results. In humans, the increase in eNOS expression by statins has been shown to be independent of its cholesterol lowering effect\textsuperscript{260}, with increases noted as early as 2 weeks following commencement of oral therapy\textsuperscript{261}. This increase in eNOS expression is reversed by geranyl geranyl pyrophosphate (GGPP) and not farnesyl pyrophosphate (FPP), implicating the involvement of small GTPases in this process (see section 1.4.3.3 below). However, there is no clear understanding as to how statins activate eNOS and increase NO production. A number of hypotheses exist, including decreased caveolin-1-eNOS interaction\textsuperscript{262} to stimulation of the PI3K/Akt activity\textsuperscript{254} and enhanced formation of eNOS-Hsp90-Akt complex in endothelial cells\textsuperscript{263}. These effects are clearly seen at lower doses of statins; higher doses have been shown to be detrimental to migratory and angiogenic properties of mature endothelial cells due to induction of apoptosis\textsuperscript{264} (see section 1.4.5).
1.4.3.2 Stimulation of PI3-kinase-Akt pathway

Statins have been shown to increase NO production through the PI3 kinase-Akt pathway (see figure 8). Stimulation of this pathway results in rapid phosphorylation of eNOS, with subsequent increased NO production. This process is blocked by wortmannin and LY294002, inhibitors of PI3-kinase. Statin-induced Akt activation is reversed by mevalonate, indicating the role of the HMG CoA reductase pathway in Akt activation and subsequent eNOS activation. Exposure of human umbilical vein endothelial cells to 0.1 microM of Simvastatin results in increased Akt phosphorylation, peaking after around one hour. Similar results have been seen with Atorvastatin, which also induces a time and dose dependent increase in early outgrowth EPC number and adhesive capacity. Dimmeler et al further extended this data by administering Simvastatin (20mg/kg daily) to mice for 3 weeks, and demonstrated an increase in early outgrowth EPCs. Further work concluded that statins exhibited these effects through the PI3-kinase/Akt dependent manner.

Figure 8: Mechanisms through which statins stimulate eNOS activity and increase NO production.
1.4.3.3 Statins and isoprenylated proteins

Statins have been also shown to increase eNOS abundance through isoprenylated proteins (see figure 8). During cholesterol synthesis, a number of isoprenoid derivatives such as farnesylpyrophosphate (FPP) and geranylpyrophosphate (GPP) are produced. These are intermediates that serve as important lipid attachments for post translational modification of various cell signalling proteins. These cell signalling proteins include members of the Rho and Ras GTPase family, and appear to be important targets for inhibition by statins. Translocation of inactive Rho from the cytosol to the membrane depends on geranyl-geranylation. Once activated, they can negatively influence eNOS mRNA half-life, and by disrupting this statins prolong eNOS mRNA half life.\textsuperscript{255, 265} Rho proteins regulate the actin cytoskeleton, changes within which can affect mRNA stability and gene transcription.\textsuperscript{266} In mouse models of ischemic stroke, inhibition of RhoA by statins increased eNOS expression and reduced severity of cerebral ischemia.\textsuperscript{267}

1.4.4 Statins and Endothelial Progenitor Cell function

Studies assessing the effect of statins on EPC biology \textit{in vitro} have demonstrated a significant increase in EPC number and an improvement in function.\textsuperscript{167} Enhanced mobilisation, migration and increased EPC survival has also been shown following \textit{in vivo} administration of statins.\textsuperscript{242}

In experiments conducted by Llevadot \textit{et al}, simvastatin was added to a chemotaxis buffer along with recombinant human VEGF (rhVEGF) and murine GM-CSF in the lower chamber of modified Boyden chamber apparatus. Human peripheral blood-
derived early outgrowth EPC chemotactic activity was increased by simvastatin in a dose dependent manner.\textsuperscript{242} A similar increase in EPC migration has been demonstrated with mevastatin and atorvastatin, an effect that was reversed by mevalonate and LY294002, a PI3 kinase inhibitor.\textsuperscript{264} Enhanced migration was noticed with increasing concentrations of atorvastatin, an effect that was not seen with mature endothelial cells.\textsuperscript{264} Studies assessing EPC function in response to oral atorvastatin in subjects with coronary artery disease showed a significant increase in migratory capacity following a 4 week course of Atorvastatin 40mg.\textsuperscript{268} This augmented migratory capacity paralleled an increase in EPC numbers. EPC migration has been shown to be dependent on PI3 kinase- Akt signalling\textsuperscript{269}, and statins have been shown to enhance the activity of this pathway.\textsuperscript{167} However, work by other groups has shown that in improvement in functional properties of EPCs may also be dependent on its lipid lowering properties, particularly its ability to reduce LDL and thus ox-LDL.\textsuperscript{270} Incubation of EPCs with ox-LDL resulted in impaired cellular migration; an effect that was reversed when cells were pre-treated with Lovastatin.\textsuperscript{270} However, given ox-LDL reduces the PI3 kinase-Akt signalling, the mechanism(s) of statin induced effects on EPC function remain debated.

As previously discussed, once mobilised from the bone marrow, EPCs home to sites of injury or ischemia and adhere to the vessel wall, eventually promoting angiogenesis and vascular repair. Statins have been demonstrated to significantly enhance adhesive properties of EPCs \textit{in vitro}, an effect that could be explained by upregulation of α\textsubscript{v} and β\textsubscript{5} integrin subunits.\textsuperscript{189}
Studies assessing the *in vivo* effects of statins have shown similar results. In a study assessing the effects of statins on reendothelialisation in carotid artery balloon-injured Sprague-Dawley rats, Walter *et al* demonstrated a significant increase in circulating EPCs in response to simvastatin therapy, an effect that was sustained through 4 weeks of therapy. Similar effects have been seen in humans EPCs. These effects have been demonstrated to be dependent on the PI3 kinase Akt pathway and eNOS.

### 1.4.5 Statins and angiogenesis

A number of animal studies have demonstrated promotion of angiogenesis by low dose statins. In a murine model of corneal injury, simvastatin augmented corneal neovascularisation via increased PI3 kinase Akt signalling. Similar results have been obtained with low dose atorvastatin.

Angiogenic effects have been described to be dose dependent, with low dose statins exerting a pro-angiogenic effect, while higher doses exert an anti-angiogenic effect. Described as a ‘double edged sword’, the pro-angiogenic effects of low dose atorvastatin on early outgrowth EPCs seems to be maintained at a higher dose; an effect that was not seen with HUVECs, which showed anti-angiogenic effects at higher doses. Similar results have been obtained with cerivastatin, which was anti-angiogenic at higher concentrations on mature endothelial cells. This variable effect is likely to be due to a differing dose-response curve between the two cell types. At higher doses, statins induce endothelial cell apoptosis, an effect that is not
seen at equivalent doses in early outgrowth EPCs. Furthermore, increased stimulatory Akt phosphorylation is observed in EPCs when compared to ECs with high dose statins, which could explain the difference in apoptotic sensitivity. However, what this effect translates to is not fully clear as we are unsure if culture derived EPCs are in fact generated in vivo: furthermore, the doses of statin administered are orders of magnitude greater than pharmacological doses. Urbich et al demonstrated that at therapeutically relevant concentrations of atorvastatin, the overall effect upon early outgrowth EPCs was pro-migratory and pro-angiogenic. Vincent et al’s study assessing cerivastatin would translate to a 10-fold higher plasma concentration than maximal therapeutic dose, perhaps explaining the anti-angiogenic effects described. In short, this would mean the low concentration of statins in vivo associated with standard clinical doses of statin therapy would resemble low concentration in vitro, producing an overall beneficial effect on EPC function and neovascularisation.

1.4.6 Statins, South Asians and endothelial progenitor cells

As previously discussed, atherosclerotic cardiovascular disease is highly prevalent in South Asian population groups. There are no published studies looking at the effects of statins on EPCs derived from South Asian men. However, post-hoc analysis of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT) has shown Atorvastatin to reduce LDL levels to a similar extent in white Europeans, Blacks (Afro-Caribbean and African) and South Asians, though how this translated into changes in EPC number is unclear. Work performed by our group has assessed basal CPC and EPC number and function, and the effect of exercise on CPC mobilisation. We have
demonstrated reduced CPC number and impaired EPC number/function in South Asian men, and in particular shown a blunted mobilisation of CPCs after exercise. However, contradictory reports have been published, and work by Hughes et al demonstrated no difference between circulating progenitor cells, late outgrowth colony numbers and non-senescent EPCs between European and South Asian subjects. However the study had a number of limitations; in particular some subjects were on regular medications such as aspirin and antihypertensive agents, suggesting that confounding factors may interfere with the assessment of links between South Asian ethnicity and EPC number/function.277

This thesis aims to probe a number of outstanding questions noted in the introduction regarding endothelial progenitor dysfunction in South Asian men, its underlying pathophysiology, and to evaluate the effects of statins on these parameters.
Chapter 2

Aims and hypotheses
2. Aims and hypotheses

As has been discussed extensively in the above introduction, South Asian ethnicity is associated with impaired indices thought to reflect endogenous endothelial repair mechanisms and which are linked with increased prevalence of atherosclerosis and subsequent coronary artery disease. However, there are only two studies that have studied this in more detail, both of which have been performed by our group. While the data is undoubtedly useful and insightful, a clear mechanistic explanation into this dysfunction is lacking. In order to address this, we have planned this project to focus on the following broad themes:

1. Is the finding of South Asian ethnicity being associated with reduced basal *in vitro* EPC numbers reproducible?
2. Is early outgrowth EPC function (migration, adhesion, and tube forming capacity) impaired in South Asian men?
3. Do pharmacological stimuli (e.g. Simvastatin) improve early outgrowth EPC functional properties?
4. Are late outgrowth EPCs derived from South Asian men dysfunctional?
5. What are the molecular mechanisms underlying EPC dysfunction in South Asian men?
6. Are South Asian EPCs also dysfunctional when assessed using *in vivo* models of vascular injury?
7. Can molecular mechanisms involved in EPC dysfunction in South Asian men be modulated to improve their functional properties?

In essence, these objectives are aimed at validating previous findings and expanding on a small body of evidence supporting impaired EPC function in South Asian men.
Furthermore, by studying the molecular mechanisms involved in this dysfunction, potential therapeutic strategies may be studied in the future.

Based on the above aims, the following key null hypotheses can be made, which will be addressed in the respective subsections of the results chapter. The first aspect covers early outgrowth EPCs and effect of Simvastatin on their function.

1. Early outgrowth EPC function is not impaired in South Asian men
2. Simvastatin does not improve the function of early outgrowth EPCs from South Asian men

The second aspect deals with late outgrowth EPCs.

1. Late outgrowth EPC function in vitro is not impaired in South Asian men
2. Late outgrowth EPC function in vivo is not impaired in South Asian men
3. Dysfunction of late outgrowth EPC from South Asian men is not amenable to restoration by manipulation of key intracellular signalling cascades.
Chapter 3:

Materials
3. Materials

3.1 Demographics and biochemical assessment

- Human Insulin ELISA – Mercodia #10-1113-10
- Sphygmomanometer – Omron MX3 plus
- Blood analyses was performed in the biochemistry laboratory of Leeds General Infirmary:
  - Full blood count
  - Renal function tests
  - Liver function tests
  - Fasting plasma glucose
  - Thyroid function tests
  - Fasting lipid profile including total cholesterol, HDL and LDL

3.2 General laboratory supplies

- Phosphate buffered saline – Sigma
- Ficoll paque plus – GE healthcare
- Fetal calf serum – Biosera

3.3 Endothelial progenitor cell culture

- Biocoat fibronectin coated plastic culture vessels in 6-well, 24-well, T25 flask and T75 flask format – Becton Dickinson
- Polycarbonate Boyden chambers with 8µm pore size - Becton Dickinson
- Trypsin EDTA 0.025% solution – Gibco BRL
- EGM-2 culture medium ± EBM2 Singlequots growth factor supplements – Lonza
- Vascular Endothelial Growth Factor-165 – R&D systems
• Olympus CKX41 Fluorescence microscope with 'Cell F' software
• Cryomedium (CryoSFM) – Promocell
• Haematoxylin and Eosin – Sigma
• Formalin 4% (b/v) in distilled water – Sigma
• Dil-AcLDL – Invitrogen
• Ulex Europeus-FITC conjugate – Invitrogen
• CM-Dil CellTracker C-7001 - Invitrogen

3.4 EPC intracellular and protein assessment (Western blot)
• BCA protein assay ELISA kit (Thermo-scientific)
• Phospho-eNOS(ser 1177) rabbit mAb – Cell signalling
• eNOS rabbit mAb – Cell signalling
• Phospho-Akt (ser 473) rabbit mAb – Cell signalling
• Akt rabbit mAb – Cell signalling
• Immobilon Western Chemiluminiscent HRP substrate - Millipore

3.5 EPC senescence assay
• Senescence β-galactosidase staining kit – Cell signalling

3.6 EPC in vitro angiogenesis assay
• Matrigel (growth factor reduced) - BD biosciences #356231

3.7 Simvastatin
• Simvastatin 5mg – Sigma

3.8 Western blot assay
• TBS tween
  • 20 mM Tris-HCl, pH 7.4
- Stripping buffer
  - Thermo Scientific Restore Western Blot stripping buffer #21059
- Running buffer
  - 25 mM Tris base
  - 190 mM glycine
  - 0.1% SDS
- Radioimmunoprecipitation assay (RIPA) buffer
  - 150 mM NaCl
  - 0.1% Triton X-100
  - 0.5% sodium deoxycholate
  - 0.1% SDS (sodium dodecyl sulphate)
  - 50 mM Tris-HCl pH 8.0
- Protease Inhibitors
  - Leupeptin 10 mg/mL (1000x)
  - Aprotinin 10 mg/mL (1000x)
  - Phenylmethylsulfonyl fluoride, 200mM in ethanol (100x)
- Phosphatase inhibitors
  - 100 mM NaF
  - 50 mM NaVanadate
  - 800 mM β-glycerol phosphate
- Non-fat dried milk
- Chemiluminiscent substrate
  - Millipore – Immobilon Western Chemiluminiscent HRP substrate
3.9 Mouse model of femoral artery injury

- Microscopes
  - Nikon SMZ1500 Stereo microscope for dissecting tissue
  - Olympus SZ61 with up to 45x magnification with QiCam Olympus digital camera
- Fluovac Anaesthetizing chamber with gas scavenging system, Harvard apparatus
- Compact Anaesthesia System AN001 (Vet Tech Solutions Ltd, UK) Fluovac system, Harvard apparatus
- Isoflurane Key fill applicator AN003B
- Heating plate (Vet Tech Solutions Ltd, UK)
- Thermal cage (HE-011 (Vet Tech Solutions Ltd, UK)
- Light source Schott KL1500 LCD
- Guide Wire 0.014” (0.010” (0.25mm) at 3 cm on the top) (Hi-Torque Cross-IT 200XT, AbbotVascular, USA)
- Micro serrifine curved (Inter Focus, #18055-05)
- Forceps type clip applicator (Inter Focus, #18057-14)
- Needle holder (World Precision Instrument, #14109)
- Forceps and scissors:
  - Vannas scissors (WPI, #501778) for making arteriotomy
  - Vannas scissors (Inter Focus, #91501-09) for cleaning vessels
  - Fine Iris scissors (Inter Focus, #14094-11) for making skin cut
  - Tweezers Dumont, WPI (500339)
  - Tweezers Dumont, WPI (500234)
- Syringes
- Terumo Insulin syringe 0.3ml with needle 29G x 13mm  613-4900
- Terumo Insulin syringe 0.5ml with needle 27G x 13mm  TERUBS - 05M2713
- Terumo Insulin syringe 1ml with needle 29G x 13mm  613-4904

• Surgical sutures
  - VICRYL absorbable surgical suture, 6-0, ETHICON, W9575, (Johnson & Johnson)
  - VICRYL absorbable surgical suture, 8-0, ETHICON, W9577, (Johnson & Johnson)

• Microscope slide (Cell Path, #MAE-1000-03P)

• Microscope Cover-slip 22 x 22 mm (SLS, # MIC 3114)

• Others
  - Cotton buds
  - Gauze
  - Veet hair removal cream
  - Hair shaver
  - Seller tape

• Reagents
  - Euthanal (Pentobarbital sodium Ph.Eur.)
  - 1% DPBS
  - 0.9% NaCl (saline)
  - 4% paraformaldehyde (Paraformaldehyde, Fisher (P/0840/53)
  - Surgical scrub Providone-Iodine (Vetasept®, Animal Care, York)
o Rodent No.1 maintenance diet RM1(E); code 801002, SDS (Specialist Diet Service), UK

o Evans Blue (Fluka, 46160)

o Buprenorphine (Buprenorphine 0.3 mg/ml as hydrochloride (Ph.Eur.) Alsatoe Anima Health, York, UK

3.10 Lentiviral Transduction of constitutively active Akt

- QuickTiter™ Lentivirus Titer Kit #VPK-107 - Cell Biolabs
- Akt Kinase assay kit #9840 - Cell signalling
- FuGENE® HD transfection reagent - Promega #E2311
Chapter 4:

Methods
4. Methods

4.1 Subject identification and recruitment

Ethical approval for subject recruitment was obtained from the Leeds (East) Research Ethics committee (Ref No 08/H1306/27). Recruitment posters were displayed over the University campus. South Asian ethnicity was defined as individuals who identified themselves as of Indian, Pakistani, Bangladeshi, Nepalese or Sri Lankan origin based on UK National Health Service approved lists.278

Healthy South Asian male subjects with no previous medical history were recruited. All subjects were between the ages of 18 to 40 years, and were non smokers with no diabetes mellitus (defined by the WHO as fasting plasma glucose > 7 mmol/l), obesity (defined as BMI > 30) hypertension (defined as a blood pressure over 140 mmHg systolic and/or 90 mmHg diastolic) or hypercholesterolemia (total cholesterol>7mmol/L). These exclusions were applied to avoid the impact of their confounding influence upon EPC function.170, 279, 280 Due to the effects of the menstrual cycle and estrogens on EPC number and function,281 females were not recruited in any of the studies. All potential subjects were screened for the presence of any of the exclusion criteria through a questionnaire, clinical examination and blood tests.

Blood tests were performed after an overnight fast, and included a full blood count, full lipid profile, liver function, renal function, thyroid function, insulin and blood glucose. Blood pressure, BMI (weight in kg/height in m²) and waist: hip ratio was obtained. All samples other than the fasting insulin levels were analysed in the Leeds Teaching Hospitals NHS Trust chemical pathology laboratory at Leeds General
Infirmary. Insulin concentrations were obtained using Human insulin ELISA kit as per manufacturer's instructions. Standard curve calibration was only accepted if R>0.99 with samples analysed in duplicate; intra-assay coefficient of variation (CV) was required to be <10% for optical density >0.1 arbitrary units. In house quality controls were used in all experiments to ensure inter-assay CV <5%. The homeostasis index of insulin resistance (HOMA-IR) score was calculated using the formula insulin (mU/L) x fasting glucose (mmol/L) / 22.4 \textsuperscript{282}, with higher values indicating greater degree of insulin resistance.

4.2 Early outgrowth EPCs and Simvastatin

4.2.1 Culture of Early outgrowth EPC's

Whole peripheral blood was drawn from subjects into EDTA coated tubes and transferred to 50ml Falcon tubes. An equal volume of Dulbecco Phosphate buffered saline (DPBS) was added, and the resultant mixture was layered onto Ficoll paque plus. As per manufacturer's instructions, density gradient centrifugation was carried out, and peripheral blood mononuclear cells (PBMCs) were aspirated from theuffy coat layer. These were washed and re-suspended with DPBS and subject to re-centrifugation at 300g for 10 min at 18°C. The cell pellet obtained was suspended in EGM2 growth medium with EGM-2 single bullet kits and 10% fetal calf serum (FCS) (v/v), with an equivalent of 5 million cells per 2 ml of medium placed per well of a 6-well fibronectin coated plate. Cells were then incubated at 37°C with 5% CO\textsubscript{2}. After 3 days, cells were washed with DPBS and fresh medium replaced. The non-adherent cells were thus removed, leaving behind cells firmly adherent to the
fibronectin. The cells firmly adherent between 4 - 7 days were predominantly early
toutgrowth EPCs. The phenotype of early EPC’s was confirmed by assessing co-
staining with Dil - AcLDL and Ulex Europeus lectin – FITC conjugate using
fluorescence microscopy (see section 4.2.2.3).

4.2.2 In vitro exposure of endothelial progenitor cells to Simvastatin

In order to assess the effect of statins on endothelial progenitor cell number and
function, Simvastatin at 0.1 µM was added to each culture well as described below.
Previous experiments conducted with Simvastatin and EPCs have used varying
concentrations of Simvastatin ranging from 0.01 µM to 1 µM. On reviewing the
pharmacokinetic data of Simvastatin, an oral dose of 40mg (normally prescribed
for therapeutic effect in hypercholesterolemia) achieves a maximum concentration of
16.2 ng/ml at 2.3 hours. Based on this data, and taking into consideration the
molecular weight of Simvastatin of 418, a 40 mg oral dose equivalent would amount
to an in vitro concentration 0.04 µM. When pilot work was performed with 0.1 µM and
1 µM of Simvastatin with early outgrowth EPCs, cell death was seen with 1 µM
concentrations (see section 5.2). Given the use of 0.1 µM is broadly comparable to
pharmacological concentration, and cell death was seen at higher concentrations,
we opted to use 0.1 µM in our experiments.

4.2.2.1 Preparation of Simvastatin

Simvastatin was purchased from Sigma-Aldrich. 4mg Simvastatin was dissolved in
100 µL ethanol. 150 µL of 0.1M sodium hydroxide (NaOH) was added to this, and
the mixture was incubated in a water bath at 50 ºC for 2 hours. Following this the
solution was returned to pH 7 with hydrochloric acid (HCl) and the stock solution was
made up to 1 ml with ethanol to obtain a final concentration of 4mg/ml. 0.5 µL of this solution added to 2ml EGM-2 medium resulting in a concentration of 0.1 µM Simvastatin. In order to assess the effect of Simvastatin alone, vehicle solution was prepared for control experiments by incubating 100 µL ethanol with 150 µL of 0.1M NaOH in a water bath at 50 °C for 2 hours. Following this the solution was brought up to pH 7 with hydrochloric acid and the stock solution was made up to 1ml with ethanol.

4.2.2.2 Cell culture assays

PBMC’s were plated as described above into 6 well fibronectin coated plates at a concentration of 5 million cells / well. On day 3, control wells received standard medium with appropriate volume of vehicle, and intervention wells were exposed to 0.1 µM Simvastatin in standard EGM-2 medium. On the following day, cells were enumerated and used in functional studies.

4.2.2.3 Confirmation of early outgrowth EPC phenotype

Following aspiration of culture medium, cells were washed twice with DPBS and stained with Dil - AcLDL 10 µg/ml in supplemented EGM-2 medium. The plate was incubated for 3 hours at 37°C with 5% CO₂. Following two wash steps with DPBS, cells were fixed with 4% paraformaldehyde (v/v) for 15 minutes, and rewashed. Further staining with 10 µL Ulex Europeus Lectin - FITC conjugate in 990 µL in DPBS was performed for one hour, prior to washing with DPBS. Cells were then analysed using phase contrast microscopy, along with corresponding red (DiI) and green (FITC) fluorescence microscopy using an Olympus CKX-41 fluorescence microscope at 200x magnification and Olympus Cell F software. Cells appearing yellow on overlay images were defined as early outgrowth EPCs according to
standard definitions (see figure 9). Cells were counted in 10 random high power fields (200x) and expressed as number of cells per high power field. Mean inter-assay CV in 3 identically prepared experiments was 10%.

Figure 9: EPC enumeration assay. Images of early EPCs were taken using phase contrast (A) microscopy and Dil AcLDL (B) and Ulex Europeus lectin (C) fluorescent microscopy. Final EPC number was determined by counting cells seen in the overlaid image per high powered (200x) field (D).

4.2.2.4 Migration assay

Early EPC’s treated with vehicle or simvastatin were suspended in un-supplemented EGM-2 and 5 x 10^4 cells in 500 µL medium placed in the upper compartment of modified Boyden chambers with a polycarbonate membrane base and 8 µm pores. The chambers were placed in a non-coated 24 well plate, each well containing 750
µL of un-supplemented medium in triplicate in control wells; 50 ng/ml Vascular Endothelial Growth Factor in EGM-2 was used as chemotactic stimulus to migration in the remaining wells. Cells were incubated for 24 hours at 37°C with 5% CO₂, and then fixed with 70% ethanol for 1 hour at -20°C. After removing the cells from the upper surface of the membrane and washing with DPBS, membranes were stained with Haematoxylin and Eosin for 30 seconds each. Migrant EPC’s per 10 high power fields (715 x 530 µm) were counted (200x) and results expressed as mean cell number per high power field. Pilot work in 3 identically prepared experiments performed previously by our group has shown an intra-experimental CV of 7.8%.

Figure 10: Representative control, VEGF-stimulated and statin-treated EPC migration assays. Hematoxylin and Eosin stained EPCs adhere to the lower surface of the modified Boyden chamber. EPCs and pores are labelled.
4.2.2.5 Adhesion assay

Both untreated and pre-treated (with Simvastatin or vehicle) early EPCs were suspended in EGM-2 with growth factors and plated in a 24-well fibronectin coated plate in triplicate at a concentration of $5 \times 10^4$ cells / well. These were incubated at 37°C with 5% CO$_2$ for one hour. The non-adherent cells were then gently washed twice with DPBS, and the adherent cells were counted (10 high powered fields 715 x 530 µm). Results were expressed as mean cell number per high powered field. Mean inter-assay CV in 3 identically prepared samples was 17%.

Figure 11: Representative control and Simvastatin-treated EPC adhesion assays. Phase contrast microscopy shows EPCs adherent to fibronectin coated surface of culture plates.

4.2.2.6 Tubule formation on matrigel

Tubule formation on Matrigel was used to assess paracrine stimulation of in vitro angiogenesis by EPCs. Matrigel was thawed overnight on ice, and the following day 300 µL of Matrigel was coated onto the surface of each well of a 24 well culture plate. The plate was incubated at 37°C with 5% CO$_2$ for 30 minutes to facilitate gelling of Matrigel. $5 \times 10^4$ HUVECs were suspended in 500 µL EPC conditioned
medium (derived from both pre-treated and untreated cells) and placed into wells in duplicate. The plate was incubated for 24 hours at 37°C with 5% CO₂ and tubules were counted in 5 high powered fields (x100 - 1430x1060μm). Results were expressed as the number of tubules per high powered field.

**Figure 12:** Tubule formation on Matrigel

### 4.3 Late outgrowth EPC culture and conduit vessel re-endothelialisation

#### 4.3.1 Culture of late outgrowth EPCs

PBMC’s derived from 50ml whole blood were harvested as described above, and plated in 12 ml of EGM-2 medium with EGM-2 bullet kit and 10% FCS in T75 fibronectin coated flasks. Cells were washed with DPBS and medium changed daily for the first week, followed by alternate day half medium changes over the next 3-4 weeks. At approximately 3-4 weeks, larger cells with a cobblestone like morphology were noted to develop in colonies. Colonies were identified by their specific microscopic appearance of a spatially distinct group of cells.
These cells eventually expanded into larger colonies and exhibited contact inhibition, with a capability of serial passaging as previously reported\textsuperscript{152}. The endothelial phenotype of these cells has been previously confirmed by my colleague Dr Richard Cubbon (as a part of his PhD dissertation) using FACS analysis demonstrating co-expression of KDR and CD31 in 89% of cells (figure 14). All specimens derived in this project underwent assessment for the presence of eNOS by Western blotting, also supporting their endothelial phenotype. Once confluent in a T75 flask, the cells detached with trypsin, suspended in cryopreservation medium and stored in liquid nitrogen for future use (see section 4.3.3).
Figure 14: FACS analysis of late outgrowth EPCs – co-expression of KDR (PE) and CD31 (FITC) is seen in 89.8% of cells (from unpublished work by Dr R M Cubbon)

4.3.2 Population doubling time assay

In order to calculate cell population doubling time, EPCs derived from PBMCs were counted at every passage until fourth passage (P4), then cryo-preserved. The time point at which the cells were passaged was noted as was the total cell number prior to each re-plating. Population doubling time was calculated using software available online capable of calculating doubling times over a series of cell passages and time points (www.doubling-time.com/compute.php). In brief, given two cell counts at 2 separate time points, and assuming a constant growth rate, the population doubling time ($T_d$) was calculated using the equation:

$$T_d = (t_2 - t_1) \times \frac{\log (2)}{\log (q_2/q_1)}$$
where q1 is the cell count at time t1, and q2 the cell count at time t2.

4.3.3 Cryostorage of late outgrowth EPC’s

Growth medium was removed from T75 flasks, and cells were washed twice with 10 ml DPBS in order to remove all latent trypsinases present in foetal calf serum. Pre-warmed trypsin-EDTA 0.025% was added to the flask at 40 µL/ sq cm, and the flask was incubated at 37°C for 90 seconds. Once removed from the incubator, the flask was tapped gently on all sides for 30 seconds to dislodge any cells that remain weakly adherent. EGM-2 with additives and FBS was then added to deactivate the trypsin. The resultant cell suspension was centrifuged at 300g for 10 min at 18°C. The cell pellet obtained was washed with DPBS and then re-centrifuged. The final cell pellet obtained was re-suspended in cryomedium at a concentration of 1 x 10^6 cells/ml and stored in cryo-vials. These were cooled at a rate of <1°C/min overnight (in order to reduce cell attrition from ice crystal formation) in a -80°C freezer, following which they were transferred to liquid nitrogen storage. When required, cryovials were removed from liquid nitrogen and rapidly warmed to 37°C in a water bath. Cells were then suspended in EGM-2 medium with EGM-2 bullet-kit and re-plated on either a 6 well fibronectin coated plate or a fibronectin coated T75 flask. Medium was changed completely within 24 hrs in order to remove latent cryo-medium which is potentially toxic to the cells. Cells stored in liquid nitrogen retained their proliferative capacity and functional properties after more than 6 months of storage.
4.3.4 Western Blot assay

4.3.4.1 Cell harvesting

Cells were serum deprived in un-supplemented EGM-2 with 1% FCS for 24 hours in order to assess basal signalling activity. Cells were then removed with a cell scraper following the addition of 70 µL of lysis buffer (containing Tris 50mM, NaCl 150 mM, SDS 0.1 %, Na.Deoxycholate 0.5 %, Triton X 100 or NP40 1% and protease and phosphatase inhibitors). The procedure was performed on ice. Lysates were either stored at -80°C or subject to centrifugation. The supernatant was aspirated and transferred to a fresh eppendorf, and the pelleted debris discarded.

4.3.4.2 Total protein quantification

The sample total protein concentration was first estimated using the Bicinchoninic Acid assay to ensure equal protein loading when later performing Western Blot analysis. 8 µL of each lysate sample was diluted 8-fold with 56 µL of milliQ distilled water. The samples were vortexed to ensure optimum mixture, and 25 µL of the sample was loaded in duplicate on a 96 well plate. Standards were prepared by serial dilution of 1mg/ml BCS protein assay kit as per manufacturer’s instructions and loaded onto the same plate in duplicate. 200 µL of a 50:1 dilution of Bicinchoninic acid: 4% copper (II) sulphate was then pipetted into each of the wells with the standards and samples. This initiates a two-step reaction – the first reaction to occur is the ‘biuret reaction’ - chelation of copper in an alkaline environment resulting in a light blue complex. The second step results from the reaction of the cuprous cation produced from step one with bicinechinonic acid, resulting in a light purple coloured
complex. These reactions occur when the plate is incubated at 37°C for 30 minutes after covering with sealing foil. Absorption spectra are then measured by colorimetric assay at 562nm. The protein content is then calculated in reference to a standard curve generated with known concentrations of bovine serum albumin, with correction for the initial 8-fold dilution.

4.3.4.3 Protein gel resolution and transfer

From the protein concentrations obtained above, the volume of lysate containing 50 µg of protein was calculated. Any shortfall in volume under 30 µL was made up with lysis buffer. Loading dye is added to achieve a 1:4 dilution, followed by reducing buffer to obtain a 1:10 dilution. The lysate was boiled at 95 degrees C for 5 min. The reduced samples were then cooled on ice, vortexed and pulse centrifuged, then loaded onto a 4-12% pre-cast polyacrylamide gel. One well was loaded with a protein standard ladder for reference. Running buffer was loaded into the gel running system and samples were resolved by electrophoresis at 160V for 1 hour. Once completed, the gel with resolved protein was removed from the gel-case and placed over the PVDF membrane pre-soaked in methanol and transfer buffer. These were then sandwiched between 3mm filter paper, ensuring firm contact, and then placed in a transfer cassette in an orientation which ensured correct direction of transfer. This is then connected to a power pack supplying a constant 100V for 45 min. Upon completion, the membrane was removed and air dried for future immunostaining.
4.3.4.4 Immunostaining

When required, membranes were refreshed by washing in methanol for 10 seconds, and rinsing in distilled water. Stripping buffer was added for exactly 5 minutes. This was then washed with TBS Tween three times for 15 minutes in total. The membrane was then blocked using 5 ml of 5% milk buffer (25 ml TBS Tween + 1.25g dried skimmed milk powder) in a universal container on a roller for 1 hour. Blocked membranes were then transferred to new containers containing milk buffer and primary antibody (See table 2). These were probed overnight at 4°C on a roller. The following day, buffer and antibody were washed off with TBS Tween for 3 cycles of 5 minutes each. Membranes were then probed by an appropriate secondary antibody conjugated to horseradish peroxidase (see table 2) for 1 hour.

<table>
<thead>
<tr>
<th>Antibody Target</th>
<th>Antibody Type</th>
<th>Source</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>Monoclonal Mouse</td>
<td>BD Biosciences</td>
<td>1:1000</td>
</tr>
<tr>
<td>Serine&lt;sup&gt;1177&lt;/sup&gt; phospho-eNOS</td>
<td>Monoclonal Mouse</td>
<td>Cell Signaling</td>
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<tr>
<td>p16 INK 4a</td>
<td>Rabbit</td>
<td>Cell Signaling</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

Table 2: Antibodies directed toward specific antigens for Western blot protein assay
Once probed, the membranes are washed again with TBS Tween three times for 15 minutes each followed by low salt TBS solution for 15 minutes (or even longer if desired) to remove excess antibody. The membrane was then covered with chemiluminiscent substrate solution for 5 minutes, after which the solution was allowed to run off and the membrane sandwiched between two translucent acetate films. This was then analysed with a digital camera and the band densities analysed with the accompanying proprietary software. In order to standardise results, all band densities were normalised to the corresponding sample beta-actin density.

4.3.5 Senescence β-galactosidase assay

EPC senescence was measured using a commercially available β-galactosidase assay kit. As confluent cells exhibit enhanced senescence due to contact inhibition, sub confluent late outgrowth endothelial progenitor cells in one well of a 6-well plate were washed twice with DPBS and then fixed with 1ml of supplied fixative (20% formaldehyde and 2% glutaraldehyde in 10x DPBS) for 15 minutes. While the cells were being fixed, the working staining solution was prepared using the supplied stock staining solution (400 mM citric acid/sodium phosphate (pH 6.0), 1.5 M NaCl, 20 mM MgCl$_2$), X-gal (5-bromo-4-chloro-3-indolyl-βD-galactopyranoside powder), staining solution A (500 nM potassium ferrocyanide) and staining solution B (500 nM potassium ferricyanide) as per manufacturer’s guidance. The fixative was then washed and replaced with a 1ml of working staining solution and the 6-well plate placed in an incubator overnight at 37°C with standard atmospheric air in order to maintain the pH of the solution at 6.
The following day, with the β-galactosidase still on the plate, cells were photographed at 200x magnification (715 x 530 µm) for the development of deep blue nuclear colour. Senescent cells were expressed as a percentage of blue cells in the total number of cells. As is seen in figure 15, cells that are senescent stain a deep blue, while the non-senescent cells stained a lighter shade of blue or did not stain at all. Acknowledging that this made analysis of the images subjective and could result in poor reproducibility, inter-observer agreement was assessed (see section 6.3).

4.3.6 Polymerase chain reaction

In order to study mRNA expression of Akt and eNOS, polymerase chain reaction (PCR) was performed. The experiments were performed with Dr Piruthivi Sukumar. Late outgrowth EPCs in 6 well plates were washed with DPBS, and lysed using 500 µl TriReagent. The lysate was transferred to an eppendorf and stored at -80°C for future use.
The lysates were subject to centrifugation at 13000 rpm at 4°C for 10 min to pellet out the cell debris. The supernatant was mixed thoroughly with 50 µl of bromochloropropane and left at room temperature to allow phase separation. The mixture was then subject to centrifugation at 13000 rpm at 4°C for 15 mins to complete phase separation. The topmost layer containing the RNA was carefully pipetted into a separate eppendorf, and the RNA was precipitated with isopropanol and glycogen by further centrifugation. The pellet is then washed with ethanol and dissolved in water.

Following this stage, the sample was treated with DNase mastermix to remove genomic DNA content. The quantity of RNA was measured using Nanodrop controlled by ND-1000 software.

In order to perform the reverse transcription stage of PCR, the quantity of the sample containing 1000ng of RNA was reverse transcribed by high capacity reverse transcriptases to cDNA mastermix. Using the forward and reverse primers specific to the Akt and eNOS gene, real-time PCR was performed with SYBR green base detection system in Applied Biosystems' 7900HT controlled by SDS 2.2 software. Results were presented after normalising to house-keeping (β-actin) gene. Primer sequences were as follows -
4.4 Re-endothelialisation following mouse femoral artery wire injury

In order to study the *in vivo* functional properties of late outgrowth EPCs derived from both SA and WE men, a series of transfusion experiments after femoral artery wire injury were conducted in nude mice.

4.4.1 Deriving late outgrowth EPC

Late outgrowth EPCs were derived as described in section 4.3.1. Cells were thawed from cryopreserved cell banks and placed in T75 flasks. Cells were harvested for transfusion studies once confluent.

4.4.1.1 Harvesting late EPCs

Prior to harvesting late EPCs from the T75 flasks, a fluorescent cell tracker solution was prepared by dissolving 50μg of CM-Dil in 50μL of DMSO, and then dissolving this in 50ml of standard supplemented EGM-2 growth medium. Medium was
discarded from the T75 flask, and replaced with cell tracker enriched medium for 5 min. This medium was then discarded and the cells washed twice with DPBS, and subsequently removed from the plate using trypsin as described earlier. The total number of cells was counted, and the volume of medium containing $3.5 \times 10^5$ cells was aliquoted. These cells were then subject to centrifugation at 300G for 10 min at 18°C. The cell pellet was then washed and suspended in 200μL of standard supplemented EGM-2 growth medium. This was drawn into a sterile 300μL microsyringe and transported in a sterile container on ice to the clinical sciences building at St. James’s hospital, where transfusion experiments were carried out.

4.4.2 Vascular injury protocol

Animal experiments were carried out under the regulation of the Animal (scientific procedures) Act 1986. Murine surgery was performed by Dr Nadira Yuldasheva. Male nude CD1$^{-/-}$ mice (9-11 weeks of age; weighing 26.5-32.0 g) were chosen as recipients of human LEPC in order to avoid alloimmune reactivity with donor material. Mice were fed a standard chow diet, and were given ad libitum access to food and water. All animals were in good health. Prior to vascular injury, and the later harvesting of vessels, the mice were anaesthetised with volatile isoflurane saturated with oxygen via nose mask. A regulator was used to titrate the content of Isoflurane (~1.5%) and oxygen.

The mouse was placed in supine position with paws fixed on the mask tube and its lower extremities abducted and extended. It was given an intra-peritoneal injection of
0.25 mg/kg buprenorphine in 0.9% NaCl. The lower abdomen and both groins are cleaned with 3% H₂O₂ and povidone-iodine. The operation performed with the assistance of surgical microscope (Nikon SMZ1500) under appropriate magnification (~ x7.2 to x19.2). A 1.2 cm longitudinal groin incision is made, crossing the inguinal ligament. The femoral vessels were exposed at two sites: near the inguinal ligament and distal to the bifurcation of the saphenous artery from the femoral artery. The structure between these two sites, particularly the fat pad containing the epigastric vessels was not dissected. Sparing these structures minimizes traumatic consequences and preserves collateral flow after injury. The segment below bifurcation of femoral artery (to the saphenous artery) was dissected free from vein and nerve and encircled with 8-0 vicryl suture (two ligatures). The external iliac artery and vein were occluded temporarily using an atraumatic vascular mini clamp placed just below inguinal ligament or external iliac artery, dissected from vein, or encircled with 8-0 vicryl suture. An arteriotomy was made approximately 1 mm distal to bifurcation of femoral artery. A 0.010” (0.25mm) guide wire was introduced into the arterial lumen, the clamp was removed (or ligature was freed) and then the wire was advanced ~1.5 cm, to the level of aortic bifurcation. The wire was advanced and pulled back three times through femoral artery, and the external iliac and common iliac were abraded three times over their entire length (~1.5 cm). Prior to removal of the wire the blood flow was stopped and the arteriotomy site was ligatured by tying the proximal and distal sutures placed previously. The same procedure was carried out on the contra-lateral side without arteriotomy (sham operation). The skin incision was closed with continuous, 6-0 vicryl suture and povidone-iodine was applied on the wound. The mouse was injected intra-peritoneally with 150 µl 0.9% NaCl solution.
4.4.3 Vessel harvesting, imaging and analysis of results

On the fourth day following vascular injury, the mice were anesthetised with volatile isoflurane (as described above) and an incision was made on the linea alba to access the inferior vena cava (IVC). The IVC was then injected with 75 µL of 5% Evans blue in 0.9% NaCl. After a few minutes, the thoracic cavity was opened and the mouse was perfused-fixed by injecting the left ventricle with 0.9% NaCl solution followed by 4% paraformaldehyde in DPBS (pH 7.0 to 7.2). The femoral, external iliac and common iliac arteries along with the distal aspect of the abdominal aorta (at the region of the common iliac bifurcation) were carefully dissected from the surrounding tissue. Arteries (injured and contralateral uninjured) were separated from attached fascia and fat by careful dissection, and a longitudinal incision extending from the exposed abdominal aorta to the femoral artery was made. The dissected vessel was then mounted between a histological slide and a cover slip.

![Figure 16: Estimation of re-endothelialisation of injured femoral artery following EPC transfusion.](image)

The specimen was digitally photographed and analysed using Image Pro software version 7.0 (Media Cybernetics, Bethesda, MD) to calculate the percentage of the stained and unstained area. The measured area was standardised for all samples as extending from 5mm from the proximal suture. Results were analysed using student’s t test.
4.4.4 Confocal microscopy

Following *en face* assessment of the proportion of re-endothelialisation, confocal microscopic assessment of late EPCs adherent to the vessel wall was performed. The vessel was mounted with Vectashield DAPI prior to assessment using a confocal microscope (Zeiss LSM 510 META Axioplan 2).

4.4.5 Akt transduction using Lentiviral vector

In order to study the effect of augmenting Akt activity function of late outgrowth EPCs, transduction studies using lentiviral vector gene delivery of constitutively active Akt-1 mutant were performed. The lentiviral vectors used are based on human immunodeficiency virus-1 (HIV-1) given its advantage and suitability of use in both dividing and non-dividing cells. The experiments were performed with assistance from Dr Hema Viswambharan and Dr Sam Stephen, who performed the viral titration pilot work and lentiviral delivery of constitutively active Akt.

a. SIN-Lentiviral production

Recent reports have described mutation in AKT1 to be linked to various forms of human cancer. In particular, substitution of the glutamic acid by lyseine at amino acid 17 (E17K) results in altered morphology of the Pleckstrin Homology domain, promoting localisation of AKT1 to the plasma membrane and so subsequent phosphorylation of amino acids serine 473 (Ser473) and threonine 308 (Thr308). In our experiments, the EGFP lentivirus, pSINC6GWdINot1 was a kind gift of Dr.
Yashiro Idea (Mayo Clinic, Minnesota, USA). The E17K Akt mutant constructs was kindly donated by Dr JM Askham and SIN-lentiviral vector pHV-SLS 2 expressing E17K mutant was produced by Dr SL Stephen by cloning E17KAkt in place of the enhanced Green Fluorescent Protein (EGFP) cassette in pSINCSGWdlNot1. The SIN-Lentiviral vectors were generated as described by Zufferey et al.287

b. Titration of Lentiviral samples

The Lentiviral vectors were titred using a p24 ELISA kit as per manufacturer protocol (QuickTiter™ Lentivirus Titer Kit #VPK-107). In brief, for each lentiviral sample, the HIV p24 standard, blank and control medium were all assayed in duplicate. 100μL of inactivated lentiviral sample or p24 antigen standard was added to anti p24 antibody coated plate. This was incubated at 37 degrees C for 4 hours.

The plate was then washed with supplied wash buffer and 100μL of diluted FITC-Conjugated anti-p24 monoclonal antibody was added to each well. This was incubated for 1 hour at room temperature on an orbital shaker, and then washed three times with wash buffer. 100μL of diluted HRP-Conjugated anti-FITC Monoclonal antibody was added to all wells, and this was again incubated at room temperature for 1 hour, followed by a similar wash. The substrate solution was brought to room temperature and 100μL of this was added to all wells. Following incubation at room temperature for 30 minutes, 100μL of stop solution was then added, and the absorbance results were read immediately using a spectrophotometer at 450 nm. These data were then used to infer the concentration of lentiviral particles (infectious or non-infectious), as per manufacturer’s instructions.
c. Late outgrowth EPC culture

Late outgrowth EPCs were cultured as described in section 4.3.1. In brief, PBMC were subject to density layered centrifugation and cells were placed on fibronectin coated plates. These were cultured for periods of 4 to 6 weeks till late outgrowth EPCs were obtained. Cells were cryo-stored for use at a later date. When required, cells were rejuvenated and plated at a concentration of $10 \times 10^4$ cells/well in a 6 well fibronectin coated plate.

d. Lentiviral transduction and Akt activity assay

Late outgrowth EPCs were infected by lentiviral vectors encoding EGFP or constitutively active Akt (E17KAkt) in 6 well plates for a period of 72 hours.

Pilot work was conducted to determine adequate multiplicity of infection (MOI) i.e. ratio of viral agent to target late EPCs. Varying concentrations of virus was used to infect late EPCs, and Akt kinase activity was determined using western blotting (Cell signaling Akt Kinase assay kit #9840). In brief, Akt was immuno-precipitated from lysates, prior washing with recombinant Glycogen Synthase Kinase-3 (GSK-3) substrate and ATP; Akt activity is then defined by Western blotting for phospho-GSK-3. The results are shown in figure 17.
Figure 17: A) Schema of pro-viral vectors pHVSL2 and pSINCSGWdlNot1; B) Representative Akt activity data used to define viral load used in subsequent experiments (one arbitrary infectious unit per $10^5$ LEPC), with Akt activity immunoblots corresponding to points on graph.

Based on above results, one infectious unit defines as the volume of E17Akt lentiviral sample sufficient to enhance Akt kinase expression 3-fold, and this concentration was used for all subsequent experiments.
e. Transfusion of transduced late EPCs into nude mice

Late outgrowth EPCs expressing EGFP and E17Akt were transfused into femoral arteries of nude mice as described previously. Femoral arteries were harvested on day 4 and re-endothelialisation was assessed by means of Evans blue staining as previously discussed.

4.5 Statistical methods

Continuous data are presented as arithmetic mean and standard error of the mean unless otherwise stated. Continuous data between the study cohorts are compared with Student’s t-test; paired data are compared with paired t-tests. Statistical significance was accepted at p<0.05. The co-efficient of variation describes the standard deviation of a sample divided by its arithmetic mean and is used as an index of reproducibility. Statistical calculations were performed using Microsoft Excel 2007.
Chapter 5: Results

Part One

In vitro studies of Simvastatin and circulating endothelial progenitor cell number and function
5. Results Part One: In vitro studies of Simvastatin and circulating endothelial progenitor cell number and function

5.1 Study cohort demographics and cardiovascular risk profile

A total of 9 white European and 9 South Asian men were recruited for the study. These subjects were well matched for age and systolic blood pressure. Mean haematological and biochemical parameters fell within ‘normal’ range as indicated by Leeds Teaching Hospitals NHS trust laboratories, although white cell count was higher in South Asian men. Biochemical parameters including LDL and blood glucose were well matched. BMI showed a trend of being greater in South Asian men. HOMA-IR, an index of insulin sensitivity, was calculated using fasting plasma glucose and insulin concentrations. South Asians were significantly insulin resistant when compared to white European counterparts (1.2 [0.19] vs. 0.51 [0.08]; p=0.003).

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<td>15.4 [0.3]</td>
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<td>Creatinine (µmol/L)</td>
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<td>96.7 [1.9]</td>
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<td>ALT (iu/L)</td>
<td>23.9 [2.3]</td>
<td>20.3 [1.5]</td>
<td>0.22</td>
</tr>
</tbody>
</table>
Table 3: Subject demographics and blood results for experiments with early outgrowth EPCs

5.2 Pilot work

In the initial stages of the project, we planned to administer oral Simvastatin 20mg daily for 1 month to 20 healthy South Asian men, and assess EPC function prior to and after Simvastatin exposure. Unfortunately, we were only able to recruit 5 subjects and due to poor subject compliance, this part of the experiments had to be abandoned. Further experiments were limited to obtaining blood samples and performing in vitro studies of EPCs exposed to clinically relevant concentrations of Simvastatin.

Pilot work was conducted with early EPCs derived from two white European and two South Asian subjects. Simvastatin at concentrations of 0.1 µM and 1.0 µM was added to the EPC culture medium on day 3 and numerical and functional assays as previously described were conducted after 15 hours. Endothelial progenitor cell
death was consistently noted with Simvastatin 1.0 µM in South Asian group, as demonstrated by loss of adhesion to the fibronectin coated plate and alteration in morphology suggestive of cell death (see figure 18).

![Altered EPC morphology](image)

**Figure 18:** Altered EPC morphology with Simvastatin 1.0 µM

Further experiments were therefore conducted with 0.1 µM Simvastatin only and the results are as described below; as discussed earlier, this represents a clinically relevant concentration.
5.3 Early outgrowth EPC numerical assay

5.3.1 Enumeration

Basal EPC numbers at day 4 were defined by dual staining with Dil-acetylated LDL and Ulex Europeus lectin-FITC conjugate. The number of cells, expressed in cells per high power field, was significantly reduced in SA males when compared to WE males (12 [2.4] vs. 27 [5.6]; p = 0.009) (See figure 19).

![Figure 19: Basal EPC numbers in SA and WE subjects. SA EPC count is significantly lower than WE subjects.

Interestingly, HOMA – IR score negatively correlated with EPC numbers, as seen in figure 20 (R² = -0.55, p < 0.05)
Figure 20 – Negative correlation between HOMA-IR score and number of EPCs per high powered field.

Following treatment with 0.1 µM Simvastatin on day 3, a significant increase was noted in EPC numbers (cells/HPF) in SA group (10 [2.3] vs. 19 [4.0]; p=0.005), a change which was also seen in the WE group (26.7[5.6] vs. 38.5 [6.1]; p=0.005). The total EPC count recorded following Simvastatin treatment in SA subjects did not exceed the basal EPC numbers seen in WE subjects, though the difference was not statistically significant (19[4.0] vs. 26.7[5.6]; p=0.20) (See figure 21).
**Figure 21:** EPC numbers in SA and WE men following treatment with 0.1 µM Simvastatin. A significant increase in EPC numbers is seen in both groups.

### 5.4 Early outgrowth EPC functional assays

#### 5.4.1 Migration in response to VEGF

Early EPCs exhibited migration in response to vehicle medium (i.e. haptotaxis) and VEGF (i.e. chemotaxis). Early EPCs that had been treated with Simvastatin 0.1 µM also demonstrated migration to both vehicle medium and VEGF. Basal haptotaxis was initially analysed, as was the response to VEGF as a chemo-attractant factor. Cells that were treated with Simvastatin were then analysed similarly.
There was a significant increase in migration in response to VEGF in both SA men (1.5 [0.36] vs. 2.6 [0.64]; p=0.01) and WE men (2.1 [0.65] vs. 3.8 [0.91]; p=0.007), as compared with basal haptotaxis. EPC pre-treated with Simvastatin 0.1 µM alone also demonstrated an increase in haptotaxis in both groups; this increase was significant in SA men (1.5 [0.36] vs. 2.4 [0.3]; p=0.04) but non-significant in WE men (2.1 [0.65] vs. 2.6 [0.64]; p=0.54) (See figure 22).

**Figure 22:** Basal and Simvastatin pre-treated EPC migration towards VEGF. A significant increase in EPC migration is seen in both groups in response to VEGF, but only seen in SA EPCs following pre-treatment with Simvastatin.

In order to assess specific migratory effect to VEGF, data was corrected for each individual subject for haptotaxis of cells to vehicle solution. Migration in response to VEGF was greater in WE men, though the increase was not significant (1.1 [0.4] vs. 1.7 [0.52]; p=0.28). Cells that were treated with 0.1 µM Simvastatin were analysed similarly. Migration of cells treated with Simvastatin 0.1 µM to VEGF was
interestingly reduced in both groups, compared with non-statin exposed cells. The degree of reduction was lesser in WE EPCs (1.7 [0.52] vs. 1.1 [0.56]; p=0.43) when compared with SA EPCs (1.1 [0.4] vs. 0.35 [0.56]; p=0.26), though the difference was not statistically significant.

Figure 23: Effect of Simvastatin 0.1 µM pre-treatment on EPC migration to VEGF. No difference is seen in overall migration to VEGF or following pre-treatment with Simvastatin.

5.4.2 Adhesion of Early EPCs to fibronectin

EPC derived from SA and WE men were plated at a concentration of 5 x 10⁴ per well into 3 wells of a 24 well fibronectin coated plate. The plate was washed twice with DPBS after 1 hour, and the number of adherent cells was counted. At baseline, there was no significant difference in adhesion to fibronectin of non-stimulated EPCs derived from South Asian males to the white European subjects (13.7 [1.9] vs. 15.5 [3.1]; p=0.61) (See figure 24).
Figure 24: Basal EPC adhesion to fibronectin. No difference is seen between SA and WE EPCs.

However, following stimulation with Simvastatin, South Asian EPCs demonstrated a significant improvement in adhesion to fibronectin (13.7 [1.9] vs. 21.28 [3.1]; p=0.009), while in the WE group there was a smaller non-statistically significant increase noted (15.4 [3.0] vs. 21.54 [7.4]; p=0.26) (See figure 25).
Figure 25: Enhanced adhesion of EPCs following treatment with Simvastatin. A significant increase is seen in EPC adhesion in SA group but not in WE group.

5.4.3 *In vitro* angiogenesis assay

As previously discussed, early outgrowth EPCs are believed to release a number of paracrine factors that stimulate angiogenesis. In order to assess this further, conditioned medium derived from both un-treated and pre-treated (with Simvastatin 0.1 µM) cells was used to perform an *in vitro* angiogenesis assay. HUVECs were suspended in this medium and then placed onto a thin layer of Matrigel, and tubular structures formed after 24 hours were counted.

The number of tubules formed was significantly lower in the South Asian subject’s EPC conditioned medium when compared to matched white Europeans, as shown in figure 26 (1.1 [0.89] vs. 4.9 [1.5]; p = 0.049).
Figure 26: Basal tubule formation in conditioned medium from SA and WE EPCs. Significantly fewer tubule are formed with SA EPC derived conditioned medium.

However, conditioned medium derived from EPCs pre-treated with Simvastatin showed a non-significant increase in tubule formation in both South Asians (1.1 [0.89] vs. 2.5 [1.8]; p=0.34) and WE subjects (4.9 [1.5] vs. 5.0 [1.7]; p=0.98) (See figure 27). Importantly, this conditioned medium did not contain Simvastatin.
Figure 27: Tubule formation in conditioned medium derived with or without pre-treatment of early outgrowth EPCs with Simvastatin. No increase in tubule formation was noted in either group following pre-treatment with Simvastatin.

5.5 Discussion

This section of the thesis presents an assessment of in vitro functional potential of early EPCs in South Asian men. Early EPC function has been assessed at baseline and following the treatment of cells with Simvastatin 0.1 µM. Before discussing the individual findings in detail, the overall findings in these groups of experiments will be summarised first.

Limited work has been carried out assessing functional properties of early EPCs in South Asian men. We have, however corroborated findings of Murphy et al\textsuperscript{132} confirming a significant reduction in basal early EPC numbers in South Asian men compared to white European men. Our method of enumerating early EPCs involves...
counting adherent cells that are dual positive for Dil-acetylated LDL and Ulex Europeaus Lectin, whereas Murphy et al enumerated EPC-CFUs instead. The subjects in both are healthy non-obese South Asian males compared with a well matched control group. Studies analysing early EPC numbers in groups of human subjects using dual staining in South Asian men are relatively limited, though work performed in white European subjects by Dimeller et al has shown this to be a reliable way of performing a numerical assay, with confirmatory phenotyping performed using FACS. Flow cytometry was not used to enumerate EPC using cell surface markers which could be viewed as a limitation of this study. However, our group has performed similar work and validated the conclusion of Murphy et al that CPC numbers are also reduced in South Asian men, using FACS analysis. We have also demonstrated impairment in basal functional properties of South Asian EPCs, with reduced migration to VEGF and induced endothelial tubule formation as a measure of paracrine activity, when compared to white Europeans. However, the in vivo significance of these observations remains unclear, as will be mentioned at different points in the discussion.

5.5.1 Basal South Asian EPC function

5.5.1.1 South Asian EPCs demonstrate impaired migration

Our work has demonstrated a reduction in migratory capacity to VEGF in early outgrowth EPCs derived from South Asians when compared with white European subjects. In vivo, this would translate into impaired capacity of the EPCs to migrate to site of vascular injury or tissue ischemia. VEGF has been demonstrated to be released following vascular injury and thus appears to be physiologically relevant as
a stimulus for migration, though it would be interesting to study the effect of other molecules such as SDF-1. Our group has previously demonstrated similar responses of EPCs to VEGF and IGF-1 (Dr Richard Cubbon: unpublished data). As VEGF and IGF-1 signal through the PI3k/Akt signalling pathway to promote migration, further mechanistic work will need conducting in future to analyse signalling properties in these cells.

5.5.1.2 South Asian EPCs demonstrate normal adhesion

Our finding of non-impaired EPC adhesion to fibronectin would suggest that South Asian EPC adhere optimally at sites of vascular endothelial damage. Work by Tepper et al. studied adhesion of EPCs derived from subjects with type II diabetes and control subjects to fibronectin. They found no significant difference in adhesion between the groups. Our findings in the insulin resistant SA group are in keeping with their findings, though it must be borne in mind that the diabetic phenotype is a lot more advanced and complex when compared to the insulin resistance phenotype in this study. This is in contrast to studies in mice where adhesion of diabetic EPCs was significantly reduced to numerous extracellular matrices including fibronectin. Our findings in this study are in contrast to previous unpublished work by our group that demonstrated significantly impaired adhesion of early EPCs when seeded on Matrigel. However, the SA group whilst only showing a trend towards insulin resistance did not show any significant difference in the HOMA-IR scores from WE men. How these contradictory findings would therefore translate in vivo is unclear, and ideally future experiments should be conducted under shear stress to better mimic arterial injury. Also, the lack of difference in adhesive capacity in our groups...
could be explained by the small sample size, and studies in a larger cohort may demonstrate a statistically significant difference. As discussed in the introduction to this thesis, adhesion of EPCs to denuded vessel wall is dependent to some extent on integrin expression \textsuperscript{186}, and future work into this could provide valuable insights into the adhesive capabilities of SA early EPCs. Attachment of circulating EPCs to denuded endothelium on an injured arterial wall is critical in mediating re-endothelialisation, and understanding the exact mechanisms would aid future modulation of adhesive capacity of EPCs.

5.5.1.3 South Asian EPCs demonstrate impaired paracrine function

\textit{In vivo}, early outgrowth EPCs are believed to release numerous paracrine mediators that stimulate local vascular cells to repair injured blood vessels\textsuperscript{161,195}. Hence, analysing this effect \textit{in vitro} using HUVECs cultured in conditioned medium derived from early EPCs is helpful to assess the reparative properties of these cells. We found that tubule formation in Matrigel was significantly lower in HUVECs exposed to South Asian specimens. Work by other research groups has shown a majority of this effect to be dependent on VEGF\textsuperscript{161}, though other secreted factors may also play a role. However, it must be noted that the Matrigel assay is a 2-dimensional assay which does not replicate the actual 3-dimensional process involving endothelial cells, pericytes and smooth muscle cells that occurs \textit{in vivo} in response to shear stress. In our assay we have used conditioned medium containing paracrine mediators derived from early outgrowth EPCs as stimulatory factors for tubule formation. Data by different groups regarding the ability of EPCs to form tubules in Matrigel is variable and conflicting.\textsuperscript{289,290} In addition, the pattern of tubule formation seen on microscopy
may not be representative of capillary network formation in vivo. While the release of paracrine mediators by early outgrowth EPCs has been clearly established in existing research, it may be worthwhile probing the specific alteration in selected growth factor (e.g. VEGF) secretion in future studies.

5.5.2 Effect of Simvastatin on basal EPC function

5.5.2.1 Simvastatin increases early EPC numbers

In keeping with research by other groups, we found a significant increase in EPC numbers in both ethnic groups following stimulation with Simvastatin 0.1 µM. However, we believe that the finding of increased EPC numbers in SA men is novel. Interestingly however, this change did not numerically match basal EPC number in white Europeans, and was significantly lesser than EPC numbers following pre-treatment of EPCs with Simvastatin in white Europeans. Given this increase in numbers, one may speculate that statins exert this effect by stimulating the release and expression of numerous angiogenic growth factors like VEGF and IGF-1, with resultant autocrine and paracrine effects. However, it appears that statins can induce differentiation of CD34 positive hematopoietic precursor cells into EPCs, even when critical aspects of VEGF/IGF-1 downstream signalling are inhibited. Future work is required to dissect the mechanisms involved.

5.5.2.2 Simvastatin treated EPCs may demonstrate reduced migration

Migration to VEGF was assessed with EPCs that had been pre-treated with Simvastatin 0.1 µM. Our findings demonstrate that un-treated EPC migration to
vehicle (haptotaxis) was reduced in SA men versus WE men, and increased significantly in both groups, as expected, in response to VEGF. On assessing migration of Simvastatin treated EPCs, a significant increase in haptotaxis was noted in SA group (compared to untreated EPCs); a similar increase was also seen in WE group. Interestingly, we found a reduction in net migration of Simvastatin treated EPCs to VEGF in both groups (i.e. after accounting for haptotaxis); the reduction however, was not statistically significant.

The above findings are in contrast to work published by other groups. EPCs pre-treated with Lovastatin demonstrated enhanced migration when compared to control groups.270 In a study group of 15 patients with angiographically evident coronary artery disease, a 3 week course of oral Atorvastatin augmented migration of circulating EPCs.292 It is important to note that subject ethnicity was not indicated in these studies, and as far as we are aware no such work has been conducted in SA men to date. Unpublished work by our group has shown reduced basal migration of untreated SA early EPCs to VEGF and IGF-1. It remains unclear how statins impact on EPC migration toward other physiologically relevant stimuli such as IGF-1, however.

Surprisingly, in our study, a reduction in Simvastatin pre-treated EPC migration to VEGF was noted in both groups, though to a non-significant degree. A possible explanation for this could be that both VEGF and statins appear to promote PI3K/Akt signalling.88,167 It is possible that the Simvastatin pre-treatment has maximally stimulated PI3K/Akt activity, and so VEGF is unable to produce its usual chemotactic effect, which, as outlined earlier, is PI3K/Akt dependent. Another possibility is that
the power of the study is insufficient to make an accurate analysis of the overall effect of Simvastatin on migration. Repeated analyses and other migration assays (e.g. scratch wound assay) are needed in order to more confidently define the impact of statins on early outgrowth EPC migration to VEGF.

5.5.2.3 Simvastatin enhances EPC adhesion

Following treatment with Simvastatin, early EPCs demonstrated an enhanced degree of adhesion to fibronectin. In particular, a statistically significant increase in EPC adhesive capacity was noted in South Asian specimens. An increase of similar magnitude was also seen in white European specimens, though this increase was not statistically significant.

Adhesion of EPCs is thought to play a key role in their promotion of neo-angiogenesis. Research assessing the adhesive capacity of early EPCs following treatment with statins is limited. Unpublished work by our group assessing EPC function in South Asian men demonstrated an incidental finding of significantly reduced adhesion of early EPCs to Matrigel following 24 hours of seeding. However, most adhesion assays are performed following an hour of seeding onto fibronectin or matrigel coated plates, and hence the results could be construed as inaccurate. Previous work by Tepper et al assessing the function of EPCs derived from subjects with type 2 diabetes found no significant difference in adhesion to fibronectin when compared to control subjects. Our study has shown similar results in an insulin resistant SA group.
Adhesion studies of EPCs treated with simvastatin is limited. Human EPCs pre-treated with Lovastatin showed increased EPC adhesion in a dose dependent manner\textsuperscript{270}. However, the ethnicity of the subject population in the study population was not specified. Our study has shown a significant increase in adhesion to fibronectin in South Asian subjects. Adhesion of EPCs is mediated by integrins – in particular β2 integrins.\textsuperscript{186} Further study of expression of these integrins and inhibition of their function with blocking antibodies, in our study population may provide valuable insights into statin-mediated effects upon EPC adhesion.

5.5.3.3 Simvastatin enhances paracrine function

As discussed above, tubule formation from HUVECs cultured in conditioned medium derived from early EPCs demonstrated significantly greater promotion of angiogenesis in white European subjects when compared to South Asians. We found that when HUVECs were cultured in conditioned medium derived from early EPCs pre-treated with simvastatin, there was an increase in tubule formation of 60% seen in south Asians, though this increase was not statistically significant. No material change was seen in HUVEC angiogenesis when exposed to conditioned medium from white European EPCs pre-treated with Simvastatin.

The existing literature on the impact of statins on angiogenesis offers no clear data on their mechanism of action. Most of the \textit{in vitro} work performed is with murine EPCs, but did not address the impact of statins on their secretory paracrine function. There appears to be no data to date on the effect of statins on EPC conditioned medium in humans.
Future work should address the inter-ethnic differences in secretion of specific pro-angiogenic compounds by early EPCs at baseline and following treatment with Simvastatin; this may provide valuable insights into our observations and suggest methods of translating into novel therapeutic strategies.

5.6 Common underlying mechanisms

The *in vitro* studies presented in this chapter have highlighted numerous functional impairments in early EPCs derived from South Asian men. Early EPCs once released from the bone marrow home to sites of injury or ischemia, release paracrine factors, and mediate endothelial repair. Impairment in any of these steps could result in defective endothelial repair, a process likely to be a required in retarding the progression of atherosclerosis. From the work we have described above, two main factors seem to play an important role in impaired EPC function in SA men.

5.6.1. Insulin resistance

As discussed in the introduction to this thesis, insulin resistance forms a central role in a complex group of cardiovascular risk factors collectively termed metabolic syndrome. We have previously shown reduced EPC numbers in SA insulin resistant subjects. More recent work by Jialal et al has also shown the same in subjects with metabolic syndrome, though a majority of their subjects were female. Our study has corroborated these findings and gone on to show associated dysfunction of these cells. We have also shown the HOMA-IR score to be negatively correlated
to basal EPC numbers. While such indices of whole body insulin resistance are associated with increased cardiovascular risk, insulin resistance at a cellular level may also be relevant, by retarding EPC PI3K/Akt signalling, whilst sparing the MAP kinase signalling. However, the existence of insulin resistance at EPC level remains speculative.

5.6.2. Blunted PI3 kinase/Akt signalling

In addition to showing reduced basal SA EPC numbers, we have also demonstrated reduced migration of SA EPCs toward VEGF which could reflect impaired migration toward damaged vascular tissue in vivo. Once progenitors have mobilised in vivo, they migrate to site of injury and ischemia and initiate endothelial repair and angiogenesis. Tubule formation in vitro on Matrigel aims to reflect the angiogenic process in vivo, and in our studies required the release of paracrine mediators by EPC (producing conditioned medium). We have demonstrated reduced promotion of endothelial tubule formation by conditioned medium derived from SA EPCs. One of the key secreted molecules in early outgrowth EPC conditioned medium is VEGF, although many others are co-secreted. The mechanism of EPC VEGF secretion is unclear, although it is interesting to note that insulin resistance has been shown to reduce vascular cell and myocardial VEGF secretion via reduced PI3K/Akt signalling.

The available evidence and our study included have shown that Simvastatin increases basal EPC numbers and may improve functional properties of early EPCs. Previous work has shown this increase in numbers to be due to stimulation of the PI3K/Akt signalling pathway by Simvastatin, and thus blunting of
this pathway could explain reduced basal signalling in untreated early EPCs, especially those derived from South Asian men. It may be speculated that Simvastatin increases EPC numbers by enhancing the expression of paracrine angiogenic growth factors such as VEGF and thus act through the PIK/Akt pathway, though unpublished work by Dimeller et al has shown this not to be the case.\textsuperscript{167} As was discussed in the introduction, isoprenylated proteins and Rho Kinases play a role in eNOS production. Blocking Rho kinases with atorvastatin and another pharmacological inhibitor HA 1077 did not increase EPC numbers, thus implying that statins are unlikely to exert its effects on EPCs through this mechanism.\textsuperscript{167}

It is noteworthy that while we have shown increased SA EPC numbers and enhanced adhesion to fibronectin following pre-treatment with Simvastatin, we did not notice a statistically significant increase in EPC migration. This is in contrast to available evidence where Simvastatin enhanced EPC migration, though this was not in a South Asian population group.\textsuperscript{242} It is likely that this is due to our small sample size. In addition, studies that have been performed with statins and EPCs are with different statins and murine EPCs.\textsuperscript{254} For example, work by Shao et al explored the effect of Fluvastatin on EPC migration as a part of their experiments assessing the effect of statins and SDF-1 on human EPC function and angiogenesis.\textsuperscript{296} They demonstrated enhanced migration with low dose Fluvastatin, while this was inhibited with a higher dose of Fluvastatin. This was further increased when EPCs were treated with both Fluvastatin and SDF-1 when compared to statin or SDF-1 alone. This is in contrast to our results with Simvastatin. They then proceeded to examine Akt phosphorylation following statin treatment, and demonstrated a statistically significant increase in Akt phosphorylation with low dose statins, while no increase
was noted with a higher dose of statin. Work with Cerivastatin by Vincent et al demonstrated reduced endothelial migration to chemotactic factors, though the migratory capacity of un-stimulated endothelial cells was preserved. Our basal EPC function results and those obtained after treatment with Simvastatin may support a role for PI3k/Akt signalling in impaired function of SA EPCs. However, this requires further work with western blot analysis of Akt protein expression, phosphorylation and activity in early EPCs.

However, it must be borne in mind that while this might be one possible mechanism, other factors cannot be conclusively ruled out. In particular, early EPC dysfunction in South Asians could be related to enhanced apoptosis and premature senescence, as demonstrated and discussed later with late outgrowth EPCs. If this were to be the case, then the impaired basal functional properties of early EPCs may not be regarded as a true impairment, but instead be all due to enhanced early cell death. While we do not have this data at present, future studies could be designed to address this issue.

5.7 Study limitations

Our data, as with any scientific work, has both strengths and weaknesses. It is important to identify these limitations in order understand the implications of our observations and to plan future studies.
5.7.1 Defining ethnicity

As evident from our preceding discussion, defining ethnicity accurately is difficult, and in most published papers is arbitrary. Criteria remain unclear, and can include parameters ranging from geographic location to religious beliefs. Using one particular criterion may promote standardisation; however this system would ignore numerous other heterogeneous factors within each ethnic group (culture and religion). This heterogeneity would no doubt impact cardiovascular risk. Furthermore, migration patterns would imply a greater spread of the population globally, resulting in difficulty applying commonly used descriptors to a fewer number of people in this ethnic group. In an attempt to avoid this difficulty in defining ethnicity, we opted to consider geographic origin and self defined ethnicity sufficient to represent our study population similar to previous studies. Of course, this would unfortunately exclude other parameters that may impact on cardiovascular risk (such as genetics), and given the lack of a clear solution to this, we decided that this would be the clearest and simplest way to define our study groups.

5.7.2 Sample size

Our study has a relatively small sample size which has potential implications for the entire study. The in vitro studies assess the numerical and functional properties of EPCs at basal level and following treatment with Simvastatin. While at basal level a few parameters show significant differences between these properties, those that have been described as being no different between the two groups or showing no correlation between various factors cannot be conclusively stated as fact. When the project was designed, the main context of the project was to generate hypothesis as to how differences in ethnicity and thus endothelial repair mechanisms in these two
groups could explain a difference in prevalence of cardiovascular events. In our study, we have collected a large amount of data for a small sample size, which we consider to be more effective than collecting a limited amount of information from a larger sample size. Furthermore, consumable expenditure for this study was limited, as was the availability of resources and time. We have mentioned this fact clearly where relevant, in addition to the small sample size and the requirement for further experiments.

5.7.3 Defining EPCs

In the introduction to this thesis, I have discussed the difficulty in clearly defining EPC phenotype based on surface markers and appearance in culture, as no unique marker has been identified.

The extrapolation by the other groups of Asahara's seminal work on 'progenitor endothelial cells' resulting in a definition of circulating EPCs as cells expressing endothelial and hematopoietic progenitor markers has confused the wider literature and is now agreed to be inappropriate. Studies have shown alteration in the number/function of these cells to be associated with cardiovascular disease; however no clear causal association has been demonstrated. Our study focuses on culture derived early outgrowth EPCs. We have previously discussed the confusion that prevails over the link between in vitro and in vivo studies. In addition, it is unclear if EPCs derived in culture are solely an artificially derived phenotype. As research has progressed over the years, other groups described these cells as being monocytic in origin. In particular, this group demonstrated the transfer of endothelial surface markers to monocytes by platelet microparticles that are generated in
However, whether monocytes that do not bear these ‘transferred’ surface markers exhibit properties such as angiogenesis *in vivo* is uncertain. Furthermore, the *in vivo* therapeutic role of the cells derived in this project are yet to be defined; a series of infusion studies after arterial injury in immuno-deficient mice (akin to the next chapter) would go some way to addressing this issue.

In our study, we have also examined the effect of Simvastatin on EPCs *in vitro*. While the results are interesting, there still remains the question as to what the *in vivo* relevance of this would be. We attempted to study this by administering oral Simvastatin to healthy male subjects, but encountered difficulties with subject compliance and recruitment and hence abandoned the study. Studies looking at oral administration of Simvastatin in C57BL/6 male mice have demonstrated an increase in circulating progenitor cells number. Similar results have been observed in human studies as well, though are mostly in patients suffering from coronary artery disease.
Chapter 6: Results

part two

Late outgrowth
endothelial progenitor cell function
6. Results part two - Late outgrowth endothelial progenitor cell function

The work in this chapter forms part of a jointly authored manuscript which is currently under revision (see non-revised manuscript in appendix 1). I acknowledge the contribution of all authors to this work, and have highlighted the role of some authors in this chapter, where relevant.

6.1 Subject demographics

A total of 8 SA and 8 WE subjects were studied. Parameters such as systolic blood pressure and BMI appear statistically comparable. HOMA-IR score was significantly higher in SA when compared to WE (1.19 [0.22] vs. 0.44 [0.08]; p = 0.01). Interestingly, total cholesterol and LDL concentrations were both significantly higher in SA men.

<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>30.6 [1.5]</td>
<td>29.4 [1.3]</td>
<td>0.54</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>116.3 [3.2]</td>
<td>115.8 [3.5]</td>
<td>0.91</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>24.2 [1.0]</td>
<td>22.3 [0.6]</td>
<td>0.12</td>
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<tr>
<td>Waist :hip ratio</td>
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<td>0.8 [0.0]</td>
<td>0.86</td>
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<td>Haemoglobin (g/dL)</td>
<td>15.4 [0.2]</td>
<td>15.4 [0.4]</td>
<td>0.94</td>
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<td>White cell count (10⁹/L)</td>
<td>6.0 [0.2]</td>
<td>4.8 [0.2]</td>
<td>0.01</td>
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<tr>
<td>Creatinine (µmol/l)</td>
<td>97.8 [0.2]</td>
<td>96.8 [2.5]</td>
<td>0.22</td>
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</table>
### Table 4 - Subject demographics and blood results for experiments with late EPCs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value 1</th>
<th>Value 2</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>ALT (iu/L)</td>
<td>24.6 [2.8]</td>
<td>19.7 [1.3]</td>
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<td>Cholesterol (mmol/L)</td>
<td>4.9 [0.1]</td>
<td>4.4 [0.2]</td>
<td>0.02</td>
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<td>LDL (mmol/L)</td>
<td>3.1 [0.2]</td>
<td>2.7 [0.2]</td>
<td>0.04</td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.2 [0.1]</td>
<td>1.2 [0.1]</td>
<td>0.53</td>
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<tr>
<td>Triglyceride (mmol/L)</td>
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<td>0.9 [0.1]</td>
<td>0.37</td>
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<tr>
<td>Glucose (mg/dL)</td>
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<td>4.6 [0.1]</td>
<td>0.81</td>
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<tr>
<td>Insulin (mU/L)</td>
<td>5.6 [0.9]</td>
<td>2.7 [1.0]</td>
<td>0.004</td>
</tr>
<tr>
<td>HOMA-IR score</td>
<td>1.19 [0.22]</td>
<td>0.44 [0.08]</td>
<td>0.01</td>
</tr>
</tbody>
</table>

#### 6.2 Population doubling time

Population doubling time was calculated as described in section 4.3.3.

South Asian EPCs (n=7) demonstrated a significantly greater population doubling time when compared to WE subjects (n=6) (5.25 [0.81] vs. 2.92 [0.3]; p = 0.019).
Figure 28: Population doubling time in SA EPCs vs. WE EPCs. Significantly higher population doubling times are seen in the SA group.

Interestingly, a positive correlation was noted between HOMA-IR scores and population doubling times (n=13), suggesting a trend toward association between insulin resistance and EPC proliferation ($R^2 0.31$, $p=0.15$).

Figure 29: Scatter plot and line of best fit demonstrating an association between HOMA-IR score and EPC population doubling times. Proliferation assays were performed in a subset of samples (SA n= 7, WE n=6)
The number of population doublings occurring per unit time (days) i.e. the growth rate of SA EPCs was significantly lower than WE subjects (0.16 [0.02] vs. 0.28 [0.03]; p=0.014).

**Figure 30**: Late outgrowth EPC growth rate. A significantly lower growth rate is seen in the SA EPCs.

### 6.3 EPC senescence

At passage 4, cells were stained with β-galactosidase to measure senescence. The total number of cells counted per high powered field was noted, as were the number of cells that had stained blue. Results are expressed as a percentage of total cells that β-galactosidase staining. There was good inter-observer agreement when analysed by 2 separate observers (R² 0.99, p=0.95)
Figure 31: Inter-observer variability in measuring EPC senescence

A

South Asian

White European
Figure 32: β-galactosidase staining of late EPCs at passage 4. A greater number of EPCs stain blue in SA when compared to WE (A). A significantly greater percentage of β-galactosidase positive senescent cells are seen in South Asians (B)

6.4 Molecular signalling pathways

Previous unpublished work performed by our group has demonstrated reduced colony formation, migration to VEGF / IGF-1 and reduced angiogenic tubule formation by late outgrowth EPCs derived from SA men, compared with matched white European controls (see appendix 1). Given this, these experiments were not repeated, but mechanisms involved in this dysfunction were investigated further.
6.4.1 Expression of phospho S1177-eNOS and eNOS

Late outgrowth EPCs were harvested from fibronectin coated plates when confluent and were subject to western blot analysis of phospho-eNOS and eNOS protein expression.

6.4.1.1 Expression of phospho-eNOS

In order to standardise protein expression, the ratio of phospho-S1177eNOS to β-actin expression was calculated for each sample. A significantly higher phospho-eNOS : β-actin protein ratio was noted in WE men when compared to South Asian men (0.15 [0.01] vs. 0.05 [0.02]; p=0.015).

A

![Image of western blot analysis](image1)
Figure 33: Western blot analysis of phospho-eNOS protein in EPCs derived from SA and WE men. A representative immunoblot clearly demonstrates denser phospho-eNOS bands in WE EPCs (A). A significant difference is observed between the 2 groups (B).

6.4.1.2 Expression of eNOS

In order to standardise individual values, a ratio of total eNOS to β-actin was calculated. eNOS : β-actin ratio was significantly higher in WE men when compared to SA men (1.83 [0.62] vs. 0.11 [0.05]; p=0.018).
Figure 34: Western blot analysis of eNOS protein in EPCs derived from SA and WE men (expressed as eNOS: β-actin). A representative immunoblot clearly demonstrates denser bands in WE EPCs (A). A significant difference is observed between the 2 groups (B).
6.4.2 Expression of phospho- S473 Akt and total Akt

6.4.2.1 Expression of phospho-S473-Akt

A significantly higher expression of phospho-S473-Akt protein was noted in WE men when compared to South Asian men (0.81 [0.2] vs. 0.14 [0.05]; p=0.005).

A

β-actin

SA

WE
Figure 35: Expression of phospho-Akt in South Asian vs. WE men. A representative immunoblot demonstrates denser bands in WE EPCs (A). Phospho-Akt expression is significantly greater in WE EPCs (B).

6.4.2.2 Expression of Akt

A significantly higher expression of total Akt protein was noted in WE men when compared to South Asian men (1.19 [0.13] vs. 0.69 [0.16]; p = 0.049).
Figure 36: Akt expression in SA and WE LEPCs. A representative immunoblot clearly demonstrates denser bands in WE EPCs (A), and this is significantly greater as well (B).
6.4.3 Polymerase chain reaction

eNOS and Akt mRNA was quantified by PCR. eNOS mRNA concentrations were significantly higher in WE LEPCs when compared to SA LEPCs. However, there was no difference between Akt mRNA concentrations (n=6 in both groups).

![Graph showing relative expression of Akt and eNOS mRNA in South Asian (SA) and White European (WE) LEPCs.](image)

**Figure 37**: Akt and eNOS mRNA concentration in SA and WE LEPCs. eNOS mRNA concentrations in SA EPCs are significantly lower than in WE EPCs. No difference is seen in Akt concentrations.

6.4.4 EPC mediated in vivo arterial repair following femoral wire injury

Late outgrowth EPCs derived from South Asian and white European men were cultured until confluent. They were then fluorescently tagged, detached and transfused into nude mice subject to femoral artery wire injury. The arteries were harvested on day 4 post transfusion, and stained with Evan’s blue to assess the degree of re-endothelialisation. Non-re-endothelialised sections of the artery remained blue, while re-endothelialised sections were marked by lack of staining.
Comparisons were also made to vessels from mice receiving vehicle only (i.e. medium without EPCs) after wire injury.

EPCs derived from SA subjects demonstrated significantly impaired capacity to re-endothelialise injured femoral arteries when compared to white European EPCs, with a significantly smaller area of the artery demonstrating endothelial regeneration (36.9% [4.5%] vs. 54.2% [8.4%]; p=0.03). Interestingly the percentage of endothelial regeneration was no different between vessels exposed to SA EPC and vehicle alone (acellular) (36.9% [4.5%] vs. 34.7% [5.7%]; p=0.70). Images were analysed as described in section 4.4.3.

![Images](A,B,C)
Figure 38: Evans blue staining of representative injured conduit artery 4 days after EPC transfusion. The degree of endothelial regeneration is no different between vehicle (A) and SA EPCs (B), but is significantly greater with WE LEPCs (C). Re-endothelialisation is significantly promoted by WE, but not SA LEPC (D).

6.4.5 Confocal microscopy of injured vessel

A subset of injured vessels were analysed using confocal microscopy. Fewer late EPCs derived from SA men were found adherent to the intima of the healing vessel when compared to WE late EPCs.
**Figure 39**: Representative images on confocal microscopy of fluorescently tagged late EPCs derived from SA and WE men. Fewer LEPCs are adherent to the intimal wall in mice receiving SA LEPC (A) when compared to WE LEPC (B). Red denotes CMDii tracked LEPC; Blue denotes DAPI stained nuclei.

6.4.6 *Viral titre data and induced change in Akt content*

Given the above data, and the background literature presented in the introduction, we speculated reduced Akt signalling may promote SA EPC dysfunction and so elected to augment late EPC Akt activity in further *in vivo* studies. Using a constitutively active E17K Akt1 mutant delivered with lentiviral vectors we demonstrated 3-fold augmented Akt activity within SA EPC (see appendix 1). Prior to late EPC transfusion, Akt activity in native SA late EPCs and EGFP expressing SA late EPCs was also compared - no significant difference was found in the between the two.

Intravenous transfusion of SA late outgrowth EPCs expressing EGFP was associated with no change in femoral artery re-endothelialisation in CD1 immunodeficient mice. However, E17K Akt expressing SA late EPCs demonstrated
significantly augmented re-endothelialisation to a degree comparable with non-transduced WE late EPCs (See figure 40).
**Figure 40**: Re-endothelialisation following transfusion of EGFP (A) and E17KAkt (B) transduced SA LEPCs. Re-endothelialisation is significantly enhanced with E17KAkt transduced EPCs to a magnitude comparable to non-transduced WE LEPCs (C). * denotes p < 0.05, NS denotes p≥0.05.

### 6.5 Discussion

This section of my thesis discusses functional properties of late outgrowth EPCs, which are considered by many to be the ‘true EPC’, and also to represent an attractive autologous cell-based therapeutic modality. In addition to this, we have attempted to assess the molecular mechanisms involved in late outgrowth EPC dysfunction in South Asian subjects by looking at basal protein expression and phosphorylation, with subsequent manipulation using lentiviral transduction. Once again, this is an area in EPC biology where limited information is available. In
particular, information relating to late outgrowth EPC biology and South Asian ethnicity is extremely scarce.

6.5.1 South Asian EPCs demonstrate reduced proliferative potential

Previous unpublished work by our group has shown the late outgrowth EPC colony number to be significantly lower in South Asian men. In keeping with these findings, we found the population doubling time of EPCs derived from SA males was significantly higher than WE counterparts. Cell cultures grow and multiply through a process of binary fission, with each cell splitting into two at a constant rate. Population doubling time refers to the time taken for a particular number of cells to double in total number. A higher population doubling time indicates a lower growth rate which in turn implies a reduced number of population doublings over a fixed period of time. Our findings may simply reflect the fact that the lower colony number in SA samples requires more rounds of LEPC division in order achieve a particular population size than WE samples, resulting in a more senescent slow-growing population at any point in time.

Previous work by Zhang et al in cord blood derived late outgrowth EPCs demonstrated increased senescence following treatment with TNF-α, a pro-inflammatory cytokine. Further analysis showed that chronic treatment of EPCs with TNF-α resulted in a dose dependent reduction in cumulative population doublings. In the results seen above, higher mean population doubling time seen in SA men would imply a slower growth rate and thus a lower aggregate of population
doublings. This could indicate a greater degree of senescence at the same passage stage in SA when compared with WE counterparts (see below).

As is seen from the results, there may be a positive correlation between late EPC population doubling times and the HOMA-IR score. In addition, a link has been established between inflammatory markers such as TNF-α and insulin resistance. From this available evidence, it could be hypothesised that high population doubling times in South Asian men may be explained by insulin resistance.

6.5.2 South Asian EPCs are more senescent

We have clearly shown in figure 32 that at passage 4, South Asian EPCs are significantly more senescent than white European EPCs. As described in section 5.6.1, this is linked with a higher population doubling time in EPCs derived from South Asians.

EPC senescence has been linked to a number of cardiovascular risk factors. In this study, we have shown South Asian population groups to be insulin resistant, in keeping with studies conducted by other groups. As discussed above, pathway specific insulin resistance has been implicated in endothelial dysfunction. This results in selective down-regulation of the PI3k/Akt pathway, while maintaining or promoting signal transduction via the MAPK pathway. Excessive signalling via MAPK pathway has been implicated in defective EPC survival, proliferation and differentiation. In addition to this, a close link has been established between insulin resistance and increased inflammatory markers such as TNF-α. Chronic exposure
of EPC cultures to TNF-α has shown increased levels of senescence mediated via the p38 MAPK pathway.\textsuperscript{299}

In keeping with these effects, premature senescence of EPCs derived from South Asians could also be explained by insulin resistance. Whether or not senescence is related to other cardiovascular risk factors in South Asians is unclear, and certainly our cohort showed no significant difference in systolic blood pressure readings between the two groups, though there was a difference in LDL levels between the two groups. Studies using larger cohorts may be required to assess this further. Also, future work assessing p38 MAPK activity and it’s phosphorylation in South Asian EPCs could provide useful insights into enhanced senescence and EPC dysfunction in this group. In addition, studying other principle mediators of senescence (p16 and p53) would be interesting to study, along with ROS as a driver of senescence.

6.5.3 South Asian EPCs demonstrate blunted expression and activation of pro-angiogenic signalling molecules

Previous unpublished work by our group has shown that late outgrowth EPCs derived from healthy South Asian men demonstrate impaired differentiation, migration toward VEGF and IGF-1 and reduced \textit{in vitro} angiogenesis (appendix 1). These experiments were hence not repeated, but mechanisms involved in this impairment were analysed further. In particular, expression of Akt, phospho-Akt,
eNOS and phospho-eNOS was analysed further using Western blotting and polymerase chain reaction.

We found that basal eNOS, phospho-eNOS, Akt and phospho-Akt protein expression were all significantly blunted in South Asian EPCs. This is a novel finding in late outgrowth EPCs derived from South Asian populations; in fact such changes have not been addressed to date in late outgrowth EPC from any insulin resistant cohort..

Murphy et al have previously demonstrated reduced circulating progenitor cell numbers, and impaired flow mediated dilatation in response to brachial artery shear stress.\textsuperscript{132} Vasodilatation in response to shear stress is mediated by NO, and as previously discussed; NO production secondary to shear stress is dependent on PI3k/Akt signalling. Furthermore, our group has also shown that exercise induced circulating progenitor cell mobilisation is blunted in SA men as a result of reduced NO bioavailability.\textsuperscript{133} In keeping with these findings, we have now shown reduced basal expression of eNOS and Akt in late outgrowth EPCs from SA men. Quon et al have demonstrated pathway specific insulin resistance as a mechanism for reduced NO production by selective downregulation of the PI3k/Akt pathway.\textsuperscript{122} In our study and in previous work, we have clearly demonstrated SA men to be metabolically insulin resistant, and can hypothesise this as a mechanism of blunted PI3K/Akt signalling in this group. Of course, our findings are derived from experiments on ex-vivo expanded late EPCs, and it remains to be established regarding the in vivo relevance of these studies. However, cumulative data from our studies suggests
downregulation of PI3k/Akt signalling \textit{in vivo}, resulting in reduced NO production which may cause blunted angiogenesis and vascular repair in SA men.

6.5.4 \textit{South Asian late EPCs demonstrate impaired re-endothelialisation capacity following arterial injury}

We have clearly demonstrated impaired injured femoral artery re-endothelialisation following transfusion of native (non-manipulated) SA late EPCs when compared to WE late EPCs. This finding is novel, and to our knowledge there is no evidence to date other than our data demonstrating that human late outgrowth EPCs can promote vascular repair.

Impaired vascular repair plays an important role in the pathogenesis of atherosclerosis. Circulating progenitor cells may mediate cellular repair in vascular injury and so be essential to maintain the integrity and function of the vasculature. Endothelial dysfunction is an early event in atherosclerosis, and studies assessing the pathogenesis of atherosclerosis in ApoE\textsuperscript{-/-} mice have certainly demonstrated this. Seminal work by Wassmann \textit{et al} in splenectomised ApoE\textsuperscript{-/-} mice fed a high fat diet demonstrated a significant impairment in endothelium dependent vasodilatation when compared to wild type mice.\textsuperscript{239} This impairment was rescued by intravenous transfusion of mononuclear cells, CPCs or culture-derived early outgrowth EPCs derived from wild type mice.
We have demonstrated, in keeping with previous studies\textsuperscript{132}, that South Asian subjects are insulin resistant. While data assessing murine vascular repair following transfusion of EPC derived from this particular group is unavailable, there is a plethora of evidence suggesting impaired EPC function in insulin resistant phenotypes.\textsuperscript{233, 288} Our group recently performed a study investigating the effects of insulin resistance on EPCs and endothelial regeneration in Insulin receptor knock out (IRKO) mice.\textsuperscript{233} In addition to demonstrating impaired mobilisation in response to VEGF and reduced bone marrow eNOS expression in IRKO mice, we demonstrated significantly delayed endothelial regeneration following femoral arterial injury in insulin resistant IRKO mice when compared to wild type mice. Vascular repair was rescued following transfusion of MNCs derived from wild type mice. This appears to be the only data to date that assesses the impact of insulin resistance \textit{per se} on vascular repair, though experiments addressing insulin resistant diabetes have reached similar conclusions.\textsuperscript{220}

While the work described above involved EPCs derived from mice, there remain limited studies assessing re-endothelialisation capacity of EPCs derived from insulin resistant humans. Sorrentino \textit{et al} derived EPCs from subjects with diabetes and demonstrated severely blunted re-endothelialisation capacity when compared to EPCs derived from healthy subjects.\textsuperscript{220} This effect persisted even after 7 days of transplantation. EPCs derived from the subjects with diabetes also demonstrated significantly elevated levels of superoxide production and reduced NO availability.
Keeping in mind the high possibility of development of an immune reaction in mice following transfusion of human EPCs, we used immunodeficient nude mice for our work. Our findings are similar to the findings of Sorrentino et al., and we have clearly demonstrated impaired re-endothelialisation of injured femoral arteries following transfusion of EPCs derived from insulin resistant SA subjects. The underlying mechanism appears likely to be impaired PI3K/Akt signalling and possibly via reduced NO production, in view of our rescue of EPC mediated vascular repair after E17K Akt transduction. Of course, we have not assessed the role of other factors such as oxidant stress or probed the reasons for altered PI3K/Akt signalling, and these could be pursued in future studies.

6.5.5 Impaired re-endothelialisation capacity is rescued by constitutively active Akt expression in South Asian EPCs

As we demonstrated impaired re-endothelialisation of injured conduit vessels following transfusion of South Asian EPCs which exhibited reduced PI3K/Akt signalling, we elected to upregulate Akt activity using lentiviral vector constructs. A significant improvement in EPC-mediated repair was noted following E17K Akt transduction, to a magnitude comparable to non-transduced WE EPCs. This corroborates our hypothesis that reduced PI3K/Akt signalling may mediate SA EPC dysfunction.

Previous work by Ackah et al. has demonstrated a crucial role for Akt in EPC function. In addition, loss of Akt1 has been demonstrated to result in increased
release of pro-inflammatory mediators resulting in atherosclerosis. While the concept of augmenting Akt activity in SA EPCs to improve their function appears promising, it is important to bear in mind that chronic hyperactivation of Akt signalling may be detrimental to EPC function. Work by Wang et al in mice with a mutant circadian rhythm gene demonstrated increased Akt signalling, which was associated with greater EPC senescence and impaired proliferation. Inhibition of Akt signalling rescued impaired cellular function in these mice. In addition, Nishi et al demonstrated endothelial dysfunction due to excessive Akt activity following VEGFR-1 deletion.

Our study is the first to demonstrate improvement in late outgrowth EPC function following promotion of Akt activity in insulin resistant human. While we have used lentiviral technology to modify EPCs in our study, we appreciate the difficulty of replicating this in a clinical setting due to safety concerns described later. Of course, Akt activity can be augmented in EPCs with statins, and this has been shown to improve their function. However, these agents have many other pharmacological effects and much more work is required to determine their role in this capacity. Furthermore, it is unknown as to whether long term statin use and hence chronic Akt upregulation is beneficial or detrimental to EPC function. These caveats notwithstanding, it maybe that novel pharmacological agents could promote vascular repair, by normalising endothelial and/or progenitor PI3K/Akt signalling.
6.6 Study limitations

As was observed earlier, this aspect of our study has certain limitations. As already discussed in chapter 4 defining ethnicity accurately is impossible, and the small sample size of these experiments limits statistical power and may not be reflective of the entire SA population group. These limitations remain applicable to this aspect of our study.

6.6.1 Origin of and defining ‘late EPCs’

Despite extensive research being conducted in the field of endothelial progenitor cell biology, there is so far no clear agreement on what constitutes an ‘EPC’, how to define such cells and whether they exist in vivo. To this date, these issues continue to be a matter of debate, with different groups culturing and identifying EPCs by different methods.\(^{147}\)

From a functional point of view, while culture derived late outgrowth EPCs may represent true endothelial progenitors, the possibility that these cells might have acquired or lost properties during culture which could influence experimental results should not be ruled out. In other words, the results we have described above may, to some extent, be artefacts due to cell culture. This of course is relevant to any in vitro experiment. However, independent of the in vivo relevance of these data, it is apparent from our work that late outgrowth EPCs offer an attractive therapeutic tool, providing their reparative function can be restored.
The origin of late outgrowth EPCs also remains uncertain. These cells appear in culture after 14 – 21 days of plating mononuclear cells, at which point early EPCs have died. However, given the lack of a unique marker for late outgrowth EPCs the precursor of these cells remains uncertain and so cannot be sought in vivo. Case et al described cells expressing surface markers CD34,CD45,CD133, and VEGFR-2 to be hematopoietic progenitors and not EPCs. Work by Timmermans et al identified late outgrowth EPCs to lie within a cell fraction that express CD34 and lack CD45 on flow cytometry. From the available evidence, it appears late outgrowth EPCs are CD34⁺CD45⁻CD133⁻.

Certain groups have described late outgrowth EPCs as different from HUVECs due to their greater proliferative potential and resistance to oxidative stress. However, contradictory reports have been published, which adds to the uncertainty of the origin of late EPCs. To add to this uncertainty, some groups have speculated whether late outgrowth EPCs are in fact sloughed off mature endothelial cells that have expanded in culture. We have previously described these cells as CECs, which possess poor proliferative potential. However, vessel wall derived mature ECs such as HUVECs also possess high proliferative potential, which could imply the origin of culture derived late EPCs to be CECs. However, there are no clear studies clarifying this, and hence this possibility remains uncertain. More recently, Mund et al attempted to define and characterise circulating endothelial cells. Polychromatic flow cytometry (PFC) has now shown these not to be endothelial cells but mostly platelet and extracellular vesicles. Upon culture, it appears that a proportion of these CECs bear high clonogenic potential, and appear to be true EPCs. However, this study also identified EPCs as bearing cell surface
markers CD34+CD146+CD45-, which was also present on mature CECs. It thus appears that despite advanced flow cytometric methods, it still remains difficult to accurately define circulating EPCs and CEC, except based on their clonogenic capacity.

6.6.2 Potential problems with oncogene transfer using lentiviral vectors into EPCs

The introduction of an oncogene into EPCs using lentiviral vectors poses potential problems if applied to clinical use. As late EPCs possess high proliferative potential and are capable of serial passages, there remains the possibility that these cells can form or contribute to tumour-like lesions. Lentiviral vectors bear an advantage in that they can integrate into the target cell genome in a stable fashion, with the added capability of transducing non-dividing cells. However, using HIV as a lentiviral vector bears the risk of viral replication into wild type-recombinants or interaction between lentiviral vectors and subsequent wild type viral infection.307, 308 In addition to this exists the possibility of insertional oncogenesis if vectors with long terminal repeats (LTR) are used. These problems are addressed by using self-inactivating (SIN) lentiviral vectors which consist of inactive long terminal repeats (LTR)309 that prevent vertical and horizontal transmission of the wild type virus. However, there still remains a risk of the SIN vectors forming competent viral transcripts capable of encapsidation and integration into the host cell genome.307 This risk would need addressing when lentiviral vector mediated gene delivery is considered for clinical use. So far there do not appear to be any human clinical studies, probably due to the
lack of sufficient number of preclinical models to predict the likelihood of insertional oncogenesis.\textsuperscript{310} Furthermore, most mouse models that have used lentiviral gene delivery are short studies, which could make it difficult to predict the likely long term outcomes if similar studies are conducted in humans. Designing such a clinical study would require careful analysis of the potential risks of oncogenesis versus the benefits. SIN vectors may bear promise, but more studies need to be conducted before clinical application.

In addition to the problem addressed above, we have used constitutively active Akt in our experiments. We have previously discussed the potential problems with over-expression of Akt leading to enhanced EPC senescence. Wang \textit{et al} studied Per2\textsuperscript{m/m} mice (a circadian regulatory gene) and demonstrated enhanced senescence in these specimens to be due to over-expression of Akt.\textsuperscript{208} To add to this, Nishi \textit{et al} demonstrated that premature senescence in VEGFR-1 deleted endothelial cells was secondary to Akt activation, implying a detrimental role of endothelial cells in postnatal angiogenesis in the event of excessive Akt activation.\textsuperscript{209} This should be borne in mind when titrating the ‘dose’ of constitutively active Akt if performing \textit{in vitro} experiments. Furthermore, Akt over-expression has been linked to certain cancers\textsuperscript{311,312}, which may pose a risk if \textit{in vitro} studies are translated into clinical trials in the future.

\textbf{6.7 Study implications}

From the data presented in this chapter, it is evident that there appears to be a link between SA ethnicity, insulin resistance and impaired endothelial progenitor Akt
signalling and endothelial progenitor function. While this is evident in a research setting, the clinical implications of this are not clear.

Unpublished work by our group has shown late outgrowth EPCs derived from SA men demonstrate impaired migration to angiogenic stimuli, impaired adhesion to fibronectin and impaired in vitro angiogenesis. We have gone on to show that this is mechanistically related to impaired PI3K/Akt signalling. We translated this into an in vivo model of vascular injury, and demonstrated impaired re-endothelialisation at baseline, with a rescue of reparative function following restoration of Akt activity. This data is novel in demonstrating that human late outgrowth EPC can promote vascular repair, but are profoundly dysfunctional in a group at high risk of cardiovascular disease. While the actual existence of culture derived late EPCs in vivo remains unproven, our work still provide data around which future cardiovascular cell based therapies can be designed. However, whether isolating sufficient numbers of EPCs and optimising their function sufficiently to treat patients with vascular disease is feasible remains an issue to be addressed in future. Finally, we have identified a common pathophysiological mechanism that leads to EPC dysfunction in late (and potentially early) outgrowth EPCs from humans at risk of cardiovascular events. These data will undoubtedly generate further hypotheses around which future therapeutic studies can be designed.
Chapter 7: Conclusions and future work
7. Conclusions and future work

In summary, my thesis above has demonstrated the following findings -

1. Basal early EPC abundance is reduced and function is impaired in metabolically insulin resistant SA men, when compared to matched WE men.

2. Simvastatin exposure \textit{in vitro} increases early EPC number in SA and WE men and may improve some indices of EPC function in cells derived from SA men.

3. Human late outgrowth EPC can incorporate into the vessel wall and promote vascular repair after transfusion into immuno-deficient mice with arterial injury.

4. Late outgrowth EPC function is impaired in SA men. In particular, they exhibit early senescence and impaired capacity to promote re-endothelialisation in murine conduit artery injury studies.

5. Restoration of Akt activity in SA EPCs by lentiviral introduction of constitutively active Akt rescues their capacity for vascular repair.

In section 7.1, we shall discuss the common underlying themes linking some of these findings and also discuss future work which may translate our findings toward clinical relevance.

7.1 Common underlying themes

In chapter four we have demonstrated in a cohort of SA men that metabolic insulin resistance is associated with early outgrowth EPC dysfunction. Though the number of parameters we have evaluated to assess early EPC function are only a few, we have performed these keeping in mind the putative role of progenitors \textit{in vivo}. It is speculated that following tissue injury or ischemia, the CPCs are mobilised from the
bone marrow, home to the site of injury and then release paracrine mediators that aid re-endothelialisation and healing at the site of injury, or themselves incorporate into new vessels.\textsuperscript{174} Our group has previously demonstrated reduced number and mobilisation of CPCs in SA men, with the reduction in basal abundance being inversely correlated to the HOMA-IR score.\textsuperscript{133,313} The \textit{in vitro} studies in this thesis have extended such observations to culture derived EPCs. In particular, we have shown reduced basal EPC number, reduced EPC migration and reduced paracrine stimulation of endothelial angiogenesis. When translated \textit{in vivo}, this could imply impaired mobilisation, homing and incorporation into new vessels. However, the lack of clear evidence as to whether cultured EPCs are in fact CPCs seen \textit{in vivo} adds to the confusion of accurately interpreting the relevance of these results.

Treatment of early EPCs with Simvastatin, as shown above, resulted in increased EPC numbers, along with an improvement in adhesive capacity of SA early EPCs. Such increased have been previously been shown to be equipotent to physiologically relevant concentrations of the angiogenic cytokine VEGF, and is mechanistically related to increased PI3K/Akt activity.\textsuperscript{167} This increase in EPC abundance using Simvastatin could add to the list of their pleiotropic effects, though remains of uncertain clinical relevance in isolation, even if EPCs are proven to exist \textit{in vivo}.

While the \textit{in vivo} relevance of \textit{in vitro} studies of EPCs remains unclear, late outgrowth EPCs appear to represent an attractive autologous therapeutic tool. These cells not only have the capacity to proliferate significantly, but are also morphologically similar to endothelial cells, bear functional properties similar to ECs and share similar gene expression profiles.\textsuperscript{157,164,165} Given this, we elected to
analyse various functional properties of late EPCs derived from South Asian men. In addition to our previous findings (Appendix 1), we have clearly demonstrated reduced proliferative capacity and increased senescence of SA LEPCs. In an attempt to explain these observations, we went on to demonstrate reduced expression of the key pro-angiogenic molecules eNOS and Akt. In keeping with a mechanistic role of reduced Akt activity, re-endothelialisation following conduit artery injury was impaired, and rescued by increasing Akt activity using lentiviral manipulation of SA EPCs. These results are in keeping with the possibility that pathway specific insulin resistance, with impaired cellular PI3K/Akt signalling, is of relevance to LEPC derived from a group of insulin resistant humans.

In the introduction to this thesis, I discussed ‘pathway specific’ insulin resistance as an important concept linking whole body insulin resistance, insulin resistance at the vascular endothelial cell level, and the development of atherosclerosis. This results in selective downregulation of endothelial PI3K/Akt signalling pathway, while maintaining normal or enhanced activity of the MAP kinase pathway. As NO production is stimulated by the PI3K/Akt pathway, impairment of its activity results in reduced NO bioavailability. NO is a critical molecule in regulating angiogenesis in the setting of tissue ischemia. In addition to being an anti-atherosclerotic molecule, it attenuates senescence and enhances EPC migration, which are key factors in vascular repair. Furthermore, EPC mobilisation to VEGF is absent in eNOS knockout mice, indicating a role of NO in this process.

From the results we have seen with late EPCs (and possibly early EPCs), increasing the activity of the PI3 kinase / Akt pathway increases EPC number, enhances EPC
function and promotes vascular repair. While these findings most certainly are hypothesis generating, their application in disease preventing strategies remains to be tested. Of course, these findings could be used to direct future translational research.

### 7.2 Future work

#### 7.2.1 In vivo effect of Simvastatin on SA EPCs

Our study has shown enhanced EPC number and function following *in vitro* treatment of South Asian early EPCs with Simvastatin. While these findings are intriguing and novel in a South Asian population, they may not necessarily reflect changes that could occur in a clinical setting following oral Simvastatin. Dimmeler *et al* demonstrated a significant increase in c-kit/sca-1 cells in the bone marrow and EPC numbers in the periphery following administration of 20mg/kg Simvastatin to C57BL/6 male mice. While we attempted to study these *in vivo* effects by administering oral Simvastatin to healthy SA men, difficulties encountered with subject compliance required that the study be terminated. Of course, we only planned to recruit 20 subjects based on power calculations. This sample size may still be construed as too small to reflect the entire SA population group, so studies involving a larger cohort may be more enlightening. The cost implications and time required in evaluating important functional parameters should be kept in mind, which could be difficult in a large study group. However, there still remains the issue that these cells are not established to exist *in vivo* and the appropriateness/relevance of their use as a surrogate cardiovascular endpoint is unclear.
7.2.1.1 *Brachial artery flow mediated dilatation following Simvastatin*

Previous work by our group has shown a positive correlation between brachial artery flow mediated dilatation and endothelial progenitor cell function, suggesting reduced NO bioavailability underlies reduced CPCs in SA men.\(^{132,133,313}\) Studying an individual parameter such as flow mediated dilatation (FMD) following treatment with Simvastatin could provide interesting insights into its *in vivo* effects on endothelial function. Murphy *et al* studied vascular function in 24 SA and 25 WE men demonstrating a reduced FMD and blunted basal NO bioavailability in forearm blood flow studies in the insulin resistant SA group\(^{132}\); their FMD data was recapitulated by Cubbon *et al*.\(^{133}\) Flow mediated dilatation study is a validated technique to infer endothelial NO production secondary to shear stress. In addition, changes recorded in brachial artery FMD reflect vasomotor changes in coronary arteries.\(^{317}\) A reduction in FMD is reflective of underlying endothelial dysfunction, which is a precursor to atherosclerosis and subsequent development of CAD.

In keeping with the above findings, and the results described with early EPCs treated with Simvastatin in this thesis, designing a study based around our pilot study of oral Simvastatin in a larger study cohort of SA men would provide valuable information as to the *in vivo* effects of therapeutic doses of statins normally used in patients with hypercholesterolemia or coronary artery disease. Furthermore, FMD would offer a more validated outcome measure than early outgrowth EPC number/function in isolation. Assessing basal and Simvastatin induced changes in vascular function using FMD as a marker would be of great interest in linking altered EPC indices with changes in vascular function *in vivo.*
In chapter 5, we have described the effect of transfused human late EPCs on endothelial repair following conduit artery injury in immunodeficient mice. The findings we have described are novel in SA populations, and in addition, have allowed us to define out a likely mechanism of endothelial progenitor dysfunction.

Existing EPC transfusion studies have assessed the effect of transfusion of early EPCs on vascular repair.\textsuperscript{237} In our in vitro studies, we have demonstrated basal early EPC dysfunction in insulin resistant SA men, with some improvement following treatment with Simvastatin. It would be interesting to assess the effect of statin pre-treatment on early EPC mediated conduit artery repair. Work by Sorrentino et al clearly demonstrated a significant reduction in re-endothelialisation following transfusion of early EPCs from diabetic subjects when compared to non-diabetic subjects; this was rescued by Rosiglitazone therapy.\textsuperscript{220} Similar results have been seen in early EPCs derived from pre-hypertensive and hypertensive patients.\textsuperscript{318} We have shown that by up-regulating Akt in late EPCs, conduit artery repair can be augmented significantly in SA EPCs to a degree comparable to basal repair seen with WE late EPCs. Combining these two results together, and using similar methods to our late EPC work, early EPCs could be transfused into nude mice and re-endothelialisation can be assessed. Using a statin as pharmacological method of improving early EPC function, possibly via promoting PI3K/Akt activity, it would be possible to probe its effects on vascular repair in vivo. Of course, detailed assessment of PI3K/Akt signalling in EPC treated with statin or vehicle would be required to link altered signalling with altered function.
### 7.2.3 Other mechanisms of EPC dysfunction

While our work has offered some interesting observations regarding EPC functional impairment both \textit{in vitro} and \textit{in vivo} in SA men, it would be useful to assess other mechanisms that may be involved. In particular, an interesting avenue of future research would be to address why Akt activity is reduced in SA late EPCs. Determining this could lead to safer means of restoring EPC function, and thus avoid the potential problems that could arise using viral delivery of constitutively active Akt. Our work here has concentrated mostly on the PI3 kinase/Akt pathway. EPC function is closely linked with NO production\textsuperscript{176}, and promotion of PI3K/Akt signalling is not the sole means of augmenting NO bioavailability.

Reactive oxygen species play a role in NO degradation, and so evaluating oxidant stress as a regulator of EPC function in SA men would be helpful. While EPCs have been shown to be relatively tolerant to oxidant stress due to expression of antioxidant enzymes, excess ROS generation may play a role in endothelial progenitor cell dysfunction.\textsuperscript{217, 219, 319} In addition, they appear to play a critical role in progenitor mobilisation and tissue reperfusion in hind limb ischemia models.\textsuperscript{218, 320}

We have shown SA EPCs to be more senescent in culture, and senescence has been shown to be related to oxidative stress.\textsuperscript{321} Enhanced EPC apoptosis may also play a part in EPC dysfunction, and FACS assessed cell surface Annexin expression may be a useful way of evaluating this. Detailed study of ROS subtypes and subsequent modification with selective anti-oxidants \textit{in vitro} may provide a platform to define the relevance of ROS to SA late outgrowth EPC dysfunction.
7.2.4 Sample size and standardisation of assays

While our findings are interesting and hypothesis generating, our study cohort is small and the most relevant sub-studies should ideally be repeated in a larger sample of South Asian and White European men. However, given the financial constraints and limited resources, performing such work will be difficult. If studies are to be continued to be conducted in small samples, assays would benefit from standardisation amongst research groups to aid comparison and future meta-analysis.

7.3 Future potential therapeutic strategies

Our study has not only corroborated previous findings by our group, but has also extended these data by demonstrating rescue of endothelial progenitor cell function by augmenting Akt activity. This presents a potential therapeutic target to enhance autologous EPC function, and thus neovascularisation, should cell-based therapies reach the clinical arena.

Previous research has suggested the mechanism of impaired mobilisation and migration of diabetic EPCs to be dependent on NO. However, the therapeutic strategy used by this group to modify EPC function by enhancing NO production using hyperoxia is difficult to replicate in a clinical setting. In addition, other groups have shown that NO production is dependent on PI3 kinase/Akt signalling. We have shown improvement in EPC function by targeting this pathway. This result is of
particular significance, given our subjects are insulin resistant, and the concept of pathway specific insulin resistance downregulating NO production via the PI3K/Akt pathway would apply here.\textsuperscript{122} In particular, we have shown that increasing Akt activity in SA EPCs enhances their capability to promote re-endothelialisation. However, as has been previously discussed, chronically activated Akt may be detrimental\textsuperscript{208}, and so the longer term implications of our findings to progenitor function are unclear. While our data from Simvastatin exposure of early EPCs appears promising, we did notice cell death at higher concentrations, which could imply a role of Akt over-activation, although we have not formally assessed this. Studies assessing the effect of long term statin use in patients with coronary artery disease identified statin use as an independent predictor of reduced CPC and EPC numbers.\textsuperscript{322} From this data, it could be that while short term use of statins may improve EPC function transiently, prolonged use and high doses of statins could be detrimental to EPCs in a clinical setting. This deduction is purely hypothetical and can be investigated in future studies.

We have demonstrated enhanced vascular repair following arterial injury using lentiviral vector delivery of constitutively active Akt. While the results are novel and appear promising, there remain potential problems which we have highlighted in section 6.6.2. These hurdles will need to be adequately dealt with the confirm safety of the application of this research technique in clinical practice.
7.4 Concluding remarks

When we commenced this project, our aims were to assess EPC number and function in SA men, to corroborate previous findings by our group, assess effects of Simvastatin on EPC function, identify mechanisms of EPC dysfunction and modulate these mechanisms to improve this function.

Our data has not only corroborated our previous work $^{132,133}$, but has also clearly demonstrated impaired capability of EPCs derived from healthy SA men to promote vascular repair, when compared to white European men. These subjects were all apparently healthy, non-obese with normal lipid profile and normal blood pressure, though South Asian men were insulin resistant. Early EPC function was impaired in SA men, and some indices improved significantly with Simvastatin exposure $in vitro$. While similar findings have been described in human EPCs before, these findings are the first to be reported in South Asian men.

In addition, we have systematically studied the re-endothelialisation capacity of late outgrowth EPCs derived from WE and SA men. Beyond demonstrating for the first time that human late EPCs can promote arterial repair, we also found that SA EPCs were dysfunctional $in vivo$ and this could be rescued by restoration of Akt activity. This finding is both novel and exciting, and opens numerous avenues in modulating EPC function in subjects at risk of cardiovascular events as part of developing cell-based cardiovascular repair therapies.
Finally, our work has continued to demonstrate numerous aspects of endothelial progenitor dysfunction in SA populations. This could bear direct relevance to the higher prevalence of cardiovascular disease in South Asians. Hopefully our data in combination with future research will aid development of new strategies in the prevention and management of cardiovascular disease in this high risk population.


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Appendix 1

Restoring endothelial progenitor Akt activity in humans at high cardiovascular risk rescues vascular reparative capacity

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Classification: Biological Sciences - Medical Sciences
Abstract

Late-outgrowth endothelial progenitor cells (LEPC) are putative mediators of endogenous vascular repair, and represent an attractive tool for future cell-based cardiovascular repair strategies. However, progenitor function is impaired in potential target populations. We have conducted detailed analysis of in vitro and in vivo LEPC pro-angiogenic function in insulin resistant South Asian (SA) men and matched European controls. SA LEPC exhibited impaired colony formation, migration and in vitro angiogenesis, associated with markedly decreased expression of the pro-angiogenic molecules Akt and eNOS. Transfusion of control LEPC into immunodeficient mice after wire-induced femoral artery luminal injury augmented re-endothelialization; however, neither SA LEPC, nor vehicle, augmented re-endothelialization. Lentiviral vector gene delivery of constitutively active Akt1 (E17KAkt), but not enhanced green fluorescent protein (EGFP) control, to SA LEPC was associated with augmented Akt content and activity, and rescue of in vivo re-endothelialization capacity. These data support the role of human LEPC as a platform for future cell based vascular repair therapies, and suggest a mechanism by which autologous progenitor function can be promoted in subjects likely to benefit from cardiovascular repair therapies.
Introduction

LEPC possess the functional properties of vascular endothelium, whilst exhibiting a progenitor hierarchy and the capacity for significant ex vivo expansion (1). These properties and the capacity to easily derive autologous cells without the need to introduce multiple potentially oncogenic genes ex vivo, makes their translation as a cardiovascular repair therapy appealing. A large body of evidence supports progenitor dysfunction in diabetes and insulin resistance, disorders linked with persistently poor cardiovascular mortality and morbidity (2). Our previous work has demonstrated impaired vascular function and a reduction in basal and exercise mobilized circulating progenitor cells in healthy insulin resistant South Asian (SA) men as a result of reduced nitric oxide (NO) bioavailability (3,4). Indeed, many other studies have shown vascular dysfunction and insulin resistance from childhood onwards in people of South Asian ethnicity, and in later life this group is at high risk of major vascular events, which may be amenable to cell based preventative or therapeutic intervention in future (5-7).

Results

We recruited a cohort of apparently healthy SA men and matched white European (WE) controls (n=12 per group) to assess basal abundance and function of LEPC, prior to assessing strategies to optimize the therapeutic efficacy of progenitors. Groups were well matched for demographic factors and established cardiovascular risk factors, though insulin resistance was apparent in the SA cohort (Table 1).

LEPC colony formation was markedly reduced in SA men (Figure 1a, Supplemental data), suggesting a lower abundance of circulating progenitors; endothelial phenotype was confirmed using standard assays (see Supplemental data). The
proliferative rate and migration toward physiologically relevant stimuli (Vascular Endothelial Growth Factor [VEGF] and Insulin-like Growth Factor-1 [IGF1]) were blunted in SA LEPC (Figure 1b-c, Supplemental data). VEGF stimulated in vitro angiogenic capacity, measured by tubular structure formation on Matrigel, was also significantly reduced (Figure 1d, Supplemental data). Senescence, assessed using acidic beta-galactosidase staining, was more prevalent in SA LEPC populations (Figure 1e, Supplemental data). Concentrations of the pro-angiogenic molecules protein kinase-B (Akt) and endothelial nitric oxide synthase (eNOS) were markedly reduced in SA LEPC at protein level (including the ‘activated’ S473 phosphorylated form)(Figure 1f-i). eNOS, but not Akt, mRNA was less abundant in SA LEPC (see Supplemental data).

In order to probe the in vivo relevance of our in vitro studies, equal numbers of LEPC were transfused intravenously into CD1 immunodeficient mice immediately after wire-induced luminal injury of the common femoral artery, with vehicle medium serving as control. After 4 days re-endothelialization was assessed using Evans blue staining of the injured and contralateral non-injured vessel to indicate persistent endothelial denudation (Figure 2a). LEPC derived from WE subjects significantly augmented re-endothelialization, whereas the femoral arteries of mice exposed to SA LEPC were indistinguishable from those receiving vehicle (Figure 2b). Confocal microscopy of a subset of vessel preparations revealed markedly fewer adherent fluorescently tagged LEPC in the intima of specimens previously treated with SA LEPC (see representative images in Figure 2c-d).

Based upon the upstream location of Akt relative to eNOS, and literature demonstrating the critical role of both molecules in angiogenesis (8-10), we elected to augment LEPC Akt activity in SA LEPC using lentiviral vector gene delivery of a
constitutively active Akt1 mutant (E17KAkt – Supplementary data) (11,12). Self-
inactivating lentiviral vectors (LVHVLS2 and LVSinCSGWdlNot1) were used to
introduce E17KAkt or EGFP (control), with viral titers chosen to augment SA LEPC
phospho-Akt S473 content approximately three-fold, in order to achieve levels
comparable to control European LEPC (Supplementary data). E17KAkt expressing
SA LEPC exhibited increased phospho-Akt S473 content, Akt activity as measured
using a cell free GSK phosphorylation assay, and phospho-eNOS S1177 content
(Figure 3a-c). Akt activity in EGFP expressing and native SA LEPC was comparable
(Figure 3c). Intravenous transfusion of EGFP expressing SA LEPC was associated
with unchanged femoral artery re-endothelialization, whereas E17KAkt expressing
LEPC augmented re-endothelialization to a magnitude comparable to non-
transduced European cells (Figure 3d). Confocal microscopy of injured femoral
artery specimens from mice exposed to EGFP expressing SA LEPC revealed
persistent in vivo expression of EGFP in tracked LEPC (see Supplemental data).

Discussion

Our data are the first to demonstrate the dysfunction of LEPC derived from
apparently healthy South Asian men using a detailed array of in vitro and in vivo
assessments; furthermore, these functional deficits have been linked to molecular
abnormalities relevant to vascular repair. In particular, by demonstrating improved
SA LEPC function after lentiviral augmentation of Akt activity, we have causally
implicated this molecule in the EPC dysfunction related to South Asian ethnicity.
Moreover, our work provides the first demonstration of rescued in vivo LEPC function
in cells derived from subjects at high risk of major cardiovascular events, an
important step in optimizing future cell based cardiovascular repair strategies.
LEPC are recognized as representing an attractive form of autologous cell based cardiovascular repair therapy, given their close mimicry of endothelial phenotype and capacity for significant ex vivo expansion and manipulation (1). Indeed, they have already been demonstrated to augment myocardial angiogenesis and stimulate beneficial left ventricular remodeling in large animal models of myocardial infarction (13). Hence, these cells may offer the prospect of preventing or retarding cardiovascular morbidity in groups of patients at high risk of adverse events. As far as we are aware, our data is the first to demonstrate that human LEPC are capable of conduit artery repair, an important proof of principle for human autologous cell based vascular repair therapy.

A growing body of evidence supports the elevated cardiovascular risk associated with South Asian ethnicity, compared with European ethnicity (6,14); insulin resistance and diabetes have been strongly implicated in this phenomenon. Our previous work has demonstrated important abnormalities in the vascular biology of apparently healthy, but insulin resistant, South Asian men (3,4). Endothelial dysfunction/damage were suggested by impaired flow mediated vasodilatation and elevation of circulating endothelial microparticles, whilst marked reductions in circulating progenitor subsets were apparent. Furthermore, physiological mobilization of progenitors appeared reduced in SA men, as a result of reduced nitric oxide bioavailability (3). Our current data complements these observations, demonstrating clear perturbation in the function of ex vivo expanded LEPC and mechanistically implicating impaired Akt signaling in this process. The causality of such impaired signal transduction in SA LEPC is unclear, though abundant data support the role of Akt, and downstream eNOS, signal transduction in promoting vascular repair and abrogating atherogenesis (8-10,15,16). Importantly our data also support the
capacity of E17KAkt to augment phospho-eNOS S1177, a surrogate of eNOS activity, and so nitric oxide generation.

If LEPC are to become a therapeutically useful cardiovascular repair tool, it is likely they will be introduced in patients at the highest risk of cardiovascular morbidity and mortality. Our data is therefore important in demonstrating that in a group of high cardiovascular risk subjects, LEPC function is impaired and can be ‘normalized’ by modulation of Akt signaling. It is tempting to speculate that our observations may also extend to insulin resistant subjects within other ethnic groups, though this hypothesis requires ongoing assessment. It is also important to direct future studies toward identifying the causation of reduced Akt content and signaling in SA LEPC, since this may hint at more effective strategies to augment LEPC function ex vivo, or even modulate endogenous vascular repair.

In conclusion, we have demonstrated that LEPC derived from healthy, but insulin resistant, South Asian men exhibit profound blunting of vascular reparative function in vitro and in vivo, which is mechanistically linked to reduced Akt signaling. By augmenting Akt signaling using lentiviral vector gene delivery, we have corrected these functional deficits, and so have demonstrated a potential means of augmenting the efficacy of cell based autologous vascular repair in this group at high risk of major vascular events.

Materials and Methods

*Subject recruitment:* Healthy white European and South Asian men were recruited by poster advertisement. Ethnicity was defined by subjects from a list used in UK
healthcare monitoring (17); subjects defining their ethnicity within subdivisions of ‘Asian or British Asian’ were defined as South Asian; those within the subdivisions of ‘White’ were defined as white European. Subjects were eligible for inclusion if aged 18-40, free from any chronic illness, not taking prescription medication, non-smokers within the past year and free from hypertension (BP>160/90mmHg), diabetes and hypercholesterolaemia (Total cholesterol>7mmol/L). A total of 12 white European and 12 South Asian men were included. All participants provided written informed consent, according to the declaration of Helsinki; ethical approval was provided by the Harrogate and Leeds (Central) research ethics committees.

**LEPC culture:** Peripheral blood mononuclear cells (PBMC) were harvested using density gradient centrifugation (Ficoll), and suspended in EGM-2 medium with EGM-2 Bullet kit (Lonza) and 10% Fetal Calf Serum (FCS). Cells were seeded on fibronectin and medium replaced daily for the first week, then on alternate days. After 3-4 weeks cells with cobblestone morphology developed in LEPC colonies (18); cells exhibited contact inhibition and were capable of serial passage. Endothelial phenotype was confirmed using flow cytometry, demonstrating co-expression of the vascular endothelial growth factor receptor-2 (KDR) and platelet-endothelial cell adhesion molecule (CD31) in >90%.

**Flow cytometry:** LEPC were washed twice in phosphate buffered saline (PBS), then incubated with FcR blocker (Miltenyi Biotech). Appropriate concentrations of FITC conjugated anti-CD31 (Miltenyi Biotech), PE conjugated anti-KDR (R&D systems), or equivalent isotype control non-fluorescent antibodies were added. Cells were analyzed using a FACSCalibur cytometer with CellQuest software (Becton Dickinson).
Migration: $4 \times 10^4$ LEPC suspended in basal EGM2 medium (1% FCS, without other additives) were placed in the upper compartment of modified Boyden chamber apparatus. The lower compartment contained EGM2 with 50ng/ml Vascular Endothelial Growth Factor (VEGF$_{165}$), 50ng/ml Insulin like Growth Factor-1 (IGF-1), or vehicle alone (control). Wells, in triplicate, were incubated for 24 hours. Membranes were fixed in 70% ethanol before mechanical removal of cells on the upper surface, and Haematoxylin/Eosin staining; migrant LEPC were enumerated in 10 high power fields (HPF: 1430x1060µm).

In vitro angiogenesis: LEPC ($5 \times 10^4$ cells/ml in EGM-2 with 50ng/ml VEGF$_{165}$ and 1% FCS) were seeded in triplicate on Matrigel plates for 24 hours. tubule formation was defined as participation in contiguous circular structures; mean number of tubular structures per HPF was calculated from 10 HPF.

Senescence: Sub-confluent 3rd passage LEPC were studied using a senescence associated β-galactosidase staining kit (Cell Signaling); senescent EPCs were counted by two blinded independent operators (inter-observer agreement: r=0.99) using Image J software (National Institutes of Health, USA) and expressed as % total cells.

Western blotting: 30mcg total protein from cellular lysates was loaded onto 4-12% SDS-PAGE gels (Invitrogen) and electrophoresed under reducing conditions before transfer to PVDF membranes. Membranes were probed with primary antibody (anti-Akt [Cell Signaling], anti-eNOS [BD], anti-phospho S473 Akt [Cell Signaling], anti-phospho S1177 eNOS [BD], or anti-beta-actin [Santa Cruz]) overnight in 5% skimmed milk in TBS-Tween buffer, followed by HRP-conjugated secondary
antibody for an hour. Proteins were visualized using enhanced ECL kit (Amersham Biosciences).

Polymerase chain reaction: Total RNA was extracted from cells using TRIZOL (Sigma). Equal quantities of RNA were reverse transcribed using reverse transcription kit (AB Systems) following manufacturer's protocol. Real-time PCR was performed using the following primers using SYBR based assay (AB systems: 7900HT), and beta-actin normalization:

eNOS forward, 5'-CTG-GAG-CAC-CCC-ACG-CT-3'

eNOS reverse, 5'-AGC-GGT-GAG-GGT-CAC-ACA-G-3'

Akt1 forward, 5'-CCT-TCC-TCA-CAG-CCC-TGA-AGT-3'

Akt1 reverse, 5'-CCG-GGA-CAG-GTG-GAA-GAA-C-3'

Beta-actin forward, 5'-CGT-GAA-AAG-ATG-ACC-CAG-ATC-A-3'

Beta-actin reverse, 5'-TGG-TAC-GAC-CAG-AGG-CAT-ACA-G-3'

Akt activity: Akt kinase activity was analyzed by non-radioactive immunoprecipitation-kinase assay according to manufacturer's protocol (Cell Signaling Technology). 30mcg cell extracts were incubated with immobilized Akt 1G1 monoclonal antibody. After extensive washing, the kinase reaction was performed at 30°C for 30 min in the presence of 200µM cold ATP and GSK-3 substrate. Phospho-GSK-3 was measured by Western blot, using phospho-GSK-3α/β (Ser-21/9) antibody.

Animals: 9-13 week old (weight 25-33g) male immuno-deficient CD1 nude mice (Charles River Labs) housed in isolators with 12 hour light-dark cycle were used in
all experiments; standard diet was provided *ad libitum*. Experiments were performed under license from the UK Home Office, observing standard animal welfare regulations.

**Vascular injury and analysis:** As published (19), mice were anaesthetized with isoflurane prior to femoral arteriotomy and three 1.5cm passages of a 0.014-inch-diameter angioplasty guidewire (Hi-Torque Cross-IT 200XT, AbbotVascular, USA); the vessel was then ligated, and skin closed. The contralateral artery underwent sham operation, without arteriotomy or wire injury. Animals received postoperative buprenorphine 0.25mg/Kg. 3x10^5 CMDiI (Invitrogen) labeled LEPC in EBM2 (or EBM2 without cells, as control) were transfused into the external iliac vein immediately after injury. Injured and contralateral femoral arteries were explanted 4 days later after injection of 50µL Evans Blue dye via the inferior vena cava and perfusion fixation with 4% paraformaldehyde in PBS. Re-endothelialization was assessed in *en face* specimens, defined as the region of absent Evan's blue staining in relation to a total area of a 5mm section of vessel, commencing 5mm distal to the aortic bifurcation; vessels were then mounted with Vectashield DAPI for confocal microscopic (Zeiss LSM 510 META Axioplan 2) assessment of adherent LEPC. All murine studies and analysis were performed by researchers blinded to ‘treatment’ allocation.

**SIN-Lentiviral vector production and transduction:** pSINCsgWdlNot1 was a kind gift of Dr. Yashiro Idea (Mayo Clinic, Minnesota, USA) (20). The EGFP cassette of pSINCsgWdlNot1 was replaced by E17KAkt to generate pHVSLS2. The SIN-lentiviral vectors were generated in accordance to established protocols (21), and were titred using QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24 ELISA kit [Cell Biolabs]) in accordance with manufacturer’s protocol. Semi-confluent
LEPC were transduced at one moi (multiplicity of infection) of appropriate lentiviral vector for use 4 days later.

**Statistical analysis:** Data are presented as mean (standard error of mean) and groups compared with Student’s t-tests or paired t-tests as appropriate. Statistical significance is defined as p<0.05.

Acknowledgements: We would like to thank Dr. Yasuhiro Ikeda (Mayo Clinic, Minnesota, USA) for donation of the pSinCSGWdINot1 plasmid. This work was supported by the British Heart Foundation and the Diabetes Wellness & Research Foundation, UK.

Conflict of interests: All authors have no conflict of interests to declare

Authorship:

Study conception and design: RMC, NYY, HV, SS, JA, KEP, SP, SBW, DJB, MTK

Data acquisition: RMC, NYY, HV, VB, BNM, SS, PS, AS, MG, MR

Data analysis and interpretation: RMC, NYY, HV, VB, BNM, SS, PS, HI, MG, JL, KEP, SP, SBW, DJB, MTK

Drafting, critical analysis, revision and final approval of manuscript: All authors
References


Figure 1: *In vitro* characterization of LEPC abundance and function

In South Asian men, compared with white European controls, LEPC: A) colony formation is reduced (n=8); B) population doubling time is prolonged (n=8); C) Migration to VEGF and IGF-1 is impaired (n=8); D) In vitro angiogenesis is reduced (n=8); E) Senescence is increased (n=8); F-G) Total and phospho-S473 Akt are reduced (n=7); H-I) Total and phospho-S1177 eNOS are reduced (n=7). All data are displayed as mean ± SEM; p<0.05 is denoted by *. 
Figure 2: In vivo assessment of LEPC mediated arterial re-endothelialization

A) Injured (top) and non-injured (bottom) femoral arteries demonstrating endothelial denudation, indicated by Evan’s blue staining, in the injured vessel (Scale bar denotes 5mm); B) Femoral artery re-endothelialization is augmented in mice receiving WE, but not SA LEPC, nor vehicle (n=7 in all groups; data is displayed as mean ± SEM); C-D) Representative confocal microscopic images of injured intima, showing sparse tracked LEPC in vessel from mouse receiving SA LEPC (C) and abundant tracked cells in vessel of mouse receiving WE LEPC (D) [Scale bars denote 100µm; Blue – DAPI, Red – CMDil cell tracker labeled LEPC]. p<0.05 is denoted by *; p≥0.05 is denoted by NS.
Figure 3: In vitro and in vivo assessment of LEPC expressing constitutively active Akt

A) Phospho-S473 Akt content is increased in E17K expressing cells, relative to EGFP expressing, or untreated SA LEPC (representative immunoblots included; n=6 in all studies); B) Phospho-S1177 eNOS is increased in E17K expressing cells, relative to EGFP expressing, or untreated SA LEPC (representative immunoblots included; n=6 in all studies); C) Akt activity is increased in E17K expressing cells, relative to EGFP expressing, or untreated SA LEPC (representative immunoblots included; n=6 in all studies); D) Lentiviral vector mediated gene delivery of constitutively active Akt1 (E17kAkt), but not EGFP, to SA LEPC rescues capacity to re-endothelialise injured femoral artery, when compared with unmodified WE LEPC (n=6 in E17KAkt and EGFP groups, and n=7 in all other groups). All data are displayed as mean ± SEM; * denotes p<0.05.
Table 1: Demographic and cardiovascular risk data

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<th>South Asian</th>
<th>White European</th>
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<tr>
<td>Age (years)</td>
<td>30.9 (1.2)</td>
<td>29.7 (1.1)</td>
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<td>Body Mass Index (Kg/m2)</td>
<td>24.1 (0.7)</td>
<td>22.4 (0.5)</td>
<td>NS</td>
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<td>0.84 (0.01)</td>
<td>NS</td>
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<td>Systolic Blood Pressure (mmHg)</td>
<td>116 (2.3)</td>
<td>116 (2.5)</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mmHg)</td>
<td>70 (1.6)</td>
<td>69 (1.6)</td>
<td>NS</td>
</tr>
<tr>
<td>Total Cholesterol (mmol/L)</td>
<td>4.6 (0.1)</td>
<td>4.7 (0.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
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<td>1.2 (0.1)</td>
<td>NS</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
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<td>NS</td>
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<tr>
<td>Insulin (mu/L)</td>
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<td>0.009</td>
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<td>HOMA-IR</td>
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<td>0.6 (0.1)</td>
<td>0.008</td>
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All data are expressed as mean (SEM). NS denotes non-significant.