The role of Endothelial Progenitor Cells in the pleiotropic effects of Atorvastatin

Dr Kulwinder Singh Sandhu

Keele UNIVERSITY

Doctor of Medicine in Clinical Medicine

June 2023

Dedications

This thesis is dedicated to my father Tarsem Singh Sandhu who has always been a great source of inspiration, encouragement, and strength.

Thanks dad.

Contents

Acknowledgements, abstract, index, list of tables, figures, and abbreviations

Acknowledgements

I am grateful to Dr Robert Butler for giving me the opportunity to carry out and complete this thesis. Dr Butler has been a continuous source of guidance, support and more importantly a great mentor not only in research but also in my clinical training.

I would like to thank Professor Ying Yang and Dr Wanjiku Njoroge from Guy Hilton Research Centre with their invaluable advice and assistance on the bench-based studies.

I would like to express my utmost gratitude to Jill Wain and Ian Massey for their advice, assistance in recruitment and arrange follow up.

I would like to thank Sheila Brookes for her technical support in my laboratory work and Mr David Sowter for his expert knowledge and guidance in setting up flow cytometer sequential gating protocol for the enumeration of the endothelial progenitor cell analysis. I would also like to thank Dr Ivan Olier for help with statistical analysis.

Above all, I would like to thank all the recruited patients for their cooperation and willingness to be involved in the studies featured in this thesis.

Abstract

The following thesis began by discussing current controversies surrounding EPC identification then studied the effect of atorvastatin on EPC numbers in bench and bed side studies.

Atorvastatin was hypothesised to increase chemokine CXCL-12 its receptor CXCR-4, adhesion molecule e–selectin and EPCs on damaged endothelium. The study found atorvastatin had a significant increase in CXCL-12 concentration ($P<0.05$) an effect also seen with greater incubation time also had a significant effect on CXCL-12 concentration ($P<0.05$) and maintained elevated expression of CXCR-4 and E-selectin on denuded intima. The study confirmed the feasibility of performing flow cytometric analysis of whole blood samples at Royal Stoke University hospital and that samples may be analysed up to 12 hours after venesection if stored at 4^0 C. An inverse trend was found between EPC count and coronary artery calcium score, converse to pre study hypothesis of a linear relationship between EPC count and coronary artery calcification - a marker of coronary artery disease. The thesis also expected to find that a greater number of EPCs in patients admitted with acute coronary syndromes and given the higher dose of 80 mg atorvastatin when compared with patients diagnosed with stable angina given 20mg atorvastatin. However, despite no statistical difference there appeared to be a peak of EPC numbers by 48 hours with return to baseline levels at day 3. Finally, the study found no significant difference in EPC counts over the 28 day study period in patients treated with 80mg atorvastatin.

Index

List of figures

List of tables

Table of abbreviations

°c	Degrees Celsius
%	Percentage
3D	Three dimensional
7AAD	7 amino actinomycin D
Ml	Micro-litre
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid
ACS	Acute coronary syndrome
AG	Angina
Akt	Protein kinase B
APC	Allophycocyanin
acLDL	Acetylated Low density lipoprotein
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
Avidin-HRP	Avidin horseradish peroxidase
BSA	Bovine serum albumin
BP	Blood pressure
CAC	Circulating angiogenic cells
CAD	Coronary artery disease
CCS	Calculate calcium scores
CCU	Coronary care unit
CD	Cluster of differentiation marker
CD ^{dim}	Diminished cluster of differentiation marker
CFU	Colony forming units
CFSE	Carboxyfluorescein succinimidyl ester
c-kit	Proto-oncogene or tyrosine-protein kinase (Kit) or CD117
CO_2	Carbon dioxide
COPD	Chronic obstructive pulmonary disease
CST beads	Cytometer set up and tracking
CS&T beads	Suspension of fluorospheres with uniform and stable size and fluorescence intensity
CTCA	Coronary computed tomography
CXCL-12	Chemokine C-X-C motif chemokine 12
CXCR-4	Chemokine (C-X-C motif) receptor 4
DAPI	4′,6-diamidino-2-phenylindole) staining solution
DiOC6	3,3′-dihexyloxacarbocyanine iodide
DM	Diabetes mellitus
DMF	Dimethyl formamide
DNA	Deoxyribonucleic acid
ECFC	Endothelial colony forming cells
ECG	Electrocardiograph
EDTA	Ethylene diamine tetra acetic acid
eGFR	Estimated glomerular filtration rate
ELAM-1	Endothelial-leukocyte adhesion molecule 1
ELISA	Enzyme linked immunosorbent assay quantification

eNOS	Endothelial-type nitric oxide synthase
EO-EPC	Early outgrowth endothelial cell progenitor cells
EPC	Endothelial progenitor cells
EPC-CFU	Endothelial progenitor cells colony-forming units
E-selectins	Endothelial selectins
EudraCT	European Union drug regulating authority's clinical trial
FACS	Fluorescence-activated cell sorting
$FeCl_3$	Ferrous chloride
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
Flk-1	Foetal liver kinase – 1 (also known as KDR)
FMO	Fluorescence minus one control
FRET	Fluorescence resonance energy transfer
FSC	Forward scatter
G-C	Guanine-cytosine content in DNA structure
G-CSF	Granulocyte colony-stimulating factor
G proteins	Guanine nucleotide-binding proteins
GPCR	G protein-coupled receptors
GRAFS	Glutamate, rhodopsin, adhesion, frizzled/taste2 and secretin
GTPase	Guanosine triphosphate hydrolase enzymes
Hb	Haemoglobin
HCASMC	Human cardiac artery smooth muscle cells
HDL-C	High-density lipoprotein cholesterol
HMG CoA reductase	3 hydroxy-3-methylglutaryl Coenzyme A reductase
HRA	Health and Research Authority
HRP	Avidin horseradish peroxidase conjugate.
HTN	Hypertension
HUVEC	Human umbilical vein endothelial cells
IHD	Ischaemic heart disease
IL	Interleukin
Ig	Immunoglobulin
IRAS	Integrated Research Application Service
ISHAGE	International Society for Hematotherapy and Graft Engineering
KDR	Kinase insert domain receptor
LDL	Low-density lipoprotein cholesterol
LECAM-2	Leukocyte-endothelial cell adhesion molecule 2
LSGS	Low serum growth supplement
LO-EPC	Late outgrowth endothelial cell progenitor cells
LSGS	Low serum growth supplement
LV function	Left ventricular function
MAC	Myeloid angiogenic cells
MACE	Major adverse cardiovascular events
MCP-1	Monocyte chemo-attractant protein 1
MHRA	Medicines and Healthcare advisory authority

miR	Micro non-coding ribonucleic acid
mg	Milligram
mmHg	Millimetres of mercury
mmol/L	Millimoles per litre
MNC	Mononuclear cells
MPTP	Mitochondrial permeability transition pore
MRI	Magnetic resonance imaging
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NAD(P)H oxidase	Nicotinamide adenine dinucleotide phosphate oxidase
NICE	National Institute for Health and Care Excellence
nm	Nanometer
NSTEMI	Non-ST segment elevation myocardial infarction
NO	Nitric oxide
NRES	National Research Ethics Service
OA	Osteoarthritis
OCT	Optical coherence tomography
PI3K/Akt/MTOR pathway	Phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signalling pathway
PAF	Paroxysmal atrial fibrillation
PBS	Phosphate buffered saline
PCI	Percutaneous coronary intervention
PDGF	Platelet derived growth factor
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
PIS	Patient information sheet
pg/ml	Picograms per millilitre
Pharm Lyse (BD) 10%	Lysing solution used throughout the study
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PLA	Polylactic acid nanofibres
PPCI	Primary percutaneous coronary intervention
PTL	Pre-test likelihood
RACPC	Rapid access chest pain clinic
Ras	family of **genes** that make proteins involved in cell **signalling** pathways that control cell growth and cell death.
RCT	Random controlled trial
RISK	Reperfusion injury salvage kinase pathway
RNA	Ribonucleic acid
ROCK	Rho associated protein kinases (ROCK)
ROS	Reactive oxygen species
rpm	Revolutions per minute
SDF-1	Stromal cell-derived factor 1
SSC	Side scatter
SMGS	Smooth muscle growth supplement

STEMI	ST-segment elevation myocardial infarction
TEBV	Tissue engineered blood vessel
TEIL	Tissue engineered intimal layer
TEM	Tissue engineered models
TEML	Tissue engineered medial layer
TG	Triglycerides
TGF	Transforming growth factor
TIMI	Thrombolysis in Myocardial Infarction
TNF	Tumour necrosis factor
UC	Ulcerative colitis
UK	United Kingdom
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
vWF	Von Willebrand factor

Chapter 1

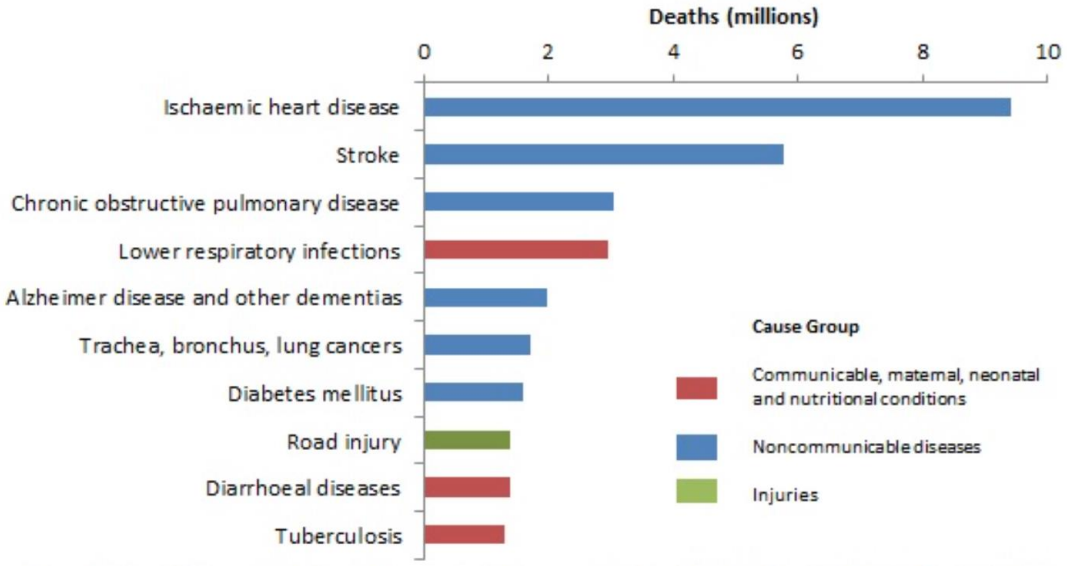
Introduction and literature review

1.1 Coronary artery disease epidemiology and pathophysiology

Epidemiology

Ischaemic heart disease is the leading cause of death and morbidity worldwide and accounting for over 9 million deaths each year. [1] Figure 1.1.

Figure 1.1; Leading causes of death worldwide (WHO 2018) [1]



In the United Kingdom (UK) there are an estimated 7 million people living with heart and circulatory diseases. [2] Cardiovascular disease, coronary artery disease and stroke, causes 1 death every 3 minutes, 420 deaths per day, 150,000 deaths each year

and accounts for approximately 25% of all deaths. [2] Coronary artery disease (CAD) is responsible for over 66,000 deaths each year, on average 180 deaths per day or 1 death every 8 minutes [2] and 540 daily admissions in to hospital with an acute heart attack. CAD has also a large economic burden with the annual health cost for cardiovascular disease estimated at £9 billion each year rising to £19 billion if including premature deaths, disability and informal costs. [2] However, the disease and economic burden is likely to increase when we take into consideration our ageing population and improved survival rates from heart and circulatory events.

Pathophysiology

CAD is due to atherosclerosis, a chronic inflammation of coronary arterial walls. Atherosclerotic lesions are initiated by dysfunction of the endothelial layer and endothelial cell damage lining the arterial wall, caused by irritation caused by dyslipidaemia. This results in endothelial activation, subsequently leading to monocyte adhesion to, and migration through, the endothelium. Monocyte-derived macrophages in the arterial wall take up cholesterol-rich LDL particles, leading to the formation of foam cells. The progression of atherosclerotic lesions causes smooth muscle cells migration from the media to the intima and propagate synthesis of extracellular elastin, collagen and proteoglycans. This eventually leads to formation of a necrotic core constituting of extracellular lipids derived from necrotic and apoptotic foam cells forms in advanced plaques, along with a fibrous cap consisting of collagen and smooth muscle cells (SMCs). This results in eventual narrowing of coronary artery disease that gives rise to angina if flow limiting. However, if the

plaque ruptures complete vessel occlusion through thrombus formation may occur. [3, 4] Figure 1.2. This pathophysiological process gives rise to a spectrum of disease from angina to acute myocardial infarction. The differentiation of each syndrome relies on clinical history, ECG changes and biochemical markers of troponin levels that correspond to any evidence of myocardial damage. Figure 1.3. The management of CAD includes optimisation of medical therapy and if required, coronary revascularisation. This had led to a reduction of UK death rate from heart and circulatory diseases by more than three quarters. [2] Figure 1.4.

Figure 1.2; Pathophysiology of Coronary artery disease [5]

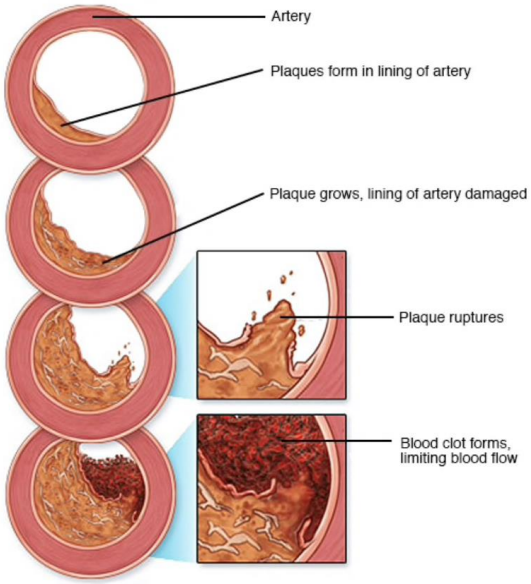


Figure 1.3; Diagram illustrating the spectrum and pathophysiology of coronary artery disease [6]

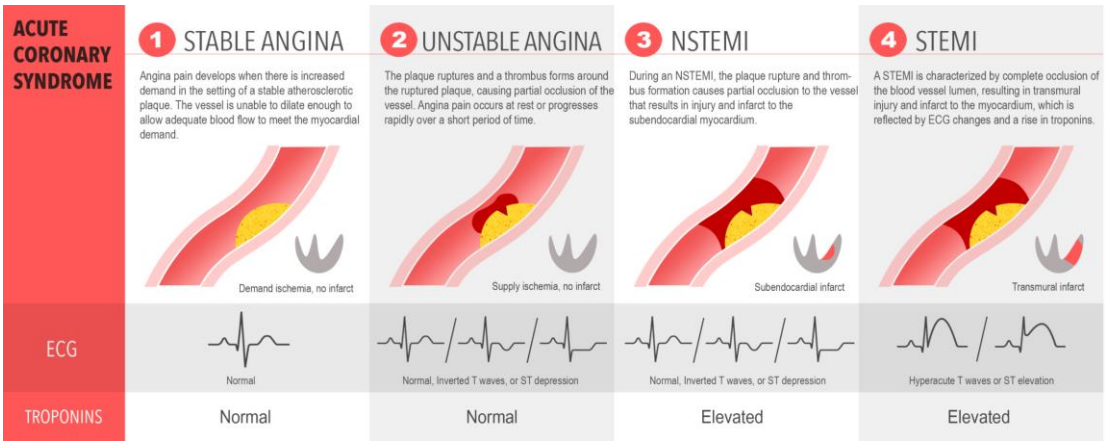
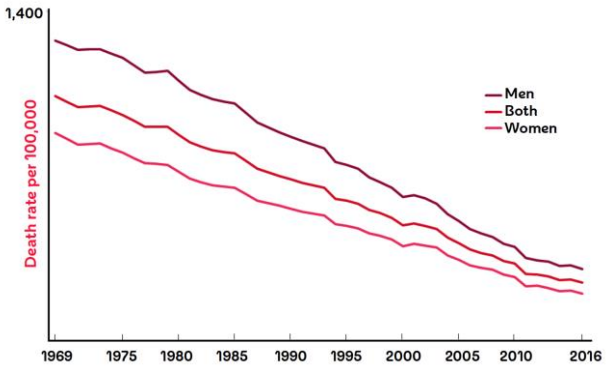


Figure 1.4; Death rate from CVD, by gender, UK, 1969 to 2016 [2]



The central step of cardiovascular disease is disruption or damage of endothelial cells lining the blood vessels. Therefore, any method of enhancing repair of endothelial cells would potentially be of great clinic benefit in terms of reducing morbidity and mortality.

1.2 Endothelial progenitor cells

Endothelial integrity depends on a balance between the extent of endothelial cell injury and the capacity for endogenous repair. Loss of endothelial integrity may cause atherosclerosis leading to coronary heart disease. [7-10] In healthy individuals, neighbouring mature endothelial cells are capable to replicate locally and replace damaged cells. [7] However local replication has limited potential and may be insufficient if the injurious stimuli remains prolonged and or is repeated. [11] Therefore an alternative mechanism is required. Vascular repair was thought to be due to migration and proliferation of fully differentiated endothelial cells, in a process called angiogenesis. [12] However an alternative proposed mechanism is dependent on undifferentiated cells migrating to sites of vascular injury [13-15] then differentiating into mature endothelial cells [16-23]. Asahara et al [28] identified putative cells with cell surface marker CD34+ or kinase insert domain receptor (KDR) markers were capable to differentiate into endothelial cells in vitro and in vivo [28-30]. Subsequent studies recognised that undifferentiated cells named endothelial progenitor cells (EPCs) migrate to sites of neovascularisation and then differentiate into endothelial cells [28, 30] in a process called vasculogenesis [31]. Figure 1.5 below.

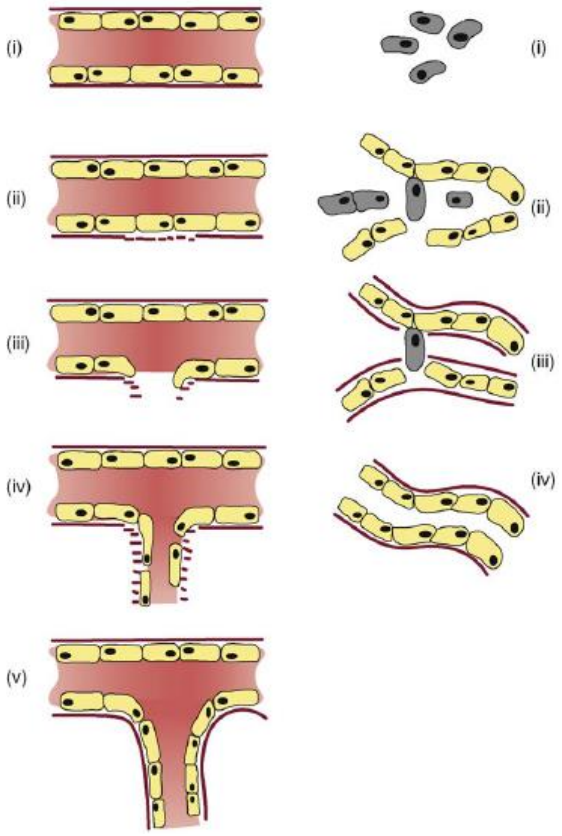
1.3 Function of endothelial progenitor cells

These undifferentiated cells [28, 33] have a central role in vascular repair due to their ability to proliferate, migrate to the site of vascular injury, then differentiate into mature vascular endothelium [23, 24] and perpetuate this cycle by secreting pro-angiogenic cytokines. [25-27] Figure 1.6 below.

It is now generally accepted that cardiovascular risk correlates with EPC number. This highlights the integral relationship between endothelium and atherosclerosis [34-38].

Several studies have shown EPC dysfunction correlate with cardiovascular events. [13, 36-38]

Figure 1.5; Comparison between angiogenesis and vasculogenesis [32]



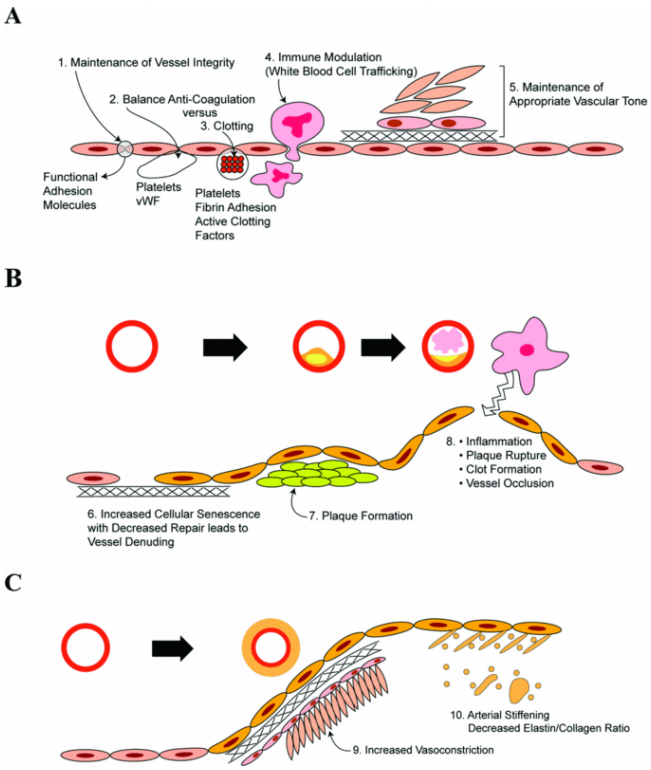
Comparison of angiogenesis and vasculogenesis. Angiogenesis originates from pre-existing vasculature (i) with secretion of matrix metalloproteases resulting in a breakdown of basement membrane (ii) allowing migration of endothelial cells with subsequent migration of proliferated endothelial cells in formation of solid endothelial cords (iv). The final stages include lumen formation and stabilization (v). In comparison vasculogenesis starts from the formation of primary vascular plexus by EPCs (i and ii) with matrix deposition (iii) and formation of the lumen (iv) by EPCs with consequent formation of immature capillaries.

Adapted from Krenning, G., M.J. van Luyn, and M.C. Harmsen, *Endothelial progenitor cell-based neovascularization: implications for therapy.* Trends Mol Med, 2009. **15**(4): p. 180-9.

. Decreased numbers have been found in patients with traditional risk factors for coronary artery disease including smoking, hypertension, [39] diabetes mellitus, [40-45] elevated low-density lipoprotein cholesterol [46, 47] and hypercholesterolemia. [46-51] Disruption of endothelial integrity by endothelial cell injury has been shown to be a stimulus for the development of atherosclerosis [10] but also as a stimulus for augmentation of EPC number and function [19, 52, 53]. Continued endothelial damage [54] may lead to an eventual reduction of the number of EPCs resulting in deficient endothelial repair and progression of atherosclerosis and myocardial ischemia[19, 46].

This has led to great interest and research in the possibility of EPC being infused in patients with intractable angina, post myocardial infarction left ventricular recovery and in chronic heart failure patients with some studies showing beneficial effect [55-64]. Therefore, the ability to augment the number and/or the function of endothelial progenitor cells may have a profound beneficial effect on vascular repair and a positive impact on outcomes and become a possible treatment strategy in patients with atherosclerotic heart disease.

Figure 1.6; The role of EPC under normal physiological conditions and disease [53]



Role in healthy vasculature (A), EPCs maintaining integrity of vasculature (1), balance between anticoagulation (2) and thrombosis (3), modulation of immune system by regulating leucocytes (4), lastly maintain vascular tone (5). Disease state (B and C) senescent EPCs with decreased capacity to repair damaged endothelium vessel denudation (6), causing plaque formation (7), rupture and thrombosis (8) with consequent vessel occlusion, vasoconstriction (9) and arterial stiffening.

Section 1.4 Endothelial progenitor cells and cytokines

Vascular injury results in the initiation of an inflammatory response. Atherosclerosis has been referred to as a chronic inflammatory disease of arterial walls characterised

by the development of lesions through continuous and progressive infiltration and accumulation of lipids and leukocytes [3, 4] These inflammatory responses involves a complex interaction between inflammatory cells (neutrophils, lymphocytes, monocytes and macrophages) but also has been associated with increased expression of adhesion molecules on endothelial cells, growth factors and cytokines. Cytokines are released from endothelial cells, vascular smooth muscle and inflammatory cells.[65] Cytokines interact with specific receptors on various cell types and may augment cell adhesion, permeability and even apoptosis. Chemokines are the largest family member of cytokines. [66] Endothelial cells interact with inflammatory cells and smooth muscle cells via a variety of chemokines and their receptors. [67] Chemokines play an integral role in proliferation, migration and re-endothelisation after injury. [68] The chemokine C-X-C motif chemokine 12 (CXCL-12), also known as stromal cell-derived factor 1 (SDF-1) has been shown to have a central role in vascular repair in cardiovascular disease. [69, 70] CXCL12 is highly expressed in human atherosclerotic plaques, endothelial cells [71] and a strong chemotactic factor for EPCs. [72-74] Human studies have shown that CXCL-12 is a possible regulatory agent in atherosclerosis with up regulation of the expression of CXCL-12 on platelets and atherosclerotic plaques. This up regulation is thought to enhance homing of EPC to sites of vascular injury [70, 71, 75] Furthermore, vascular injury or damage releases inflammatory mediators TNF- α and IL-1β that both upregulate CXCL-12 and CXCR-4 expression. [76] Plasma levels of CXCL-12 have been shown to be lowest in unstable angina followed by stable angina and normal in healthy controls. [77] CXCL-12 in high concentrations has been thought to mediate anti-inflammatory and atherosclerotic

plaque stabilising effects. [77] Some authors have therefore suggested that CXCL-12 to be superior to traditional risk factors in predicting adverse cardiovascular outcomes. [78] This has led to CXCL-12 to be considered a novel target for CAD.[77, 79] Direct injection of CXCL-12 has been shown to reduce myocardial infarct size after ischemia with the authors hypothesising that this was due to an associated increased neo-angiogenesis. [80] This effect postulated to be secondary due to mobilisation and chemo-attraction of EPCs to site of vascular injury. [73] CXCL-12 with its receptor signalling has been suggested to have a protective role after a myocardial infarction.[81] CXCL-12 binds to the cell surface receptor G-protein coupled receptor, chemokine (C-X-C motif) receptor 4 (CXCR-4) that are found on several different cell types. [82, 83] The CXCL-12/CXCR-4 axis has been shown to play a crucial role in the homing and retention of EPCs in the stem cell niches of the bone marrow. [84] In physiological conditions hematopoietic stem cells are retained in bone marrow due to high levels of CXCL-12 by stromal cells. [81] CXCL-12 secreted by endothelial cells of sinusoids within the bone marrow arrest of rolling CXCR4+ EPCs. [84] Once vascular extravasation within the bone marrow has occurred, EPCs home to areas within the bone marrow that provide optimal conditions for EPC to survive, function and potentially differentiate into other lineages. [85] During conditions of stress, EPCs begin to be released into the circulation. [84] Decreased surface expression, but increased gene expression of CXCR-4 have been found in peripheral blood mononuclear cells from patients with stable angina and patients with unstable angina [77] Modulation of CXCL-12/CXCR-4 axis has been shown to have a central role in mobilisation of endothelial progenitor cells by decreasing CXCL-12. [81, 84, 91] Studies have shown cell

surface molecular expression has been observed to last 3-5 hours before down regulation.[65]

E-selectins are cell adhesion molecules expressed only on endothelial cells activated by cytokines. [92] They are also known as CD62 antigen-like family member E (CD62E), endothelial-leukocyte adhesion molecule 1 (ELAM-1), or leukocyte-endothelial cell adhesion molecule 2 (LECAM-2). [92] E-selectins are not stored in cells and therefore must be transcribed, translated, and transported to the cell surface. The production of E-selectin is stimulated by the expression of P-selectin that is in turn, is stimulated by inflammatory mediators tumour necrosis factor α (TNF-α), interleukin-1 (IL-1) and lipopolysaccharide.[93] Studies have shown that approximately two hours are required for expression on endothelial cell surface after cytokine recognition with maximal expression taking 6–12 hours with levels returning to normal by 24 hours. [93] E-selectins are thought to be integral for EPC adhesion. [94-98] Interestingly an increased CXCR-12 in ischaemic tissue may stimulate both a local endothelial cell and EPC from bone marrow to express reciprocally E-selectin/ligand pairs and therefore enhancing the interaction between endothelial cells and EPCs. [99]

1.5 Endothelial progenitor cell identification, classification and nomenclature

Endothelial progenitors cells are accounting for only 0.001-0.0001% of peripheral blood cells in an unstressed state [100]. EPCs may be isolated from bone marrow or the circulation as mononuclear cells [28, 101, 102] expressing a variety of endothelial surface markers [103]. Currently, there remains a lack of consensus not only on phenotyping

but also on EPC function [104-109] with consequent conflicting reports regarding EPC identification and function. Confounding this, some studies have combined different cell populations and labelled cell populations as EPCs without recognition of the possible synergistic effect of different cell populations.[110] Furthermore, EPCs are often referred to as a diverse group of cells of different lineages having angiogenic potential despite some populations unable to differentiate into functional endothelial cells. [104]. Consequently this has led to a lack of understanding the role of EPCs in health and disease. [111] In recognition of this, current EPC nomenclature proposed over a decade ago is widely regarded as insufficient. [112]

The review below highlights current controversies on a general consensus on a working definition on identification of EPCs.[108] The review will approach the current controversies on identification and function of EPCs by considering limitations of the commonly used laboratory methods used in EPC identification. There are currently two laboratory methods commonly used for identification and classification of EPCs. The first a blood-based assays quantified by several specific cell surface markers using flow cytometry. The second by the number of colonies of adherent cells that can be obtained from circulating mononuclear cells (MNCs) expressing mature endothelial cell markers in-vitro by cell culture isolation. [113]

1.5.1 EPC identification by flow cytometry

This assay is through flow cytometric identification and subsequent quantification of cells of interest as defined by the presence of specific cell surface markers. Principles of flow cytometry are discussed in section 3.6.2. Cellular identification and staging of

differentiation have been made possible by specific surface receptors called epitopes that allow immunophenotyping. This process allows identification of subset of cellular surface molecule termed *cluster of differentiation* (CD). Cellular subtypes may be defined by the presence or absence of a particular CD molecule. Therefore "CD" may be "+ "or " – " denoting either presence of absence of a particular CD, and is used to describe stem cells rather than fully differentiated cell types. Certain cell types may have variable CD marker expression during maturation for example, and therefore classed as bright (high), mid (mid) or dim (low) denoting intensity of expression. [114, 115]

Flow cytometry is an integral component of this research thesis and a widespread laboratory based analytical modality. There remains controversy with no current general consensus on specific markers identifying EPCs. EPCs, thought to be derived from $CD34^+$ hematopoietic progenitor cells [16, 28, 101, 103], with co-expression of specific endothelial marker proteins [16, 101, 103]. With certain cell surface markers thought to be related to the stage of maturations of the EPC. For example, the cell surface marker CD133, a 120-kDa trans-membrane polypeptide, is expressed on bone marrow derived hematopoietic stem and progenitor cells in peripheral blood.[116] Interestingly, expression of CD133 decreases to a complete absence in mature EPCs within the peripheral circulation. The timing of the loss of expression of CD133 remains unclear [117]. However, the loss of CD133 indicates the transformation into more mature endothelial like cells [116]. The converse is true for the expression of CD34, a cell surface marker found on immature pluripotential stem cells. [103] CD34 gradually increases as the CD133 decreases as the EPC matures [116]. The value of using CD133 as a marker

of EPC remains contentious, firstly due to the rarity of cells expressing CD133 and more importantly studies suggest that CD133 are hematopoietic cell lines and therefore unable to form endothelial phenotypic EPCs. [118, 119]

Certain authors suggest a minimal antigenic profile should include at least 1 marker of immature cells, commonly CD34 and/or CD133 plus at least 1 marker of endothelial cells commonly VEGFR2^{+} (KDR/Flk-1). CD133^{+} either alone or in combination with CD34^{+}/VEGFR2^{+} has been used for identification of EPCs in some studies [103, 120]. Whereas other studies suggest expression of CD34^{+}, CD133^{+}, and/or VEGF2^{+} [100, 104, 118, 121, 122]. Table 1 summarises and compares the distinct expression of three commonly used markers within bone marrow and EPCs. Some authors propose EPCs being derived from CD45^{-} lineage. [100] Interestingly, CD34^{+}, VEGFR2^{+} and diminished CD45 (CD45^{dim}) cells have been found to have greater correlation with coronary heart disease and response to statins when compared to healthy individuals. [123, 124] With the combination of CD133, CD34 and VEGFR-2 associated with early functional EPCs [17, 103]. Therefore, EPCs may express markers of both hematopoietic stem cells (CD34 and CD133) and endothelial cells (CD146, vWF, and VEGFR2). [30, 100, 101, 103, 116, 119, 125-128] amongst other proposed markers. [28, 116, 117, 129] Hence current flow cytometric identification of EPCs remains controversial. Table 1.1 below summarises the CD markers associated with maturity of EPCs.

Table 1.1; Cell surface markers during course of maturation of EPCs (adapted from Sandhu et al [109])

	Bone marrow	Circulation	
		Early EPCs	Mature EPCs
$CD133^+$	+	+/-	-
$CD34^+$	+	+	+
$VEGFR2^+$	+	++	+++

1.5.2 EPC cell culture analysis

Cell culture allows identification by formation of colonies of cells that have a pattern of immunofluorescence identifying functioning endothelial cell lines [130]. Asahara et al first isolated and defined EPCs as circulating mononuclear cells expressing CD34 and Flk-1 with further cell culture identification by CD31, uptake of acLDL, and lectin binding. [28] Characteristics that are still commonly used to define EPC in cell culture. Cell culture definitions of EPCs also lack phenotypic specificity for several reasons. Firstly, microparticles from platelets may transfer CD31 to haematopoietic cells. [131] Secondly, CD31 and vascular endothelial growth factor receptor 2 (VEGFR2) may also be found in some monocytic subpopulations.[132] Finally, AcLDL uptake and lectin binding have been found in both macrophages and mature endothelial cells. [106]

Several studies have described two types of time-dependent cell cultured with distinct properties. The spindle shaped early outgrowth EPC (EO-EPC) in the early period of culture and late outgrowth EPC (LO-EPC) that produce colonies and tube formation in latter period of culture. [19, 28, 110, 133-136] These two populations have very different phenotypes, EO-EPC are thought of as haematopoietic and LO-EPC as endothelial cell

lines. [137] This has led to populations being named as "hematopoietic EPCs" and "non-hematopoietic EPCs". [138] Thereby supporting the hypothesis of hematopoietic EPCs giving rise to non-hematopoietic EPCs and ultimately endothelial cells.

Early outgrowth EPC (EO-EPC) are thought to be short-lived cells (< 2 weeks) and do not differentiate into endothelial cells in vivo but can restore endothelial function and enhance angiogenesis after tissue ischaemia through a paracrine mechanism. [28, 127, 139] However, they are thought to be a heterogeneous population of hematopoietic cells [139-141] and often referred to as circulating angiogenic cells (CACs). [142] CACs have been produced in vitro in cell culture conditions, with little evidence to suggest that this occurs in vivo. Leading to some authors suggesting that this cell population be termed as myeloid angiogenic cells (MACs) based on their lineage and function. [106] MACs are characterised by cell culture immune phenotyping with CD45, CD14, CD31, and negative for CD146, CD133, and Tie2. [143, 144] These cells have potent pro-angiogenic and vaso-reparative effect by a paracrine mechanism [22, 145-147]. Importantly, they are not capable of becoming endothelial progenitor cells. [108, 148] Therefore the terms MACs/CACs should not be used interchangeably with EPCs. [106]

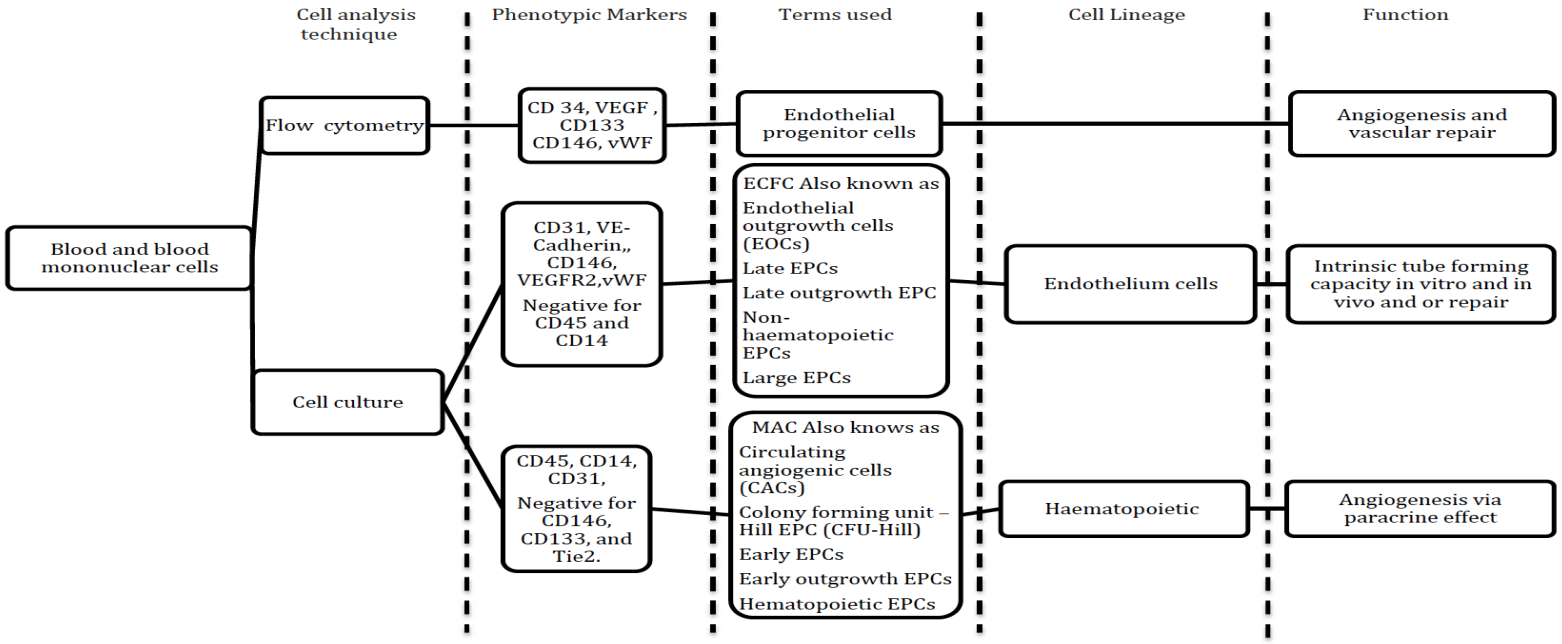
In contrast, LO-EPCs, are thought to be homogeneous endothelial-like progenitor cell population that possess a high proliferative potential, differentiate into vascular endothelial cells and form networks in vitro and in vivo. Furthermore LO-EPCs are also capable of augmenting the process by auto paracrine mechanism. [106, 110, 149-152] A mechanism noted in patients with cardiovascular risk factors.[150, 153] More recently, current recommendations suggest this population of cells perhaps should be referred to as endothelial colony forming cells (ECFCs). [102, 106] ECFCs derived from peripheral

blood mononuclear cells, or umbilical cord blood, grown in endothelial cell culture conditions are characterised by immunophenotype positive for CD31, VE-Cadherin, von Willebrand factor, CD146, VEGFR2, and negative for CD45 and CD14. CD34 expression may also be expressed, however it may decline during in vitro expansion [149, 154, 155] under flow cytometric analysis.

Interestingly, the proliferative, differentiation and tube forming ability have been found to been enhanced by laminar shear stress, [156-159] suggesting that they may contribute to autologous vascular repair. This is an important finding, raising the possibility of using these cells as viable treatment option for cardiovascular patients. [136] However any future use in as a treatment option would require an ex vivo production due to the low concentrations of LO-EPC in vivo. [133, 160]

The use of ECFCs and MACs are preferentially used terms, as this definition accurately describes the phenotype and function of these cell-types. [148, 161] Figure 1.7 adapted from Medina et al [106] summarises cellular analysis technique, phenotype markers, preferred nomenclature and function of cells often termed as EPC in current literature.

Figure 1.7; Figure summarising cellular analysis technique, phenotype markers, preferred nomenclature and function of cells often termed as EPC in current literature. [162]



Abbreviations EPC – endothelial progenitor cell, ECFC - endothelial colony forming cells, MAC - myeloid angiogenic cells, vWF - von Willebrand factor

1.6 Endothelial progenitor cells and cardiovascular risk factors

There are several well-recognised risk factors of atherosclerosis including age, physical activity, diabetes, dyslipidaemia, hypertension, smoking and family history. A number of studies have highlighted an association between these risk factors and EPCs.

An inverse relationship between age and bone marrow cells expressing endothelial progenitor markers [29] circulating EPC number [19, 46, 53, 165] and EPC function [166] has been described. $CD34^+$ $VEGFR2^+$ [46] and $CD133^+$ $VEGFR2^+$ cells [167] decrease with advancing age. One mechanism thought to be responsible for this age-related decline in EPC number is based on the premise that bone marrow EPC are subjected to oxidative stresses throughout a lifetime. These can be at least initially compensated, however these compensatory mechanisms may become exhausted resulting in a decrease in the function of EPCs [168]. Alternative explanations include down regulation of tissue hypoxia-inducible factor 1 or insufficient local expression of $VEGFR2^+$ pivotal attractors for EPC accounting for the decrease in EPC numbers with age [169]. Shaffer et al [170] showed a progressive decline in number of certain subsets of EPCs with advancing age in both healthy volunteers and patients with peripheral vascular disease [170]. Jie *et a*l found an inverse relationship between the number of circulating $CD34^+$ $VEGFR2^+$ EPCs in healthy individuals aged from 1 to 81 years [165]. However, to the contrary, Pelliccia et al [171] found no difference in absolute numbers of $CD34^+$, $CD133^+$, $CD105^+$, and $CD14^+$ cells in older patients with coronary artery disease. This study suggested that other factor(s) may be required to reduce EPCs

numbers and arguably more fundamentally the importance of a precise definition of EPC populations studied. [171]

Physical activity is known to have a beneficial effect in both primary and secondary prevention of atherosclerosis. [172] This beneficial effect is multifactorial however exercise has been shown to increases EPCs number, function and prevention of EPC apoptosis, enhance replacement of dysfunctional endothelium by EPCs in animal studies. [172, 173] These effects have been observed to last for up to 4 weeks after physical activity.[172] The mechanisms that increase circulating EPCs include acute mobilisation of EPCs from a bone marrow EPC pool as a response to shear stress [174, 175] or due to an exercise induced increase in nitric oxide bioavailability. [176] This phenomenon of a significant increase in EPC number and function after exercise has also been observed in patients with coronary artery disease [177, 178] but also in patients who have had an acute coronary syndrome. [179, 180] A complex relationship exists between diabetes, a well-known risk factor for CAD and EPCs. However, fewer circulating EPCs are seen in diabetes. [41-45] Interestingly there also appears to be a progressive decrease in EPCs levels, from impaired glucose tolerance, through to diagnosed diabetes, in patients with an acute myocardial infarction.[181] A reduction in EPC migratory capacity has been associated with hypertension [39, 46] and hypercholesterolemia. [46, 47] Hypercholesterolemia has also been observed to reduce EPC number, migration, function and inversely related to both total and LDL-cholesterol levels [46]. LDL levels have been associated not only with premature apoptosis of circulating EPCs [48, 49] but also together with oxidised LDL capable of blocking VEGF-induced EPC migration

via inhibition of NO production [47, 50, 51]. These mechanisms have also been attributed to the reduction of circulating EPCs seen in with hypercholesterolemia [47].

Smoking has a complex interaction with EPCs, with higher levels associated with cytotoxic effects [182]. Smoking has been shown to reduce EPC numbers, whereas low concentration of nicotine has been shown to have a positive effect on both EPC numbers and function [182, 183]. The use of nicotine patches increases the magnitude of EPC numbers seen after smoking cessation alone [183]. Chronic smoking is associated with a reduction in EPCs with a rapid restoration of EPC numbers seen in smoking cessation, especially in light smokers.[183]

A significant inverse relationship between age and bone marrow cells expressing endothelial progenitor markers, [29] circulating EPC number [19, 46, 53, 165] and EPC function [166] has been described. Both $CD34^+$ $VEGFR2^+$ [46] and $CD133^+$ $VEGFR2^+$ cells [167] decrease with advancing age. Alternative explanations include down regulation of tissue hypoxia-inducible factor 1 or insufficient local expression of $VEGFR2^+$ pivotal attractors for EPC accounting for the decrease in EPC numbers with age [169]. Shaffer et al [170] showed a progressive decline in number of certain subsets of EPCs with advancing age in both healthy volunteers and patients with peripheral vascular disease [170]. Jie et al found an inverse relationship between the number of circulating $CD34^+$ $VEGFR2^+$ EPCs in healthy individuals aged from 1 to 81 years [165]. However, to the contrary, Pelliccia et al [171] found no difference in absolute numbers of $CD34^+$, $CD133^+$, $CD105^+$, and $CD14^+$ cells in older patients with coronary artery disease. This study suggested that other factor(s) may be required to reduce EPCs numbers and

arguably more fundamentally the importance of a precise definition of EPC populations studied [171].

1.7 Endothelial Progenitor cells (EPCs) and atherosclerosis

There is a well-described integral relationship between impairment of endothelium repair and atherosclerosis. [34-38] A reduction in EPC numbers subsequently leads to decrease in endothelial repair and progression of atherosclerosis. [19, 46] Patients with coronary artery disease may have significantly lower EPC numbers when compared to patients without coronary artery disease. [185, 186] Several studies have also found impaired function of EPCs being associated with cardiovascular events. [13, 36-38, 46] EPCs defined as $CD34^+$ $VEGFR2^+$ cells have been associated with freedom from myocardial infarction, hospitalisation, revascularisation and cardiovascular death in patients with coronary artery disease.[185]

Werner et al measured EPCs with cell surface markers $CD34^+$ $VEGFR2^+$ in 519 patients with angiographically confirmed coronary artery disease and compared the association between baseline EPC levels and major adverse cardiac events at 12 months. The group found increased levels of EPCs were associated with a reduced risk of death from cardiovascular cause, major adverse cardiovascular events, coronary revascularisation and rehospitalisation after adjustments for age, sex and vascular risk factors [187]. Similarly, Schmidt-Lucke et al studied 120 patients (43 control subjects, 44 patients with stable coronary artery disease, and 33 patients with acute coronary syndromes) with a median follow up of 10 months. The primary outcome measures were percutaneous coronary intervention, coronary artery bypass

graft or ischaemic stroke. They found that reduced numbers of EPCs were associated with a significantly higher incidence of cardiovascular events by Kaplan-Meier analysis. [164] Several studies have found that after multivariate analysis, reduced EPC levels or reduction in function were a significant, independent predictor of poor prognosis, even after adjustment for traditional cardiovascular risk factors and disease activity. [135, 164, 187] Furthermore the combination of cardiovascular risk factors and decreased numbers of $CD34^+$ $VEGFR2^+$ EPCs appear to have a cumulative effect. [46] Interestingly, the prognostic association between EPC number and cardiac events has also been observed in patients with no known coronary disease. Hill et al studied 45 men with no cardiac history and with varying magnitude of cardiovascular risk factors and found a correlation between number of circulating EPCs and Framingham risk factor score. The group found the level of circulating EPCs was a better predictor of vascular risk than presence or absence of traditional cardiovascular risk factors and therefore suggested EPCs levels may be a marker for cardiovascular risk [19].

1.8 The effect of cardiac ischemia on EPCs

Cardiac ischemia has been considered as a primary stimulus for EPC mobilisation and has been associated with increased levels of both inflammatory and haematopoietic cytokines stimulating EPC mobilisation from the bone marrow. [188-191] Several smaller studies have also suggested an increase in the numbers of circulating $CD34^+/CD133^+$/VEGFR2 EPCs after an acute myocardial infarction. [120, 192] The timing of the increase in EPCs numbers is debatable. [189, 190, 192, 193] Shintani et al [192] was the first to describe a significant increase in $CD34^+$ EPCs by flow cytometry and

cultured clusters of endothelial cell lineage markers (CD31, vascular endothelial cadherin, and kinase insert domain receptor) plateauing on day 7 after onset of cardiac ischemia.[192] Similarly Regueiro et al found the number of EPCs increased after 7 days and peaked at 30 days in patients admitted with an acute myocardial infarction, the group defined EPCs as $CD45^-$, $CD34^+$, KDR^+, $CD133^+$.[194] Other cell subpopulations, such as $CD34^+/CD117^+$, $CD34^+/CXCR4^+$, $CD34^+/CD38^+$ and $CD34^+/CD45^+$ have also been observed to have similar patterns as that mentioned above.[188, 189] Whereas Massa et al [189] observed a 5.8 fold increase of $CD34^+$ $VEGFR^{2+}$ and circulating CD34 circulating CD34+ cells co-expressing CD33, CD38, or CD117 and in-vitro culture peaking at 3 hours after onset of symptoms of an acute myocardial infarction. This was followed by a significant decrease by day 7 and normalisation within 60 days. The group noted the increase in EPCs occurred prior to pharmacological or invasive percutaneous coronary intervention. [189]

1.9 EPCs and treatment of coronary artery disease

Percutaneous coronary intervention (PCI) has been associated with an increase in EPCs. This, in one study may be as early as 6 hours after PCI and returned to basal levels after 24 hours post-PCI following elective PCI in patients with stable angina. [195] Similarly Bannerjee et al found increased CD34/CD31 positive EPC colony-forming units (EPC-CFU) in patients undergoing elective PCI without a corresponding rise in VEGF levels 12h after PCI. However the study found patients with an acute coronary syndrome had no significant increase in EPC mobilisation 12h after PCI despite a significant rise in VEGF. [196] Perhaps unsurprisingly, the

combination of an acute coronary syndrome treated by angioplasty provokes a greater EPC response. [197]

Recruitment of EPCs to the site of vascular injury has been proposed to promote vascular healing and has been shown to inhibit neo-intimal proliferation and restenosis associated with PCI, [198, 199] this is to the contrary Schober et al. [200] The JACK-EPC trial found the use of the Genous ™ stents used in acute coronary syndrome patients were associated with lower restenosis rate than BMS at 6 months and the number of circulating EPCs was inversely correlated with in-stent re-stenosis at 6 months. [199] The Genous ™ stent (OrbusNeich Medical Technologies, Fort Lauderdale, FL, USA) was described as an EPC capturing stent coated with anti-human $CD34^+$ antibodies that bound circulating EPCs from the peripheral blood. This was hypothesised to facilitate rapid differentiation into a functional endothelial layer on the stent surface. The safety and feasibility of Genous ™ stent have been previously verified. [201, 202] The first randomised, single-center study showed a non-significant higher rate of repeat revascularisation in the Genous™ stent compared with the drug eluting Taxus Liberté™ (Boston Scientific, MA, USA) stents in 193 patients with lesions carrying a high risk of restenosis randomised to have either Genous™ or the Taxus stent. The Taxus Liberté™ stent group were noted to have higher rates of stent thrombosis. [203] As described above, atherosclerosis has been linked with a reduction in EPCs and therefore reduction in repair of vascular endothelium resulting in an increased risk of myocardial ischemia. [19, 46] This has led to several studies assessing EPCs transplantation as potential treatment modality in patients with intractable angina, post myocardial infarction left ventricular recovery and in chronic heart

failure patient. [55-64, 204] Matured endothelial cells have been associated with reduction of infarct size. [205] Initial animal studies assessing the effect of EPC transplantation had shown some favourable outcomes. This finding led to other clinical studies in ischemic heart disease patients. [206] The beneficial effects of EPCs in re-endothelialisation has raised great interest in the direct infusion of EPC or bone marrow mononuclear cells, [204, 207-209] use of colony stimulating factors to increase EPC levels [210-212] and the use of stents with EPC capture ability. [213] Further interest was raised when a greater improvement of left ventricular contractility was observed by bone marrow derived cells delivered to infarct-related coronary arteries when compared to circulating mononuclear cells. [57, 61, 62] The BOOST trial, revealed no long-term benefit on left ventricular systolic function after acute myocardial infarction when compared to the randomised group. However, left ventricular recovery was accelerated in the stem cell therapy group. [63] Similarly Janssens et al, [64] despite no improvement in global left ventricular function the group found favourable infarct remodelling with a reduction in infarct size at 4 months follow-up. [64] The REPAIR-AMI trial, a double-blind, placebo controlled multicentre study of 204 patients with myocardial infarction were randomised to bone marrow-derived progenitor cells via intracoronary infusion or placebo with the pre-specified cumulative endpoint of death, myocardial infarction, or revascularisation. The 12-month end points were significantly reduced in the bone marrow-derived progenitor cells group compared with placebo but also that the bone marrow-derived progenitor cells was an independent predictor of favourable clinical outcome [204]. Dobert et al, found intracoronary infusion of bone marrow derived or circulating blood EPCs 4 days *after*

myocardial infarction resulted in a significant increase in myocardial viability and perfusion as shown by positron emission tomography and single photon emission computerised tomography.[56] In another study by Flores-Ramirez et al intracoronary infusion of CD 133+ phenotypic EPCs into patients with New York Heart Association scale of at least II and left ventricular ejection fraction of less tha 35% showed significant improvement in both left ventricular ejection fraction and quality of life. [55] Several other small studies noted favourable outcomes of infused EPCs for management of acute coronary syndromes, intractable symptomatic angina and after recanalisation of chronic coronary artery occlusion. [57-60] A number of studies have shown the feasibility and safety of advancements in stem cell engineering augmenting the regenerative capacity of stem cells including EPCs has led to the possibility of personalised therapy cell-based therapy of patients with CAD. [214, 215]

1.10 Statin therapy in coronary artery disease

The importance of early statin therapy in acute coronary syndrome patients was highlighted in the MIRACL trial [216] that assessed effects of atorvastatin on early recurrent ischemic events. This was a double blinded randomised controlled trial from May 1997 to September 1999 cross 122 clinical centres in Europe, North America, South Africa, and Australasia. The study recruited 3086 patients with unstable angina or non-Q-wave acute myocardial infarction. The patients were randomly assigned and matched to receive either 80mg of atorvastatin or a placebo between 24 and 96 hours after hospital admission. Primary end points were all cause death, non-fatal acute myocardial infarction, cardiac arrest with resuscitation, or recurrent symptomatic

myocardial ischemia with objective evidence and requiring emergency re-hospitalisation. The trial noted a beneficial effect of high-dose statin intervention immediately after an ACS within a 4-month period compared with placebo. This consequently led to question of the benefit of high versus moderate dose statin therapy. [216]

The REVERSAL trial [217] attempted to answer this. The study randomised 654 patients with symptomatic coronary artery disease, angiographic evidence of 20% or greater stenosis in the non-culprit coronary artery, and LDL levels between 125 mg/dL (3.2 mmol/L) and 210 mg/dl (5.4 mmol/L) to either pravastatin 40 mg or atorvastatin 80 mg. Intravascular ultrasound (IVUS) imaging of the non-target coronary artery was used to assess atheroma and performed prior to randomisation and repeated after 18 months of treatment. The primary end point was percentage change in atheroma volume. The study found a significantly lower progression rate in the intensive atorvastatin (P 0.02). Secondary end points included change in total atheroma volume and change in percent stenosis volume, which also confirmed no significant change in the intensive dose atorvastatin group. The REVERSAL trial was one of the first to suggest that intensive lipid-lowering therapy may be superior to standard therapy. However, the study did not assess clinical outcomes, but rather was a mechanistic investigation. [217]

This led to the PROVE IT trial, [218] comparing standard pravastatin (40mg) dose to intensive high-dose atorvastatin (80mg) and measured the composite end point of death from all-cause and major cardiovascular events. The trial randomly assigned 4,162 ACS patients hospitalised within the previous 10 days to receive either intensive

statin therapy with 80 mg atorvastatin or standard therapy with 40 mg pravastatin. The primary end points were time to all cause death, myocardial infarction and unstable angina requiring hospitalisation, revascularisation or stroke. The intensive therapy group had lower median low-density lipoprotein (LDL) cholesterol level and a significant 16% reduction in outcome events after a mean follow up of 24 months. The benefits were observed early after initiation of statin therapy and over the 2 years mean follow up period. [218] Similarly the ASTEROID trial [23] found a reduction in cardiovascular events in high-intensity statin therapy with regression of coronary atherosclerosis. This prospective trial included 53 centres within the United States, Canada, Europe, and Australia. The study recruited 507 patients that had baseline IVUS examination and at least 1 dose of the study drug with 349 patients undergoing repeat IVUS assessment of coronary atheroma burden a after 24 months. The group found that the high-intensity statin therapy with rosuvastatin 40 mg achieved an average reduction of LDL-C of 60.8 mg/dL (1.6 mmol/L) and increased HDL-C by 14.7%. The study found significant regression of atherosclerosis burden. [23] The beneficial effect of intensive therapy was further studied by a larger prospective meta-analysis by Baigent et al, of 90,056 patients in 14 randomised trials found greater cholesterol reduction was associated with better patient outcomes.[219] These studies have shown that pre-treatment with atorvastatin may reduce peri-procedural myocardial infarction in patients with stable angina during elective PCI. This led to the ARMYDA-ACS randomized trial. [220] A total of 171 patients with non-ST-segment elevation ACS were randomised to pre-treatment with atorvastatin (80 mg 12 h before PCI, with a further 40-mg pre-procedure dose [n = 86]) or placebo (n = 85). All patients

received long-term atorvastatin treatment thereafter (40 mg/day). Multivariable analysis found that pre-treatment with atorvastatin conferred an 88% risk reduction of 30-day major adverse cardiac events. The authors concluded that even short-term pre-treatment with atorvastatin may improve outcomes in patients with ACS undergoing early invasive strategy. [220] This led on to the ARMYDA RECAPTURE study that found reloading of high dose statin, atorvastatin 80mg in 383 NSTEMI and stable angina patients on chronic therapy prior to PCI had a 50% reduction in 30-day major adverse cardiac events in both group with a greater reduction in NSTEMI group. [221] Furthermore a meta-analysis of 13 RCTs including 3321 patients compared intensive statin therapy versus low dose/no statin therapy loading prior to PCI. The authors found aggressive statin therapy was associated with reduced peri-procedural myocardial infarction and a 44% risk reduction in major adverse cardiovascular events at 30 days irrespective of clinical presentation. [222] A post hoc analysis of 1600 patients aged less than 75 with coronary artery disease showed a reduction in cardiovascular mortality over a 3 year follow up. [223]

Statin therapy rapidly enhances coronary blood flow in patients with stable coronary artery disease [224] and to reduce myocardial ischemia after an acute ischemic episode within a few weeks of treatment. [216] Statins reduce death and cardiovascular events in primary prevention of atherosclerosis, [225] stable coronary artery disease (CAD),[226-229] and acute coronary syndromes (ACS). [216, 218] Statins also appear to reduce development of atherosclerotic lesions and decrease plaque burden [23, 217, 230, 231]. Several large clinical studies have shown the safety and efficacy of statins in reducing cardiac events by decreasing serum cholesterol. [226, 232, 233]

Therefore, statins have been recommended in national and international guidelines in patients with coronary artery disease.[234, 235]

1.11 Pleiotropic effects of statin therapy

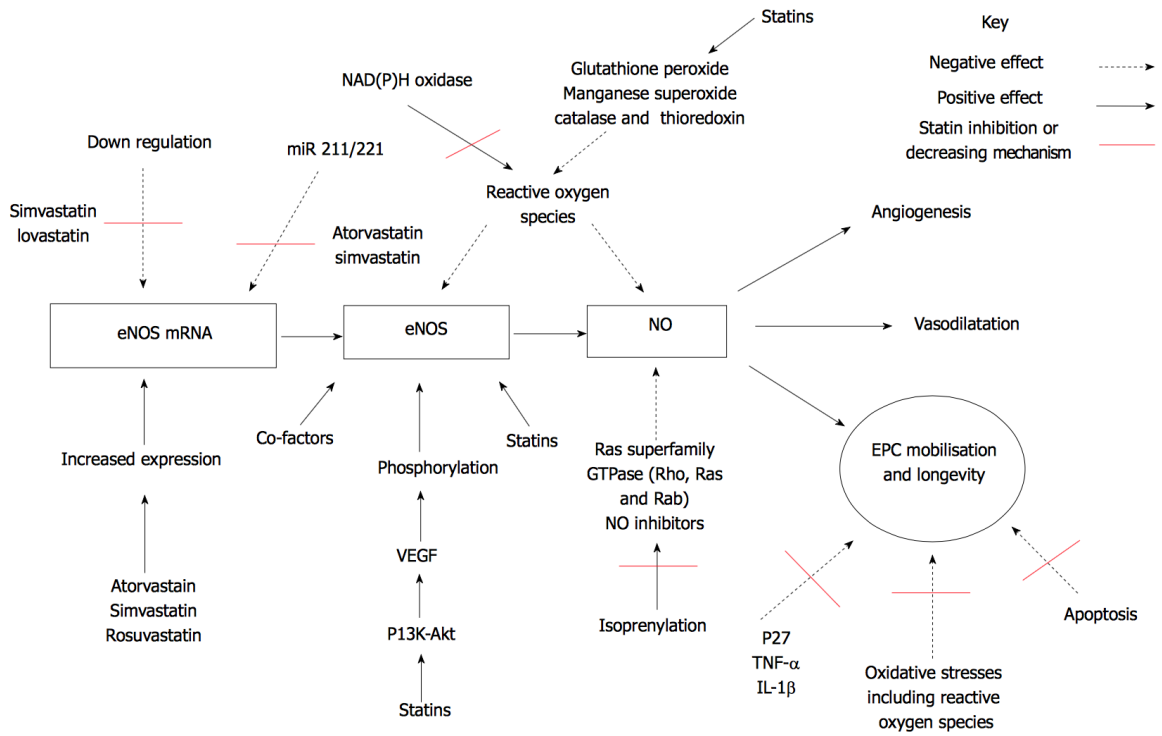
Several studies have shown the early beneficial effect of statins occurs before any significant decrease in lipid profile. This has led to the suggestion that cardiovascular benefits of statins may occur via alternative mechanisms other than reduction of cholesterol alone [219, 236]. The beneficial effect(s) of any therapeutic intervention augmenting EPC proliferation, differentiation and longevity may represent a potential therapeutic strategy for ischemic heart disease patients and therefore is of great interest. There have been a number of mechanisms proposed to account for pleiotropic effects of statin therapy. These include reduction in vascular inflammation [237], reduction of platelet agreeability and thrombus deposition [230, 238-240], enhancement of fibrinolysis [241] and increased endothelium derived NO production [242-244]. Several proposed intracellular signalling mechanisms accounting for the pleiotropic effect of statin therapy have been put forward. Figure 1.8 below summarizes the positive and negative effects on EPC proliferation, mobilisation and longevity but also the effect of statin therapy.

1.11.1 Nitric oxide pathway

The first proposed intracellular signalling mechanisms involves nitric oxide pathway. The endothelium releases nitric oxide (NO), a primary mediator of smooth muscle tone that causes vasodilatation through the activity of endothelial-type nitric oxide

synthase (eNOS) [245-248]. NO has an central role in vascular homeostasis with its bioavailability dependent on expression of endothelial eNOS [249], presence of eNOS substrate and or co-factors [250], phosphorylation of eNOS [251, 252] or due to excessive depletion of NO such as seen with presence of excessive reactive oxygen species [253]. However, the main functions of NO is as a cellular signalling molecule [245], an angiogenic factor involved in stimulation, promotion, and stabilisation of new blood vessels together with VEGFs, FGFs, angiopoietins, PDGF, MCP-1, TGF, various integrins, VE-cadherin, [254-257]. Statin therapy has been proposed to both enhance expression and activity of eNOS [258] a prerequisite stage for statin-mediated EPC mobilisation [259]. Statins are known to augment eNOS activity [260-262], increase eNOS expression and restoration of endothelial function [248, 263-265]. Statins have also been associated with increased EPC longevity via several pathways including inhibition of p27 [266], down regulating TNF α or IL-1β expression [267] and prolonging eNOS expression [266] and finally by increasing eNOS mRNA half-life [268, 269]. Kosmidou et al found simvastatin and rosuvastatin prolonged expression by increasing 3' polyadenylation of eNOS mRNA [270]. Laufs et al firstly noted simvastatin and lovastatin reversed the down-regulation of eNOS expression caused by hypoxia [242, 268] and secondly simvastatin reversed down regulation of eNOS expression induced by oxidised LDL [242, 268] a recognised cause of atherosclerosis.

Figure 1.8; Simplified diagram illustrating the positive and negative effects on EPC proliferation, mobilisation and longevity together with proposed mechanisms of action by statin therapy [109]



EPC: Endothelial progenitor cell; NO: Nitric oxide; eNOS: Endothelial nitric oxide synthase; VEGF: Vascular endothelial growth factor; mRNA: Messenger ribonucleic acid; TNF: Tumour necrosis factor alpha; IL-1: Interleukin 1; P13k-AKT: Phosphoinositide 3-kinase - protein kinase B pathway; NAD(P)H oxidase: Nicotinamide adenine dinucleotide phosphate-oxidase; miR : Micro non-coding ribonucleic acid.

1.11.2 Micro non-coding RNA (MiR 221 and miR 222)

A second observed pleotropic mechanism of statin therapy has been a decreased level of micro non-coding RNAs called miR 221 and miR 222. These negatively regulate protein expression at post-transcriptional stage [271]. This down regulating effect occurs by targeting 3' untranslated regions (UTRs) resulting in either degradation of

target mRNA or impairing translation [272] . Furthermore miR-221 and miR-222 have been observed to regulate proliferation and differentiation of CD34-positive haematopoietic progenitor cells by reducing expression of *c-kit* receptor *factor* impairs haematopoietic progenitor cell proliferation [273]. Increased miR-221 and or miR-221 expression in EPC down regulates EPC differentiation and mobilisation via c-kit and or eNOS pathways in coronary artery disease patients [271]. Atorvastatin has been shown firstly to decrease miR 221 and miR 222, and secondly increase EPC numbers [271]. Cerda et al found both atorvastatin and simvastatin increased NO levels and NOS3 mRNA expression, whereas ezetimibe did not [274]. Atorvastatin, simvastatin and ezetimibe have all been shown to down-regulate the expression of miR-221, whereas miR-222 was reduced only after atorvastatin treatment. The magnitude of the reduction of miR-221 and miR-222 after treatment with statins correlated with an increment in NOS3 mRNA levels [274]. The eNOS and miR221/222 are thought likely to be components of the same pathway [275].

1.11.3 The PI3K/Akt/MTOR pathway

The third proposed pleiotropic mechanism involves the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signalling pathway plays. The PI3K/Akt/MTOR pathway plays a central role in multiple cellular processes, including cell proliferation, angiogenesis, metabolism, differentiation and longevity. [276, 277] PI3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3) an important lipid secondary messenger which in turn plays a central role in several signal transduction pathways [278, 279] including activation of the

serine/threonine kinases PDKl and AKT. AKT controls protein synthesis and cell growth via the phosphorylation of mammalian target of rapamycin (mTOR). [280] The PI3K/AKT pathway has been associated with angiogenesis through the regulation of the nitric oxide (NO) signalling pathway. [281] The PI3K pathway releases a group of angiogenic factors including VEGF. VEGFR2 has a central role in VEGF-induced angiogenesis. [282] VEGF is required for the migration of endothelial cells and via PI3K–AKT dependent manner allows formation of capillary like structures. [51] Studies have shown that NO production may be induced by VEGF and appears to be attenuated by the inhibition of PI3K. [283] This is thought to occur via phosphorylation of eNOS at the serine 1177 residue by AKT, [251, 284] required for the VEGF induced endothelial cell migration. [285] Factors that stimulate the PI3-K/Akt protein kinase pathway, including statins, have been shown also to activate eNOS. [241, 284, 286] In turn, the expression of eNOS appears to be fundamental for mobilization of EPC and any impairment in PI3K/Akt/eNOS/NO signaling pathway may result in decreased EPC number. [287, 288]

The PI3K/AKT/mTOR intracellular pathway via inhibition of the Rho kinase has also been shown to preserves mitochondrial permeability transition pore (MPTP) preventing mitochondrial apoptosis, and therefore death, while conserving cardiomyocyte function. [289, 290]

These proposed mechanisms may account for difference in the effect of statin therapy in acute or chronic therapy. Statins given during acute ischaemic stress have been shown to firstly potentiate adenosine receptors [291, 292] eventually leading to downstream regulation of eNOS and therefore increases NO production. Secondly,

statins augment activation of the *reperfusion injury salvage kinase (RISK)* pathway [293]. This results in enhanced activity of the PI3K/AKT/mTOR intracellular signal pathways, [294] leading to preservation of mitochondrial function and cardio-protection. Short-term high dose statin therapy has shown an increase in both EPC mobilisations from bone marrow and augmented function. [288, 295-299]

Whereas chronic statin therapy has been linked to a phenomenon termed *pre-ischaemic conditioning,* protecting the myocardium against ischaemia. [300] This is believed to be secondary to statin induced NO availability by up regulation of eNOS and stabilisation of eNOS mRNA. Secondly, by increased production of NO and superoxide radicals improves vascular function and reducing vascular inflammation respectively. [242, 301] Statins also inhibit isoprenylation of a number of Ras superfamily GTPase including Rho, Ras and Rab [302] NO inhibitors resulting in increased NO bioavailability. Thirdly, by preventing mitochondrial apoptosis and preservation of cardiomyocyte function via the up regulation of the PI3K/AKT/mTOR intracellular signalling pathway by inhibition of Rho kinase. [289, 290] However, the RISK pathway has been shown the down regulated with chronic statin therapy [303] and has been shown to become reactivated by statin re-loading. [304] The latter may account for the increase in EPC count in patients on chronic statin therapy reloaded with statin therapy. [221, 305, 306]

1.11.4 Oxidative stress

Finally, EPC mobilisation and or function may also be affected by oxidative stress. [298, 307] Oxidative stress occur secondary to generation of oxygen free radicals or reactive

oxygen species (ROS). Oxidative stress has a central role in cardiovascular disease, and a pivotal role in atherosclerosis. [308] Cellular oxidative stress seen with oxidised low-density lipoprotein (ox-LDL) has a central role in the pathogenesis of atherosclerosis. LDL is oxidised by reactive oxygen species from both circulating cells and cells on vascular walls. [309, 310] In essence, LDL oxidation is a result of a chain reaction of free radicals converting polyunsaturated fatty acids into lipid peroxides and as a consequence, formation of active aldehydes.[311] The biochemical reaction forming ox-LDL have been found to cause senescence of EPCs.[312] Whereas high density lipoprotein is regarded as atheroprotective due to some part of its antioxidant properties also has a positive effect on EPC number and function.[313] There are a number of endogenous antioxidants exerting protective effects by scavenging ROS. An indirect way ROS affects EPCs includes ROS reacting with NO forming a potent oxidant [314] with a consequent decrease in NO. Decrease in NO either by excessive oxidation or impaired production decreases EPC mobilisation and/or function. [308, 315] Secondly, direct exposure to oxidative stresses or in disease conditions with high oxidative stress, for example diabetes, is associated with induced EPC apoptosis with significant reduction in EPC numbers,[315, 316] mobilisation, function [317] and reduced ability to migrate and or integrate into vasculature.[41, 308]

In an attempt to counteract the effects of oxidative stress EPCs produce superoxide dismutase.[318] Interestingly, cardiovascular risk factors have been found to alter and or reduce EPC oxidative stress. Healthy volunteers have found to express higher levels of anti-oxidative enzyme catalases including glutathione peroxidase and manganese superoxide dismutase when comparing patients with cardiovascular

disease.[319, 320] The underlying pathophysiological mechanism currently remains undetermined. The antioxidant pleiotropic effect of statins may include indirect mechanism increasing NO bioavailability accounting for antioxidant properties contributing to an increase in EPC mobilisation and or function. [258, 321] Secondly, statin therapy has also been shown to inhibit activation of NAD(P)H oxidase and ROS release, [322] but also activate catalase and thioredoxin ROS scavenging mechanisms. [322, 323] Finally, statins appear to have a direct effect by significantly reducing peroxide induced apoptosis of EPCs [316] and decreasing the oxidative damage to DNA in EPCs. [324]

1.11.5 G-proteins and G-protein-coupled receptors

G protein-coupled receptors (GPCR) are comprised of seven trans-membrane domain proteins and are a super family consisting of a large and diverse number of proteins encoded by approximately 5% of human genes.[325] There have been a number of classification systems proposed the most recent "GRAFS" (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2 and Secretin). [326] In mammals there are five main families. [327] GPCRs have an integral role in transfer of extracellular stimuli to within the cell by conformational changes in trans-membrane domain structure. [328-331] They regulate physiological responses to a myriad of endogenous ligands including amines, glycoproteins, peptides and lipids. Therefore, not surprising GPCRs have been implicated in regulation of cellular maintenance, differentiation, proliferation and migration of various stem cells. [332-334] [335]

GPCRs modulate activity of intracellular signalling via G proteins. There are currently four known G protein subfamilies each able to potentiate a number of downstream effectors triggering a number of signalling pathways. [328] These include activation of Rho associated kinases (ROCK) [336, 337] activation or inhibition of cyclic AMP production [338] and Phosphoinositide 3-kinases (PI3Ks) and therefore modulate the PI3K/Akt pathway. [339, 340] The aforementioned have been implicated in EPC proliferation and function as described above.

GPCRs have evoked great interest as a possible target for novel drug therapy[341] as an estimated 50% of all currently prescribed drugs target only a small proportion of GPCRs.[342] They are also becoming increasingly recognised as having a major role in stem cell signalling.[343] The role of GPCR in regulation and function of EPCs and the effect of statin therapy remains yet to be elucidated however current evidence suggests that they may have a pivotal role.The positive and negative effects of statin therapy on EPC proliferation, mobilisation and longevity are summarised in figure 1.8 below.

However, the proposed pleiotropic mechanism that has evoked perhaps the greatest interest is augmentation of both number and function of EPC

1.12 The effect of statin therapy on endothelial progenitor cells in patients with coronary artery disease.

The effect of statin therapy on EPCs in patients with coronary artery disease remains contentious. This ambiguity has arisen from a lack of consensus definition of EPCs, clinical scenario but also type and dose of statin therapy. However, studies have shown statin therapy being associated with greater numbers of circulating EPCs

possibly by enhancing mobilisation, differentiation, increasing longevity, enhance homing to sites of vascular injury with augmentation of re-endothelisation by enhancing expression of adhesion molecules on EPC cell surface membrane. [13, 225, 287, 288, 344, 345]

The following reviews assess the effect of statin therapy in patients with stable angina and acute coronary syndromes.

1.12.1 Stable angina

Statins are thought to have a number of advantageous pleiotropic effects. [13, 109, 126, 241, 288, 346] Since the first randomised placebo-controlled trial found a positive effect of statin therapy on endothelium-mediated response in patients with atherosclerosis [347] several studies have been undertaken. Further understanding of endothelial functional integrity and the central role of EPCs led to intense research assessing the effect statin therapy on EPCs. These have included enhancing mobilisation, differentiation, increasing longevity, enhancing homing to sites of vascular with augmentation of re-endothelisation by enhancing expression of adhesion molecules on EPC surface. [13, 225, 287, 288, 344, 345] The effect of statin therapy on EPCs in stable CAD patients remains contentious. Vasa et al found atorvastatin therapy significantly increased circulating EPC (CD34, CD133, and CD34/kinase insert) as soon as 1 week with plateauing after 3-4 weeks, and a 3-fold increase of EPCs from baseline in a stable angina population was also observed. The study included 15 patients with angiographically documented stable coronary artery disease (CAD) that were prospectively treated with 40 mg of atorvastatin per day for 4 weeks. [225] Deschaseaux

et al [346] investigated whether EPCs could be firstly detected and secondly characterized in patients receiving long-term statin therapy defined as 4 weeks. The group found a significantly greater number of $CD34^+$, $CD34^+/CD144^+$ circulating EPCs in patients receiving statin therapy compared to statin naïve patients. Interestingly, two types of EPCs were detected in culture described as early and late EPCs. The early EPCs were found to form elongated cells, whereas the late EPC population gave rise to cobblestone-like colonies with strong proliferation capacities seen in-vitro cell culture. The numbers of early EPCs were significantly higher in patients not receiving statin therapy whereas late EPCs were significantly higher in patients receiving statin therapy. The study also observed that long-term statin therapy specifically maintained late EPCs in circulation with a $CD34^+/CD144^+$ phenotype. [346]

More recently, it has been suggested that the effect on EPC mobilisation might be dose-and duration of treatment dependent. [348-350] A double blinded randomised pilot study found greater number of circulating $CD34^+$ VEGFR-2^+ EPCs after 12 weeks of therapy with pravastatin 20 mg when compared to atorvastatin 10mg in patients with risk factors including hypercholesterolemia and type 2 diabetes mellitus. [350] A study of 100 patients over a 12-month period with ischemic heart disease were treated with atorvastatin 40 mg/day (n=50) was associated with a higher EPC numbers than atorvastatin 10 mg/day (n=50). The same study found chronic statin therapy was associated with a reduced rather than increased EPC number and function. [348] The EPCs were identified after 5 days in culture. [348] Similarly, a study of 209 patients with angiographically confirmed CAD and treated with 10–40 mg/day of statins

(simvastatin or atorvastatin) for >8 weeks. The study found treatment with 40 mg/day significantly reduced EPC counts. Univariate analysis found the dose of continuous statin therapy inversely correlated with the EPC number. In a prospective analysis, initiation of statin therapy significantly reduced the number of circulating and isolated EPCs after 3 months but not after 1 month. EPC number was determined by flow cytometry directly ($CD34^+/KDR^+$, $n = 58$) and after in vitro-culture ($n = 209$). EPC function was assessed by the formation of colony forming units (CFUs). The study concluded that statin dose during continuous treatment independently predicted reduced numbers of circulating, as well as isolated EPCs in patients with CAD without effecting functional properties of EPCs tested by the CFU-Hill Assay.[349] The "Hipocrates" study included stable or unstable angina pre-treated with atorvastatin 80mg therapy (80 mg the day before PCI and 40 mg 2-4 h before PCI) versus low dose atorvastatin have been found to have higher circulating EPCs. The study defined EPCs by flow cytometry as the proportion of peripheral mononuclear cells co-expressing VEGFR-2^+ $CD133^+$ and VEGFR-2^+ $CD34^+$ and the capacity of the cells to form colony-forming units (CFUs) after 7 days of culture. [351] The study included statin naïve or treated chronically with low-dose statins and either stable or unstable angina who underwent PCI [351]. The authors found that the mean number of EPC-CFUs before PCI was higher in patients treated with high-dose atorvastatin vs. low-dose statins. However, after 24 hours after PCI, numbers of EPC-CFUs were similar between both groups. No statistically significant differences in flow cytometric analyses were found. [351] Similarly the REMEDY-EPC early substudy that defined EPCs as $CD34^+$ /$CD45^-$ cells, deriving from several combination of endothelial

markers (CD144, CD146 and CD309), by flow cytometry found no significant increase of EPCs after pre-procedural administration (within 24 h) of different statins, at higher doses compared with moderate statin dose before an elective PCI. [352] However, other studies have suggested that statin reloading in patients on moderate statin therapy undergoing percutaneous coronary intervention has been shown to increase EPC count, [305, 306] this correlates with the beneficial effect of statin reloading of high dose statin in patients on chronic therapy. [221]

The studies highlight the contentious effect of statin therapy on EPCs in stable coronary artery disease. One main factor accounting for this may be the lack of a universal definition of EPCs. Secondly, the analytic method used to quantify any response as flow cytometry or cell culture. For example, Deschaseaux et al [346] described two types of EPCs detected in culture described as early and late EPCs. The study inadvertently described two different types of cells derived from different cell lineage. These late EPCs, are thought to be homogeneous endothelial-like progenitor cell population that possess a high proliferative potential, differentiate into vascular endothelial cells, and form networks in vitro and in vivo. Furthermore, these cells are also capable of augmenting the process by autocrine mechanism. [106, 110, 149-152] More recent recommendations have suggested that this population of cells perhaps should be referred to as endothelial colony forming cells (ECFCs). [102, 106] In contrast, early outgrowth EPC are thought to be short-lived cells, usually less than 2 weeks. These cells are also known as early EPCs/CFU-ECs/ EPC-CFU/early EPCs/early outgrowth EPCs. However, they are not capable to differentiate into endothelial cells in vivo, as the term EPC would imply. Rather, they can restore endothelial function

and enhance angiogenesis after tissue ischemia through a paracrine mechanism. [28, 127, 139, 351] They are thought to be a heterogeneous population of haematopoietic cells [139-141] and more recent recommendations suggest these cells to be referred to as circulating angiogenic cells. [142] Perhaps this explanation may account for the positive effect on EPCs by intense therapy statin measured by cell culture seen in other studies. [221, 305, 306, 349, 351]

High dose statins appear to inhibit angiogenesis possibly by promoting endothelial cell apoptosis [349, 353]. It has been suggested that this effect is dependent on the duration of treatment, with the initial increase in EPC number being followed by a fall, possibly due to EPC depletion as result of continuous stimulation or enhanced incorporation into the endothelium [354]. The initial increase in EPC number is thought to be partly due to augmented mobilisation [354]. There also appears to be a dose response effect of statin therapy and EPC numbers. In vitro animal studies by Urbich et al found a dose dependant increase in EPC migration [345]. Therefore, the dose of statin is of great importance to have the desired pleotropic effects. Shao et al noted EPC proliferation, migration and inhibition of apoptosis augmented at lower doses of fluvastatin however at higher concentrations significant inhibition [355].

1.12.2 Acute coronary syndromes

Shintani et al were the first to propose an increase of EPCs after an acute myocardial infarction. The group defined EPCs by both flow cytometric and culture, peripheral blood mononuclear cells that formed multiple cell clusters with endothelial cell lineage markers (CD31, vascular endothelial cadherin, and kinase insert domain

receptor). The group found circulating $CD34^+$ mono-nuclear cell counts significantly increased in patients with acute myocardial infarction (n=16), peaking on day 7 after onset, whereas they were unchanged in control subjects (n=8) who had no evidence of cardiac ischemia. In patients with acute myocardial infarction, more cell clusters and EPCs developed from cultured peripheral blood mononuclear cells obtained on day 7 than those on day 1. Plasma levels of vascular endothelial growth factor significantly increased, peaking on day 7, and they positively correlated with circulating $CD34^+$ mononuclear cell counts.[192] Whereas other studies found EPCs were maximally mobilised either at 3–4 days [356] or peak on presentation and fall in the days following myocardial infarction. [189, 357-359]

The STRAP trial studied forty ST-segment elevation myocardial infarction (STEMI) patients undergoing a successful primary or rescue PCI were randomised to receive atorvastatin 80 mg immediately after the admission or atorvastatin 20 mg from the day of the discharge. Patients randomised to atorvastatin 80mg after primary or rescue PCI was associated with greater EPC count at 4 months follow up as compared to 20mg atorvastatin. EPCs were defined by flow cytometry as $CD34^+/KDR^+$ (VEGF 2). The authors however, found no beneficial effect in an improvement of LV function. [360]

Kim et al found high dose atorvastatin (80mg) verses 20mg prior to primary PCI in STEMI patients had no significant reduction of MACE, however improved immediate coronary flow was observed after primary PCI in the high dose atorvastatin group [361]. High dose statin therapy may also aid vascular healing after drug eluting stent implantation after OCT 3 and 12 months [362]. A randomised placebo controlled multi-

centre trial of 185 patients compared high dose statin therapy (rosuvastatin 40 mg plus maintenance for 7 days) compared to low dose (placebo before treatment plus rosuvastatin 10-mg maintenance for 7 days) in STEMI patients with serial cardiac MRI at 3-7 days and 3 months. The study found no differences between the groups for peri-procedural micro-vascular circulation assessed by Thrombolysis In Myocardial Infarction (TIMI) flow grade, ST-segment resolution, micro-vascular obstruction on cardiac MRI, reduction in infarct volume measured by MRI or clinical outcomes [363]. Acute coronary syndrome patients' intensive statin therapy with atorvastatin 80mg after primary or rescue PCI was associated with greater EPC count at 4 month follow up as compared to 20mg atorvastatin. The authors however, found no beneficial effect in an improvement of LV function [360].

1.13 Summary

The maintenance of endothelial integrity is essential for the preservation of a healthy vasculature resulting from a balance between endothelial damage and the rate of vascular repair. [7, 27, 364] Disruption of endothelial integrity has been shown to be a stimulus for the development of atherosclerosis and myocardial ischemia. [10, 34-38] In healthy individuals, neighbouring mature endothelial cells replicate locally to replace damaged cells. [7] This has limited potential and therefore an alternative repair mechanism must be present. [11] This may include undifferentiated cells migrating to sites of vascular injury [13-15] then differentiating into mature endothelial cells [16-28, 52, 53] These undifferentiated cells have been termed endothelial progenitor cells (EPCs) [28, 33] The ability of EPCs to repair blood vessels has a number of potential clinical

applications and therefore any therapy augmenting EPC number or function has evoked great interest. [365] EPCs can be isolated from bone marrow or the circulation as a sub-population of mononuclear cells [28, 101, 102] expressing several cell surface markers [100, 103, 118, 119, 121] and account for only 0.001-0.0001% of peripheral blood cells. [100] Studies have shown the early beneficial effect of statins occurs before any significant decrease in lipid profile. Therefore, cardiovascular benefits of statins may occur via alternative mechanisms other than reduction of cholesterol alone. [109, 219, 236] These beneficial pleiotropic effect(s) of statins may include augmenting with the greatest interest being augment number and/or function of EPCs. [13, 109, 225, 287, 288, 344, 345]

This thesis will assess the effect of atorvastatin therapy on EPC in bedside studies and the potential mechanisms in bench side studies.

1.14 Aims and hypothesis

The first challenge was to define EPCs as there are currently no consensus. Therefore, I began by reviewing the current literature, to define EPCs used in this thesis. This was followed by bed and bench side studies – a brief description follows below.

Chapter 3 a bench side study to assess the effect, if any, of atorvastatin, on chemokine CXCL-12 its receptor CXCR-4, adhesion molecule e–selectin and EPCs on damaged endothelium. The study hypothesis was that atorvastatin would increase CXCL-12 its receptor CXCR-4 and of adhesion molecule e–selectin resulting in greater EPC numbers on the denuded endothelium. *Chapter 4* confirmed the feasibility to perform

whole blood flow cytometric assay for EPCs. The second objective of the study was to assess if whole blood samples could be stored safely for analysis later time. The study hypothesis was that samples could be store at 4^0C for accurate flow cytometric analysis for some time after venesection. *Chapter 5* aimed to find an association between coronary artery calcium score and EPC count. The hypothesis being the greater the calcium score would be associated with greater number of EPCs. *Chapter 6* aimed to assess the EPC response to moderate (20mg) and high (80mg) atorvastatin doses in patients presenting with stable angina and acute coronary syndromes respectively. Extrapolating from other studies 80mg atorvastatin would have a greater effect on EPC numbers than 20mg. *Chapter 7* measured the effect of 80mg atorvastatin over a 28-day period The study investigated the effect of high dose atorvastatin loading on *real world* patients that have been inadvertently precluded from other studies. The study hypothesis was the earlier the administration of atorvastatin the greater the EPC count.

Chapter 2

Context, Methodology and Strategy

2.1 Introduction

The following chapter describes experimental design principles, methodology and description of the laboratory techniques used in both bench based and clinical studies. The described techniques allowed quantitative analyses of endothelial progenitor cells, cell surface receptor CXCL-12 (SDF-1) its ligand CXCR-4, cell culture and tracking of EPCs. Summarised in table 2.1 below.

Table 2.1 Summary table of the study setting, relevant chapters in thesis, method of analyses and particular markers studied.

Study setting	Full description of analytical technique	Chapter(s) in thesis	Marker	Source	Method of analyses
Bench side study	Chapter 2 Section 2.3	3	Quantification of CXCL-12 (SDF-1)	TEIL construct Culture fluid	Enzyme Linked Immunosorbent Assay (ELISA)
Bench side study	Chapter 2 Section 2.4	3	CXCR-4 and E-selectin	TEIL construct	Immuno-staining
Bench side study	Chapter 2 Section 2.5	3	Tracking of EPCs	TEIL construct	Staining of cultured cells
Clinical studies	Chapter 2.6	4,5,6,7	EPC Immuno-phenotyping of Cluster of differential (CD)	Whole blood samples	Flow cytometric analyses

Tissue engineered blood vessels and more precisely tissue engineered intimal layers were central to bench based studies in chapter 3.

2.2 Tissue engineered constructs

2.2.1 Introduction

Quantification of CXCL-12 (SDF-1), CXCR-4, E-selectin and tracking of EPCs required use of tissue mimicking endothelial layer of blood vessels. Modern advancements have led to 3D tissue engineered constructs mimicking true physiologically responses. This thesis presents a summary of the main principle of tissue engineering relies on a triad of appropriate cell source, scaffold, and signal media [367-369]

2.2.2 Tissue engineered models

Historically cellular biology studies were performed on two-dimensional tissue cultures. These were associated with several limitations including an artificial "polarising effect" with one side of the cell exposed to wall of petri dish with the other facing culture media causing to reduction of cell to cell and cell to matrix interactions. [370, 371] Langer and Vacanti first coined the term tissue engineering in 1993. [372] This led to development of three-dimensional tissue engineered models (TEM) allowing both cell to cell and cell to matrix interactions. Modern TEMs have increased the understanding of cellular response, but also a greater and detailed understanding of the functioning physiological system being studied in real time. TEMs more closely

resemble pathophysiological responses and perhaps more importantly overcome a number of ethical issues encountered with human and animal based research. [373]

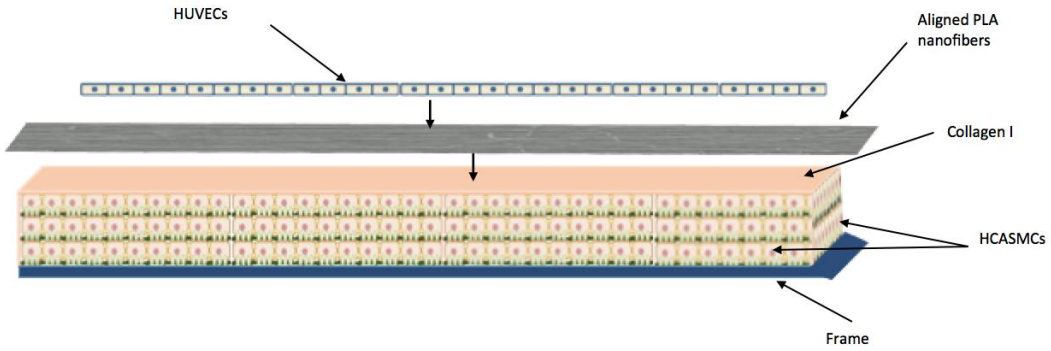
2.2.3 Principles of tissue engineered blood vessel constructs

Modern tissue engineering has made possible formation of tissue constructs mimicking intimal (endothelial), medial and adventitial layers that form blood vessels. Please see table 3.1 below for the abbreviated terms used. Tissue engineered blood vessel (TEBV) constructs allow real time, controlled studies assessing the effect of different physiological factors and or drugs on intimal (endothelial), medial and endothelial layers. TEBV allow adventitia, medial and intimal layers to be represented by fibroblasts, human cardiac artery smooth muscle cells (HCASMCs) and human umbilical vein endothelial cells (HUVECs) respectively. Figure 3.2 below. This thesis used only tissue engineered intimal layers.

Table 2.2; Summary of the abbreviated terms for tissue-engineered constructs used in this thesis

Tissue engineered constituent	Abbreviation
Tissue engineered blood vessel	TEBV
Tissue engineered adventitial layer	TEAL
Tissue engineered medial layer	TEML
Tissue engineered intimal (endothelial) layer	TEIL

Figure 2.1 - Tissue engineered blood vessels (TEBV)



Key

HUVEC - human umbilical vein endothelial cells

HCASMC - human coronary artery smooth muscle cells

2.2.4 Construction of tissue engineered intimal layers

2.2.4.1 Materials

Fabrication of tissue engineered blood vessels were based on current standard methodologies [374-376] Poly-l,d-lactic acid (96% l/4% d) (Purac BV Gorinchem, the Netherlands). Rat-tail collagen type I (BD Biosciences). Mouse anti-human collagen type I and III IgG primary antibodies and goat anti-mouse IgG FITC (Santa Cruz Biotechnology). Primary human coronary artery smooth muscle cells (HCASMCs), human umbilical vein endothelial cells (HUVECs) pooled, medium 200, medium 231, low-serum growth supplement (LSGS), smooth muscle growth supplement (SMGS), cell tracker (CMAC), and 5-(and-6)-carboxyfluorescein diacetate, succinimidyl ester, mixed isomers (CFSE) (GIBCO, Life Technologies). Fura-2/AM and prostaglandin I2

(Cambridge Biosciences). Thrombin was obtained from Merck Chemicals. Chloroform, dimethyl formamide (DMF), rhodamine B, Sudan black B, Tween 20, fibronectin from bovine plasma, 3,3′-dihexyloxacarbocyanine iodide (DiOC6), and apyrase (Sigma Aldrich).

2.2.4.2 Construction of tissue-engineered intimal layer (TEIL)

The tissue engineered intimal layer (TEIL) comprised of human umbilical vein endothelial cells (HUVECs) mimicking physiological blood vessel intimal layer supported by aligned poly lactic acid (PLA) nanofibers sheet. The fabrication of each construct was along standard published techniques. [374] Figure 3.2 below.

Figure 2.2; tissue-engineered intimal layer (TEIL)

BD type I collagen solution (0.2 mL neutralised 3 mg/mL) was added to a square frame made by filter paper of an area of 1 cm^2 forming an acellular collagen gel base. On to this a fibronectin-coated PLA nanofiber mesh was placed on a non-adhesive PTFE plate. 1mL of 10 ng/mL concentrated fibronectin solution was added to the nanofibers and incubated for 1 h at room temperature. Following this the solution was removed and the mesh allowed to dry. HUVECs 4 × 104 cells per sample were then seeded onto the dried mesh and incubated at 37°C, 5% CO_2, for 1 h to allow attachment. This formed the TEIL which was supplemented with 200 medium with further incubation for 4 days at 37°C and 5% CO_2. The media was changed after 48

hours. This cycle continued for 10 days to ensure attainment of normal cell morphology and surface coverage.

2.2.4.3 Electro-spinning of poly lactic acid (PLA) nanofibers

These nanofibers provide structural support and allow passage of supplement media throughout the TEIL. The PLA nanofiber meshes were fabricated as per established laboratory protocol.[375] The technique was modified to allow visualisation of each layer and nanofibers by confocal microscope by addition of rhodamine B to the 2% PLA solution at a final concentration of 0.1% (w/v). In summary, aligned nanofibers were made by dissolving Poly-L,D-lactic acid (96% L/4% D, inherent viscosity of 5.21 dL/g, Purac BV, Gorinchem, the Netherlands) (PLA) in a 7:3 mixture of chloroform and dimethylformamide (DMF) (Sigma,Welwyn Garden City, UK) into 2% solution. 2% PLA solution was deposited onto detachable metal collectors that comprised of two partially insulated steel blades (30 cm x 10 cm), with a gap of 5 cm between the gaps that allowed the deposition of the nanofibers. The blades were connected to a permanent copper plate with a steel wire. Deposition of the fibres involved connecting the permanent plate to a negative electrode, and a syringe containing the solution was connected to a positive electrode. The PLA extruded through an 18G needle and at a rate of 0.025 mL/min. Nanofibers were collected and affixed onto acetate frames followed by sterilisation using UV irradiation (over 3 cycles each lasting 90 seconds) prior to use in culture. The nanofiber diameter was measured as ~500 μm and the mat

thickness ~3 μm. The porosity was less than 1 μm therefore no endothelial cells were seen to migrate through the nanofiber layer

2.2.5 Lesion formation

To mimic vascular injury as seen in coronary artery disease required denudation of the intimal layer. There were two potential methods, chemical and mechanical injury. Chemical injury used a 1x1mm (0.1x0.1cm) square of filter paper in 10% ferrous chloride ($FeCl_3$) and placing this onto TEIL HUVECs for 1 minute. After this, the TEIL was washed with PBS and topped up with fresh media as per standardised ratio Section 3.2.5 below. The second potential method was mechanical injury by use of 1x1mm (0.1x0.1cm) square of filter paper alone paced on the TEIL HUVECs for 5 seconds. Studies performed by Njoroge et al. suggested the use of $FeCl_3$ was extremely toxic and difficult to reproduce. This was secondary to toxic nature of the 10% $FeCl_3$ that caused the central denuded area, that may also damage the underlying collagen perpetuating the detrimental effects. Secondly this technique had no clear margin, rather a bleeding effect that dissipated outwards along the endothelial layer was observed. This also increased the number of endothelial cells damaged. Both result in an unpredictable and varied response. Whereas the mechanical injury results in a well demarcated area without any bleeding effect.

Therefore, mechanical injury may more closely mimic physiological injury seen in coronary artery disease. Secondly mechanical injury was much more reproducible in

our laboratory. This was particularly important as chapter 4 studied concentration of CXCR-4 and E-selectin in denuded areas. Please refer to chapter 3.

2.2.6 Supplemented media

The supplemented media contained essential nutrients for cellular maintenance and growth as summarised in table 3.3 below.

Table 2.3; Description of the incubation solution used for tissue engineered intimal (TEIL)

Tissue engineered media	Cells	Supplemented media (Gibco, Life technology USA)
Intimal layer (TEIL)	Human Umbilical Vein Endothelial Cells (HUVEC)	HUVEC media 200 with low serum growth supplement (LSGS)

In this study, we defined the control sample perfusion media being composed of methanol dilated 20mg/mL in 5ml media made of 70% smooth muscle cell and 30% HUVEC media fluid. The supplemented test media had the addition of 60µg/mL atorvastatin (section 2.2.7 for concentration selection rationale).

The TEIL constructs were stained immunologically after 1, 3, 5, 7 and 9 hours in either standard media or standard media with atorvastatin and incubated at 37^0 C and 5% CO_2. The TEIL constructs were used in the following experiments described in sections 3.3, 3.4 and 3.5 and summarised in figure 2.3 below.

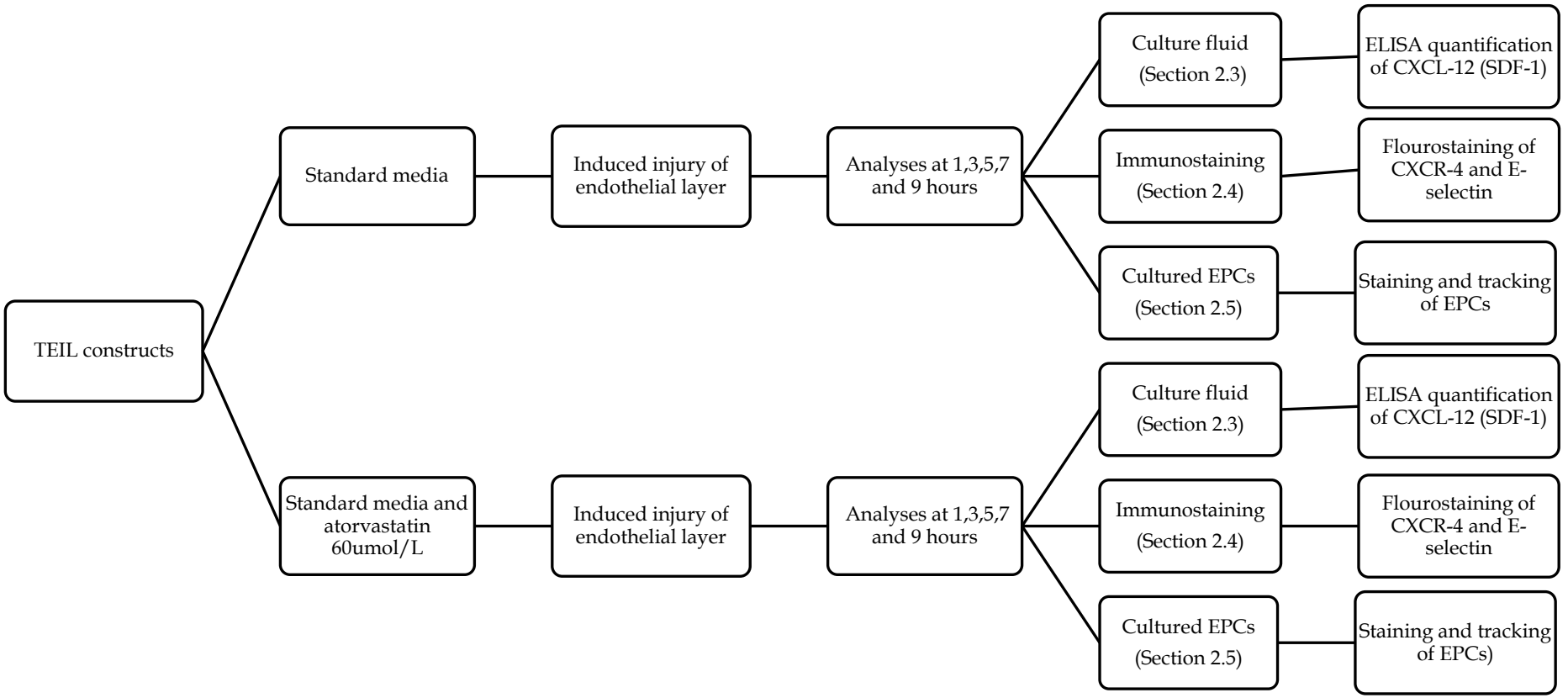
2.2.7 Concentration and type of statin used in bench side studies

The concentration of atorvastatin were based on previous publications [377] and based on manufacturers recommendations based on current standard therapy. Therefore, atorvastatin calcium trihydrate (5g) (Active Pharma Supplies Ltd) diluted with 100% methanol and kept at less than 1% of total media volume at a concentration of 60ug/mL.

2.3 Principles of Enzyme Linked Immunosorbent Assay (ELISA) quantification

The effect of atorvastatin on CXCL-12 (SDF-1) within denuded endothelium was analysed by ELISA of tissue engineered intimal layer (TEIL) supplemented incubation media. The protocol was based on manufacturers protocol on the standard PeproTech Human SDF-1α (CXCL12) Mini ABTS ELISA Development Kit (Catalog# 900-M92, Lot# 0215092-M).

Figure 2.3; Summary of experimental plan



2.3.1 Materials

Materials and solutions for ELISA quantification of CXCL-12 based on the standard PeproTech Human SDF-1α (CXCL12) Mini ABTS ELISA Development Kit (Catalog# 900-M92, Lot# 0215092-M). ELISA microplates (Nunc MaxiSorp Prod. # 439454, or Corning Prod # 3590); Tween-20 (Sigma Cat. # P-7949); BSA (Sigma Cat # A-7030); ABTS Liquid Substrate Solution (Sigma Cat. # A3219); Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075). ELISA microplates (Nunc MaxiSorp Prod. # 439454, or Corning Prod # 3590); Tween-20 (Sigma Cat. # P-7949); BSA (Sigma Cat # A-7030); ABTS Liquid Substrate Solution (Sigma Cat. # A3219); Dulbecco's PBS [10x] (Gibco BRL Cat. # 14200-075).

Wash Buffer: 0.05% Tween-20 in PBS Block Buffer: 1% BSA in PBS Diluent: 0.05% Tween-20, 0.1% BSA in PBS. PBS: dilute 10xPBS to 1xPBS, pH 7.20 in sterile water. Wash Buffer: 0.05% Tween-20 in PBS Block Buffer: 1% BSA in PBS. Diluent: 0.05% Tween-20, 0.1% BSA in PBS. Reconstitution and storage were as per supplier's recommendations for Capture Antibody (Rabbit Anti-Human CXCL12), Detection Antibody (rabbit Anti-Human CXCL12 Human CXCL12 Standard and), Avidin-HRP Conjugate. Atorvastatin calcium trihydrate (5g) (Active Pharma Supplies Ltd) diluted with 100% methanol and kept at less than 1% of total media volume at a concentration of 60ug/mL.

2.3.2 Supplemented incubation media

The supplemented incubation media was composed of methanol dilated 20mg/mL in 5ml media made of 70% smooth muscle cell and 30% HUVEC media fluid. This was the control. The test fluid had the addition of atorvastatin at a concentration of

60µg/mL. The media was then collected and analysed by ELSA to allow quantification of CXCL-12.

2.3.3 Plate preparation

The plate was prepared as per manufacturers recommendations. The capture antibody was diluted with PBS to a concentration of 2.0µg/m with immediate addition of 100µl to each to ELISA plate. The ELISA plate was then sealed and incubated overnight at room temperature. The cells were then aspirated to remove liquid and washed 4 times with 300µl of wash buffer per well. The plates were then inverted and blotted on blotted on paper towel to remove residual buffer. 300µl block buffer was added to each well and incubated for at least 1 hour at room temperature. Finally, the plates were then aspirated and washed a further 4 times and as per manufacturers recommendations prior to ELISA. The collected supplemented media could be stored if required within Eppendorf tubes and stored at -80^0 C. Once ready for analysis, the Eppendorf tubes were left to thaw at room temperature for subsequent ELISA analysis.

2.3.4 ELISA protocol

The standard was diluted from 4000pg/ml to zero in diluent with immediate addition of 100µl of sample to each well in triplicate obtaining numerical values. 100µl of the dilute detection antibody at a concentration of 0.50µg/ml was added to each well and incubated at room temperature for 2 hours. The wells were then aspirated and washed 4 times. 100µl of 5.5µl of Avidin-HRP Conjugate diluted to 1:2000 for total volume of

11ml was added to each well and incubated for 30 minutes at room temperature. The plate was aspirated and washed 4 times. 100µl of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) substrate solution was added to each well and incubated at room temperature for colour development. The plate reader was set at 405nm with wavelength correction set at 650nm to obtain absorbance of each sample as per manufacturer recommendations. The absorbance valves for each triplicate for each concentration may then be tabulated. Table 2.4.

Table 2.4; ELISA triplicate as recommended by manufacturer

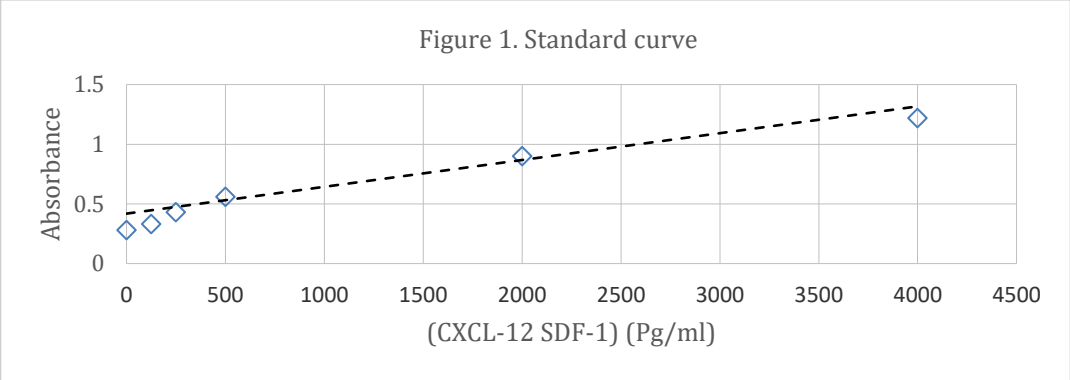
Triplicate Sample number	CXCL-12 (pg/ml)											
	4000	2000	1000	500	250	125	63	31	16	8	4	2
1												
2												
3												
Average												

Plater reader set at 405nm

Wavelength correction 650nm

The average values were then plotted with absorbance (y axis) and CXCL-12 concentration (x axis). This allowed a "line of best fit" to be derived. Figure 3.4 below demonstrates hypothetical average value from the triplicates as plotted "dots" and line of best fit (dashed line).

Figure 2.4; Standardised plot for ELISA analysis with "line of best fit"



Key

- - - Line of best fit ◇ true values

This allowed concentration of CXCL-12 in Pg/ml to be calculated. From figure 1 and application of equation of straight line:

$$y=mx+c$$

Where

Y axis = absorbance m and c - defined constants X axis = concentration (pg/ml)

Therefore:

Absorbance = mX Concentration of CXCL-12 (pg/ml) + c

Concentration of CXCL-12 (pg/ml) = absorbance/m – c

The CXCL-12 (SDF-1) concentrations were then calculated.

2.4 Immunostaining

The effect of atorvastatin on CXCR-4 and E-selectin within denuded endothelium was analysed by immunostaining of tissue engineered intimal layer (TEIL).

2.4.1 Materials

Materials and solutions for immunostaining atorvastatin calcium trihydrate (5g) (Active Pharma Supplies Ltd) diluted with 100% methanol and kept at less than 1% of total media volume at a concentration of 60ug/mL. Human umbilical vein endothelial cells (HUVEC) media medium 200 supplemented with low serum growth supplement (LSGS) (GIBCO, Life Technologies). Primary human coronary artery smooth muscle cells (HCASMC) media 231 supplemented with smooth muscle growth supplement (SMGS) (GIBCO, Life Technologies). 5% bovine serum albumin (BSA). E-selectin pre-conjugated with flourochrome alexafluor 488 (stains green) Santa Cruz. Rabbit monoclonal antibody required a secondary antibody (mouse anti-rabbit SC-3917 Ig G-TR Santa Cruz) DAPI (4′,6-diamidino-2-phenylindole) staining solution (ab228549). Olympus confocal microscope (Fluoview FV1200).

2.4.2 Method and protocol

The study compared standard media with test media that had addition of atorvastatin calcium trihydrate (5g) (Active Pharma Supplies Ltd) diluted with 100% methanol and kept at less than 1% of total media volume at a concentration of 60ug/mL The constructs had lesion formations as described above.

The TEIL constructs were incubated for 3, 5, 7 and 9 hours in either standard supplemented media (Table 3.3) or test solution and incubation at 37^0 C and 5% CO_2. On completion of the incubation time, the constructs were fixed with 4% paraformaldehyde for 20 minutes, after which they were stained immunologically. Once immunostaining was ready to be performed, the tissue construct had PBS changed to 5% bovine serum albumin (BSA) and left in ambient room temperature for 1 hour. The BSA avoided non-specific binding of antibodies.

E-selectin was identified by a mouse antibody conjugated with flourochrome alexafluor 488 staining green (Santa Cruz).

However, rabbit monoclonal CXCR4 ab124824 antibody was the primary antibody for CXCR-4. The primary antibody CXCR-4 were added at a dilution of 1:100 (1 µL of primary antibody to 100 µL of PBS) and incubated overnight at 4°C. The primary antibody was then removed, and the sample washed three times with PBS at 5 minutes per wash. The secondary antibody was conjugated donkey anti-rabbit IgGNL493 that stained green.

The constructs were then incubated at room temperature for a further two hours and thereafter washed three times with PBS. The use of rabbit and mouse antibodies minimised any cross reactivity between CXCR-4 and E-selectin. Finally, the constructs

were then incubated with 4′,6-diamidino-2-phenylindole (DAPI) staining. DAPI confirms cellular staining due to its strong affinity to adenine–thymine rich regions in DNA. Therefore, any artefactual material could be easily identified and distinguished from CXCR-4 and E-selectin. DAPI staining solution (ab228549 Abcam Switzerland) was applied to the constructs for 10 minutes after which the stain was removed, and the sample washed with PBS. The constructs were then viewed under Olympus confocal microscope (Fluoview FV1200) with images stored by Olympus Fluoview version 4.2b software.

The obtained images assessed for firstly the presence and secondly effect of atorvastatin on CXCR-4 within denuded endothelium.

2.5 EPC extraction, culture and cell tracking staining

This experiment assessed if atorvastatin was a stimulus for attraction of EPCs on denuded endothelial/intimal layer. Human EPCs were extracted from healthy volunteers and cultured in cellular supplemented media. The culture media had daily microscopic reviews and daily changes of supplemented media. Once ready, trypsin was added to the culture media to allow separation of extra-cellular matrix and EPC. EPCs are then stained with fluorescent dye. The EPCs are then transferred onto prepared denuded tissue engineered intimal layer (TEIL). The TEIL are either intact or have an area of denuded simulating damages vascular endothelium as *described in section* 3.2.5 above. These are then placed on a rocker to replicate in-vivo environment for 1 hour in either standard or test solution-containing atorvastatin 60ug/mL. The constructs are then fixed and reviewed under a microscope. The images were then

analysed with specific computer software (ImageJ) to calculate number of EPCs and assess if atorvastatin increased number of EPCs.

2.5.1 Materials

Fibronectin (2.5µg/cm^2), Anticoagulant (acid-citrate-dextrose (ACD)), PBS, Separation medium (Ficoll paque), Falcon tubes (50 mL and 15mL), 1x 12 wells plate, penicillin/streptomycin, Complete EPC media (7.5ml supplement: 42.5ml basal media). atorvastatin calcium trihydrate (5g) (Active Pharma Supplies Ltd) diluted with 100% methanol and kept at less than 1% of total media volume at a concentration of 60ug/mL.

2.5.2 Method

Ficoll-Paque solution (Sigma-Aldrich), PBS and EPC media were warmed to room temperature. The well plates were prepared by adding 0.5mL of 2.5µg/cm^2 fibronectin to and coating 8 wells of the 12 well plate and incubated for 1 hour. Thereafter any residual fibronectin was aspirated, and the well plates left to dry. EPC were obtained from whole blood samples. 60 ml of whole blood were venesected, and then split into 2 falcon tubes that contained 5ml of ACD solution. The blood/anticoagulation mixture was then equally divided into 15ml Falcon tubes then centrifuged at 2800 rpm for 8 minutes. The layers seen then corresponded from top to bottom plasma, enriched cell fraction (interphase consisting of lymphocytes / PBMC's) and finally erythrocytes and granulocytes. The plasma layer and minimum remnant of 0.5-1mL above the interphase was aspirated thus ensuring prevention of contamination of enriched cells

with platelets. The enriched cells were then pipetted with care into a tube and PBS at a ratio or 1:1 added. The diluted enriched cell fractions were carefully added to 2 tubes containing 15 mL of Ficoll Paque solution ensuring no mixing of the two components. Ficoll Paque increases density of red blood cells (RBCs) and granulocytes therefore sink to lower layer and prevent mixing of different solutions. These were then centrifuged (GE Healthcare Bio Sciences) at 400G for 30 minutes.

The layers after centrifugation (top to bottom) consisted of supernatant fluid, Ficoll Paque and the cell pellet at the bottom of the tube consisting of granulocytes. The supernatant fluid above the Ficoll Paque layer were pooled and diluted with PBS then underwent further centrifugation at 400g for 15minutes. This resulted in a cell pellet containing EPCs. The cell pellet was mixed with 2mL of complete EPC media and mixed thoroughly by pipetting up and down. 1mL of this mixture were added to 2 wells of the prepared 12 well plates. The well plates were incubated at 37^0 C and 5% CO_2.

On day 1, the contents of the plate were agitated and transferred to new wells. This was repeated over the preceding 72 hours. This step allows as many un-adhered EPCs to be collected as possible. The media was changed daily for the first 7 days then every 2-3 days for the duration of culture. The culture media had daily microscopic reviews and daily changes of supplemented media. Once the EPCs reached maturity, seen as elongated spindle shaped cells with depletion of other cells, trypsin was added to the culture media to allow separation of extra-cellular matrix and EPC. EPCs were then stained with carboxyfluorescein succinimidyl ester (CFSE) fluorescent dye. The non-specific CFSE dye stains cell membranes fluorescent green. However, as we used only purified sample, any stained cells corresponded to EPC number.

The preformed TEILs were then lesioned as described in section 3.2.5 above. Fresh media, either with or without 60ug/mL atorvastatin was then added. TEIL constructs were then placed in an incubator set at 37^0 C and 5% CO_2 for 1, 3, 5, 7 and 9 hours. After incubation period the TEIL constructs were transferred to a non-adherent well plate and the stained EPCs were added at a density of 1000 cells/100µl, and placed on rocker for 1 hour to simulate physiological stress forces. Following this the tissue constructs were then fixed with 4% paraformaldehyde for 20 minutes. The excess paraformaldehyde was removed, and phosphate buffer solution added and incubated at 37^0 C at 4^0 C. The samples were then ready to be viewed under fluorescence dissection microscope (Leica microsystems Switzerland model MSV269) at 2.5 magnifications.

Quantification of attached cells was performed by imageJ software [378] that in essence changed fluorescent stained cells into grey scale permitting calculation of number of EPCs within the sample.

ImageJ [378] software is a commonly used image analyser that allows quantification of number of cells. There are two methods available manual or automated counting. This thesis used automated counting firstly to exclude any operator counting error and secondly differentiation of overlapping cells by use of advanced functions such as pixel intensity and cell diameter. Table 2.5 summarises the step involved in converting images to numbered cell count.

Table 2.5; Summary ImageJ [378] for automated cell counting of single colour images (basic)

Open image to be counted

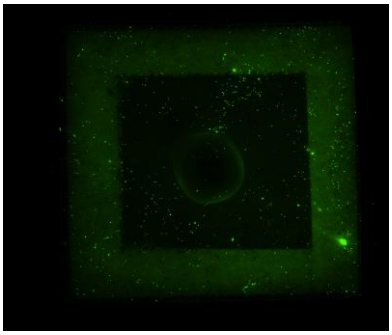
Convert to grey scale (Edit – Options – conversions to scale converting)

Image –Type – 8 bit to convert to grey scale

Image – Adjust – Threshold (Cntl+shift+T) to highlight structures that require counting

To count grouped or overlapping cells more advanced functions such as changing binary number of grey scale may be performed. [379] A brief description can be seen in figures 2.5.

Figure 2.5; Summary of conversion of fluorescent image seen on microscopy to inverted image allowing calculation of number of cells.



(a) Original image from fluorescence dissection microscope (Leica microsystems Switzerland model MSV269) at 2.5 magnifications.

(b) Final image to perform cell count by ImageJ. The ImageJ then summarises the calculated data including total number of cells.

Once the numbers of EPCs were determined they were analysed to assess for any significant change in the test sample with atorvastatin. Chapter 3.

2.6 Flow cytometry

2.6.1 Introduction

Cellular identification and staging of differentiation have been made possible by specific surface receptors called epitopes that allow immunophenotyping. This process allows identification of subset of cellular surface molecule called *cluster of differentiation* (CD). These can be detected by flow cytometry. Fundamentally flow cytometry relies on a light source, usually a laser to analyse the physical and chemical characteristics of particles suspended in a fluid medium. Flow cytometry may be used for cell counting; biomarker detection and separating different cell lines in a process called cell sorting. The ability to cell count, detect specific biomarkers and cell sort is made possible bypassing suspending cells within a stream of fluid along an electronic detection system. Modern flow cytometry machines can perform real time multi-parametric analysis at a rate of several thousand particles per second. This acquired data can then be stored and displayed as either "dot and plot" or alternatively "contour "plots. These fundamental qualities have led to the routine and ubiquitous use of flow cytometry in modern pathology and research laboratories.

2.6.2 EPC flow cytometric method

Flow cytometry is a well-established laboratory technique. [380-381] The flow cytometric protocol used in this thesis has been verified from earlier studies [385-388] and based on *ISHAGE* guidelines for flow cytometric enumeration of $CD34^+$ hematopoietic stem cells. [389] Based on these adaptations, Hristov et al further refined the analytic technique, [390] by simplifying earlier flow cytometric techniques [123, 124, 389] by using a

"lyse/no-wash procedure" with determination of absolute cell counts by simple gating and with the use of common routinely used staining techniques.[390] This modification allowed measurement of absolute counts as defined by those cells with intact cell membranes and therefore excluding dead cells. Secondly, use of fluorochromes with greater staining capacity for anti-CD34 monoclonal antibodies. Thirdly, with the addition of the use of *fluorescence minus one* (FMO) control that allows identification of cells of interest and therefore increasing accuracy. These modifications are particularly important in flow cytometric studies with low/rare events of interest, [391] such as EPCs that only account for 0.001-0.0001% of peripheral blood cells in an unstressed state. [100] Therefore, the Hristov et al technique optimised matching of high intensity fluorochromes for example allophycocyanin (APC) or phycoerythrin (PE) to low-density markers CD34 and the use of FMO control to accurately assess the cut-off point for *true* positive by subtracting any, *noise* artefact - recommended by earlier studies. [387, 392, 393] Furthermore we also sequentially gated for cell size, clustering and viability. [390] These gating measures allowed elimination of background noise, spurious erroneous results due to potential antibody binding to platelets or red cell debris.

The same immunostaining techniques were universally performed in all samples. To ensure consistency throughout the study only monoclonal antibodies from BD Biosciences were used. In accordance with manufacturer's recommendations, we allowed at least 30 minutes warm up period for the flow cytometer and calibration on each day of analysis.

2.6.3 EPC Immunostaining protocol

EPCs were stained immunologically by pipetting 20 µl CD45- Fluorescein isothiocyanate (FITC) (clone 2D1, no. 345808, 5 µl CD34-allophycocyanin (APC) (clone 8 G12, no. 345804), 20 µl CD309/VEGFR2- Phycoerythrin (PE) (no. 560494) monoclonal antibodies and 5 µl 7 amino actinomycin D (7-AAD) (BD Biosciences, no. 559925) into a Trucount tube (BD Biosciences, no. 340334).

The fluorescence-minus-one (FMO) control was formed from pipetting 20 µl CD45-FITC and 5 µl CD34-APC monoclonal antibody into a polystyrene round-bottom snap cap tube.

Monocyte assessment was performed by pipetting 20 µl CD45-peridinin chlorophyll protein (PerCP) (clone 2D1, no. 345809), 20 µl CD14- FITC (no. 345784) and 20 µl CD16-PE (clone 3 G8, no. 555407) monoclonal antibodies into a Trucount tube. 50 µl of well-mixed blood were added to each tube by reverse pipetting. The sample tubes were then incubated for 20 min at room temperature in the dark. Red blood cell lysis was achieved by adding 450 µl of 10% Pharm lysing solution (BD Biosciences, no. 555899) to each tube. Thereafter each tube was incubated for a further 10 minutes in the dark at room temperature. The tubes were then placed immediately on wet ice (4^0C) and protected from light in preparation for analysis [390].

2.6.4 Flow cytometric analysis and sequential gating

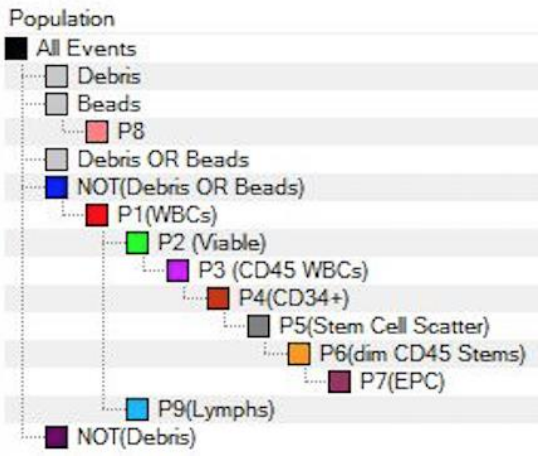
Sample acquisition was performed within 1 hour of red cell lysis on a FACSCanto II flow cytometer (2-laser, 6-color configuration) with FACS Diva 6.1.2 software (BD Biosciences). Instrument performance checks were performed daily with CST beads

(BD Biosciences, no. 641319). This ensures optimal daily set up of the flow cytometer for our study. The CST beads were safely stored in the dark and kept cold. The instrument was allowed to warm up for at least 30 minutes between fluidics start-up of the flow cytometer and acquisition of samples or QC beads as recommended by the manufacturer. Sample tubes were acquired at a medium flow rate < 10,000 events per second by setting the threshold on CD45 positive white cell events. We obtained at least 200,000 $CD45^+$ events. This would allow identification of low/rare events.

The gating strategy was a modification of the *ISHAGE* guidelines for stem cell enumeration [389] and modified from the paper of Histrov. [390]

This was based on a hierarchy (family tree) of sequential gating from all cells in the sample being reduced to EPCs alone. Figure 2.6 below.

Figure 2.6 – Figure of hierarchy (family tree) of sequential gating



Sequential gating was used to selectively remove any non-specific events so the final gate has an accurate measurement of cells of interest, in this thesis EPCs. Figure 2.7,

2.8 and 2.9 below are sequential gating steps used in this thesis. A summary follows below and described in figure 2.9.

All samples had leucocytes first gated on a forward scatter/side scatter (FSC/SSC) dot plot (P1) this allowed separation of white cells into granulocytes, monocytes lymphocytes and stem cells.

The cells in gate P1 were displayed on an SSC/7-AAD dot plot. This is used to identify viable living cells (7AAD negative) and non-viable dead leucocytes (7AAD Positive) that express $CD45^+$. The non-viable leucocytes have a porous membrane allowing 7-AAD into the cell whereas the viable leucocytes had a non-porous membrane excluding the dye. 7AAD binds to G-C rich regions within DNA. The viable leucocytes are seen on the left side of the dot plot and were subsequently gated as P2. This is a dye exclusion assay; therefore, only dead cells take up the dye and live cells do not.

P1 – total white cell population

This P2 viable live population were then gated by SSC/CD45 dot plot and the $CD45^+$ events are gated as P3 that showed $CD45^+$ viable cells.

The P3 population were further gated to $CD34^+$ cells in the P3 population events by SSC/CD34 dot plot identifying $CD34^+$ cells and termed P4 population.

These represented leucocytes that were viable having both $CD45^+$ and CD34 cell surface markers. CD34 is a stem cell marker expressed on progenitor cells. The EPCs have be reported to be within this population. [390] The P4 population events were then gated on a FSC/SSC dot plot. This was an important part of the analysis to ensure identification of the stem cell scatter region but also to remove residual debris and termed P5 events. Figure 3.20. P5 - $CD45^{dim}$ cells

The population in P5 was then gated and shown on a SSC/CD45 dot plot allowing identification of $CD45^{dim}$ cells and were gated as P6. Immature Stem Cells are known to express lower levels of CD45 compared to lymphocytes.

These two latter steps were essential in identifying cell size and clustering of $CD45^{dim}CD34^{+}$ stem cells. The $CD45^{dim}CD34^{+}$ stem cell events were shown on a CD34/VEGFR2 dot plot and accurate cut-off point for $VEGFR2^{+}$ events (P7) were made possible by the fluorescence minus one (FMO) control tube. The FMO control underwent the same steps as the sample however no PE- flourochrome was added to the tube therefore defining the background fluorescence in the PE channel. This allowed accurate gating of the VEGF positive population, maximising objectivity and minimised subjectivity in placing the gate. This allowed justification of setting the gate defining positive and negative EPC events. Every cell to the right of the gating marker corresponds to cells of interest (EPCs). Stem cells are larger than and have a more complex internal structure than lymphocytes, [390] thus allowing identification of $CD45dim/CD34^{+}$/ VEGFR2 cells. The beads were measured on the red laser as they have a good signal to noise ratio, therefore minimising non-bead artefact being included in the gating. The number of cells in each gating step is recorded below the dot plots. Figure 3.20 describes in greater detail the sequential gating steps.

In summary, we modified the earlier techniques for this thesis to maximise the accuracy of results in enumeration of $CD45dim/CD34^{+}$/ VEGFR2 EPCs by sequential gating. This allowed determination of the percentage of stem cells in total white cell population to be calculated. The optimisation of sequential gating analyses was critical due to the *rare true events* recalling that EPCs constitute only approximately

0.02% of total blood cells in an unstressed state. The sequential gating to exclude debris and include non-debris and bead counts. This decreased cell numbers from millions to hundreds of thousands. This allowed accurate discrimination of true events in subsequent sequential gating and secondly transfers of smaller volume of data files to other software used such as FlowJo. This non-debris and bead count (Cells) were used in the calculation of dispersion, section 2.6 below.

Figure 2.7; Dot graph with sequential gating, hierarchy (family tree) of sequential gating (bottom left) and table of numbers seen in some of the sequential gated steps (bottom right).

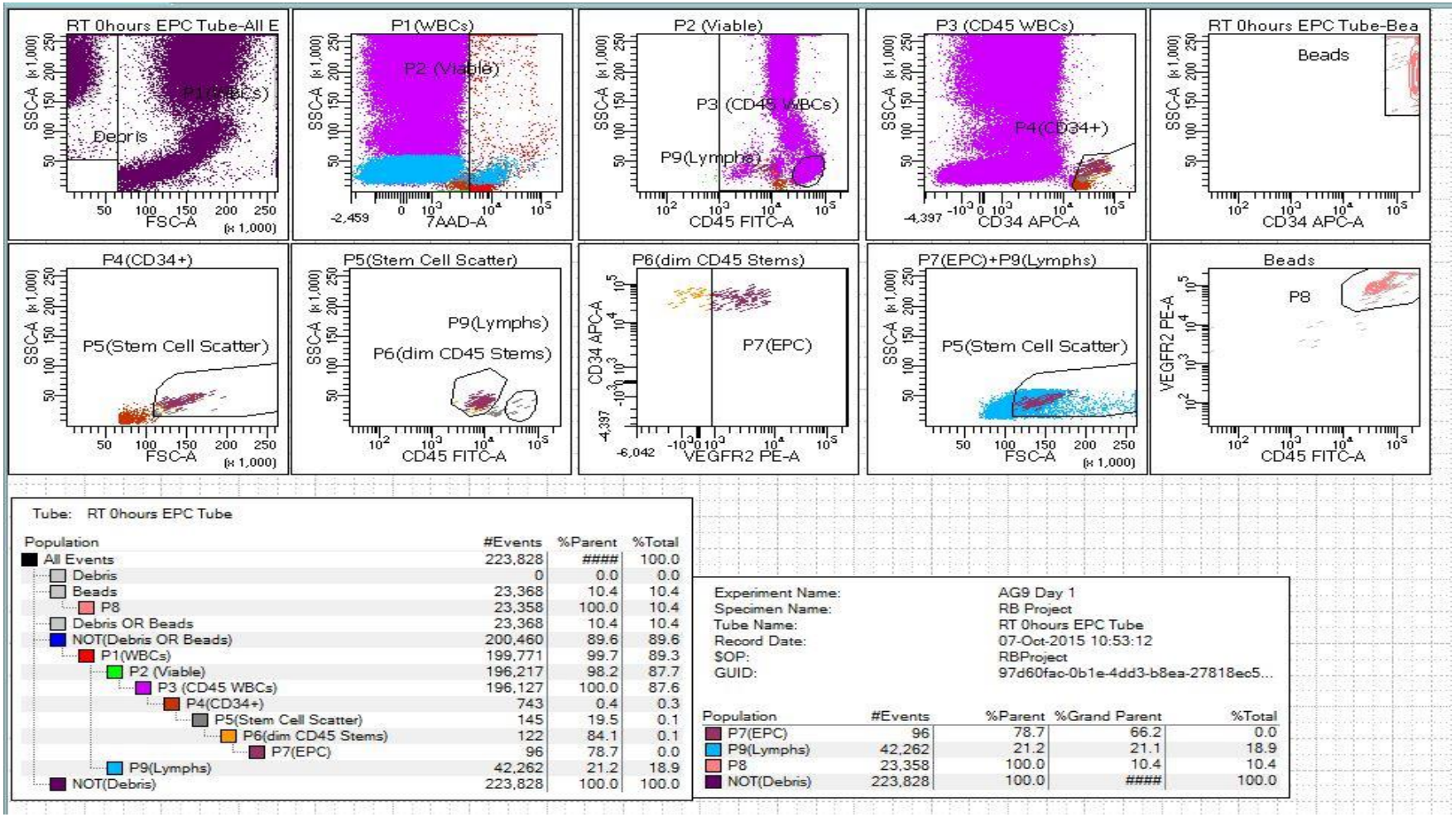
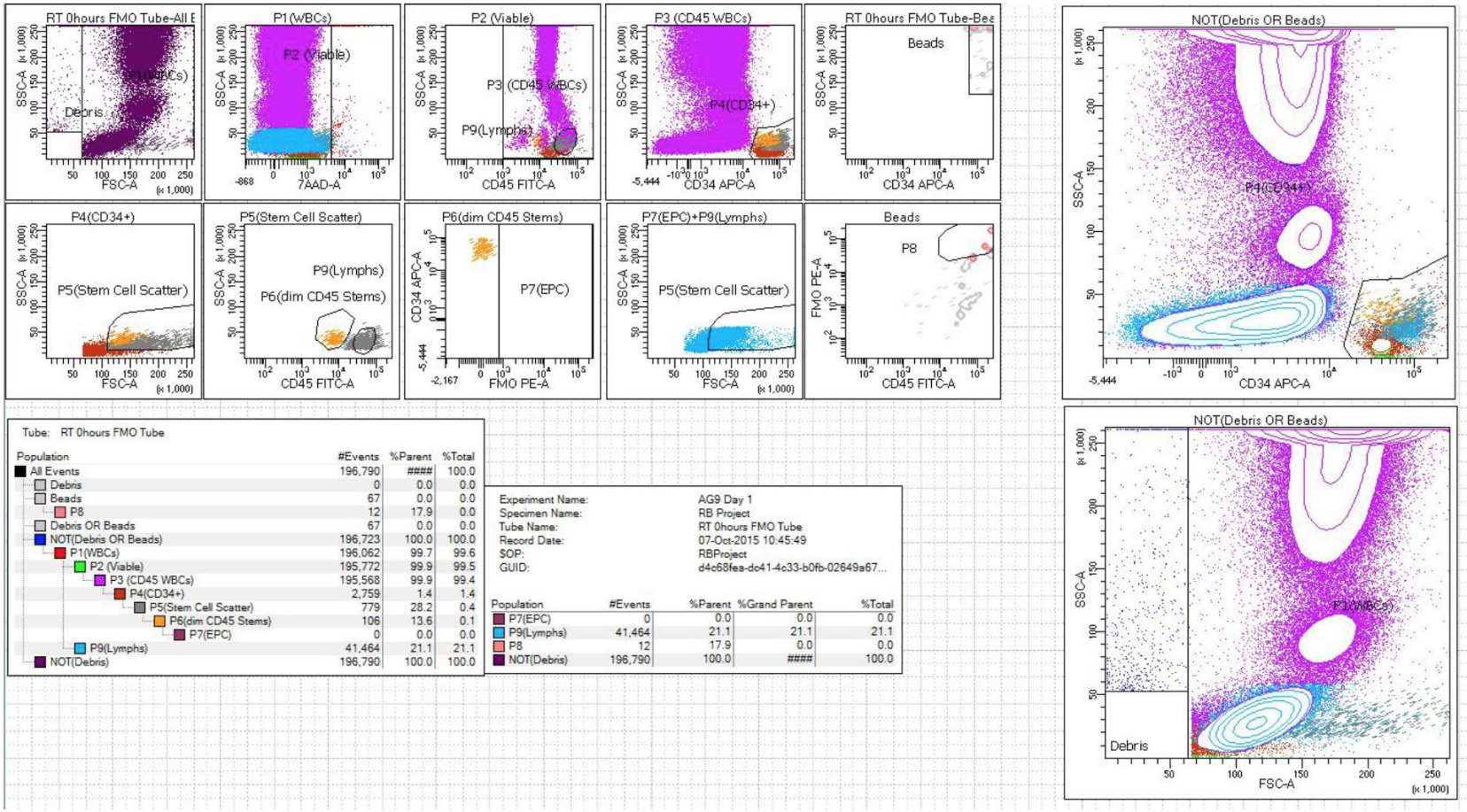


Figure 2.8; FMO sample dot plot with sequential gating, hierarchy (family tree) of sequential gating (bottom left) and table of numbers seen in some of the sequential gated steps (bottom right) and contour plot (right column) Please note dot graph with sequential gating FMO –no EPC. Therefore no cells seen (EPCs) in P6 or P7.



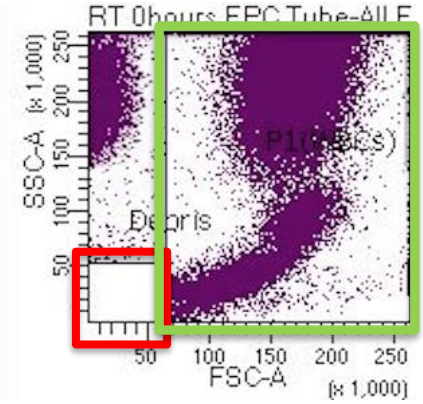
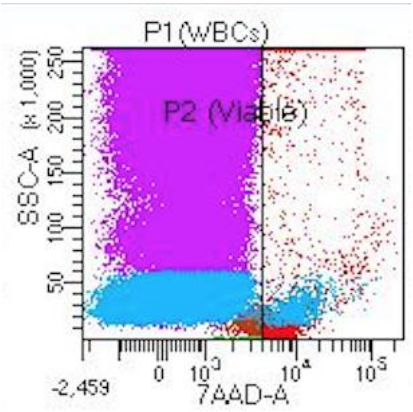
Tube: RT 0hours FMO Tube

Population	#Events	%Parent	%Total
All Events	196,790	####	100.0
Debris	0	0.0	0.0
Beads	67	0.0	0.0
P8	12	17.9	0.0
Debris OR Beads	67	0.0	0.0
NOT(Debris OR Beads)	196,723	100.0	100.0
P1(WBCs)	196,062	99.7	99.6
P2 (Viable)	195,772	99.9	99.5
P3 (CD45 WBCs)	195,568	99.9	99.4
P4(CD34+)	2,759	1.4	1.4
P5(Stem Cell Scatter)	779	28.2	0.4
P6(dim CD45 Stems)	106	13.6	0.1
P7(EPC)	0	0.0	0.0
P9(Lymphs)	41,464	21.1	21.1
NOT(Debris)	196,790	100.0	100.0

Experiment Name:	AG9 Day 1
Specimen Name:	RB Project
Tube Name:	RT 0hours FMO Tube
Record Date:	07-Oct-2015 10:45:49
$OP:	RBProject
GUID:	d4c68fea-dc41-4c33-b0fb-02649a67...

Population	#Events	%Parent	%Grand Parent	%Total
P7(EPC)	0	0.0	0.0	0.0
P9(Lymphs)	41,464	21.1	21.1	21.1
P8	12	17.9	0.0	0.0
NOT(Debris)	196,790	100.0	####	100.0

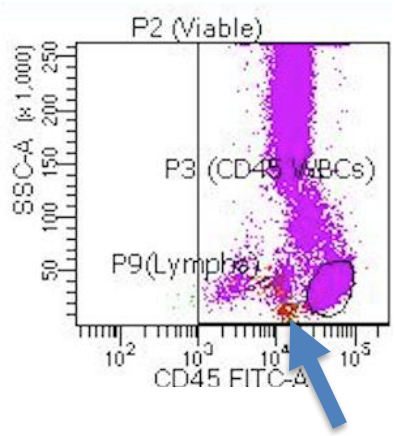
Figure 2.9; Description of each step of sequential gating

Gated P number	Cell surface marker identified	Image
Debris and P1	This initial gate called the *debris gate* allowed the separation and exclusion of debris. Therefore, only white cells from whole blood sample are seen on the right side of the dot plot. Debris is the small square gate in the bottom left corner highlighted by the red square. Drawn on the FSc (size) versus SSc (complexity) plot. The P1 gate (green rectangle) identifies the total white blood cell fraction. The population in the top left of this plot are the counting beads. .	
P2	The plot title shows the population shown in the plot, so in this case this plot shows P1 WBCs. The cells to the left represent the viable (living cells). This gating step separated viable living and non-viable dead leucocytes expressing CD45⁺. 7AAD traverses the porous cell membranes and therefore enters dead non-viable cells and unable to transverse intact membranes of living cells. Viable living (7AAD negative) and non-viable dead leucocytes expressing CD45⁺ (left side – dye exclusion cells). Therefore, dead cells are on the right side of dot plot. The red coloured population represents debris in the WBC gate that is outside the debris gate on the previous plot	

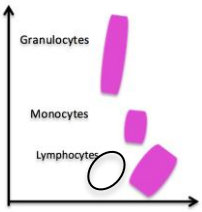
P3

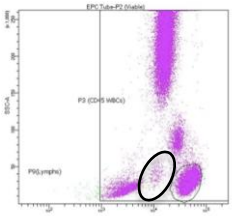
CD45 is a pan white cell marker, so cells expressing this antibody are white cells including granulocyte, monocytes, lymphocytes and stem cells. Granulocytes, monocytes, lymphocytes and stem cells express CD45 to different levels. The cells of interest were in this P3 gate on the dot plot.

Some non specific binding can be seen indicated by the red coloured population (Blue arrow).



Below is an idealised diagram of the positions of granulocytes, monocytes and lymphocytes.





P9

Stem Cells in the Blue area.

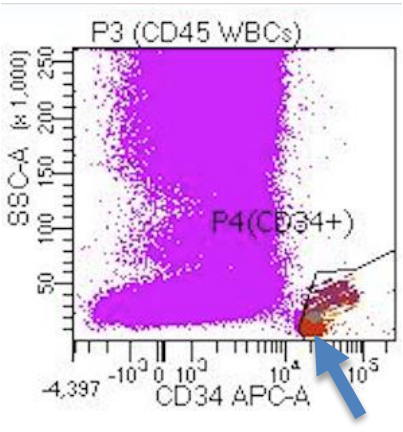
P9 Lymphocytes (indicated by the black oval ring) are used to identify any lymphocytes falling into the stem cell gate in a later plot.

P4

Viable cells expressing $CD45^{+}$ and CD34 (7AAD negative).

This plot is gated on the viable CD45 positive white cells from the previous gate. The CD34 antibody identifies the stem cell population which includes the EPC events.

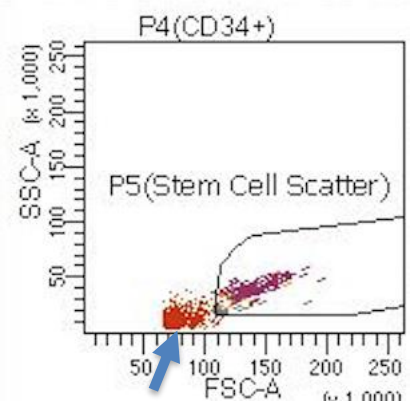
Caution has to be exercised when gating this population as debris falling out the debris gate can be seen in red (Blue Arrow). This needs to be removed by gating out on subsequent plots.



P5

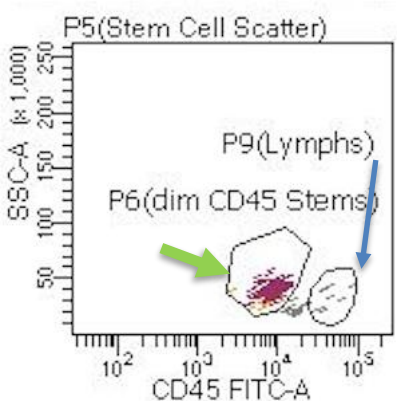
Viable cells expressing $CD45^+$ $CD34^+$ stem cells are larger and have more complex internal structure than other white cells. Therefore, forward scatter (indicator of size) and side scatter (indicator of complex internal structure) can be used to remove any further debris and identify the larger stem cells.

Further debris is removed in this plot (blue arrow)



P6 and P9

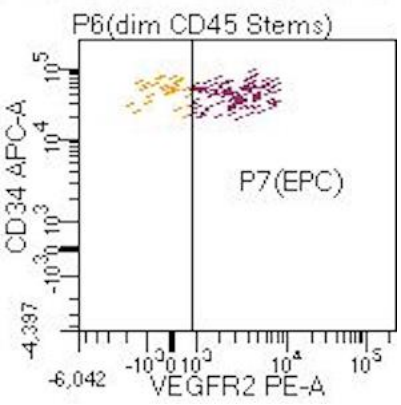
Viable cells expressing $CD45^+$ $CD34^+$ within the Stem Cell Scatter gate with residual debris excluded. Stem cells in purple (P6) indicated by the green arrow. Thin blue arrow shows a few lymphocytes (high CD45 expression compared to stem cells). This plot is also used to remove these lymphocytes which may fall into the stem cell scatter gate P5.



P7

Viable cells expressing $CD45^+$ $CD34^+$ $CD45^{dim}$ cells within lymph-blast scatter region. Y axis = side scatter x axis cd45. That is why EPCs are present higher up therefore more complex internal structure and cd45 dimmer. EPCs are reported to express VEGFR2 (CD309).

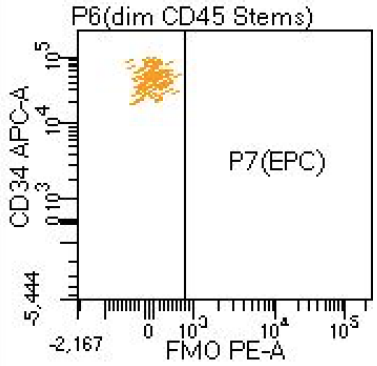
The EPCs are purple coloured to the right of the line.



P7

FMO tube

To accurately measure EPC area a fluorescence minus one (FMO) tube is required. The FMO tube is prepared identical to the sample, except for the addition of VEFG PE. Therefore on actual sample anything on the right of the line and encircled identifies true EPCs as seen in P7 above.

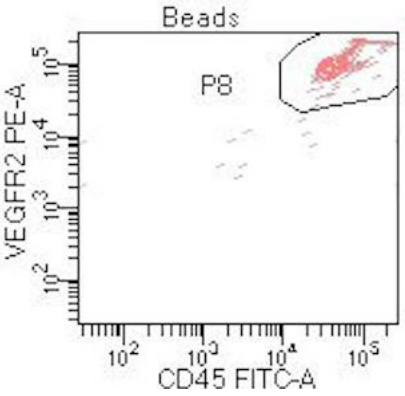


The FMO decreases errors by compensating for background fluorescence. Background fluorescence may be caused by auto-fluorescence, undesirable antibody binding and spectral overlap. [381] The required EPCs (red cluster of cells) on right side were identified and any debris removed any non-bead events to exclude stray events.

P8

You need an EPC vs SSc plot to show the first bead gate. The P8 gate is use to clean up any non bead events falling into it (the grey marks on the plot to the right.

Bead count region. This gate identified highly fluorescence beads used in the experiment.



2.6.5 Determination of ECP count

The final step is the quantification of EPCs by the following equation

$$\text{EPC count}/\mu l = \frac{\text{(events in P7) x (Trucount bead count) on pouch x1 (dilution factor)}}{\text{(Events in P8) x (sample volume uL added into tube)}}$$

This equation highlights the importance obtaining accurate sequential gating leading to P7 but also the bead count region P8. The gated P7 value will be discussed latter in more detail below 3.6.8 and chapter 5 under the assessment of dispersion of the dot and plot points with varied time and temperature.

2.6.6 Calculation of dispersion

The two most common graphical methods of data presentation are linear and logarithmic scales. The use of either linear or logarithmic graphs depends on the data. Linear scaling presents data in an evenly distributed manor with the distance between data points being proportional to the numerical distance between the values.

All data acquired on the flow cytometer (FacsCanto II BD Biosciences), used a linear amplifier, and the measured values were *binned* into 2^{18} (262144 channels) by a process called, *binning resolution*. Parameters with small differences in populations can be plotted on graphical diagrams (e.g. dot plots) directly. However, fluorescent values may cover a much larger range of magnitudes of thousands or tens of thousands such as in flow cytometric analysis, therefore linear scale to display the data would be inappropriate. Logarithmic (log) scales may overcome this limitation of linear scales and relies on an exponential difference between consecutive gradations along the axis

that represent equal changes in ratio. Therefore, the observations observed are percentage changes rather than absolute changes. This is particularly useful in presenting fluorescent flow cytometry data. Mathematically transforming the linear value using a log look up table converts the measured linear value to a log equivalent allowing large differences in the data to be shown on the same plot. The log scale has great advantage of representing data at the high end of expression however have limitations in when expressing lower end values. Therefore, transformation of the data is required in such a way so cells on the axis can be observed more accurately. On traditional log plots zero or negative values, generated after fluorescent background subtraction and introduction of compensation errors are not possible. This results in data being *pushed* into the first displayed channels and *piled up* on *x* and *y* axes on data plots. To avoid this *pile up* of data biexponential scaling algorithms have been developed which provides a more precise way to visualise the data at low fluorescence levels. An example of this is shown below. Biexponential scaling helps visualise compressed data by adding a section of linear scale to log data. This allows more precise data visualisation and allows data to be more easily interpreted as in the example below.

Despite the advantages of biexponential graphical representation the log scale needed to be changed to a linear scale for calculation of dispersion. FlowJo © Version 10.5.3 Java version (Tree Star (BD Biosciences)) was used to convert log value data values into linear scale. To allow transfer of required information the use of non-debris and bead count "dot plot" information was transferred. This reduced total number of events from millions to tens of thousands and therefore enabled quicker calculation.

The gated P7 dot plot was of interest as they included only EPCs with $CD34^+$ and VEGF on Y and X-axis respectively and were therefore converted to linear scale from biexponential logarithmic scale.

The first step was to open the raw data file in Diva. This includes a large number of irrelevant data and to decrease this the data was defined as debris and non-debris. This was defined by drawing a debris gate in the bottom left corner on the FSc versus SSc dot plot. Non-debris was an inverted gate of the debris and included the cells of interest and importantly, as they were a small fraction of the total cells from whole blood sample and therefore a smaller fcs data file. This was then imported back into Diva and subsequently underwent sequential gating as described above.

The defined EPC population (P7 from the gating) was then saved as a specific data file for transfer into FlowJo software.

The result was the linear scale data points in an Excel file format. The linear data points were then stored. These values were then used to assess dispersion of the dot and plots over time the study time period. The two mathematical models used to assess for dispersion were dispersion covariance and distance. These values formed a matrix. These models took 3 main considerations to obtain a unique value. Firstly, dispersion in horizontal axis, secondly, dispersion in vertical axis and finally dispersion related in both horizontal and vertical axis. The mathematical formulae below were used in this thesis with the assistance of Dr I Olier.

The advantage of calculation of covariance matrix allowed measure of dispersion in 2 planes. Unlike conventional standard deviation that allows calculation in a single plane only. Therefore, dispersion was described not only in horizontal and vertical

axes but also dispersion related in both horizontal and vertical axis the give a 3-dimensional position. Two plausible mathematical models were derived, dispersion covariance and dispersion distance. Dr Ivan Olier was instrumental in advice and assistance with ese models. Section 3.6.8 The first model was dispersion covariance. To determine a unique value, the following formula was used. Figure 2.10

Figure 2.10 Formulae for dispersion covariance with a 2 x 2 matrix:

$$Cov = \begin{bmatrix} S_x & S_{xy} \\ S_{xy} & S_y \end{bmatrix}$$

$$determinant = S_x \times S_y - S_{xy}^2$$

$$dispersion\ covariance = \sqrt[4]{determinant(Cov)}$$

Where:

S_x - the variance of points x.

S_y - the variance of points y

Variance calculated as $\sum (x_i - \dot{x})^2 / n\text{-}1$

Covariance - relationship between x and y = $\sum [(x_i - \dot{x})(y_i - \acute{y})] / n\text{-}1$

For each point the dispersion covariance was calculated by 4th root of the determinant. The determinant allows a matrix value to be converted into linear formulae.

The second method used was determination of dispersion distance rather than covariance. This model, based on the principles of Euclidean distance, is a measure of a straight line between 2 points in space. More specifically a Euclidean space, a geometric principle in 2 or 3-dimensional space, whereby points are defined by co-ordinates that define a specific point with the distance between these co-ordinates given by a distance formula. In our study the co-ordinates corresponded to the

distance in x-axis and distance in y axis of a particular point. This model corresponded to the distance in x axis and distance in y axis of a particular point. Figure 1.10 below.

Figure 2.11 Formulae for dispersion distance -

$$dispersion\ distance = \frac{1}{n-2}\sqrt{\sum_{i=1}^{n}(\mu_x - x_i)^2 + \left(\mu_y - y_i\right)^2}$$

μ_x – mean of all x values μ_y – mean of all y values x_i - n^{th} x value

y_i - n^{th} y value n = number of observations.

The obtained values for both these variance mathematical models were then plotted to assess for any relationship between dispersion and time with the mean (blue line) and confidence intervals are represented by the grey shaded area.

2.6.7 Statistical data analysis

On completion of each successful flow cytometric analysis, FCS files were saved with all studies exported and stored in the BD FACS database. Data analysis was performed with the FACS DIVA software. Every sample had a unique study number with no actual patient demographic including hospital or NHS identifiers used or stored within either the BD FACS database or FACS Diva software. This is in accordance with the Ethics Committee recommendations. Data analysis was performed by use of R – statistical programming language (version 3.6.1 St. Louis, Missouri, USA) or Microsoft Excel ANOVA statistical software programme.

2.6.8 Standard operating procedure for sample preparation for EPC enumeration based on Hristov et al [390] flow cytometric analysis method.

1. Allow all reagents and consumables to stabilise at room temperature for approximately 15 minutes
2. In a blank Falcon tube pipette 20µl CD45 FITC and 5µl CD34 APC. This is the fluorescence minus one (FMO) tube.
3. Using a Trucount tube pipette 20µl CD45 FITC, 5µl CD34 APC, 20µl CD309/VEGFR2 and 5µl 7ADD. This is the EPC tube.
4. Reverse pipette 50µl of well mixed whole blood into the EPC and FMO tubes
5. Incubate both tubes for 20 minutes at room temperature in the dark
6. After 20 minutes add 450µl of 10% Pharmlyse to each tube. This will lyse red cells
7. Incubate both tubes for a further 10 minutes at room temperature in the dark
8. Leave for 10 minutes then place the Trucount tube on ice in the dark
9. Analyse the FMO tube
10. All leucocytes are first gated on the FSC/SSC plot (P1)
11. The P1 population is displayed on the SSC/7ADD plot. All 7AASD negative events are gated as the P2 population
12. This P2 population is shown on the SSC/D45 plot and the CD45 positive events are then gated as P3
13. The P3 population is shown on SSC/CD45 plot and the CD45 positive events gated as P4 population
14. The events of the P4 are subsequently presented on the FSC/SSC plot and gated as P5 in order to confirm the lymph-blast scatter region and remove residual debris
15. The P5 events are then shown on the SSC/CD45 plot and only the CD45 dim cells are gated as P6
16. Finally, the CD45dim/CD34 positive events are shown on the CD34/FMO plot for the FMO tube and on the CD34/VEGFR2 plot for the EPC tube. This is the P7 population
17. The cut off for the VEGFR2 positive events- P7 is assessed by the FMO control In the EPC tube, the bead region – P8 is gated on the CD45/CD34 plot showing all events.

2.6.9 Standard Operating Procedures (SOPs) flow cytometry

1. Setting up of the FACSCanto for EPC enumeration
2. Switch on the FACSCanto analyser and leave for 30 minutes
3. Switch on the FACSCanto PC. Press Ctrl/Alt/Delete
4. Username will appear as Administrator – Password is BDIS
5. Open FACSCanto software. Enter username and password
6. Set up analyser as detailed in HST004
7. Ensure that CS&T beads Lot 60811 is used. Exit FACSCanto software
8. Open FACSDiva software Select Username RB project, no password required press ok. Select new folder (1st Icon on browser)
9. Select new experiment (2nd Icon on browser)
10. In the experiment, right click on Cytometer settings →Link set up → RB Project COMP → Link → Overwrite
11. Back to Cytometer settings. Right click → Unlink from RB Project COMP → OK
12. Back to Cytometer settings
13. Right click → Application Settings →Apply → RB Project App Settings → Apply
14. Acknowledges the messages to keep the compensation value and any other alterations to the settings
15. Right click on the Experiment and select New Specimen
16. From the Panel template, select the RB Project tab → RB project → OK and the worksheet appears. Highlight the inspector dialogue box
17. Select the specimen tab and enter the patient details
18. Select the keywords tab and enter the Trucount value of the beads from the pouch of Trucount beads currently in use
19. In the experiment, click on the RB Project icon to bring up Tube 1 – the FMO tube and Tube 2 the EPC tube
20. Position the green pointer against the appropriate tube and load the specimen onto the analyser, ensuring the tube guide is moved to the left and the aspirator arm is vertical
21. In the Experiment, click on the RB Project icon to bring up Tube 1 – the FMO tube and Tube 2 the EPC tube
22. Position the green pointer against the appropriate tube and load the specimen onto the analyser, ensuring the tube guide is moved to the left and the aspirator arm is vertical

2.7 Clinical studies

2.7.1 Study application and ethics

The study was conducted in accordance with the declaration of Helsinki.[395] The studies firstly underwent successful external independent peer review followed by Keele University Independent Peer Review Committee and finally by the Royal Stoke University Hospital Research and Development Department. All studies had Integrated Research Application Service (IRAS) and ethical approval from the national research ethical committee (NREC). A Research Ethics Committee (REC) reference number, IRAS project and European Union drug regulating authority's clinical trial (EudraCT) numbers were obtained.

2.7.2 Study Setting

This was a single centre study to be performed at Department of Cardiology, The Royal Stoke University Hospital NHS Trust and Keele University.

2.7.3 Study inclusion/exclusion criteria

Potential patients were identified for enrolment within the study by strict inclusion and exclusion criteria. Inclusion criteria included age 18 to 75 years with new onset angina. Participants with new onset angina had to be statin naïve. All patients were able to attend follow up visits and not enrolled in or planned participation in other drug trial(s).

Exclusion criteria

The exclusion criteria can be sub-divided into 6 categories. Table 2.6 below.

Exclusion criteria	Description
Clinical	• Diabetes mellitus type I or II • Stable angina group should be statin naive • All participants must have no other significant comorbidity for example malignancy on or off therapy, chronic obstructive pulmonary disease on home oxygen or nebuliser therapy, inflammatory bowel disease, on-going steroid therapy, autoimmune disease, therapy with disease modifying medications for rheumatic or autoimmune disease. • Women who are pregnant or breastfeeding • Allergies to excipients of IMP • Women of childbearing potential unless they are using a recognised effective form of contraception or are not sexually active and have no intention of becoming sexually active during the course of the trial.
Previous adverse reaction or contra-indications	• Any contraindication(s) to statin therapy • Any prior adverse reaction(s) to any statin therapy
Biochemical	• Total cholesterol greater than 7.5 mmol/L • Liver function tests deranged from normal range • eGFR less than 90 ml/min/1.73m^2
Haematological	• Concurrent haematological conditions requiring active drug or chemotherapy
Follow up	• Able to attend outpatient clinic follow up
Other	• Individuals lacking the capacity to consent for themselves • Participation in other clinical trials

Withdrawal criteria

Withdrawal criteria from the study included adverse events, inter-current illness, inability attend follow up research clinics. The data collected from withdrawn patients was analysed alongside the other process outcomes unless the patient explicitly states their wish not to contribute further data to the trial, this will be clearly noted.

2.7.4 Patient consent

Potential patientswere identified and if they fulfilled the inclusion criteria were consented. This initially included a face-to-face discussion. The potential participant was also given a patient information sheet (PIS) and at least 24 hours to decide if they wish to participate in the study. The only exceptions were patients enrolled into *pilot* "acute coronary syndrome" (*ACS) group* and *"All-comers"* studies, chapters 5 and 8 respectively. These studies involved patients with acute coronary syndromes, where treatments are given in a time sensitive manner. Therefore, these patients were given 3 hours in concordance with research ethics committee recommendations. This was to avoid any delay in potentially life-saving treatment such as coronary angiography or angioplasty. These patients were given a brief information sheet. Thereafter a full explanation of the consent form was given, allowing ample opportunity to ask questions and then if the individual wished to participate, they were asked to sign the consent form.

Chapter 7 assessing effect of EPC by timing of atorvastatin therapy in all comer ACS patients. This study assessed if the timing of statin therapy had an influence on EPC count in patients presenting with an acute coronary syndrome (ACS). This was made

possible due to three variables. Firstly, flow cytometric determination of EPC count was only possible during 9am to 5pm Monday to Friday. Secondly hospitalised patients were given atorvastatin late in the evenings at 2200. Finally, patients may be admitted with ACS any time or day. Therefore, patients admitted between 1600 – 2200 were given atorvastatin 80mg first and then had venesection for EPC count during working hours. Whereas patients admitted between 2200 - 1600 the following day had venesection for EPC counts prior to being given atorvastatin 80mg. This allowed determination of time between onset of chest pain to atorvastatin therapy, secondly time of onset of chest pain to venesection of blood for determination of EPC count. These two-time intervals allowed determination of the time difference between statin therapy and venesection for EPC count. Therefore, the time of admission of the patient influenced the timing of both venesection and atorvastatin initiation. For example, patients were admitted between 9am to 5pm then venesection and EPC count determination was performed before initiation of atorvastatin 80mg. In this way the study could determine if the timing of initiation of 80mg atorvastatin had any effect on EPC count without the need for randomisation or delaying in treatment such as coronary intervention. To consider that venesection may occur on the following day if admission was 'out' of hours the time intervals were days 1-2, 3-4 or 7-8. The day of admission was day 0. Patients then had venesection for EPC analysis on days 1-2, 3-4 and finally 7-8. ACS patients were inpatients within the cardiac unit for 3 or 4 days provided there are no complications. Therefore days 0, 1 and 3 venesections were performed whilst patients were admitted within the cardiology department.

However, outpatient cardiology research clinic follow-up arranged for day 7 to allow the final venesection for EPC analysis. Please refer to figure 7.1.

All patients requiring outpatient follow up attended the cardiology research outpatient clinic and were reviewed by a member of the cardiac research team. The outpatient clinic reviews were defined as per each study protocol however patients were allocated a time of their choice. Patients were assessed for any adverse effects of statin therapy, symptoms and any alterations to medications. Patients were verbally consented to continue in the study and if given had 3-5ml of blood aseptically venesected for EPC analysis.

Patients were refunded any travel expenses incurred to attend the outpatient clinic appointments this included either car parking charges or travel expenses.

2.7.5 Sample acquisition and transport

Analysis of all blood samples was performed within the Department of Haematology of The Royal Stoke University Hospital, University Hospitals of North Midlands NHS Trust.

All consented patients had aseptic venesection of 3-5ml of whole blood into a study labelled ethylene diamine tetra acetic acid (EDTA) bottle and immediately transported to the haematology laboratory. The *time and temperature* pilot study protocol to investigate time and temperature stability had two EDTA blood sample to be taken on day 1. One of these were stored at room temperature the other at 4^0 C. These samples then had aliquots taken at regular intervals of 0, 2, 4, 6, 9 and 12 hours for flow cytometric analysis. The Initial study protocol required immediate flow

cytometric analyses of whole blood samples. However, during the time and temperature study (chapter 5) data emerged and there was no degradation for up to 12 hours. Therefore, study protocol change was successfully applied for and changed for whole blood samples to be analysed within 2 hours of venesection. This protocol amendment allowed for greater flexibility in the use of the flow cytometer and in turn increased recruitment into the studies within this thesis.

No samples were stored or kept for greater than 24 hours and no samples were used in any other study. All samples were disposed of within the haematology department and sent for incineration as per haematology laboratory disposal protocol. The trial was closed after the final follow up on day 28 and once 20 complete patient datasets had been collected. The MHRA, HRA and Sponsor were notified within 90 days of completing the trial.

2.7.6 Clinical studies sample size

There are four clinical studies within this thesis including 83 patients. Inclusion or exclusion criteria and consenting have been described above in sections 3.7.2 and 3.7.4. The CTCA study (chapter 6) included 23 patients as the national guidelines for performing CTCA changed during the study. Table 2.7.

Table 2.7: Number of patients in each clinical study

Clinical study	Chapter	Number of patients
Time and temperature stability study	5	10
Computerised tomography coronary artery (CTCA) calcium scoring and endothelial progenitor cell count Study shortened due to changes in national guidelines	6	23
Mobilisation of endothelial progenitor cells and statin therapy: A pilot – proof of concept study	7	20
The effect on endothelial progenitor cells by statin loading in "all comers" with an acute coronary syndrome	8	40

The study design was based on power calculation as described by Ricottini et al.[306] By using power of 90% and a significance level of 0.05, with a normal distribution and a two sided significance test, this abstract reported that reloading with atorvastatin raised EPC level from 0.25±0.29% to 0.76±1.04%; a difference of means of ~0.5 and an estimated population standard deviation of 0.45 giving a sample size of 18 per group. [306]

In more detail, there were four different factors that needed to be considered when estimating the study size if the outcome is a continuous normally distributed variable:

1) Type I error rate α, or significance test size

2) Type II error rate β (related to the power of the study as $1-\beta$)

3) Anticipated standard deviation of the outcome measure, σ_{plan}

4) The anticipated effect size, δ_{plan} (difference between test and control means)

It is conventional to set $\alpha = 0.05$ and $\beta = 0.1$ (or $\beta = 0.2$, for more conservative analysis), which implies the minimum power of the test will be 90% ($1-\beta = 0.9$). The anticipated standard deviation and anticipated effect size are usually taken from previous similar studies. In our study, we assume $\sigma_{plan} = 0.45$ and $\delta_{plan} = 0.5$ as those were the values estimated [306]. Figure 2.12.

The four factors are used to estimate the study size using the following equation:

$$m = 2\theta \frac{\sigma_{plan}^2}{\delta_{plan}^2},$$

where;

$\theta = \left(z_{\alpha/2} + z_{1-\beta}\right)^2$, being $z_{\alpha/2}$ and $z_{1-\beta}$ the ordinates for the Normal distribution of values $\alpha/2$ and $1-\beta$.

In this study, $\theta = 10.5$

Therefore, the study size can be estimated as figure 2.12

$$m = 2 \times 10.5 \times \frac{0.45^2}{0.5^2} = 17.01 \approx 18$$

This was rounded up to a sample of 20 patients in the pilot –proof of concept study of mobilisation of endothelial progenitor cells and statin therapy. We decided to double the number of patients in the All-comers and CTCA studies as we included other parameters such as significant co-morbidities.

Chapter 3

The effect of atorvastatin on EPC associated chemokine CXCR-12 (SDF-1), its receptor CXCR-4 and cell adhesion molecule E-selectin

3.1 Abstract

***Introduction*:** Atorvastatin is thought to mediate chemokines, cell surface molecules and adhesion molecules as a pleiotropic mechanism to augmentation of EPCs .

***Method*:** This study assessed the effect of atorvastatin on human EPCs, chemokine CXCL-12, cell surface marker CXCR4 and an adhesion molecule E-selectin in denuded tissue engineered intimal layers. Tissue constructs were incubated for varied length of time in either standard culture media or standard culture media with the addition of atorvastatin at a concentration of 60μg/L. Human EPCs, concentrations of CXCR-4, CXCL-12 and E-selectin were analysed by cell culture, ELISA and immunostaining respectively. The study hypothesis was that atorvastatin would increase CXCL-12 its receptor CXCR-4 and of adhesion molecule e–selectin resulting in greater EPC numbers on the denuded endothelium. The aim of the study was to prove this.

***Results*:** The study found incubation time and atorvastatin significantly increased CXCL-12 concentration. The study found no significant interaction between atorvastatin and time of incubation. Atorvastatin appeared to non-significantly

increase CXCR-4 within 1 hour, an effect maintained for up to 9 hours and peak E-selectin by 5 hours. No significant difference was observed in EPC numbers.

***Conclusion*:** Atorvastatin appears to significantly increase CXCL-12 concentration, reach peak concentrations of CXCR-4 and E-selectin earlier and maintain higher steady state concentrations than standard culture media. However no significant increase of cell cultured EPCs was found.

3.2 Introduction

Several studies have shown the early beneficial effect of statins occurring before any decrease in lipid profile. A proposed pleiotropic mechanism includes enhanced release of cytokines and or expression of adhesion molecules on EPC surface. [13, 225, 287, 288, 344, 345] Cytokines are central to cell signalling. A common chemokine termed C-X-C motif chemokine 12 (CXCL-12), also known as stromal cell-derived factor 1 (SDF-1) [69] has shown to be great importance and bind to the (C-X-C motif) receptor 4 (CXCR4). [81-83] CXCL-12 plays an integral role in vascular repair during cardiovascular disease, [70] and shown to be a strong chemotactic factor for EPCs. [72-74] Therefore any pharmacological treatment shown to be capable of increasing CXCL-12 and or CXCR-4 to sites of endothelial damage could potentially be of clinical benefit. There has been some evidence that statins may enhance EPCs via increasing CXCL-12. E-selectins are cell adhesion molecules expressed only on endothelial cells

activated by cytokines. [92] E-selectins have been shown to be integral for EPC adhesion. [94-98]

This study used tissue engineered intimal layer (TEIL) in assessing the effect of atorvastatin on chemokine CXCL-12 from culture, its receptor CXCR-4, cell adhesion molecule E–selectin and human EPCs form tissue engineered denuded endothelial layers. ELISA (section 3.3) and immunostaining (methods section 3.4) were used to quantify CXCL-12 and CXCR-4 respectively. Cell culture and staining (methods section 3.5) were used to identify human EPCs on TEIL layers.

3.3 Materials

Please refer to sections 2.3, 2.4 and 2.5.

3.4 Construction of tissue engineered intimal layers

Please refer to section 2.2.

3.5 Lesion formation

Please refer to section 2.2.4.

3.6 Method

Please refer to sections 2.1 to 2.5.

3.7 Enzyme Linked Immunosorbent Assay (ELISA) quantification

Please refer to section 2.3.

3.8 Immunostaining of TEIL constructs

Please refer to section 2.4

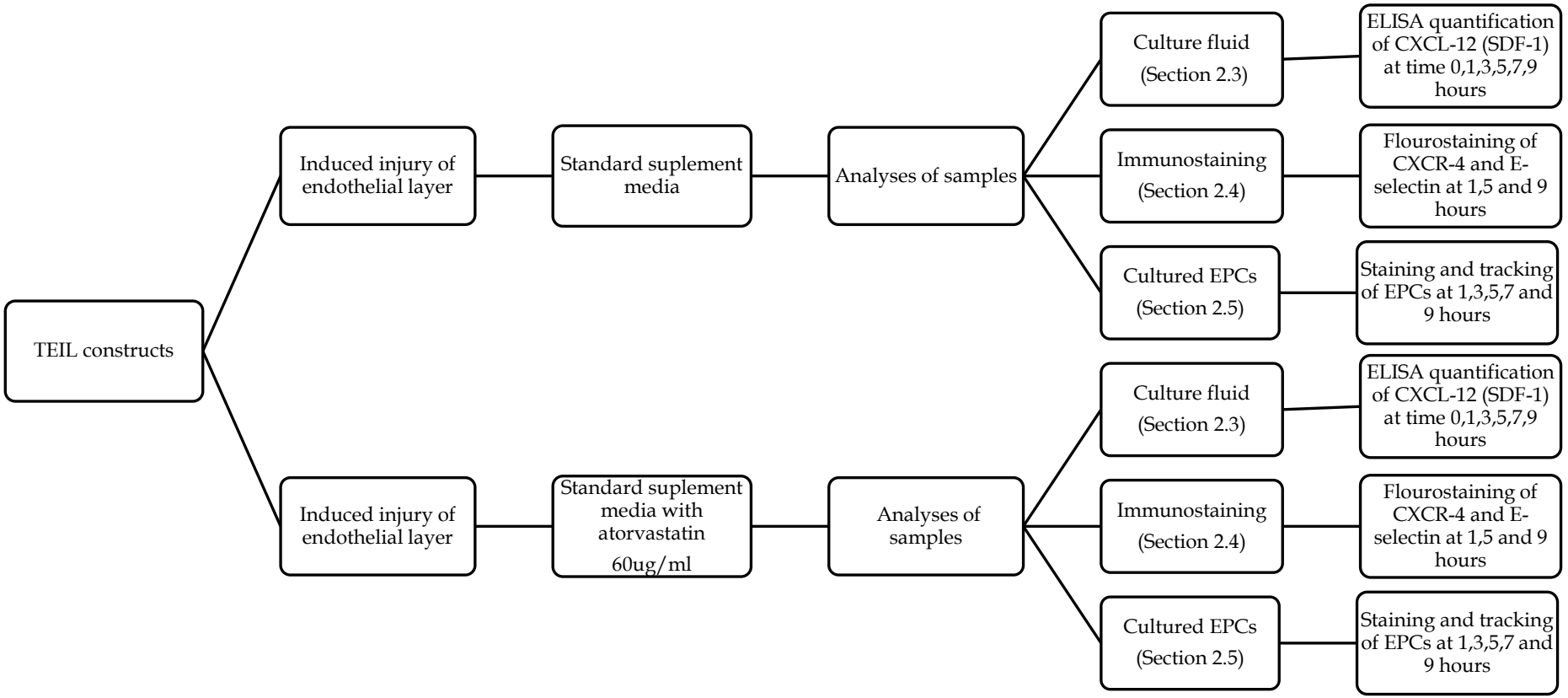
3.9 Cell tracking and staining of cultured EPCs

Please refer to section 2.5

3.10 Statistical analysis

The statistical analytical method needed to consider two important factors. Firstly, to comparison of 6 groups, time 0, 1, 3, 5, 7 and 9 hours and secondly the effect of addition of atorvastatin in standard media. Therefore, two-way replication analysis of variance (ANOVA) statistical software was used. The effect of atorvastatin, length of time of incubation or interaction between these variables would influence CXCL-12 concentration (pg/ml). The significance was assessed by F-test and P-value. The F test assumed population from F distribution, also called Fisher-Snedecor distribution. In this study chosen level of significance was α 0.05.

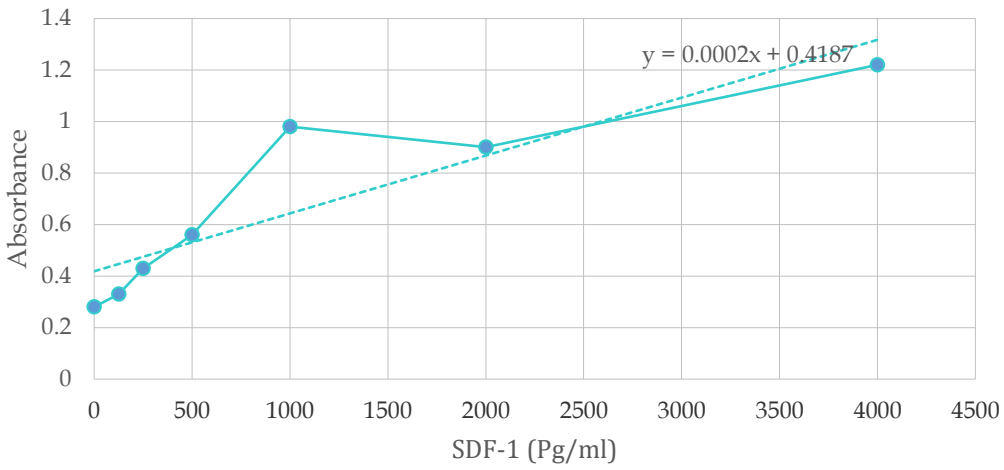
Figure 3.1. Figure to illustrate study design



3.11 Results

The effect of atorvastatin on CXCL-12 within denuded endothelium was analysed by ELISA of tissue engineered intimal layer (TEIL). Methods section 2.3. A standardised curve as per manufacturer recommendations was obtained. The obtained standardised curve can be seen in Figure 3.2 below.

Figure 3.2 - Standard curve



By application of equation of straight line, the concentration of CXCL-12 was determined.

Equation of straight line y=mx+c

Where

Y axis = absorbance

X axis = concentration (pg/ml)

m = defined constant 0.0002

c = defined constant 0.4187

Absorbance = 0. 0002.Concentration + 0.4187

Therefore:

CXCL-12 Concentration (pg/ml) = absorbance/0.0002 – 0.4187.

The equation was applied to each data point at each time interval and summarised in tables 3.1 and 3.2 below.

Table 3.1 Standard media calculated CXCL-12 concentration (pg/ml)

	Time in hours					
Number	0	1	3	5	7	9
1	1692	2657	2027	1982	1702	1807
2	1577	2597	2257	2202	1967	1722
3	1222	2477	2087	2157	1982	1817
4	1557	2287	2227	2067	2067	1692
5	1377	2527	2222	2102	1767	1792
6	1162	2542	2062	2617	1852	1717
7	1137	2012	2062	1782	1857	1477
8	1152	1807	1882	1562	1777	1587
9	1332	2527	2357	2117	1937	1867
10	1842	1957	1962	1932	1877	1892
11	1842	1957	1907	2012	1902	1837
12	1827	1937	1937	1957	1952	1912
13	1822	1952	1912	1942	1892	1852
14	1872	1937	1982	1992	1887	1877
15	1847	1927	1967	1932	1927	1942
16	1917	1982	2002	2027	1982	1932
17	1947	2067	2297	2042	1952	1972
18	1872	2122	1917	1967	2022	1917
Average	1610	2181	2059	2021	1905	1811
SD	300	290	149	206	92	130

Table 3.2 Standard media with atorvastatin calculated CXCL-12 concentration (pg/ml)

	Time in hours					
Number	0	1	3	5	7	9
1	2457	2932	3147	2977	3107	3572
2	2127	2652	2542	2632	2522	2557
3	2042	2512	2842	2572	2352	2502
4	1922	2437	2212	2457	2322	2397
5	1637	2302	2627	2702	2367	2512
6	1677	2197	2467	2247	2542	2557
7	1487	2527	2127	2037	2212	2272
8	1712	2252	2232	2047	1942	2377
9	1497	3122	2817	2577	2497	2192
10	2307	2532	2462	2487	2437	2417
11	2317	2582	2507	2507	2492	2487
12	2307	2607	2522	2462	2552	2397
13	2392	2567	2492	2502	2507	2447
14	2307	3142	2442	2467	2562	2482
15	2317	2607	2547	2512	2547	2477
16	2357	2602	2477	2577	2487	2532
17	2397	2642	2592	2597	2622	2512
18	2272	2657	2497	2412	2392	2432
Average	2085	2603	2530	2487	2470	2506
SD	337	255	236	218	225	282

Statistical analysis was performed with two-way ANOVA with replication in Microsoft Excel. Table 3.3 summarises the findings.

Table 3.3 Summary of the two-way ANOVA analysis

	Time/hours						
SUMMARY	0	1	3	5	7	9	Total
Standard media							
Count	18	18	18	18	18	18	108
Sum	28996	39271	37066	36391	34301	32611	208636
Average	1610.89	2181.72	2059.22	2021.72	1905.61	1811.72	1931.81
Variance	89875.16	84066.09	22318.30	42598.45	8505.31	16977.86	76462.58

Standard media with Atorvastatin							
Count	18	18	18	18	18	18	108
Sum	37531	46871	45551	44771	44461	45121	264306
Average	2085.06	2603.94	2530.61	2487.28	2470.06	2506.72	2447.28
Variance	113779.82	64823.94	55805.31	47374.92	50571.00	79689.62	93784.97

Total						
Count	36	36	36	36	36	36
Sum	66527	86142	82617	81162	78762	77732
Average	1847.97	2392.83	2294.92	2254.50	2187.83	2159.22
Variance	156732.60	118159.29	95084.82	99435.00	110619.29	171159.21

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Atorvastatin	14347912	1	14347912	254.551	9.85E-38	3.887
Time (hours)	6272742.6	5	1254549	22.257	8.803E-18	2.258
Interaction between atorvastatin and time	445187.04	5	89037.41	1.580	0.1672683	2.258
Within	11498558	204	56365.48			
Total	32564400	215				

Key

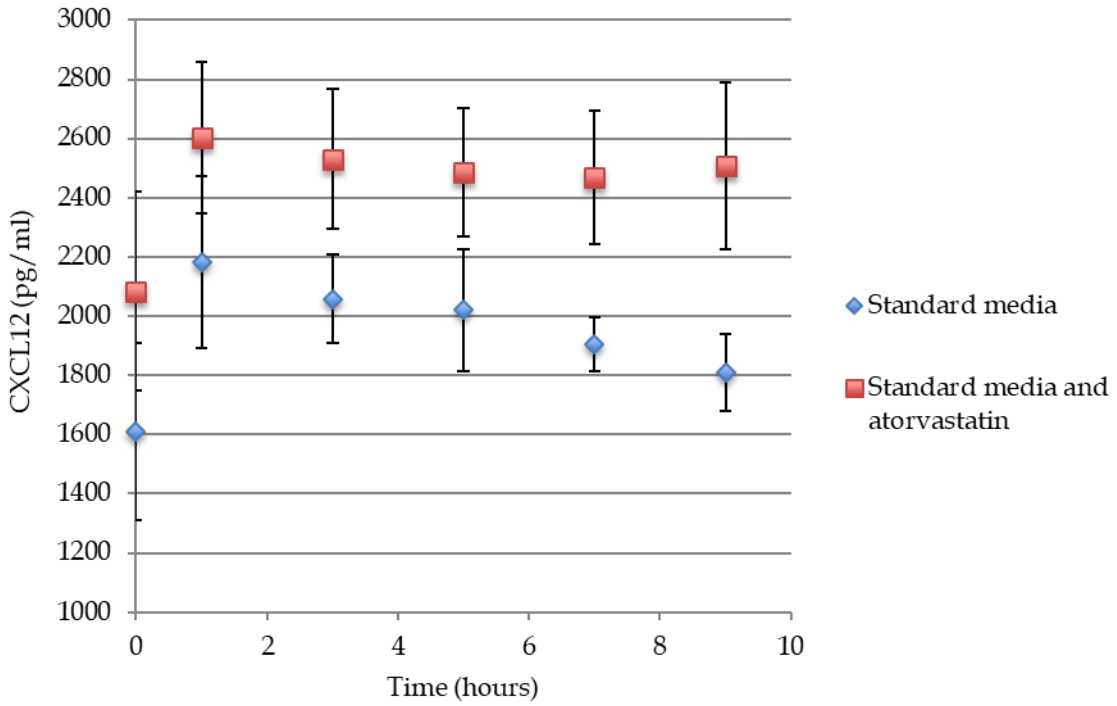
ss - sum of squares df - degrees of freedom

MS - Mean squares F statistic= MS in group/MS within groups

F crit values for α significance of 0.5

Plotting concentration of CXCL-12 (pg/ml) against time (hours), figure 4.3 below highlights the difference in CXCL-12 (pg/ml) seen between sample media and sample media with atorvastatin. The figure also shows standard deviation.

Figure 3.3; Graph comparing concentration of CXCL-12 in standard media and standard media with atorvastatin including standard deviation markers



The effect of atorvastatin on CXCR-4 within denuded endothelium was studied by immunostaining of tissue engineered intimal layer (TEIL). The effect of atorvastatin on CXCR-4 within denuded endothelium was analysed by immunostaining of tissue engineered intimal layer (TEIL). The TEIL constructs were incubated, fixed and stained immunologically by the methods previously described (methods section 2.4). The immunostaining images are shown in figure 4.4 below. CXCR-4 stained green and therefore we hypothesised a circumscribed area of denuded endothelium to become

increasingly intense correlating to increasing concentration of CXCR-4. Cellular debris and other artefacts were stained blue by *DAPI* staining.

Immunostaining for E-selectin images are shown in figure 3.5 below. E-selectin was stained green and there is blue nuclear staining with DAPI, as described previously. Figure 2.5.

The final part of this study was to assess culturing, staining and tracking of EPCs. Methods section 2.4. Figure 3.6 shows time elapsed images of EPC culture and cellular maturation over a 13-day period. Table 3.4 summarises the total EPC number and average for both standard and standard media with atorvastatin.

Table 3.4a; Total EPC number for both standard and standard with atorvastatin media obtained from J-image software.

		Time in hours				
		1	3	5	7	9
Standard media	Sample 1	90	192	97	131	139
	Sample 2	329	244	187	315	331
Atorvastatin	Sample 1	199	177	296	177	199
	Sample 2	438	286	248	152	142

Figure 3.4 Stained immunologically images for CXCR-4

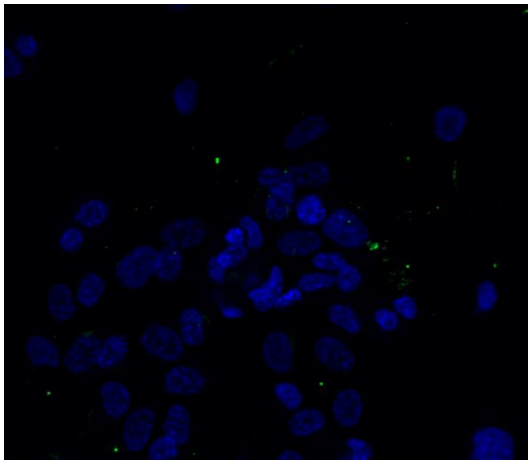
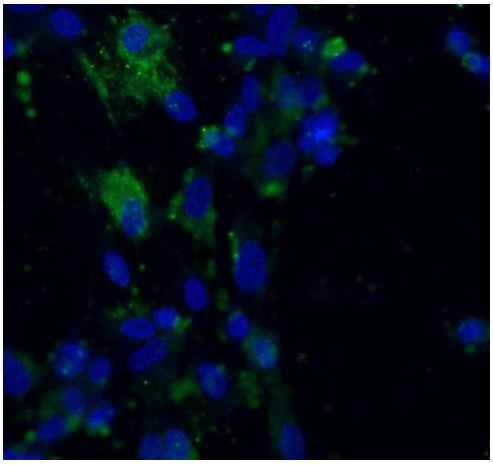
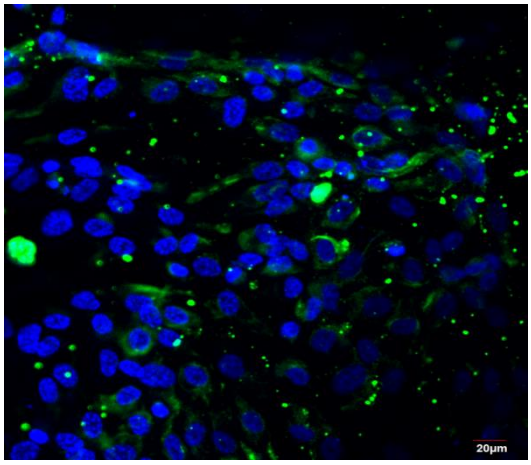
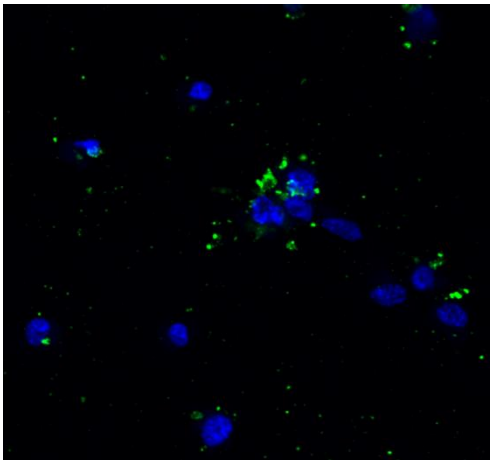
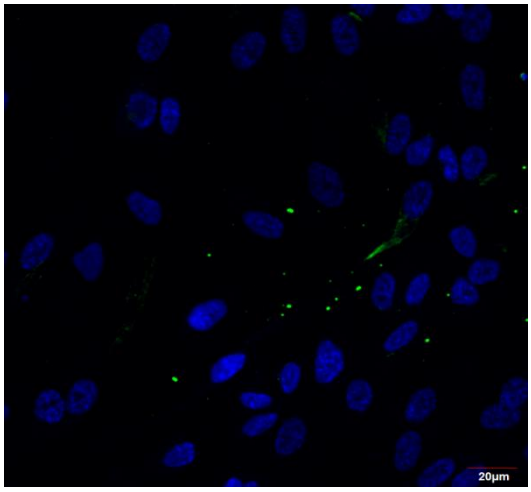
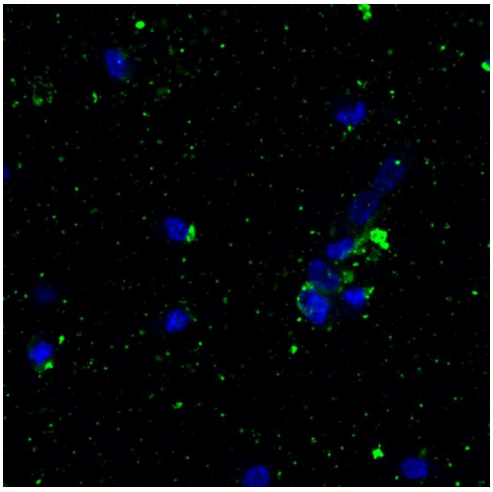
Standard supplemented media	Standard supplemented media with atorvastatin (60ug/mL)
1 hour	1 hour
	
5 hours	5 hours
	
9 hours	9 hours
	

Figure 3.5 Stained immunologically images for E-selectin

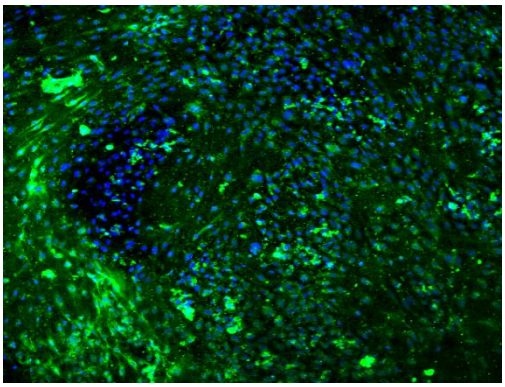
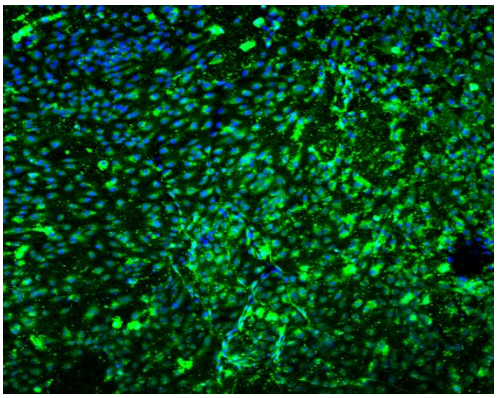
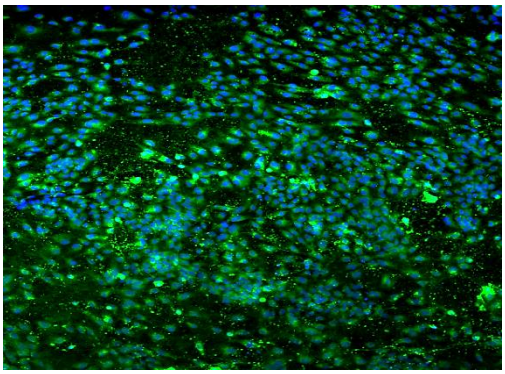
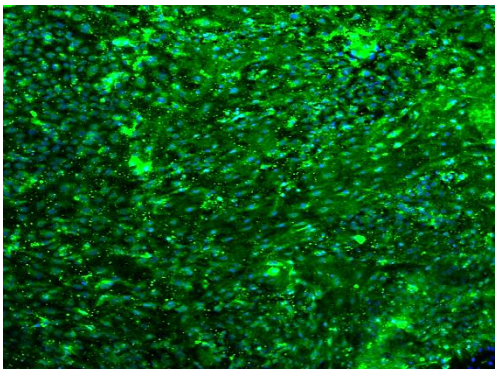
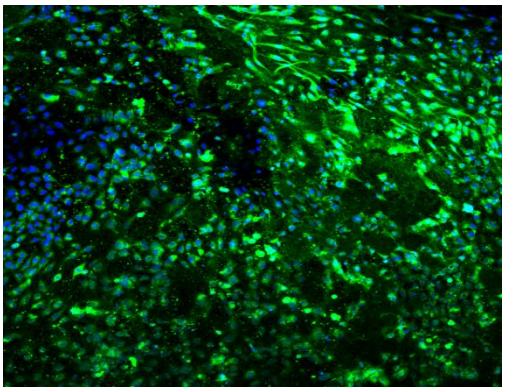
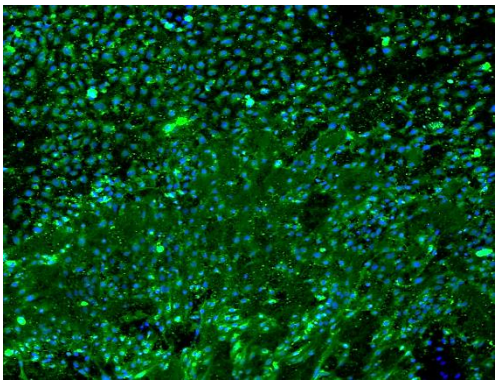
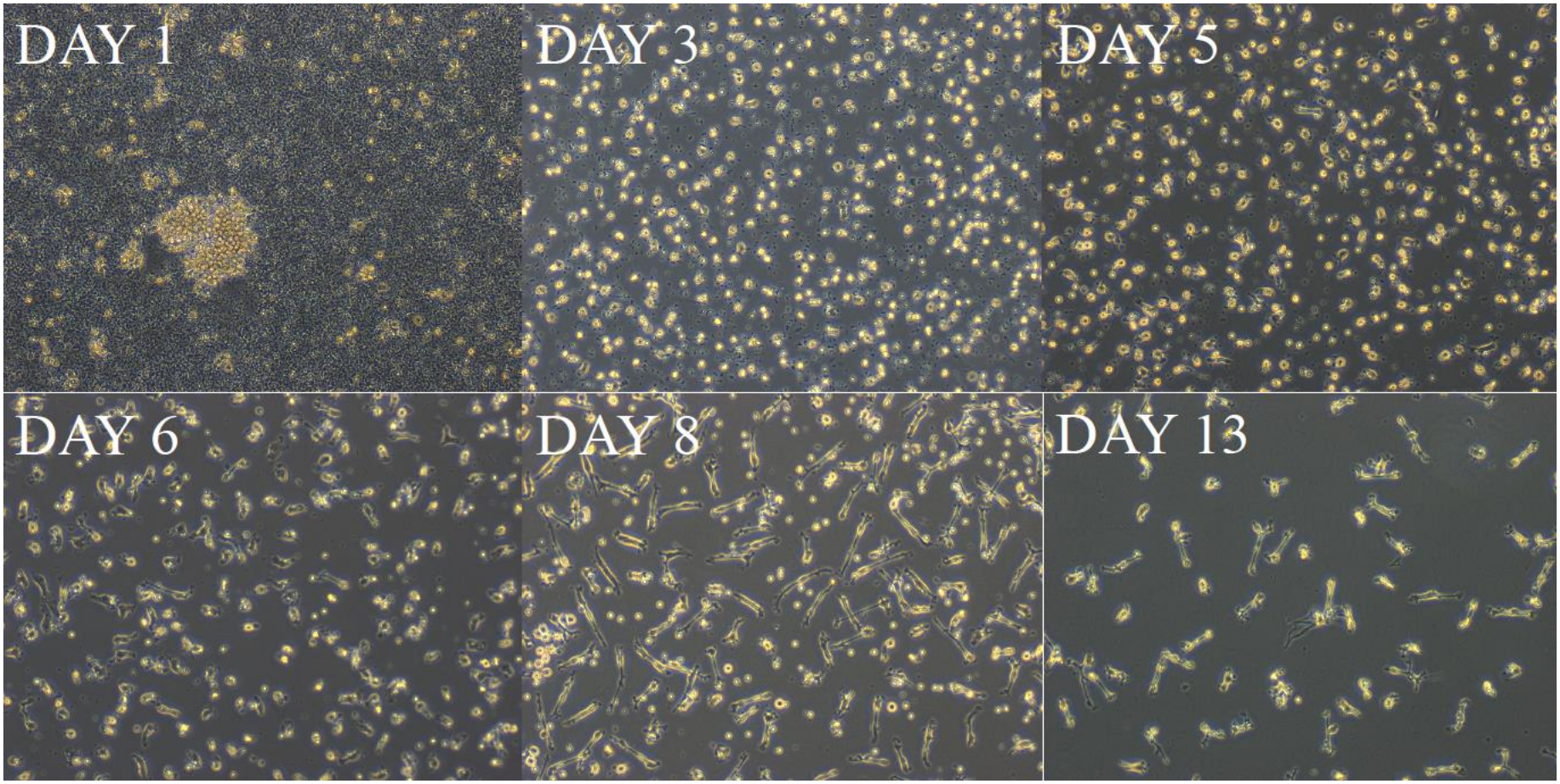
Standard supplemented media	Standard supplemented media with atorvastatin (60ug/mL)
1 hours	1 hours
	
5 hours	5 hours
	
9 hours	9 hours
	

Figure 3.6; Figure representing EPC maturation.



Days 1-8 several different cell lineage are present with immature EPCs. Day 13 shows survival of only mature EPCs identified by their elongated spindle shape. Trypsin was then added to the culture media to allow separation of extra-cellular matrix and EPC. EPCs were then stained with CFSE fluorescent dye and transferred to TEILs and placed on a rocker with either standard media or standard media with atorvastatin.

Table 3.4b Anova: Two-Factor With replication

SUMMARY	1	3	5	7	9	Total
Standard media						
Count	2	2	2	2	2	10
Sum	419	436	284	446	470	2055
Average	209.5	218	142	223	235	205.5
Variance	28560.5	1352	4050	16928	18432	8898.3

Atorvastatin						
Count	2	2	2	2	2	10
Sum	637	463	544	329	341	2314
Average	318.5	231.5	272	164.5	170.5	231.4
Variance	28560.5	5940.5	1152	312.5	1624.5	8047.6

Total						
Count	4	4	4	4	4	
Sum	1056	899	828	775	811	
Average	264	224.75	207	193.75	202.75	
Variance	23000.67	2491.583	7367.333	6887.583	8072.25	

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Atorvastatin	3354.05	1	3354.05	0.31	0.59	4.96
Time (hours)	12408.7	4	3102.175	0.29	0.88	3.48
Interaction between atorvastatin and time	33191.7	4	8297.925	0.78	0.57	3.48
Within	106912.5	10	10691.25			
Total	155867	19				

3.12 Discussion

This study compared the effect of atorvastatin added standard culture fluid compared to standard cultured fluid alone on denuded tissue engineered endothelium. This study used 60µg/mL atorvastatin corresponding to the equivalent intense statin therapy when administered orally to patients. [377]

The first part of this study compared the concentration of the cytokine CXCL-12 in culture fluid of standard media with standard media with atorvastatin group by ELISA. CXCL-12 is well a recognised chemotactic factor for EPCs. [72-74]. [68-70]

The study found that addition of atorvastatin had a significant increase in CXCL-12 concentration $P<0.05$. Secondly the greater incubation time also had a significant effect on CXCL-12 concentration $P<0.05$. The study found no significant interaction between atorvastatin and time of incubation, $P = .167$. This can be seen in figure 4.3, which demonstrates the positive effect of atorvastatin on CXCL-12 concentration, and that the atorvastatin appeared to maintain an elevated concentration of CXCL-12. Whereas in the standard media CXCL-12 peaked by 1 hour followed by a decrease in concentration. This suggests that atorvastatin firstly increased the concentration of CXCL-12 and secondly maintained a more sustained duration of effect when compared to the standard group. This is in keeping with well described up regulation of the expression of CXCL-12 on atherosclerotic plaques that enhances homing of EPC to sites of vascular injury [70, 71, 75]

Modulation of CXCL-12/CXCR-4 axis has been shown to have a central role in mobilisation of endothelial progenitor cells by decreasing CXCL-12. [81, 84, 88-91] Binding of CXCL12 to CXCR4 mediates intracellular signalling via G-protein mediate

response. One of the proposed effects is the inhibition of adenylyl cyclase activity and triggers MAPK and PI3K pathway activation, [399] which are intracellular mechanisms associated with EPC mobilisation. [400]

The study used immunostaining to assess the effect of atorvastatin on CXCR-4 at 1, 5 and 9 hours. No significant difference was observed visually in CXCR-4 between the standard sample and the standard sample incubated with atorvastatin at any time period of the study. This may be because the concentrations of CXCL-12 were insufficient to require modulation by CXCL-4 or that longer time was required to stimulate a response. Secondly, there was no identifiable circumscribed area of green immunostaining, suggesting no differentiation between denuded endothelial areas and normal endothelium. This suggested that the effect of atorvastatin was not confined only to denuded endothelium alone but also on neighbouring endothelial cells. This may have been due to 'spill-over' of the culture fluid or chemotactic factors. Despite no obvious significant difference between the standard media compared to standard media with atorvastatin there were however subtle difference within each group suggesting some effect due to atorvastatin. In the standard group, there appeared to be a modest increase of CXCR-4 by 5 hours with a visible decrease by 9 hours. This is in keeping with an earlier study. [65] In contrast the atorvastatin group, appeared to have a greater concentration of CXCR-4 by 1 hour that was maintained to 9 hours. These findings are comparable to the effect seen with atorvastatin on CXCL-12, with an acute increase at 1 hour followed by a more sustained and maintained concentration. E-selectins were included in this study as they are recognised as integral component for EPC adhesion. [94-98] We observed no circumscribed area of green immunostaining, suggesting no differentiation between denuded endothelial areas and normal endothelium. This

may be due to neighbouring cells expression of E-selectin to potentiate the effect of EPC adhesion. However, by 1 hour there appeared to be greater concentration of E-selectin in atorvastatin group. Secondly, the atorvastatin group appeared to have maximal concentration by 5 hours and by 9 hours less staining was seen. In comparison to the standard group a gradual increase to 9 hours was seen. This suggests that atorvastatin may potentiate expression of E-selectin. This delayed response may be explained by the fact that E-selectins are not stored in cells and therefore have to be transcribed, translated, and transported to the cell surface and have been found to peak between 6-12 hours. [93] Our results suggest firstly that atorvastatin may cause a more rapid transcription, translation and expression on cell surface of E-selectin. Secondly the response of E-selectin expression may not be confined only to denuded endothelium but also to neighbouring endothelial cells. Figure 4.5. The study hypothesis was a higher concentration of CXCL-4 or E-selectin within denuded endothelium.

The final part was to assess if EPCs were recruited specifically to denuded areas and secondly if atorvastatin group had a greater response. Figure 4.6 shows time elapsed images of EPC culture and maturation. Microscopic images between days 1-8 show a number of different cell lineage at varying stages of maturity. By day 13 only mature EPCs are seen and readily identifiable by their elongated spindle shape. Figure 4.6.

The study found that addition of atorvastatin had no significant increase in EPC count with P= .59. Secondly the greater incubation time had no significant increase in EPC count P= .88. These may be due to negative feedback mechanism.

This study had several limitations. The main limitation was the small sample size used for each sub-study, which is driven by the practicalities of creating the TEIL constructs. Secondly, the study only had a single concentration of atorvastatin (60ug/mL) and therefore potential effect on CXCR-4 or EPC numbers may occur at lower or higher concentrations, but this dose was picked to represent the average

tissue concentration seen with 80mg/day of atorvastatin. Thirdly immunostaining analyses of CXCR-4 and E-selectin was based on subjective visual interpretation and subjective rather than quantitative analyses, with Image-J to minimise this factor.

3.13 Conclusion

The study found atorvastatin had a significant increase in CXCL-12 concentration ($P<0.05$) an effect also seen with greater incubation time also had a significant effect on CXCL-12 concentration ($P<0.05$). No significant interaction between atorvastatin and time of incubation ($P = .167$). Atorvastatin appeared to firstly increase the concentration of CXCL-12 and secondly maintained a more sustained duration of effect when compared to the standard group. The study found a greater concentration of CXCR-4 by 1 hour that was maintained up to 9 hours. A similar effect was seen on E-selectin with greater concertation observed by 1 hour, peaking by 5 hours following by decrease by 9 hours. This suggests that atorvastatin potentiated expression of both CXCR-4 and E-selectin and maintained this effect. Interestingly the study observed no significant difference between EPC numbers by culturing in either time of incubation of addition of atorvastatin.

The study supports earlier studies that suggest atorvastatin has pleiotropic effects other than effect on lipid profile alone.

Chapter 4

Time and temperature stability study

4.1 Abstract

***Introduction*:** Current flow cytometric protocols recommend that whole blood samples are analysed within a short time interval. This may be difficult in busy haematology laboratories. The study aim was to demonstrate the feasibility of performing flow cytometric analysis of whole blood sample. The study hypothesis was that this was firstly possible and secondly that sample could be stored at 4^0C and analysed later.

***Methods*:** This study was performed in collaboration with Chapter 7. Newly diagnosed angina patients had baseline blood samples prior to starting atorvastatin 20mg as required for chapter 7 study. At day 1 two EDTA bottles each containing 5ml of blood were stored at either room temperature or at 4^0 C. From these samples, aliquots of the whole blood were then taken at time 0, 2, 4, 6, 9 and 12 hours and underwent flow cytometric analysis for quantification of $CD45^{dim}$, $CD34^+$ and $VEGFR2^+$ EPCs.

***Results*:** No statistical difference in EPC count was observed up to 12 hours at either room temperature (p=0.8) or 4^0 C (p=0.9). Significant EPC dispersion at room temperature was found (p=0.04).

***Conclusion*:** Whole blood samples may be stored up to 12 hours at 4^0 C prior to flow cytometric analyses.

4.2 Introduction

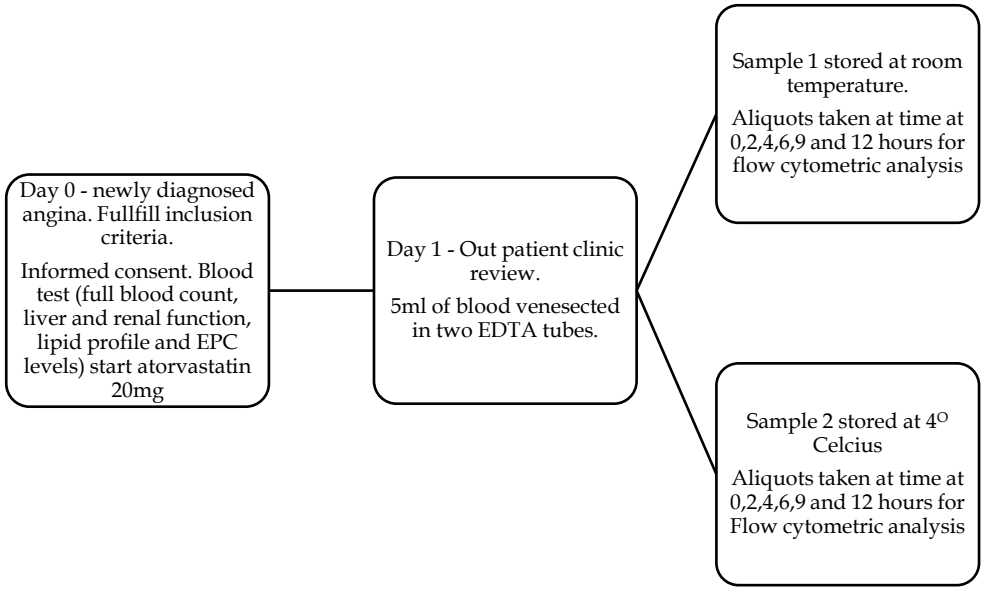
EPCs can be analysed by flow cytometry relatively easily and reliably. Current flow cytometric protocols recommend that whole blood samples analysed as soon as possible after venesection. [390] However, due to the limited availability of flow cytometers which are often located in busy haematology laboratories, often with workflows heavily weighted to oncological and immunological diseases. Many clinical units have limited flow cytometry trained staff, who are often rostered to other investigative processes, and this often precludes patients that present out of working hours, as the flow cytometer workflows are often batched.

As part of my research plan investigated EPCs in ACS, being able to capture out of hours patients was potentially important. Therefore, the first objective of this study was to assess the feasibility to perform whole blood flow cytometric assay for endothelial progenitor cells (EPCs), and perhaps more importantly to assess temperature and time stability of whole blood samples to produce accurate EPC counts. This would identify the optimal time frame from venesection to accurate EPC count analysis and possibly demonstrating that whole blood could be safely stored for analyses from patients presenting overnight.

4.3 Methods and materials

Please refer to section 2.7.

Figure 4.1. Study flow diagram



4.4 Patient recruitment

Please refer to section 2.7.1 – 2.7.4

4.5 Inclusion/exclusion criteria

Please refer to section 2.7.3.

4.6 Blood sample acquisition and transport.

Please refer to section 2.7.5

4.7 Sample preparation

Please refer to section 2.6.

4.8 Flow cytometric analysis

The same immunostaining technique was universally performed in all samples without exception and described in section 3.6.2. EPC counts were determined as described in section 3.6.5.

4.9 Statistical analysis

R–statistical programming language (version 3.6.1 St. Louis, Missouri, USA), and Microsoft excel were used for the statistical analysis in this chapter.

The study analysed results in three ways. Single ANOVA was used to assess for any significant difference between EPC count and time at either room temperature or 4^0 C. Two factor ANOVA without replication to assess the interaction between time and temperature.

Finally, dispersion characteristics were used to assess the accuracy of flow cytometric analysis of whole blood cells up to time 12 hours after venesection. The 2 models used were dispersion covariance and distance. 2.6.8.

4.10 Results

This study recruited 11 patients from the pilot study. The study included only stable angina patients. These patients had no history of previous statin therapy. The demographics summarised in table 4.1.

Table 4.1 – summary of patient demographics

		Study group of newly diagnosed angina
	Age	56.7 ±9.6
	Gender [Males]	5/10 [50%]
	Number of comorbidities	1.0 ±0.6
BP	Systolic	127.9 ±18.5
	Diastolic	73.6 ±8.7
Smoking status	Current	1/10 [10%]
	Ex-smoker	6/10 [60%]
	Never	3/10 [30%]
Renal function	Creat umol/l	71.5 ±17.2
	urea mmol/l	9.6 ±12.4
	eGFR ml/min 1.72m2	88.8 ±3.8
Liver function	ALT u/l	22.5 ±4.7
	ALP u/l	77.1 ±16.4
	Bilirubin g/l	9.9 ±3.5
	Hb g/l	150.7 ±12.8
Pre-statin	Cholesterol mmol/l	5.25 ±0.8
	HDL mmol/l	1.28 ±0.3
	LDL mmol/l	3.2 ±0.8
	Triglycerides mmol/l	1.6 ±0.5

Table 4.2: showing the EPC count/ul on day 1 with sequential analysis of time and temperature stability.

Patient	Day 0	Day 1 Time/hours											
		Room temperature						4^{O} Degrees					
		0	2	4	6	9	12	0	2	4	6	9	12
AG-1	2.2	2.9	7.6	0.6	1.1	1.0	3.4	2.1	0.6	0.6	0.8	0.2	3.8
AG-2	1.5	Excluded from study											
AG-3	1.0	1	0	0.5	0.3	0.5	0.7	0.3	0.0	0.5	0.4	0.4	0.4
AG-4	1.1	0.5	0.2	1.0	1.5	1.3	1.4	0.2	0.6	1.4	0.5	1.4	1.5
AG-5	2.7	3.3	5.8	4.7	5.3	6.0	0.5	1.3	4.4	3.6	4.5	0.5	0.5
AG-6	0.8	0.6	1.2	1.1	1.0	0.9	1.0	0.5	0.7	1.0	0.7	0.7	0.5
AG-7	0.3	0.4	0.4	0.3	0.3	0.0	0.0	0.4	0.3	0.2	0.1	0.0	0.0
AG-8	0.3	0.1	0.7	0.7	0.6	1.2	1.2	0.1	0.5	0.4	0.6	0.6	1.2
AG-9	1.9	0.7	2.2	3.4	3.1	2.6	2.9	0.9	2.1	2.2	3.7	1.7	1.3
AG-10	1.8	2.1	2.2	2.4	2.4	0.4	2	2.0	1.7	1.8	2.4	1.4	1.2
AG-11	0.7	1.2	1.3	1.6	1.1	1.9	1.6	1.3	1.3	0.7	1.4	1.5	1.5

The EPC counts verses times were plotted in figure 4.2 below. This figure highlights the variation in EPC counts in each sample over the study period for samples stored at room temperature and 4^{0} C prior to analysis at 0, 2, 4, 6, 9 and 12 hours.

Figure 4.2: Shows EPC count per uL verses time in hours for each angina patient (AG 1- 11) at 4^{0}C (top) and room temperature (below).

Figure 4.2

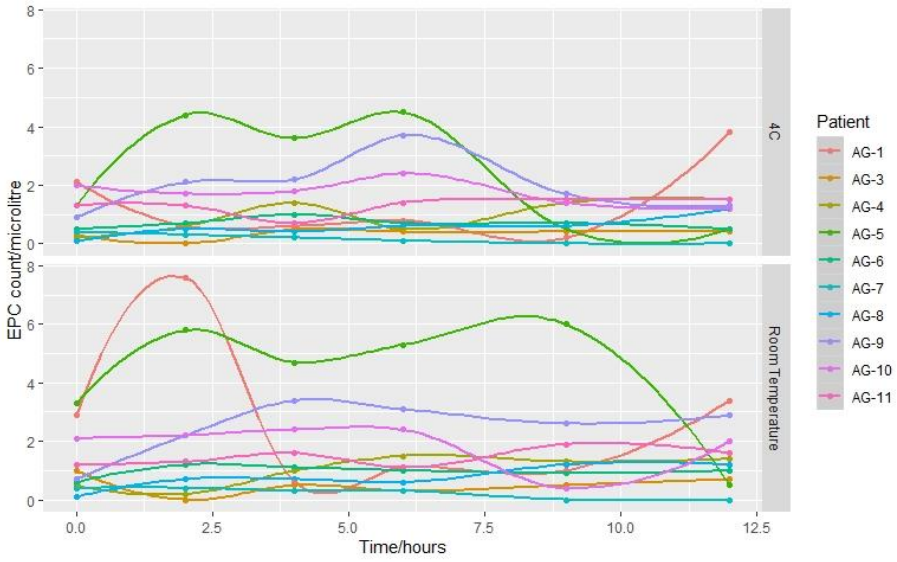


Table 4.3 summarises the mean and standard deviation of the samples at both study temperatures. Figure 4.3 illustrates the average count of EPCs at each time period. Table 4.4 a&b summarises the EPC counts for each patient at room temperature at each time period and the ANOVA statistical analysis.

Table 4.3 The derived mean and standard deviation of EPC counts of each patient at room temperature and 4^0 C

		Patient number											
	Time	1	3	4	5	6	7	8	9	10	11	Av	SD
Room temperature	0	2.9	1.0	0.5	3.3	0.6	0.4	0.1	0.7	2.1	1.2	1.3	1.1
	2	7.6	0.0	0.2	5.8	1.2	0.4	0.7	2.2	2.2	1.3	2.2	2.4
	4	0.6	0.5	1.0	4.7	1.1	0.3	0.7	3.4	2.4	1.6	1.6	1.4
	6	1.1	0.3	1.5	5.3	1.0	0.3	0.6	3.1	2.4	1.1	1.7	1.5
	9	1.0	0.5	1.3	6.0	0.9	0.0	1.2	2.6	0.4	1.9	1.6	1.6
	12	3.4	0.7	1.4	0.5	1.0	0.0	1.2	2.9	2.0	1.6	1.5	1.0
4^0 degrees	0	2.1	0.3	0.2	1.3	0.5	0.4	0.1	0.9	2.0	1.3	0.9	0.7
	2	0.6	0.0	0.6	4.4	0.7	0.3	0.5	2.1	1.7	1.3	1.2	1.2
	4	0.6	0.5	1.4	3.6	1.0	0.2	0.4	2.2	1.8	0.7	1.2	1.0
	6	0.8	0.4	0.5	4.5	0.7	0.1	0.6	3.7	2.4	1.4	1.5	1.4
	9	0.2	0.4	1.4	0.5	0.7	0.0	0.6	1.7	1.4	1.5	0.8	0.6
	12	3.8	0.4	1.5	0.5	0.5	0.0	1.2	1.3	1.2	1.5	1.2	1.0

Key

AV – Average SD – standard deviation

Figure 4.3 Day 1 Mean EPC number verses time (hours) for each temperature

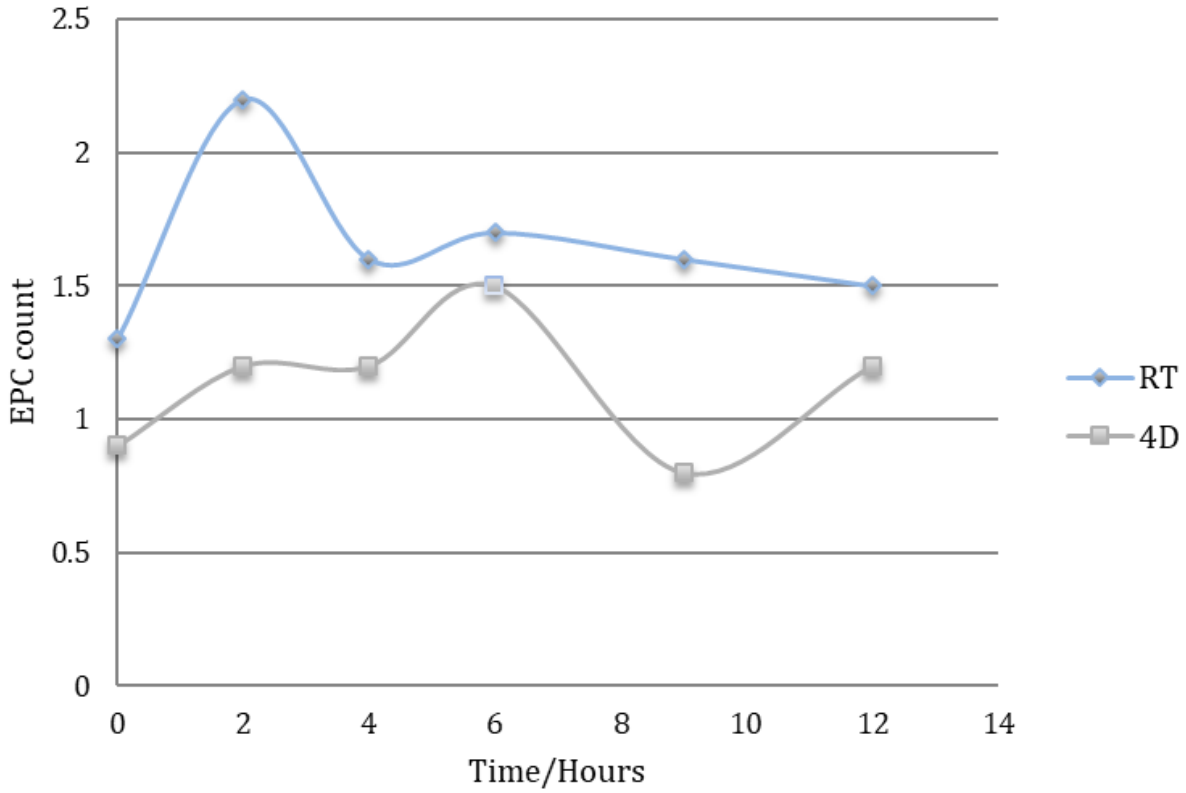


Table 4.4 Summary of EPC counts for each patient at room temperature at each time point and the ANOVA statistical analysis

(a) Summary of the EPC count at times 0, 2, 4, 6, 9 and 12 hours stored at room temperature

	Time/hours					
Patient (AG)	0	2	4	6	9	12
1	2.9	7.6	0.6	1.1	1	3.4
3	1	0	0.5	0.3	0.5	0.7
4	0.5	0.2	1	1.5	1.3	1.4
5	3.3	5.8	4.7	5.3	6	0.5
6	0.6	1.2	1.1	1	0.9	1
7	0.4	0.4	0.3	0.3	0	0
8	0.1	0.7	0.7	0.6	1.2	1.2
9	0.7	2.2	3.4	3.1	2.6	2.9
10	2.1	2.2	2.4	2.4	0.4	2
11	1.2	1.3	1.6	1.1	1.9	1.6

(b) Summary of the one-way ANOVA analyses.

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	10	12.8	1.3	1.2
Column 2	10	21.6	2.3	6.5
Column 3	10	16.3	1.6	2.1
Column 4	10	16.7	1.7	2.4
Column 5	10	15.8	1.6	2.9
Column 6	10	14.7	1.5	1.1

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F ratio*	*F crit*	*P-value*
Between Groups (b)	4.3	5	0.9	0.3	2.4	0.9
Within Groups (w)	146	54	2.7			
Total	151	59				

Key

ss - sum of squares df - degrees of freedom MS Mean squares F ratio MS between groups/MS within groups F crit values for α significance of 0.5

Table 4.5 a&b Summary of EPC counts for each patient at 4 ^{0}C at each time point and the ANOVA statistical analysis.

Table 4.5 EPC count at time 0, 2, 4, 6, 9- and 12-hours samples stored at room temperature.

	Time/hours					
Patient (AG)	0	2	4	6	9	12
1	2.1	0.6	0.6	0.8	0.2	3.8
3	0.3	0	0.5	0.4	0.4	0.4
4	0.2	0.6	1.4	0.5	1.4	1.5
5	1.3	4.4	3.6	4.5	0.5	0.5
6	0.5	0.7	1	0.7	0.7	0.5
7	0.4	0.3	0.2	0.1	0	0
8	0.1	0.5	0.4	0.6	0.6	1.2
9	0.9	2.1	2.2	3.7	1.7	1.3
10	2	1.7	1.8	2.4	1.4	1.2
11	1.3	1.3	0.7	1.4	1.5	1.5

Table X2b One-way ANOVA						
Groups	*Count*	*Sum*	*Average*	*Variance*		
Column 1	10	9.1	0.9	0.5		
Column 2	10	12.2	1.2	1.7		
Column 3	10	12.4	1.2	1.1		
Column 4	10	15.1	1.5	2.3		
Column 5	10	8.4	0.8	0.4		
Column 6	10	11.9	1.2	1.1		

ANOVA						
Source of Variation	*SS*	*df*	*MS*	*F ratio*	*P-value*	*F crit*
Between Groups	2.9	5	0.6	0.5	0.8	2.4
Within Groups	63.9	54	1.2			
Total	66.9	59				

Key

ss - sum of squares df - degrees of freedom MS - Mean squares F crit values α significance of 0.5 F ratio = MS between groups/MS within groups

Table 4.6 a&b summarises the EPC counts for each patient at each time period and the two-way ANOVA statistical analysis.

Table 4.6 a Summarising the EPC counts at both room temperature and 4^0C

	Time/ hours					
	0	2	4	6	9	12
RT	2.9	7.6	0.6	1.1	1	3.4
	1	0	0.5	0.3	0.5	0.7
	0.5	0.2	1	1.5	1.3	1.4
	3.3	5.8	4.7	5.3	6	0.5
	0.6	1.2	1.1	1	0.9	1
	0.4	0.4	0.3	0.3	0	0
	0.1	0.7	0.7	0.6	1.2	1.2
	0.7	2.2	3.4	3.1	2.6	2.9
	2.1	2.2	2.4	2.4	0.4	2
	1.2	1.3	1.6	1.1	1.9	1.6
4^0 C	2.1	0.6	0.6	0.8	0.2	3.8
	0.3	0	0.5	0.4	0.4	0.4
	0.2	0.6	1.4	0.5	1.4	1.5
	1.3	4.4	3.6	4.5	0.5	0.5
	0.5	0.7	1	0.7	0.7	0.5
	0.4	0.3	0.2	0.1	0	0
	0.1	0.5	0.4	0.6	0.6	1.2
	0.9	2.1	2.2	3.7	1.7	1.3
	2	1.7	1.8	2.4	1.4	1.2
	1.3	1.3	0.7	1.4	1.5	1.5

Table 4.6 b Two-way ANOVA analysis

SUMMARY	0 hours	2 hours	4 hours	6 hours	9 hours	12 hours	Total
Room temperature							
Count	10	10	10	10	10	10	60
Sum	12.8	21.6	16.3	16.7	15.8	14.7	97.9
Average	1.28	2.16	1.63	1.67	1.58	1.47	1.63
Variance	1.23	6.47	2.09	2.42	2.97	1.12	2.56
4^0C							
Count	10	10	10	10	10	10	60
Sum	9.1	12.2	12.4	15.1	8.4	11.9	69.1
Average	0.91	1.22	1.24	1.51	0.84	1.19	1.15
Variance	0.54	1.67	1.10	2.31	0.37	1.11	1.13
Total							
Count	20	20	20	20	20	20	
Sum	21.9	33.8	28.7	31.8	24.2	26.6	
Average	1.10	1.69	1.44	1.59	1.21	1.33	
Variance	0.87	4.09	1.55	2.25	1.73	1.08	
ANOVA							
Source of Variation	SS	df	MS	F	P-value	F crit	
Sample	6.91	1	6.91	3.55	0.06	3.93	
Columns	5.10	5	1.02	0.52	0.76	2.30	
Interaction	2.21	5	0.44	0.23	0.95	2.30	
Within	210.57	108	1.95				
Total	224.79	119					

Key

ss - sum of squares df - degrees of freedom MS Mean squares F ratio MS between groups/MS within groups F crit values for α significance of 0.5

The above calculations do not take into consideration the progressive spread of the dots in the histograms over time, but rather reports the outcome of whether a data point is within the zone classified as an EPC or not. This so-called dispersion is illustrated in Figure 4.3. The dispersion can be clearly seen when comparing the two

extremes of time 0 hours and 12 hours in figure 5.4a and b. The dot and plot have a widespread or dispersion of the data points at 12 hours (Figure 4.4a) when compared to data points at 0 hours (Figure 4.4b).

Figure 4.4 a and b dot and plot of EPC analysis at time 0- and 12-hours demonstrating dispersion.

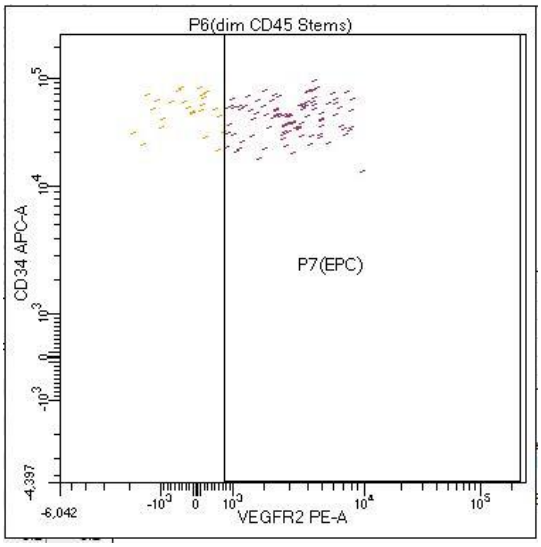


Figure 4.4a
Dot and plot of sample at 0 hours

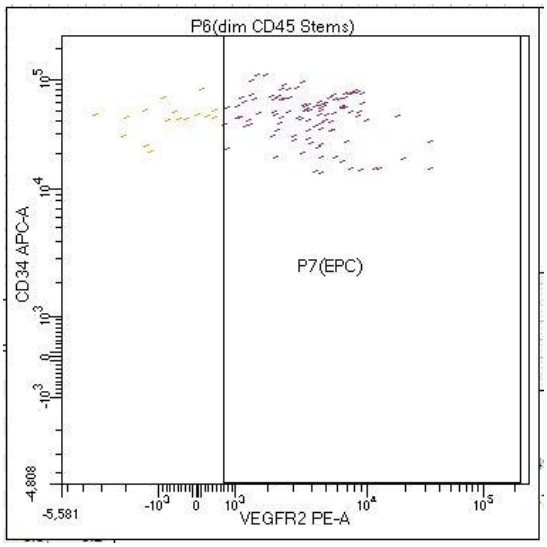


Figure 4.4b
Dot and plot of sample at 12 hours

To mathematically account for the dispersion, we derived two mathematical models. The first 'dispersion covariance' and secondly 'dispersion distance'. Please refer to discussion section.

The calculated dispersion covariance values were plotted against time (hours) Figure 4.5 below.

Figure 4.5; Relationship between dispersion verses time in hours for each patient. Dispersion measured as the determinant of the covariance matrix.

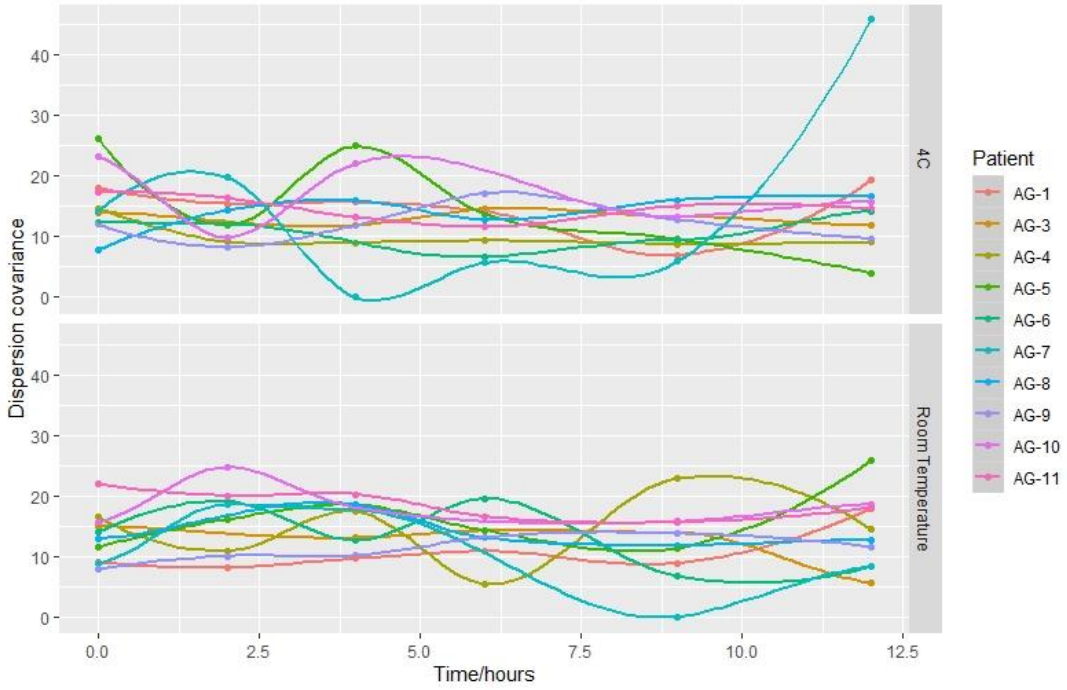
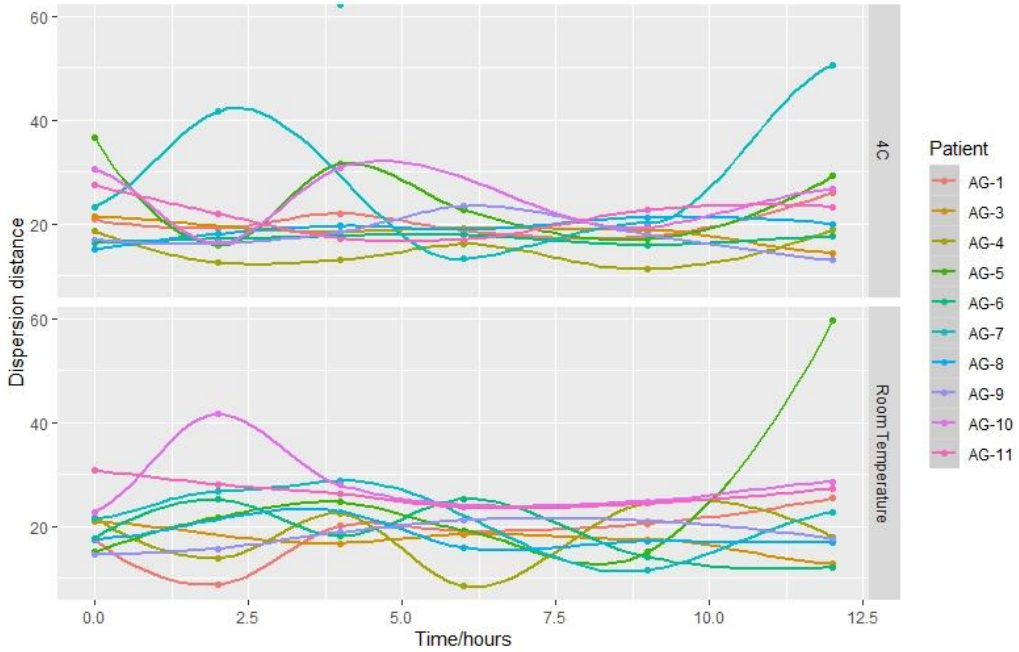


Table 4.7; EPC versus dispersion (as measured as a function of the covariance matrix), and adjusting for time and temperature

	Coefficient	Confidence interval 2.5 - 97.5	P-value
Intercept	0.96	0.16 - 1.75	0.02
Dispersion covariance	0.02	-0.02 - 0.06	0.41
Time/hour	-0.01	-0.07 - 0.05	0.75
Room temperature	0.52	0.01 - 1.04	0.04

Figure 4.6 – Figure showing dispersion verses time in hours per patient. Dispersion as measured as the 2D standard deviation (based on Euclidean distance)



4.11 Discussion

The current standardised flow cytometric protocol for EPC defined as CD45dim, CD34+ and VEGFR2+ recommends all samples should be analysed within 2 hours and stored at 4^0 C. [390] This may not always be possible for 2 main reasons. The first, patients presenting with acute coronary syndromes may be admitted out of normal working hours of haematological laboratories. Secondly, flow cytometry has become an integral part of clinical haematology and immunology and therefore may not always available on an ad-hoc basis. Therefore, the possibility of storing whole blood sample for accurate EPC analyses at greater than 2 hours could be extremely helpful. This study assessed the feasibility and accuracy of EPC count by flow cytometry after storage of whole blood samples for up to 12 hours at either room temperature or 4^0 C (ice).

The average age was 56.7 years with equal gender split. Patients had one co-morbidity including hypertension (40%), osteoarthritis, hypothyroidism, asthma or chronic pulmonary obstructive disease. Only 30% of patients never smoked, 10% were currently smoking and 60% of patients were ex-smokers. The baseline renal and liver functions were within normal range for all patients as per study inclusion/exclusion criteria. Total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL) and triglycerides were measured prior to initiation of statin therapy. Total cholesterol is the sum of HDL, LDL and triglycerides.

The pre-statin therapy total cholesterol and LDL cholesterol levels were 5.25mmol/l and 3.2mmol/l respectively. Both values were higher than the national guidelines recommendations.

Table 4.8. Current national guidelines NICE guidelines recommend

Cholesterol	Recommended value (mmol/l)
Total cholesterol	< 5
HDL	>1
LDL	<3
Triglycerides	<2.3

Recruited patients had on average raised total and LDL cholesterol, were more likely to be either current or ex-smokers and had at least 1 other com-morbidity. This is in keeping with risk factors for coronary artery disease and was expected.

The study analysed the results in three ways. Single ANOVA was used to assess for any significant difference between EPC count and time at either room temperature or 4^0 C. The second statistical analysis was ANOVA two-factor without replication to assess the interaction between time and temperature. The third method was to

describe dispersion characteristics to assess the accuracy of flow cytometric analysis of whole blood cells. The study found no clear relationship between EPC count and time was seen. The study set out to assess if accurate flow cytometric analyses was possible after storage of whole blood at either room temperature or 4^0 for as long as 12 hours. The study found no statistical difference in EPC count if samples were analysed at time 0, 2, 4, 6, 9 or 12 hours at either room temperature of 4^0 C. These results suggest that whole blood samples could be stored at either room temperature or 4^0 C for up to 12 hours prior to accurate flow cytometric analyses. However, this relates to the simple numerical value, of the data plot being bound in the zone designated of likely to represent an EPC within the flow cytometry software. Albeit reassuring that we do not appear to have to rush to the flow cytometer machine with samples on ice, and within the hour to get meaningful data about raw EPC numbers, it tells us nothing about whether the spread of those data points over the analysis time period or by storage temperature. The study found no significant difference with temperature and no interaction between temperature and time. However, there was a borderline significance p=0.06 between EPC count at 4^0C and room temperature and therefore with greater number of observations may have proven to be significant. The determination of EPC count had an inherent limitation that relied observer bias on demarcating and area on P-7 of dot and plot graphs. The above simplified statistical analysis of raw numbers with binary values for EPC counts found that samples could be stored at either room temperature or 4^0 C and analysed by flow cytometry up to 12 hours after venesection.

By looking more closely at the dot and plot values at time 0 to those at 2, 4, 6, 9 and 12 hours a wider distribution and in some cases the presence of two different subsets become apparent. Figure 4.4. This dispersion may have led to inherent accuracies in EPC count determination. Dispersion may be in part at least be due to the phenomenon of capping [401]

Therefore, the second part of the study aimed to derive mathematical models to explain the observed dispersion. These models assessed for any relationship between dispersion and time. The study hypothesis was a proportional relationship between time and dispersion. That is, the greater the time from venesection to flow cytometric analyses, the greater the dispersion of the flow cytometer data points. The dispersion as defined in this model as a covariance matrix that suggests that no significant spread or dispersion occurs over 12-hour study period. The intercept acts a base line and assesses the relation between dispersion and EPC excluding the cofounders of time and temperature. The intercept predicted EPC count at 4^0C degrees at first time point (T at 0 hours) with no dispersion. However, when adjusting for time and temperature there was a significant correlation between temperature and EPC number p=0.04. Therefore, samples stored at room temperature have greater dispersion and less accurate results than samples stored at 4^0C. This is in keeping with the study hypothesis. Dispersion distance was then plotted against time. Figures 4.5 and 4.6 illustrate dispersion covariance and distance respectively against time (hours). Both illustrations are similar. This is reassuring as both models are analysing the same data albeit as different mathematical models. With EPC versus dispersion when measured as a function of the covariance matrix and adjusting for time and temperature shows

no significant difference. Therefore this study suggests that flow cytometric analysis of whole blood samples for EPCs defined as CD45dim, CD34+ and VEGFR2+ can be accurately performed up to 12 hours after venesection. However, samples should be stored at 4^0 C. However, the limitations of this study are the small sample size. Secondly there was a lack of negative controls in this study of healthy non-patient individuals.

4.12 Conclusion

This study confirmed the feasibility of flow cytometric analysis for EPC from whole blood samples. Current flow cytometric protocols recommend flow cytometric analyses of whole blood samples within 2 hours of venesection and storage at 4^0 C. This study suggests that samples may be stored at for as long as 12 hours. This study suggests that whole blood storage at room temperature may be inferior to storage of samples at 4^0 C.

However, this pilot study paves the way for larger studies to fully elucidate the length of storage of whole blood samples.

Chapter 5

Computerised Tomography Coronary Artery Scoring and Endothelial Progenitor Cell Count

5.1 Abstract

***Introduction*:** Traditional risk factors for coronary artery disease are also know to effect EPC count, with the combinations of cardiovascular risk factors together with reduced numbers of EPCs have a cumulative effect. Earlier and current national guidelines have recommended cardiac computerised tomography and coronary calcium scoring as first line investigations for chest pain. The study aim was to find an association between computerised tomographic coronary angiography calcium score (CACS) and EPC count. The study hypothesis was a linear relationship between EPC count and CACS that is the greater the CACS the greater the number of EPCs.

***Methods*:** The study recruited 23 patients undergoing outpatient coronary artery calcium scoring (CACS) as part of clinical care. Potential participants were given an information sheet on the day of planned computerised tomography (CT) and given at least 24 hours to consider participation. On consenting and 2 weeks after CT scan a single blood test of 3-5ml of whole blood into an EDTA tube underwent flow cytometric analysis.

***Results*:** The study found an inverse trend between coronary artery calcium score and number of endothelium cells ($CD34^+$, $VEGFR2^+$ $CD45^{dim}$).

***Conclusion*:** No significant association between CACS or EPC counts was found. There were several inherent limitations to the study.

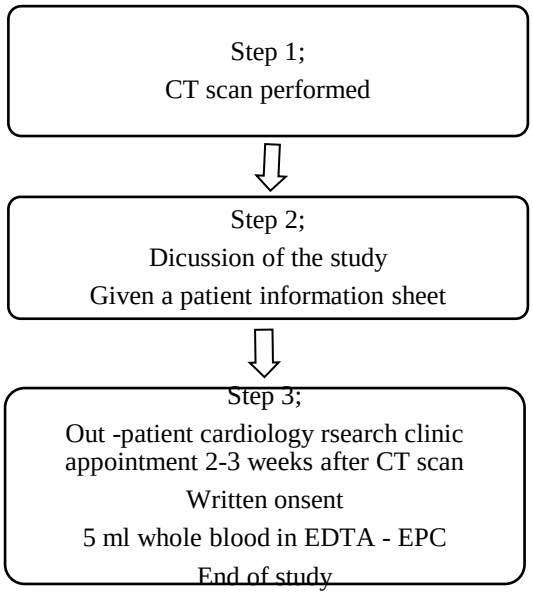
5.2 Introduction

Several studies found a prognostic value of the coronary calcium scoring in both asymptomatic [402-407] and symptomatic patients. [408-410] Coronary artery calcium scoring (CACS) has been found to be strong independent predictor of future cardiac events. [407, 411] The greater the calcium score, the greater the risk of future cardiac events. [410][412] Several studies have shown an association between calcium scoring and traditional cardiovascular risk factors. There are a number of well-recognised risk factors for atherosclerosis and a number of studies have highlighted an association between these traditional risk factors and number or function of EPCs. [19, 29, 39, 41-51, 53, 135, 164-166, 168-170, 172-181, 187] With some authors proposing circulating EPCs a better predictor of cardiovascular risks than presence or absence of traditional cardiovascular risk factors. [19] This study assessed for a possible association between CACS and EPC counts.

5.3 Study design and methodology

Please refer to section 2.7.4.

Figure 5.1: Summary of study protocol



Step 1: CTCA scan performed as planned.

Step 2: Patient information sheet given to potential participants and given at least 24 hours to consider participation in the study

Step 3: Participants had an out-patient appointment in the cardiology research clinic 2-3 weeks after the CTCA to ensure no artefactual effect on EPC count after venflon insertion for CTCA imaging. 5 ml whole blood sample taken to undergo flow cytometric analysis for EPC after written consent.

5.4 Sample preparation and transport.

Please refer to section 3.7.5

5.5 Flow cytometric analysis

Please refer to section 3.6.

5.6 Statistical analyses.

Simple linear regression was used to assess for a possible relationship between EPC count and CACS but also attempt to quantify the strength of any relationship. This was quantified by Pearson correlation co-efficient (R) and P value.

5.7 Results

The demographics of the 23 recruited patients are shown in table 5.1.

Table 5.1: Patient demographics and significant co-morbidities

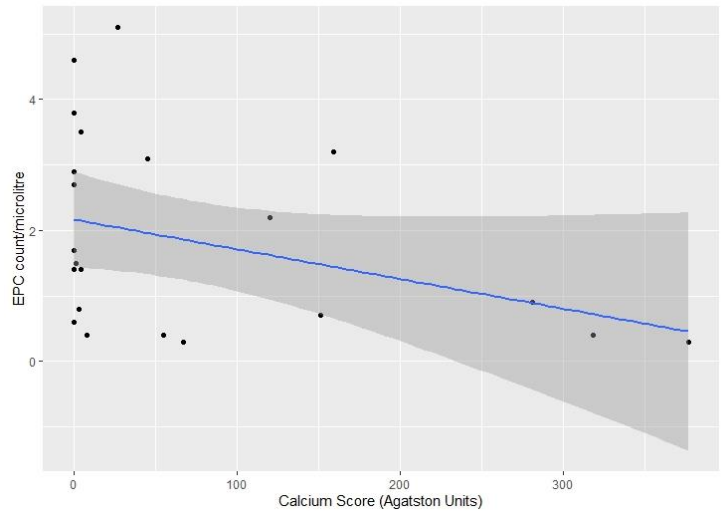
Patient demographics	Number (%)
Age (years) Average (SD)	54.7 (8.6)
Gender	
Male	16 (70)
Female	7 (30)
Smoker	
Current	3 (13)
Ex	9 (39)
Never	11 (48)
Comorbidities	
Hypertension	4 (17)
PAF	1 (4)
COPD/Asthma	3 (13)
Arthritis	1 (4)
Hyper or Hypothyroidism	3 (13)
Previous anaemia	1 (4)

The EPC count and CACS for each patient are summarised in table 5.2. These are plotted in figure 5.2 as black date points. The regression line is coloured blue with 95% confidence intervals in dark grey. Table 5.2 summarises the coronary calcium score and EPC count for each patient.

Table 5.2: EPC count and calcium score

EPC count/μl	CACS
0.6	0
1.7	0
0.8	2.8
0.6	0
3.8	0
1.4	0
3.2	159
4.6	0
2.7	0
0.9	281
0.4	318
1.5	1
3.5	4
0.4	55
0.4	8
0.7	151
5.1	27
2.9	0
3.1	45
0.3	67
2.2	120
0.3	377
1.4	4

Figure 5.2: Figure showing EPC count verses Calcium score



Key .

Black dots – data series Blue line represents the mean value Grey area – confidence interval. Note part of graph lots of points narrower gray area the fewer points larger gray area ie confidence interval.

Table 5.5 a-c summarises the regression statistical analysis of the study.

Table 5.3 (a-c)

(a)						
Multiple R	0.347					
R Square	0.120					
Adjusted R Square	0.079					
Standard Error	1.419					
Observations	23					
(b)						
	df	*SS*	*MS*	*F- stat*	*Significance F (α .05)*	
Regression	1	5.790	5.790	2.874	0.105	
Residual	21	42.307	2.015			
Total	22	48.097				
(c)						
	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	2.168	0.351	6.177	0.000	1.438	2.897
CACS	-0.005	0.003	-1.695	0.105	-0.010	0.001

Key

ANOVA analysis of variance Df = degrees of freedom Ss = sum of squares Mean sum off squares ms= ss/df F = MS regression/MS residual k = independent variable = 1 F value for alpha α 0.05 F same as p value in simple linear regression

Finally, adjusted the model was assessed for confounders of age, gender, hypertension, hypercholesterolemia and smoking status against the linear regression model. Table 5.4. These were calculated with R – statistical programming language, version 2.6.1.

Table 5.4; Correlation between EPC and Calcium adjusted for confounders

	Co-efficient	Confidence interval		P-value
		2.5	97.5	
Intercept	0.795	-3.835	5.424	0.62
CACS	-0.002	-0.009	0.005	0.50
Age	0.031	-0.047	0.111	0.40
Gender (M)	-1.224	-2.757	0.308	0.11
Hypertension	0.035	-1.853	3.537	0.97
Hypercholesterolaemia	1.312	-0.913	3.537	0.23
Current smoker	0.396	-2.013	2.805	0.99
Ex- smoker	0.405	-0.890	1.700	0.73

5.8 Discussion

Traditionally invasive coronary angiography had been the most reliable modality used to assess the severity of coronary artery disease. However invasive angiography has an associated complication rate. [414] This, and technological advancements has led to the use of non-invasive computerised tomography of coronary arteries (CTCA). One of the advantages of CTCA is the ability to calculate calcium scores, quantified in Agatston Units. Coronary artery disease results from atherosclerotic plaques within the walls of coronary arteries. This results in a spectrum of symptoms from stable

angina at one end ranging to acute myocardial infarction. Anatomical burden of coronary artery disease has also long been linked with risk of cardiac events. [415] With time calcium deposition begins to occur within the lipid laden coronary artery plaques. Coronary artery calcification (CAC) results in reduced vascular compliance, abnormal vasomotor responses, and impaired myocardial perfusion. The presence of CAC is associated with worse outcomes in the general population and in patients undergoing revascularization. There are two recognised types of coronary artery calcification (CAC), medial and atherosclerotic or intimal. Medial artery calcification has been associated with age, chronic kidney disease and diabetes. This results in arterial stiffness and may increase adverse cardiovascular events. However, of greater interest is atherosclerotic calcification that occurs within the intima. The deposition of calcium occurs in areas of high lipid content and elevated inflammatory markers within atherosclerotic plaques. CAC has been associated with greater plaque burden. This is a chronic process rather than an acute process. The result of these micro calcifications within the fibrous cap increases the risk of micro-plaque rupture, hemorrhage, and healing. Reoccurrence of this process results in obstructive fibro-calcification lesions and subsequent symptoms of angina. Studies have shown an increase in CACS in predicting progression of atherosclerotic plaques and future cardiovascular risk This study defined EPC population of interest within our study as $CD45^{dim}$, $CD34^{+}$ and $VEGFR2^{+}$ based on earlier studies [16, 28, 100, 101, 103, 104, 118, 121, 123, 124] A number of studies have highlighted an association between traditional risk factors and number or function of EPCs. [19, 29, 39, 41-51, 53, 165, 166, 168-170, 172-181] This study investigated a possible association between calcium scoring and EPC count.

Simple linear regression was used to assess for a possible relationship between EPC count and CACS but also attempt to quantify the strength of any relationship. This study found R value of 12%. In other words, only 12% of the values were on the line of best fit. The calculated multiple R or Pearson correlation co-efficient was found to be -0.34. Table 6.3 (a). $R^2_{adjusted}$ value of 0.079, therefore only 7.9% of the variation in EPC was explained by the variation in CACS of the total number of observations of 23. Adjusted R^2 was not calculated as limited value for single variable, as in this study. Therefore, the study no relationship. However, the study found an inverse trend with EPC count decreasing with higher CACS. The reason for lower EPC counts were not elucidated in this study and could be due to high coronary artery disease burden but may be due to decreased mobilisation or great consumption of due to maturation of the $CD45^{dim}$, $CD34^+$ and $VEGFR2^+$ EPC and therefore expression of different cell surface markers.

There are several well-recognised risk factors of atherosclerosis including age, physical activity, diabetes, dyslipidaemia, hypertension, smoking and family history. Table 6.4 summarised the effect of confounders of age, gender, hypertension, hypercholesterolemia and smoking status. Interestingly none of the patients included in the study had diabetes. Of note the p-values for CACS is different when comparing table 6.3 and table 6.4 at 0.105 and 0.5 respectively. This is because table 6.3 summarises the model that only considers a single variable CACS, so the values calculated are relating to only that variable and its impact on the model. However, table 6.4 shows how different covariates affect the outcome, so considering the effect of all the different variables in relation to the others. Looking at this, CACS is

contributing less to the model with multiple variables than in the single model. The confounders of age, gender, hypertension, hypercholesterolemia and smoking status had no significant effect on EPC count.

The main limitation of the study was recruitment of only 23 patients. There were three main reasons accounting for the lack of recruitment. First, the study's inclusion criteria recruited only statin naïve patients. Secondly, change in structure of rapid chest pain clinics. During the study period nurse led chest pain clinic were introduced. These specialised nurses led chest pain clinics more stringently followed the 2010 chest pain guidelines. This subsequently led to CTCA being performed in low-risk patients that tended to have lower CACS and limiting any conclusion regarding possible relationship between EPC count and higher calcium scores. Secondly, the clinics initiated angina treatment based on clinical judgement as recommended by the 2010 guidelines. [413] Therefore patients with typical angina symptoms were started on statin therapy prior to CTCA. This led to exclusion of these patients within the study. Finally, the study was initiated during 2010 NICE guidelines [413] that were updated during the study recruitment period to the 2016 NICE guidelines that no longer necessitated use of pre-test probabilities to guide further investigations. Despite cost saving projected [416] the 2010 NICE guidelines raised several concerns. Firstly, the guidelines overestimated the prevalence of CAD particularly in females secondary to the increased use of therapy for risk factor modification.[417] Secondly, the guidelines based the use of CACS on studies with asymptomatic patients.[418] Thirdly CTCA was only performed in patients with an elevated Agatston score. Fourthly, the guidelines recommended functional imaging in patients with intermediate PTL. This inherently

led to an increased number of both false positive and false negative results based on sensitivity and specificity of these techniques. [419] However the 2016 guidelines recommended functional imaging tests being reserved for the assessment of patients with chest pain and known CAD and for patients where the CTCA is equivocal or has shown CAD of uncertain significance. [414] These guidelines' [414] have been validated by large-scale randomised controlled trials **[420-423]** With one study finding cardiac CT in addition to standard care in patients with stable chest pain resulted in a significantly lower rate of death from coronary heart disease or nonfatal myocardial infarction at 5 years. [424]

5.9 Conclusion

The study found no significant correlation between EPCs, identified by $CD34^+$, $VEGFR2^+$ and $CD45^{dim}$ and Coronary artery calcium score. However there appeared to be a trend of decreased EPCs with higher CACS. The study did not elucidate the cause for the observed trend of lower EPC count with higher coronary artery disease burden. However, may include decreased mobilisation, great consumption, maturation of the $CD45^{dim}$, $CD34^+$ and $VEGFR2^+$ EPC and therefore expression of different cell surface markers and/or a combination of all these factors. The inherent limitation of this study was inclusion of only 23 patients that inherently included only low risk population with few traditional risk factors for coronary artery disease, driven by guideline changes nationally.

Chapter 6

Mobilisation of Endothelial Progenitor Cells and statin therapy: A pilot – proof of concept study

6.1 Abstract

***Introduction*:** Guidelines recommend intense and moderate therapy with atorvastatin 80mg and 20mg in patients with acute coronary syndrome (ACS) or angina (AG) respectively. This study aimed to identify any differences in EPC response between intense and moderate statin therapy over a 28-day period. The hypothesis was that ACS patients given 80mg atorvastatin would have greater number of EPCs

***Method*:** New onset AG participants were statin naïve. Whereas ACS participants were either statin naïve or established on moderate dose of atorvastatin. All ACS participants were loaded with 80mg atorvastatin. Both groups had their first blood test done at the time of diagnosis (day 0) and repeated after 1, 3, 7 and 28 days. The first blood test on day 0 also included full blood count, lipid profile, liver and kidney function. Two amendments were made during the study. The first amendment was flow cytometric analyses within 2 hours rather than immediately after venesection. However, all whole blood samples were stored at 4^0C. The second amendment was

inclusion of participants with eGFR ≥ 60 ml/min/1.73m^2 rather than ≥ 60 ml/min/1.73m^2.

***Results*:** In total 21 patients were recruited with 11 AG patients enrolled into the study and 10 patients completed follow up. Eight out of 10 patients completing follow up in the ACS group. The study had equal gender split in AG group with a male bias (90%) within the ACS group. No significant difference in the number of comorbidities between ACS and AG group were found. There was a significant difference in ALT in the ACS group. No significant difference in full blood count or renal function was observed between AG and ACS patients. There was borderline significant difference between cholesterol and LDL levels at the end of the day at day 28 between ACS and AG patients. No patient reported any adverse events with atorvastatin therapy. The study showed a non-significant initial increase in the number of EPCs within the first 3 days post atorvastatin therapy. There was no relationship between EPC count and ACS, age, gender, number of comorbidities or systolic blood pressure when adjusting area under the curve for these variables. Interestingly, cholesterol was found to have no significant effect on EPC count.

***Conclusion*:** No significant increase in EPC count was found within or between AG and ACS patients. However, the study observed an initial increase of EPC counts within the first 72 hours with gradual decrease by 28 days. A significant and borderline difference were seen in ALT and cholesterol/LDL levels respectively. The study observed no adverse reactions to atorvastatin therapy.

6.2 Introduction

This study aimed to identify any differences in EPC response between ACS treated with intense statin therapy (atorvastatin 80mg) and AG patients treated with moderate statin therapy (atorvastatin 20mg) over a 28-day period, both international guidelines driven dose choices.

6.3 Study design

This study had full Integrated Research Application Service, Research and Development Department of Royal Stoke University Hospital and ethical approval. Please refer to sections 2.7.1, 2.7.2 and 2.7.3, summarised in table 6.1a and 6.1b below

6.4 Blood sample acquisition

Please refer to section 2.7.5.

6.5 Study protocol amendments

Two protocol amendments were successfully granted. The amended flow diagrams are demonstrated in Figure 6.2a&b.

6.5.1 Time and temperature stability

The first amendment related to time and temperature study. The initial protocol was to perform flow cytometric analysis of whole blood samples straight after venesection. This was amended to all samples being stored at 4^0C and analysed within 2 hours after

venesection. This was from chapter 5 in this thesis that demonstrated validity of cytometric analyses of whole blood samples.

6.5.2 Renal function (eGFR)

The second amendments related to renal function. The initial exclusion criteria included an eGFR less than 90 ml/min/1.73m^2. Patients may be initiated with atorvastatin 80mg and secondly undergo coronary angiography with eGFR ≥ 60 ml/min/1.73m^2. This amendment allowed a greater number of patients to be eligible for recruitment into the study.

Figure 6.1a: Flow diagram for patients admitted with ACS

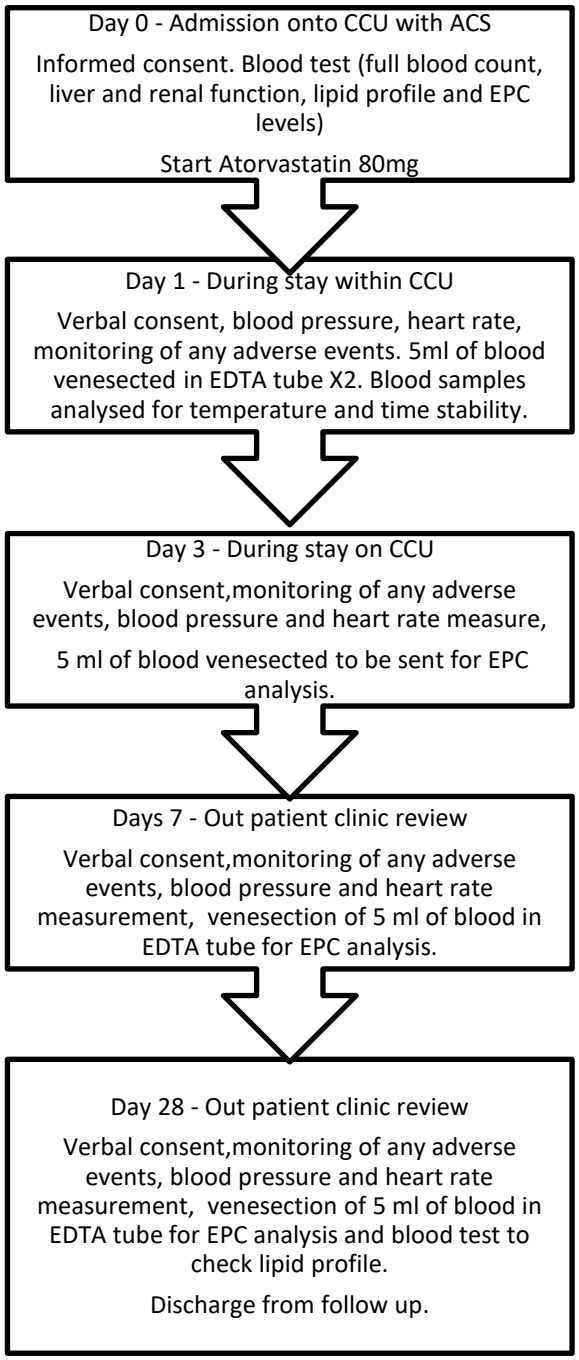


Figure 6.1b: Flow diagram for patients of newly diagnosed angina

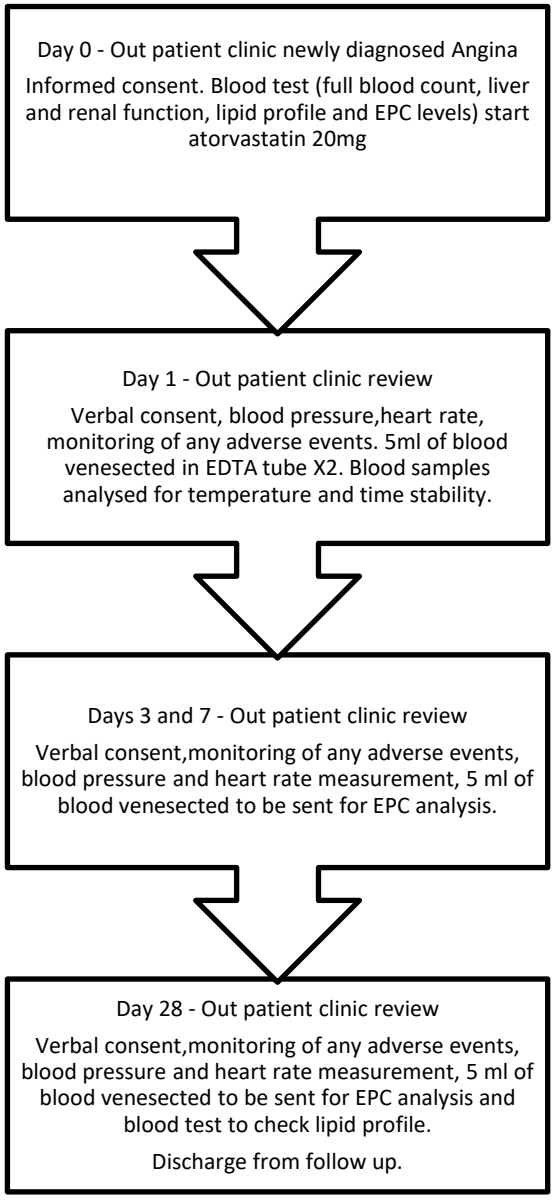


Figure 6.2a: Amended flow diagram for patients admitted with ACS

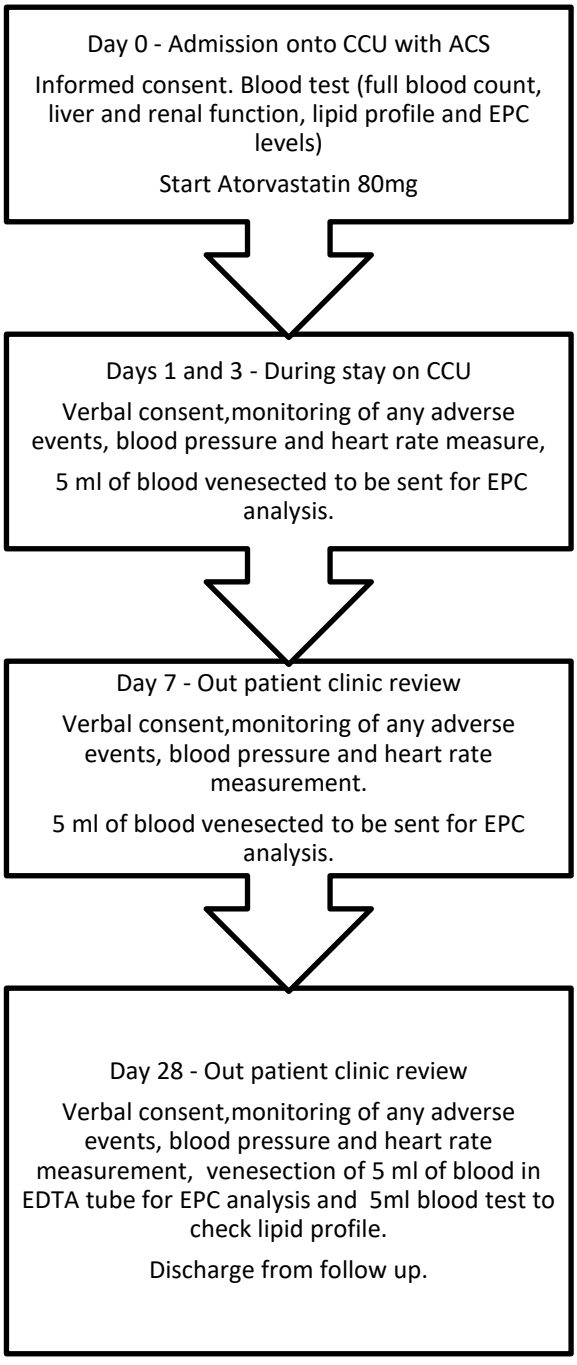
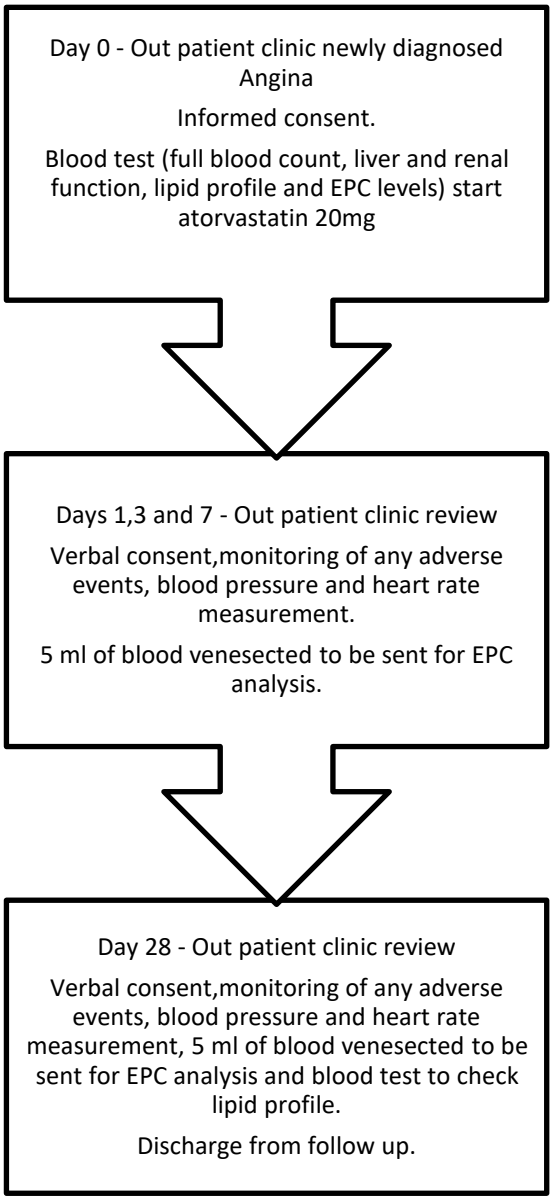


Figure 6.2b: Amended flow diagram for patients of newly diagnosed angina



6.6 Sample preparation

The same immunostaining technique was universally performed in all samples without exception and described in section 3.6.5.

6.7 Flow cytometric analysis

The same immunostaining technique performed in all samples without exception and described in section 3.6. The EPC counts were calculated as described in section 3.6.5.

6.8 Statistical analysis

The study used Microsoft Excel software for calculating means, standard deviation and P-values, and under the curve AUC box and whisker charts.

The increase in EPC values were calculated as:

Relative increase day 1 (RD1) = EPC day 1 – EPC day 0

Relative increase day 3 (RD2) = EPC day 3 – EPC day 0

Relative increase day 7 (RD7) = EPC day 7 – EPC day 0

Then, the area under the curves were estimated as follows:

$$0.5*(RD1) + 0.5*(RD3 + RD1)/2 + 0.5*(RD7 + RD3)/4$$

R – statistical programming language (version 3.6.1 St. Louis, Missouri, USA) was used for all interpolation curves and for deriving correlation between of EPC between AG and ACS adjusted for confounders.

SPSS 27 (IBM, USA) was used to calculate the one-way ANOVA with repeated measures and Friedman's Test, both using the Bonferroni correction for multiple comparisons.

6.9 Results

In total 21 patients were recruited into the study with 11 and 10 patients in the AG and ACS groups respectively. One patient in the AG group was excluded from the study due to the inability to attend follow clinic. Therefore 10 patients in each group were studied. Patient demographics are summarised in table 6.1.

Table 6.1. Study patient demographics

		Angina	ACS	P-value
Age		56.7 ±9.6	58.8 ±6.1	0.57
Gender [Males]		5/10 [50%]	9/10 [90%]	0.14
N. Comorbidities		1.0 ±0.6	0.6 ±0.9	0.29
BP	Systolic	127.9 ±18.5	122.0 ±13.6	0.42
	Diastolic	73.6 ±8.7	74.3 ±11.1	0.87
Smoking status				
Current		1/10 [10%]	5/10 [50%]	
Ex-smoker		6/10 [60%]	4/10 [40%]	
Never		3/10 [30%]	1/10 [10%]	
Renal function	Creatine (umol/L)	71.5 ±17.2	76 ±15.1	0.54
	Urea (mmol/L)	9.6 ±12.4	5.8 ±1.6	0.35
	eGFR (ml/min 1.72m2)	88.8 ±3.8	86.5 ±6.5	0.35
Liver function	ALT (u/L)	22.5 ±4.7	62.8 ±39.8	0.01
	ALP (u/L)	77.1 ±16.4	67.6 ±14.7	0.19
	Bilirubin (g/L)	9.9 ±3.5	8.3 ±1.9	0.23
	Hb (g/L)	150.7 ±12.8	144 ±10.3	0.21
Pre-statin	Cholesterol	5.25 ±0.8	5.23 ±0.9	0.95
	HDL	1.28 ±0.3	1.15 ±0.5	0.49
	LDL	3.2 ±0.8	3.5 ±1.1	0.55
	TG	1.6 ±0.5	1.4 ±0.9	0.62
End of study	Cholesterol	3.6 ±0.8	3.1 ±0.5	0.09
	HDL	1.12 ±0.25	1.03 ±0.39	0.55
	LDL	1.99 ±0.76	1.46 ±0.33	0.08
	TG	1.23 ±0.37	1.3 ±0.48	0.75

The EPC counts per micro-litre for the AG and ACS groups over the 28 days study are summarised in table 6.2. Whereas EPC curves of all of the patients are shown in figure 6.3.

Table 6.2: EPC count per micro-litre (µl) over the study period for both angina (AG) and acute coronary syndrome (ACS) group.

Group	Patient number	Day 0	Day 1	Day 3	Day 7	Day 28
AG	1	2.2	2.9	0.2	0.2	0.7
	2	1.5	-			
	3	1.0	1.0	0.8	0.3	0.0
	4	1.1	0.5	1.8	0.6	2.6
	5	2.7	3.3	2.5	3.1	2.3
	6	0.8	0.6	0.4	0.3	0.4
	7	0.3	0.4	0.3	0.6	1.2
	8	0.3	0.1	0.3	1.2	1.3
	9	1.9	0.7	2.6	2.3	1.7
	10	1.8	2.1	2.4	2.7	1.4
	11	0.7	1.2	1.6	1.7	1.2
ACS	1	0.8	2.3	0.2	1	1.5
	2	0.8	0.7	0.4	-	
	3	2.7	1.2	4	0.7	0.7
	4	1.8	2.1	1.6	1.7	2
	5	0	1.7	2	0.6	0.9
	6	1.1	0.5	1.1	0.2	0.5
	7	0	0.4	0.9	0.9	0.5
	8	0	2.4	2	1.5	2.2
	9	1.4	2.2	0.7	1.4	-
	10	0.3	2.1	0.8	1.4	1.2

Key

- Excluded from the study failure of patient to attend follow up appointments

Figure 6.3 shows the heterogenic response to atorvastatin in both ACS and AG groups.

Figure 6.3; Interpolated EPC curves of all patients.

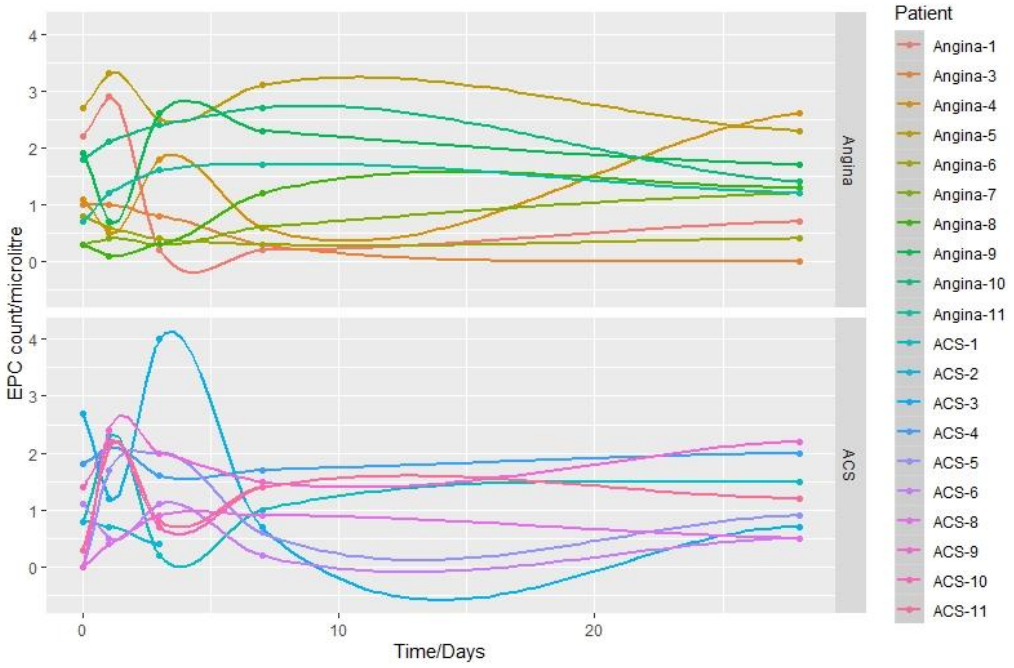


Table 6.3 statistically summarises the data.

6.9.1 Within group analysis

Visually each group looked broadly similar when evaluated over time. This was confirmed by both parametric (repeated measured analysis of variance) and non-parametric testing (Friedman's Test).

The small group size suggest that the data will not be normally distributed, and that nonparametric testing would be more logical. Therefore, a Friedman's Test was undertaken. There was no difference with either the angina group (p=0.98) or the ACS group (p=0.19)

The one way repeated measured ANOVA showed no change over time in the angina group (p=1.00) or the ACS group (p=0.27).

This confirms no difference in EPC levels within each group over time with both parametric and non-parametric testing.

6.9.2 Between groups analysis

Table 6.3 Summarises average, standard deviation and P values for EPC counts between AG and ACS groups

Study day	AG	ACS	P-value
Day 0	1.28 ±0.82	0.89 ±0.89	0.32
Day 1	1.28 ±1.11	1.56 ±0.79	0.52
Day3	1.29 ±1.00	1.37 ±1.11	0.86
Day7	1.29 ±1.1	1.04 ±0.49	0.51
Day 28	1.28 ±0.80	1.19 ±0.66	0.79

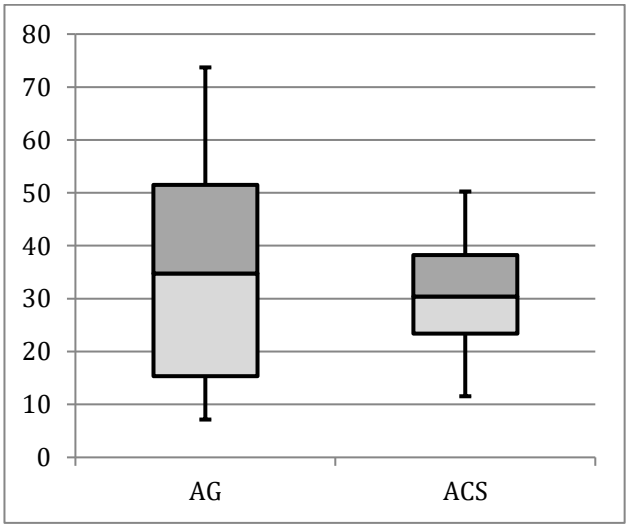
P - Two tail t test – 2 sample assuming unequal variances.

No significant statistical difference between EPC count for AG day 0 and day 28 or ACS days 0 and day 28. The area under the curves for EPC counts (AUC-EPC) were calculated for each group over the 28-day study period for the patients that completed the entire study. A box and whisker plot was constructed - figure 6.4.

Table 6.4 Summary of the area under the curve for entire study

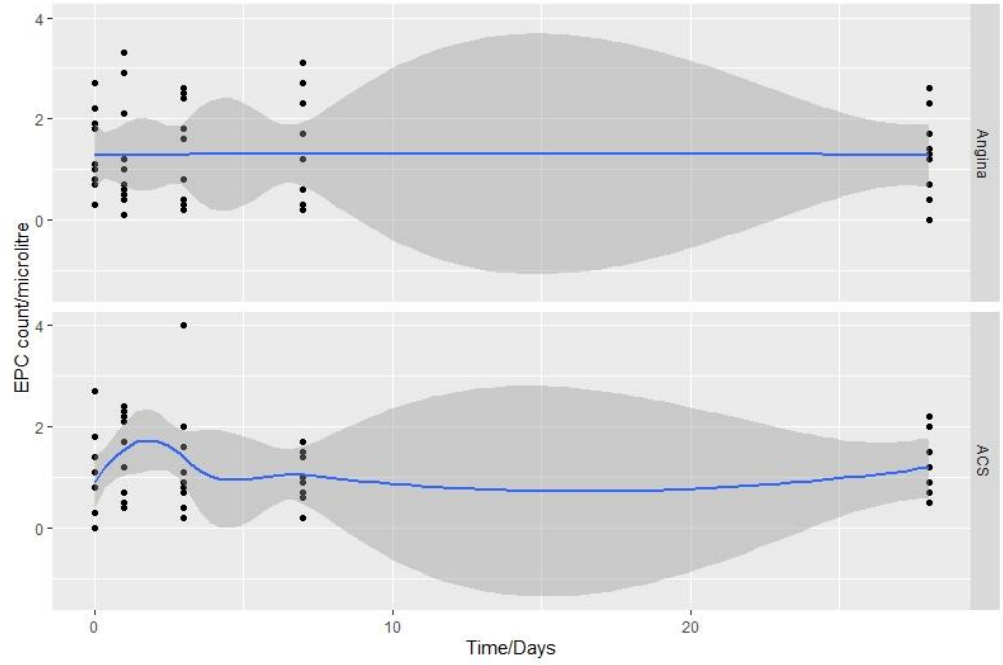
	Patient number	Area under the curve (AUC)
AG	1	13.35
	2	-
	3	7.15
	4	40.7
	5	73.7
	6	9.75
	7	21.4
	8	29.65
	9	55.1
	10	57.75
	11	39.85
ACS	1	31.5
	2	-
	3	29.3
	4	49.15
	5	24.65
	6	11.55
	7	19.6
	8	50.25
	9	-
	10	34.6

Figure 6.4: Comparison of the Area under the EPC curves (AUC-EPC) between the ACS and AG groups.



Key The boxes represent 1st and 3rd quartiles and line within the box following T-student distribution

Figure 6.5 Interpolation curves for AG (upper graph) and ACS (lower graph) groups



Key Blue line represents the mean value, grey area – confidence interval

The final table showing the correlation between EPC count and ACS when adjusted for the co-variables of age, gender, number of comorbidities, blood pressure, smoking status and pre-statin therapy cholesterol levels.

Table 6.5 Correlation between of EPC and ACS when adjusted for confounders

		Confidence interval (%)		
	Estimate	2.5	97.5	P Value
Intercept	2.497	-2.485	7.481	0.290
ACS	0.641	-0.373	1.657	0.189
Age	-0.043	-0.089	0.002	0.059
Gender	-0.778	-1.744	0.186	0.102
Number of co-morbidities	-0.484	-1.007	0.037	0.065
Systolic	0.012	-0.019	0.045	0.395
Diastolic	0.042	-0.001	0.086	0.058
Current smoker	-1.763	-3.208	-0.319	0.021
Ex-smoker	-1.755	-2.931	-0.579	0.007
Cholesterol (pre-statin therapy)	-0.145	-0.595	0.304	0.486

6.10 Discussion

In total 21 patients were recruited into the study. In total 11 AG patients enrolled into the study with only 10 patients completed follow up. Eight out of 10 patients completed follow up in the ACS group. The study had equal gender split in AG group with a male bias (90%) within the ACS group. This is perhaps not surprising given the prevalence of coronary artery disease in men. The co-morbidities were represented by numerical values rather than category due to the low number of patients in the study. Interestingly, no significant difference in the number of comorbidities between ACS and AG group were found. There was a significant difference in ALT in the ACS group. This could be secondary to an acute inflammatory response seen in ACS patients. The use of ALT/AST has some historical importance. Prior to troponin assays, AST/ALT were part of a panel of enzymes, including CK-MB to assess for myocardial injury. No significant difference in full blood count or renal function was observed between AG and ACS patients. There was borderline significant difference between cholesterol and LDL levels at the end of the day at day 28 between ACS and AG patients, likely due to the differing doses used in each group; 20mg vs 80mg angina vs ACS respectively. No patient reported any adverse events with atorvastatin therapy in the study, in keeping with larger scale studies concluding the safety of statin therapy. [226, 232, 233]

Figure 6.3 is a graphical representation of the data and clearly demonstrate the wide distribution of the EPC counts within each patient group. However, an overall impression is that of an initial increase in the number of EPCs within the first 3 days post atorvastatin therapy followed by a plateau with return to basal levels by 28 days

in both angina and ACS patients. Of note, in the angina group AG 3 had an initial decrease followed by a larger increase followed by a decrease in EPC count. However, there was no significant difference within each group observed, looking between baseline and all other time periods within either the AG or the ACS groups. There was no significant difference between EPC counts between AG and ACS groups but a marginal difference between the groups at each study day within the EPC count between ACS and AG group. This may be due secondary to the higher dose of atorvastatin or represent part of an inflammatory response in ACS patients. Due to the low numbers in both groups and the difference in number of patients completing the study between AG and ACS, ANOVA analysis was not possible. Dot and whisker plots constructed to illustrate findings. Therefore, to better understand and illustrate study findings an area under the curves for EPC counts (AUC-EPC) for each group over the 28-day study period. The study found a wide distribution of the EPC values of the AG group. The median value lies in the middle of the 'box' shape suggests normal distribution of data. Comparing the medians, the AG group appears to be greater than ACS group, however the value is within the interquartile range of ACS group therefore not significant. The maximum and minimum values of the ACS group are almost within the interquartile range and therefore no significant difference between groups. The trend appears to be a lower EPC count in ACS group. This could also be due to increased consumption or secondary to inhibitory effect of larger dose atorvastatin.

The AG group shows a static line with no change in average EPC counts throughout the study. The ACS group shows an initial increase with a peak at approximately day

2 followed by decrease in EPC counts within first 3 days. This suggests that either higher dose of atorvastatin or acute response following an ACS or both increase EPC count. The decrease could be auto feedback to decrease EPC release from bone marrow or represent consumption of EPCs. This is followed by a gradual plateauing phase.

Finally, a linear model for EPC-AUC adjusted for age, gender, number of comorbidities and smoking. Current smoking had borderline, whereas ex-smoking (P=0.014) had a significant effect on EPC count. However, no relationship between EPC count and ACS, age, gender, number of comorbidities and systolic blood pressure was found. Interestingly, cholesterol was found to have no significant effect on EPC count.

This study has several limitations. The main limitation is the low number of patients enrolled or completing the study. There appears to be a peak of EPC count within the first 48 -72 hours in ACS patients. However, no blood tests were performed at day 2. Therefore, a daily blood sample for the first 72 may be considered in future studies. The effect of atorvastatin may more pronounced in other EPC populations rather than EPC population defined by CD45dim, CD34+ and VEGFR2+ used in this study or other subsets grown in cell culture. However, the study does show an initial increase of EPC numbers with a subsequent fall with gradual plateauing. The cause of the initial increase may be due to an inflammatory response of an ACS or due to atorvastatin 80mg loading. The subsequent decease may be due to consumption of the EPCs, negative feedback on the release from bone marrow or toxic effect of

atorvastatin 80mg. There was also lack of negative controls and small number of patients. Therefore, a larger study could be performed to address these questions.

7.11 Conclusion

The study found no significant difference in the endothelial progenitor cell (EPC) count between angina (AG) group loaded with atorvastatin 20mg and acute coronary syndrome (ACS) patients loaded with 80mg atorvastatin, either within or between groups. However, this study appears to show a peak in EPC count within first 48 hours followed by a fall by day 3 in ACS patients. The causes of the increase may be inflammatory or due to the higher dose of atorvastatin with the subsequent fall due to consumption of the EPCs, negative feedback of release from bone marrow or toxic effect of the dose of atorvastatin or a combination of the aforementioned.

The main limitation of the study was the low number of patients in both groups. But this pilot study supports larger scale studies.

Chapter 7

The effect on endothelial progenitor cells by timing of atorvastatin loading in "all comers" with acute coronary syndromes.

7.1 Abstract

***Introduction*:** This real-world study of patients with ACS and eligible to undergo coronary intervention assessed the effect of the timing of atorvastatin on EPC count. The study aim was to assess for any link with the hypothesis being an inverse relationship between time interval between symptom onset and administration of atorvastatin and EPC count.

***Methods*:** Patients presenting with an ACS had the time from pain onset to statin therapy with atorvastatin 80mg, time from pain onset to EPC sample and time difference between statin therapy to EPC count calculated. The study hypothesis was an inverse relationship between EPC count and time difference between symptom onset and administration of atorvastatin 80mg.

***Results*:** 40 patients with an average age of 60 and 83% male gender were recruited into the study. 68% of patients were either current or ex-smokers, 1 patient had a history of previous percutaneous coronary intervention. Significant risk factors for coronary artery disease including history of diabetes and hypertension were observed in 10% and 35% patients respectively. This study included 4 patients that were admitted with a STEMI, 36 patients with an NSTEMI of which only 2 patients had no stents implanted. No statistical difference was found between EPC count and time over the study period as seen in earlier studies in this thesis. Adjusting for confounders including age, gender, number of comorbidities and smoking status shows a trend towards significance (P=0.06) in time difference and number of co-morbidities. Age and gender had significant differences with P=0.009 and P=0.01 respectively. This study found a trend towards a significance relationship between relative EPC area under the curve and a negative time difference. In other words, administration of atorvastatin 80mg sooner after pain was associated with a greater relative increase in EPCs in acute coronary syndrome patients.

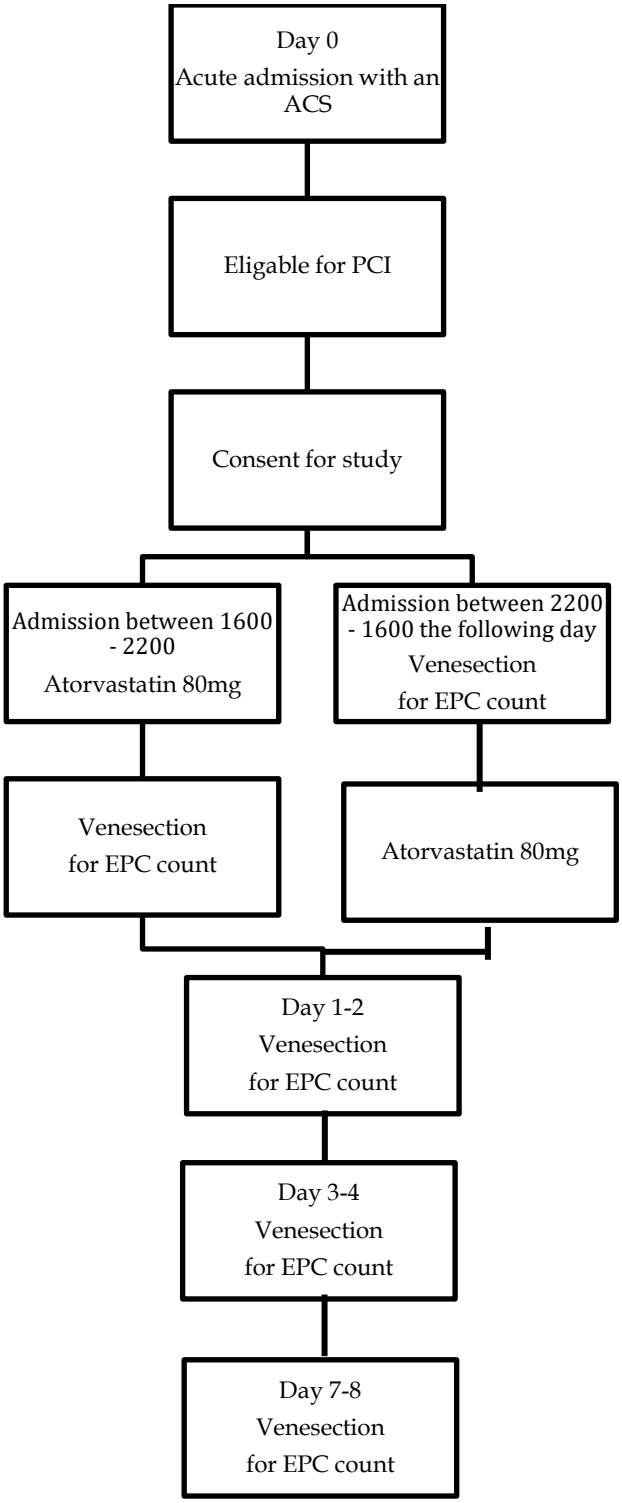
***Conclusion*:** A trend towards significance for an inverse relationship between EPC count and time difference between symptom onset and administration of atorvastatin 80mg. However, this study had several limitations. Therefore, further larger scale studies are required to further investigate the effect of timing of intensive statin therapy in ACS patients on EPC count, but at this time there is no evidence to suggest earlier intensive statin administration during an ACS admission.

7.2 Introduction

Several studies have shown a link between endothelial progenitor cell (EPC) count, coronary artery disease and statin therapy or loading. However, many of these have excluded patients with significant comorbidities and therefore have not truly represented the patients we manage day to day. This study therefore represents "real world" patients. If the study found that atorvastatin increases EPC count this may lead on to larger studies to assess outcomes for up front statin therapy to be initiated by paramedics in the community along with the current recommended aspirin therapy.

7.3 Study design

Fig 8.1 Flow diagram of study and patient-participant pathway



7.4 Inclusion and exclusion criteria

Please refer to 2.7.2.

7.5 Patient consent and follow up

Please refer to section 2.7.4 and 2.7.5.

7.6 Sample preparation

Please refer to section 2.6.5.

7.7 Flow cytometric analysis

Please refer to sections 2.6.5, 2.6.6 and 2.6.7.

7.8 Statistic analysis

Microsoft Excel 2010 software was used for calculating means, standard deviation P-values, relative area under the curve values with box and whisker plots. R – Statistical programming language (version 3.6.1 St. Louis, Missouri, USA) was used for all interpolation curves and for calculation of significance.

The effect of atorvastatin 80mg on EPC were illustrated on box and whisker plots of relative area under the curve. Relative area under the curve for each patient was calculated by the following: The first step was to calculate relative count at each of the study dates of 1,3 and 7 days.

$$\text{Relative EPC count at day 1 } (R_1) = \text{EPC count day 1- day 0}$$

$$\text{Relative EPC count at day 3 } (R_3) = \text{EPC count day 3 - day 0}$$

$$\text{Relative EPC count at day 7 } (R_7) = \text{EPC count day 7 - day 0}$$

This allowed determination of the relative area under the curve for each patient.

$$\text{R-AUC} = [(0.5 \times R_1) + 0.5\,(R_3 + R_1)/2 + 0.5\,(R_7 + R_3)/4]$$

7.9 Results.

Table 7.1 summarises patient demographics including presence of co-morbidities and smoking status.

Table 7.1 – Patient demographics

Patient demographics	Number (%)
Age (Mean/SD)	60 (+/- 11.5)
Gender	
Male	33 (82.5)
Female	7 (17.5)
Comorbidities	
Nil	14 (35)
Previous PCI	1 (2.5)
Previous CABG	0 (0)
Angina	1 (2.5)
DM type 2	4 (10)
Hypertension	14 (35)
COPD/ emphysema/ asthma	5 (12.5)
Colitis/UC	2 (5)
Previous malignancy	2 (5)
Current malignancy	1 (2.5)
OA	1 (2.5)
Previous cholecystectomy	2 (5)
Smoking status	
Current	22 (55.5)
Ex	5 (12.5)
Never	13 (32.5)

Table 7.2 summarises the EPC count for every patient over the study period and the derived relative area under the curve.

Patient	EPC count / µl				
	Day				
	0	1	3	7	Relative AUC-EPC
1	2.3	2.9	2.7	2.5	0.63
2	2.1	1.3	2.4	1.6	-0.55
3	0.3	0.7	1.7	1	0.91
4	1	0.4	0.4	0.6	-0.73
5	2.2	0	0.4	-	-2.33
6	0	1.2	-	-	0.90
7	0.2	1	-	-	0.60
8	2.4	2.6	2.4	2	0.10
9	0.5	0.5	0.8	0.9	0.16
10	1.8	1.3	0.7	0.6	-0.94
11	0.5	1	0.8	1.1	0.56
12	0	1	0.7	0.3	1.05
13	0.5	2.8	1.3	2.6	2.29
14	1.5	3.6	1.9	2.3	1.83
15	0.9	0.8	2.1	2.3	0.55
16	0.9	3.1	-	2.6	1.86
17	0.5	0.8	2.7	-	1.05
18	1.9	1.6	3.7	1.5	0.40
19	1.8	1.7	2	3	0.15
20	4.3	1.1	2	1.8	-3.58
21	2.3	-	2.7	2.5	0.18
22	1.9	2.2	1.3	0.7	-0.15
23	1.1	2.7	3.7	1.2	2.19
24	1.6	1.8	2.4	3.1	0.64
25	3.7	3.1	3.6	2.5	-0.64
26	3.9	4.1	2.8	1.9	-0.51
27	3.2	1.2	1.6	1.2	-2.35
28	3.5	1.4	2.3	4.2	-1.94
29	1.6	3.3	2.8	2.6	1.85
30	2	3.4	1.5	2.5	0.93
31	0.7	1.1	2.1	1.2	0.89
32	2.9	2.7	2.4	-	-0.34
33	2.1	2.4	1.2	1.8	-0.15
34	1.2	2	5.4	4.8	2.63
35	2.6	2.1	1	2.3	-1.01
36	1.6	3.7	-	-	1.58
37	4.7	3.7	1.3	2	-2.36
38	0.8	1.5	0.6	0.1	0.36
39	3.7	-	-	-	0.00
40	0.8	1	0.2	-	-0.08

This study had 4 patients that were admitted with a STEMI, all of whom were stented, and 36 patients with an NSTEMI of which only 2 patients had no stents implanted. The impact of an invasive coronary strategy on the EPC numbers was not therefore further considered in this study, as almost all patients had an invasive procedure, which is a well-recognised stimulus to EPC release

Table 7.3 (a) and (b) shows the single ANOVA calculation to assess for any significant difference in the EPC counts over the 7-day study period.

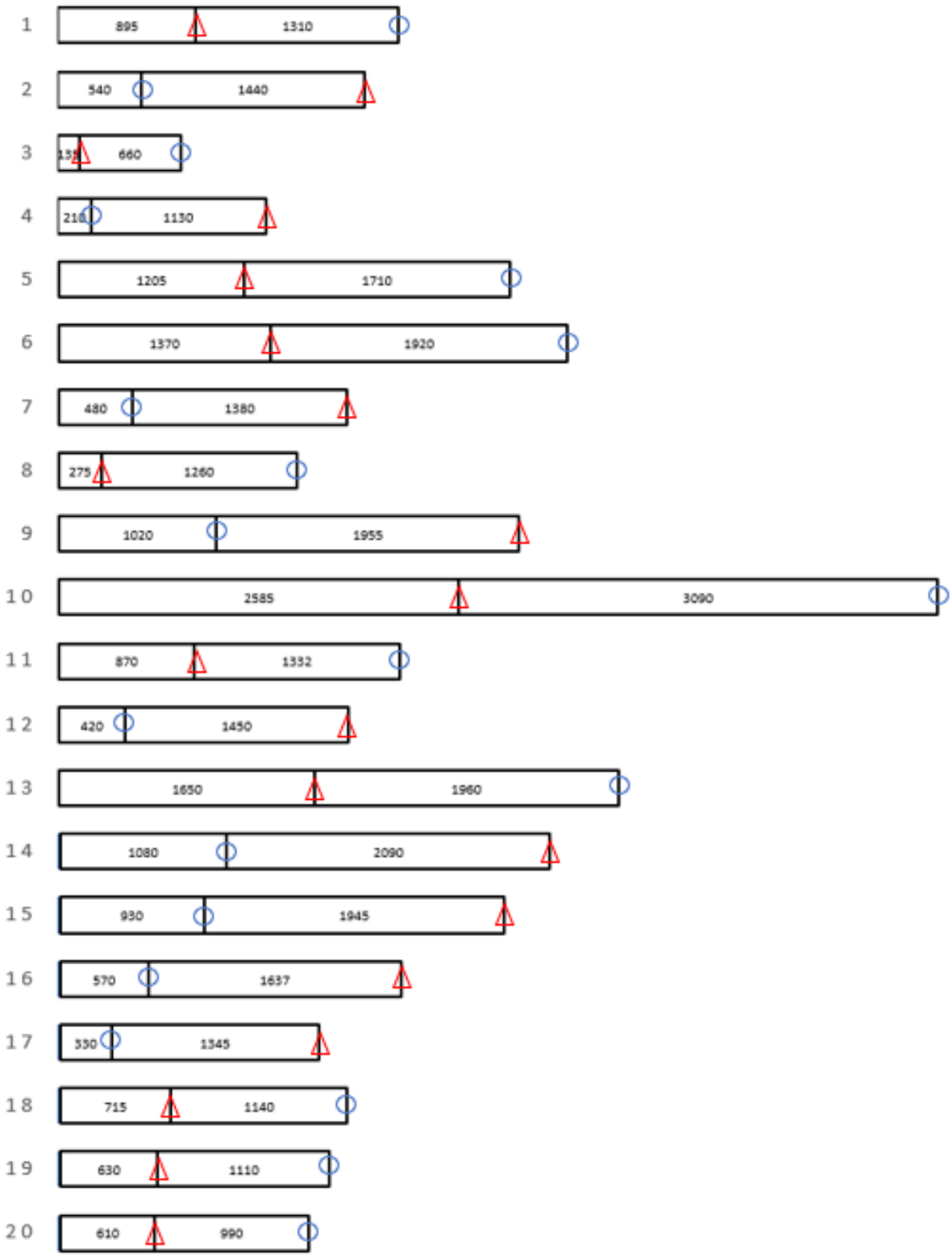
Table 7.3 (a) and (b)

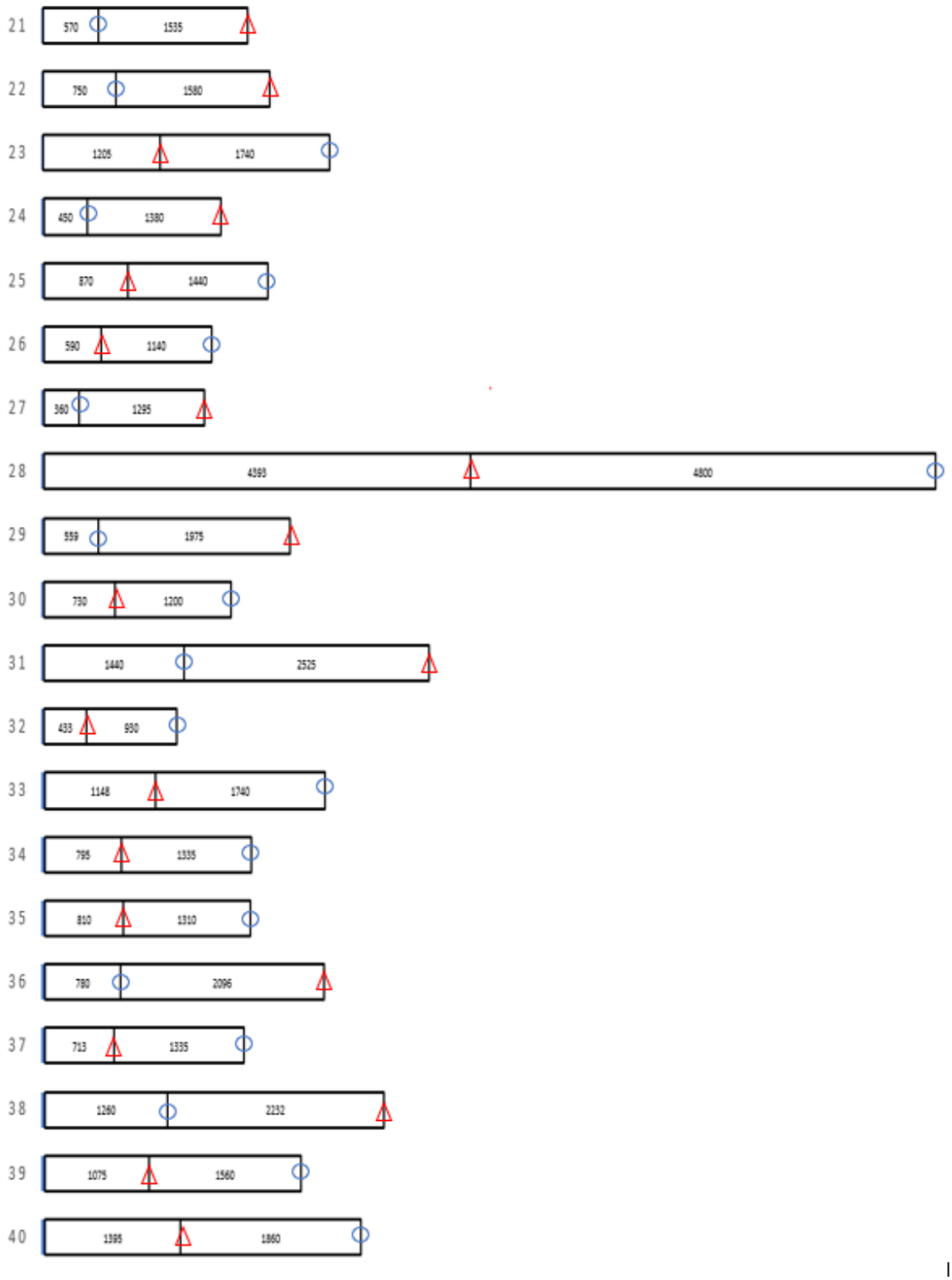
Days	*Count*	*Sum*	*Average*	*Variance*
0	30	56.4	1.88	1.52
1	30	59.3	1.98	1.08
3	30	59.2	1.97	1.26
7	30	56.2	1.87	1.16

Source of Variation	*SS*	*df*	*MS*	*F ratio*	*P-value*	*F crit*
Between Groups	0.29	3	0.10	0.08	0.97	2.68
Within Groups	145.70	116	1.26			
Total	145.99	119				

Figure 7.3 illustrates the time from pain to administration of atorvastatin (blue circle) and venesection for EPC count (red triangle) from start of chest pain experienced by the patient. Statin therapy with atorvastatin 80mg was administered at a fixed drug round at 10pm every day of the week. Secondly EPC count was only possible during 9am to 5pm Monday to Friday. Therefore, as can be seen in figure 8.2 that some patients had atorvastatin before venesection for EPC analysis and visa-versa. This was dependant on time the patient was admitted into hospital. This gives rise to 'negative' values seen in table 4 that summarises time difference between statin therapy to EPC count.

Figure 7.2 Summary of the time of administration of atorvastatin and venesection for each patient from the onset of pain (time in minutes with in the boxes)





Key - Time from onset of pain to administration of atorvastatin (blue circle)
Time from onset of pain to venesection for EPC count (red triangle)

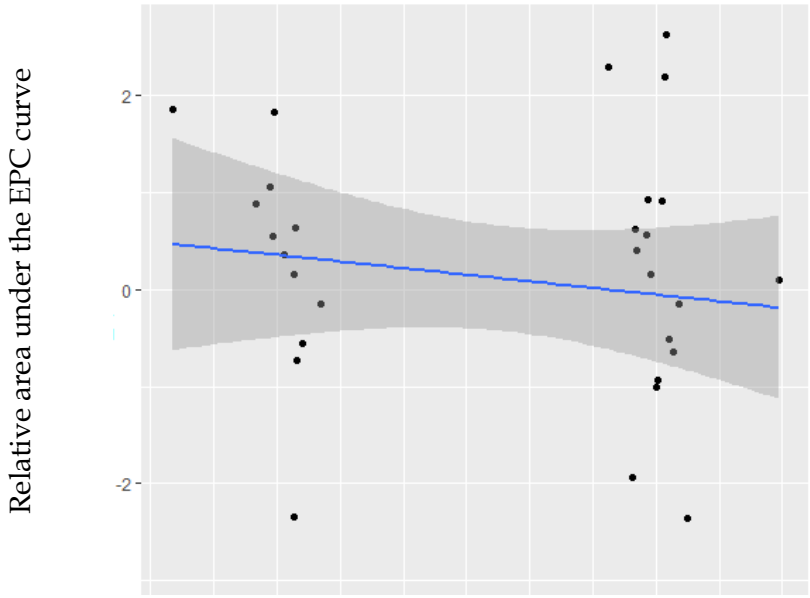
Table 7.4; Summarises the time from onset of pain to atorvastatin therapy and venesection for determination of EPC count.

	Time in minutes		
Patient	Time from pain onset to statin	Time from pain onset to EPC sample	Time difference between statin therapy to EPC count
1	1310	895	415
2	540	1440	-900
3	660	135	525
4	210	1130	-920
5	1710	1205	505
6	1920	1370	550
7	480	1380	-900
8	1260	275	985
9	1020	1955	-935
10	3090	2585	505
11	1332	870	462
12	420	1450	-1030
13	1960	1650	310
14	1080	2090	-1010
15	930	1945	-1015
16	570	1637	-1067
17	330	1345	-1015
18	1140	715	425
19	1110	630	480
20	990	610	380
21	570	1535	-965
22	750	1580	-830
23	1740	1205	535
24	450	1380	-930
25	1440	870	570
26	1140	590	550
27	360	1295	-935
28	4800	4393	407
29	559	1975	-1416
30	1200	730	470
31	1440	2525	-1085
32	930	433	497
33	1740	1148	592
34	1335	795	540
35	1310	810	500
36	780	2096	-1316
37	1335	713	622
38	1260	2232	-972
39	1560	1075	485
40	1860	1395	465

Table 7.5 Relative area under the curve and time difference between statin therapy to time of EPC count.

Patient	Relative AUC-EPC	Time difference between statin therapy to EPC count
1	0.63	415
2	-0.55	-900
3	0.91	525
4	-0.73	-920
8	0.10	985
9	0.16	-935
10	-0.94	505
11	0.56	462
12	1.05	-1030
13	2.29	310
14	1.83	-1010
15	0.55	-1015
18	0.40	425
19	0.15	480
20	-3.58	380
22	-0.15	-830
23	2.19	535
24	0.64	-930
25	-0.64	570
26	-0.51	550
27	-2.35	-935
28	-1.94	407
29	1.85	-1416
30	0.93	470
31	0.89	-1085
33	-0.15	592
34	2.63	540
35	-1.01	500
37	-2.36	622
38	0.36	-972

Figure 7.3 Linear model shows an inverse relationship between relative area under the EPC curve values and time difference from table 7.5.



Key;

Blue line - mean value Grey area – confidence interval Black dots – data values

Tables 7.6 and 7.7 summarise the association between timing of atorvastatin and venesection for EPC count when unadjusted and adjusted for confounders.

Table 7.6; Association between EPC curve and time from statin therapy to EPC count (unadjusted). AUC relative to day 0 (baseline)

		Confidence interval (%)		
	Estimate	2.5	97.5	p-value
Intercept	0.08	-0.46	0.62	0.76
Time difference	-0.0003	-0.001	0.0004	0.44

Table 7.7 Association between EPC curve and time from statin therapy to EPC count adjusted for confounders.

		Confidence interval (%)		
	Estimate	2.5	97.5	*P* Value
Intercept	2.009	-0.451	5.38	0.15
Time difference	-0.00069	-0.0012	$3.32x10^{-6}$	0.06
Age	-0.1005	$-1.58x10^{-2}$	0.045	0.009
Gender (M)	1.600	0.427	2.77	0.01
Number of co-morbidities	0.530	-0.0137	1.073	0.06
Current smoker	-0.934	-1.498	$5.81x10^{-1}$	0.15
Ex-smoker	0.458	-2.834	4.878	0.37

7.10 Discussion

The importance of early intense statin therapy and reloading prior to percutaneous coronary intervention in ACS patients has been highlighted in several studies. [216, 218-223] This study attempted to assess the effect of the timing of atorvastatin 80mg loading on EPC count in patients presenting with acute coronary syndrome.

In total 40 patients were recruited into the study. The average age was 60 years of age and 83% of patients were male. 68% of patients were either current or ex-smokers, 1 patient had a history of previous percutaneous coronary intervention. Significant risk factors for coronary artery disease including history of diabetes and hypertension were observed in 10% and 35% patients respectively. Patient demographics are summarised in table 8.1. In total 30 patients fully completed the study. Table 8.2 summarises the EPC count at days 0, 1, 3, 7 and the calculated relative area under the

curve. Relative area under the EPC curve was used in the analysis of the effect of the timing of atorvastatin on EPC count. This reflected any actual change in EPC count from baseline but also at each time period throughout the study for each patient. No statistical difference was found between EPC count and time (0, 1, 3 and 7) in days as seen in earlier studies in this thesis. The study had certain inherent time constraints. Firstly, statin therapy with atorvastatin 80mg was administered at a fixed drug round at 10pm every day of the week. Secondly EPC count was only possible during 9am to 5pm Monday to Friday. This therefore allowed the derivation of time from pain onset to statin therapy, time from pain onset to EPC sample and time difference between statin therapy to EPC count. Time difference between statin therapy to EPC count could be either positive or negative valves. Positive valves corresponded to patients having venesection first and then atorvastatin therapy. Conversely negative values corresponded to atorvastatin therapy prior to venesection for EPC analysis. Table 8.5 was used to plot Figure 8.3 a linear model showing an inverse relationship between relative area under the EPC curve values and time difference. No significant difference of the timing of atorvastatin therapy was found.

However, adjusting for confounders including age, gender, number of comorbidities and smoking status the association of EPC and count becomes insignificant. This model shows a trend towards significance (P=0.06) in time difference and number of co-morbidities. Age and gender had significant differences with P=0.009 and P=0.01 respectively. Both known risk factors for ischaemic heart disease. No significant difference was noted with smoking status another risk factor of ischaemic heart disease. ACS is an umbrella term that includes patient presenting with non-ST

elevation myocardial infarction (NSTEMI) and ST-elevation myocardial infarction (STEMI). The latter group are a medical emergency and therefore have percutaneous coronary interventions and stents. Conversely stable NSTEMI patients may undergo coronary intervention 24-48 hours after admission. Please refer to sections 1.1,1.6 and 1.7. This study had only 4 patients that were admitted with a STEMI, 36 patients with an NSTEMI of which only 2 patients had no stents implanted. This was a very skewed population as the study only included patients consenting to participate and was not 40 consecutive patients. Patients initially diagnosed as a NSTEMI who after angiography had no significant coronary artery disease and could be discharged did not wish to participate in the study due to the need to attend follow up. Second group of NSTEMI patients were those that on angiography were found to have severe three vessel coronary artery disease and required coronary bypass surgery. The study did not assess clinical outcomes. The dose of atorvastatin was based on current national guidelines. However, the effect on EPCs may be either insufficient or inhibitory. The study identified EPC as $CD45^{dim}$, $CD34^{+}$ and $VEGFR2^{+}$ and further studies would include other flow cytometric EPC nomenclature.

This study had several limitations. Firstly, the study only had 40 patients in the study with only 30 patients completing the study. The primary reason for patients not completing of the study was the requirement of follow up with a blood test. Most patients were discharged at day 3 therefore required at last 1 further follow up appointment at day 7. Royal Stoke University Hospital provided emergency tertiary cardiac service to large geographic region and this hampered ability of patients to return for follow up, despite significant encouragement and the offer of free

transportation. The study had no control patients that were non hospitalised healthy subjects, and no dose responses were performed. Moreover, the timing of administration of atorvastatin was governed by the drug round on the ward.

7.11 Conclusion

This study found no significant difference in EPC counts throughout the study period as with earlier studies in this thesis. In keeping with the study hypothesis, the study found a trend towards early administration of atorvastatin 80mg and association with a greater relative increase in EPCs in acute coronary syndrome patients. Male gender and increasing age were found to significantly increase EPC counts when adjusted for confounders. Larger studies are required to assess the effect of atorvastatin 80mg in acute coronary syndrome patients. Further studies should also include other defined nomenclature for EPCs. Therefore, based on current evidence there are no indications to support earlier administration for statin therapy in ACS patients.

Chapter 8

General summary

The premise of the thesis was based on the importance of maintaining endothelial integrity. This integrity being a balance between endothelial damage and mechanisms of repair. The first mechanism of repair having limited potential with neighbouring endothelial cell replication. The second relying on undifferentiated EPCs migrating to site of vascular damage to form endothelial cells. An effect that may be potentiated by atorvastatin therapy. The study defined EPCs by flow cytometric co-expression of $CD34^{+}$ /$VEGFR2^{+}$ and diminished (dim) CD45 ($CD45^{dim}$).

This thesis used bench side studies to assess the effect of atorvastatin on chemokine, chemokine receptor and adhesion molecules CXCL-12, CXCR-4 and E-selectin respectively to account for increase in EPCs in chapter 3 of the thesis. The study hypothesis was that atorvastatin would increase CXCL-12 its receptor CXCR-4 and of adhesion molecule e–selectin resulting in greater EPC numbers on the denuded endothelium. The study found atorvastatin significantly increased the concentration of CXCL-12 and secondly maintained a more sustained duration of effect when compared to the standard group. Although no significant difference visually in either CXCR-4 or E-selectin when compared with standard sample. Atorvastatin was associated with greater concentration of CXCR-4 and E-selectin by 1 hour and an effect seen to be maintained. The peak levels of CXCR-4 and E-selectin were 1 and 5 hours

respectively. Despite effects on CXCL-12, CXCR-4 or E-selectin, no significant increase in cultured EPCs was observed.

The remaining studies were bed side based. Chapter 4 aimed to demonstrate the feasibility of flow cytometric analysis of EPC at RLUH but also to study if whole blood samples could be stored to be undergo flow cytometric analysis at a later time. The study hypothesis was that accurate flow cytometric analysis would be possible in our haematology laboratory and that it would be possible to safely store whole blood samples for analysis at a latter time. The study confirmed feasibility of EPC analysis. Secondly the study found that whole bloods samples stored at 4^0 C could undergo flow cytometric analysis up to 12 hours after venesection. This led to protocol amendment for further studies allowing samples to be stored at 4^0 C and analysed by 2 hours after venesection.

Chapter 5 assessed for any association between computerised tomography of coronary arteries calcium scoring and EPC count in patients undergoing CT coronary angiography as part of clinical review. The hypothesis being that the greater the coronary artery calcium score the greater the number of EPCs. The study found an inverse non-significant trend with EPC count decreasing with higher CACS. The reason was not elucidated in this study however may be due to decreased mobilisation or great consumption of EPCs.

Chapter 6 assessed the effect of moderate (20mg) and intense dose (80mg) atorvastatin on EPC count given to newly diagnosed angina and acute coronary syndrome patients respectively. The study hypothesis was that atorvastatin 80mg would be associated with a greater number of EPCs. The study found no significant difference in number

of co-morbidities, full blood count or renal function between AG and ACS patients. There was borderline significant difference between cholesterol and LDL levels at the end of the day at day 28 between ACS and AG patients. There were no reports of any adverse events with atorvastatin therapy in the study. The study found an overall trend of an initial increase in the number of EPCs within the first 3 days post atorvastatin therapy followed by a plateau with return to basal levels by 28 days in both angina and ACS patients. There appeared to be a trend of lower EPC count in ACS group, in contrary to study hypothesis.

Chapter 7 assessed the effect of atorvastatin 80mg loading on EPC count in patients presenting with an ACS. The study hypothesis was an inverse relationship between EPC count and time difference between symptom onset and administration of atorvastatin 80mg. In other words, the sooner the atorvastatin given the greater the number of EPCs. No significant difference of the timing of atorvastatin therapy was found. However, the study found a trend towards significance in both time difference and number of co-morbidities when adjusted for comorbidities. This study suggests administration of atorvastatin 80mg may be associated with a greater relative increase in EPCs in ACS patients with earlier administration.

The studies had several limitations. The bench side studies had lack of dose response studies. The bedside studies had no control patients that were non hospitalised healthy subjects. The most significant limitation was the small number of patients recruited in this thesis. Larger studies would be required if any changes to current guidelines are to be proposed.

Publications and poster related to this thesis

Publications

2018 Endothelial Progenitor Cell Identification, Classification and Nomenclature: A Review. J Blood Lymph 8: 221. Doi: 10.4172/2165-7831.1000221
Sandhu K, Njoroge W, Yang Y, Harper AGS, Butler R

2017 Endothelial progenitor cells: exploring the pleiotropic effects of statins
Sandhu K Mamas M Butler R
World Journal of Cardiology
World J Cardiol. 2017 Jan 26; 9(1): 1–13.

Posters

2015 10th Annual ISTM Post Graduate Symposium Keele University
Sandhu K, Butler R

Abstracts

2019 The 14th international symposium in Biomechanics in Vascular Biology and Cardiovascular Disease
Atorvastatin effect on homing of MSCs and EPCs on a 3D blood vessel models
Njoroge W, Harper A, Butley R, Sandhu K, Yang Y

References

1. https://www.who.int/news-room/fact-sheets/detail/the-top-10-causes-of-death.
2. https://www.bhf.org.uk/for-professionals/press-centre/facts-and-figures.
3. Hansson, G.K. and Hermansson, A., *The immune system in atherosclerosis.* Nat Immunol, 2011. **12**(3): p. 204-12.
4. Weber, C. and Noels, H., *Atherosclerosis: current pathogenesis and therapeutic options.* Nat Med, 2011. **17**(11): p. 1410-22.
5. https://www.mayoclinic.org/diseases-conditions/arteriosclerosis-atherosclerosis/symptoms-causes/syc-20350569.
6. https://www.grepmed.com/images/3707/definitions-cardiology-diagnosis-unstable-nstemi-angina-acs.
7. Deanfield, J.E., Halcox, J.P., and Rabelink, T.J., *Endothelial function and dysfunction: testing and clinical relevance.* Circulation, 2007. **115**(10): p. 1285-95.
8. Widlansky, M.E., Gokce, N., Keaney, J.F., Jr., et al., *The clinical implications of endothelial dysfunction.* J Am Coll Cardiol, 2003. **42**(7): p. 1149-60.
9. Munzel, T., Sinning, C., Post, F., et al., *Pathophysiology, diagnosis and prognostic implications of endothelial dysfunction.* Ann Med, 2008. **40**(3): p. 180-96.
10. Ross, R., *Atherosclerosis--an inflammatory disease.* N Engl J Med, 1999. **340**(2): p. 115-26.
11. Op den Buijs, J., Musters, M., Verrips, T., et al., *Mathematical modeling of vascular endothelial layer maintenance: the role of endothelial cell division, progenitor cell homing, and telomere shortening.* Am J Physiol Heart Circ Physiol, 2004. **287**(6): p. H2651-8.
12. Folkman, J., *Angiogenesis in cancer, vascular, rheumatoid and other disease.* Nat Med, 1995. **1**(1): p. 27-31.
13. Walter, D.H., Rittig, K., Bahlmann, F.H., et al., *Statin therapy accelerates reendothelialization: a novel effect involving mobilization and incorporation of bone marrow-derived endothelial progenitor cells.* Circulation, 2002. **105**(25): p. 3017-24.
14. Griese, D.P., Ehsan, A., Melo, L.G., et al., *Isolation and transplantation of autologous circulating endothelial cells into denuded vessels and prosthetic grafts: implications for cell-based vascular therapy.* Circulation, 2003. **108**(21): p. 2710-5.
15. Fujiyama, S., Amano, K., Uehira, K., et al., *Bone marrow monocyte lineage cells adhere on injured endothelium in a monocyte chemoattractant protein-1-dependent manner and accelerate reendothelialization as endothelial progenitor cells.* Circ Res, 2003. **93**(10): p. 980-9.
16. Bhattacharya, V., McSweeney, P.A., Shi, Q., et al., *Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells.* Blood, 2000. **95**(2): p. 581-5.
17. Gehling, U.M., Ergun, S., Schumacher, U., et al., *In vitro differentiation of endothelial cells from AC133-positive progenitor cells.* Blood, 2000. **95**(10): p. 3106-12.
18. Hu, Y., Davison, F., Zhang, Z., et al., *Endothelial replacement and angiogenesis in arteriosclerotic lesions of allografts are contributed by circulating progenitor cells.* Circulation, 2003. **108**(25): p. 3122-7.
19. Hill, J.M., Zalos, G., Halcox, J.P., et al., *Circulating endothelial progenitor cells, vascular function, and cardiovascular risk.* N Engl J Med, 2003. **348**(7): p. 593-600.
20. Takahashi, T., Kalka, C., Masuda, H., et al., *Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization.* Nat Med, 1999. **5**(4): p. 434-8.
21. Carmeliet, P., *Angiogenesis in health and disease.* Nat Med, 2003. **9**(6): p. 653-60.

22. Urbich, C., Heeschen, C., Aicher, A., et al., *Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells.* Circulation, 2003. **108**(20): p. 2511-6.
23. Nissen, S.E., Nicholls, S.J., Sipahi, I., et al., *Effect of very high-intensity statin therapy on regression of coronary atherosclerosis: the ASTEROID trial.* JAMA, 2006. **295**(13): p. 1556-65.
24. Rafii, S. and Lyden, D., *Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration.* Nat Med, 2003. **9**(6): p. 702-12.
25. Mukai, N., Akahori, T., Komaki, M., et al., *A comparison of the tube forming potentials of early and late endothelial progenitor cells.* Exp Cell Res, 2008. **314**(3): p. 430-40.
26. Deanfield, J., Donald, A., Ferri, C., et al., *Endothelial function and dysfunction. Part I: Methodological issues for assessment in the different vascular beds: a statement by the Working Group on Endothelin and Endothelial Factors of the European Society of Hypertension.* J Hypertens, 2005. **23**(1): p. 7-17.
27. Dong, C. and Goldschmidt-Clermont, P.J., *Endothelial progenitor cells: a promising therapeutic alternative for cardiovascular disease.* J Interv Cardiol, 2007. **20**(2): p. 93-9.
28. Asahara, T., Murohara, T., Sullivan, A., et al., *Isolation of putative progenitor endothelial cells for angiogenesis.* Science, 1997. **275**(5302): p. 964-7.
29. Stump, M.M., Jordan, G.L., Jr., Debakey, M.E., et al., *Endothelium Grown from Circulating Blood on Isolated Intravascular Dacron Hub.* Am J Pathol, 1963. **43**: p. 361-7.
30. Asahara, T., Masuda, H., Takahashi, T., et al., *Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization.* Circ Res, 1999. **85**(3): p. 221-8.
31. Risau, W., *Mechanisms of angiogenesis.* Nature, 1997. **386**(6626): p. 671-4.
32. Krenning, G., van Luyn, M.J., and Harmsen, M.C., *Endothelial progenitor cell-based neovascularization: implications for therapy.* Trends Mol Med, 2009. **15**(4): p. 180-9.
33. Yoder, M.C., *Human endothelial progenitor cells.* Cold Spring Harb Perspect Med, 2012. **2**(7): p. a006692.
34. Padfield, G.J., Newby, D.E., and Mills, N.L., *Understanding the role of endothelial progenitor cells in percutaneous coronary intervention.* J Am Coll Cardiol, 2010. **55**(15): p. 1553-65.
35. Schachinger, V., Britten, M.B., and Zeiher, A.M., *Prognostic impact of coronary vasodilator dysfunction on adverse long-term outcome of coronary heart disease.* Circulation, 2000. **101**(16): p. 1899-906.
36. Suwaidi, J.A., Hamasaki, S., Higano, S.T., et al., *Long-term follow-up of patients with mild coronary artery disease and endothelial dysfunction.* Circulation, 2000. **101**(9): p. 948-54.
37. Halcox, J.P., Schenke, W.H., Zalos, G., et al., *Prognostic value of coronary vascular endothelial dysfunction.* Circulation, 2002. **106**(6): p. 653-8.
38. Gokce, N., Keaney, J.F., Jr., Hunter, L.M., et al., *Risk stratification for postoperative cardiovascular events via noninvasive assessment of endothelial function: a prospective study.* Circulation, 2002. **105**(13): p. 1567-72.
39. Pirro, M., Schillaci, G., Menecali, C., et al., *Reduced number of circulating endothelial progenitors and HOXA9 expression in CD34+ cells of hypertensive patients.* J Hypertens, 2007. **25**(10): p. 2093-9.
40. Fadini, G.P., Miorin, M., Facco, M., et al., *Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus.* J Am Coll Cardiol, 2005. **45**(9): p. 1449-57.
41. Tepper, O.M., Galiano, R.D., Capla, J.M., et al., *Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures.* Circulation, 2002. **106**(22): p. 2781-6.
42. Ii, M., Takenaka, H., Asai, J., et al., *Endothelial progenitor thrombospondin-1 mediates diabetes-induced delay in reendothelialization following arterial injury.* Circ Res, 2006. **98**(5): p. 697-704.

43. Chen, Y.H., Lin, S.J., Lin, F.Y., et al., *High glucose impairs early and late endothelial progenitor cells by modifying nitric oxide-related but not oxidative stress-mediated mechanisms.* Diabetes, 2007. **56**(6): p. 1559-68.
44. Imanishi, T., Hano, T., and Nishio, I., *Angiotensin II potentiates vascular endothelial growth factor-induced proliferation and network formation of endothelial progenitor cells.* Hypertens Res, 2004. **27**(2): p. 101-8.
45. Min, T.Q., Zhu, C.J., Xiang, W.X., et al., *Improvement in endothelial progenitor cells from peripheral blood by ramipril therapy in patients with stable coronary artery disease.* Cardiovasc Drugs Ther, 2004. **18**(3): p. 203-9.
46. Vasa, M., Fichtlscherer, S., Aicher, A., et al., *Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease.* Circ Res, 2001. **89**(1): p. E1-7.
47. Chen, J.Z., Zhang, F.R., Tao, Q.M., et al., *Number and activity of endothelial progenitor cells from peripheral blood in patients with hypercholesterolaemia.* Clin Sci (Lond), 2004. **107**(3): p. 273-80.
48. Ito, H., Rovira, II, Bloom, M.L., et al., *Endothelial progenitor cells as putative targets for angiostatin.* Cancer Res, 1999. **59**(23): p. 5875-7.
49. Li, D., Yang, B., and Mehta, J.L., *Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas.* Am J Physiol, 1998. **275**(2 Pt 2): p. H568-76.
50. Chavakis, E., Dernbach, E., Hermann, C., et al., *Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial cell migration by an inhibitory effect on the Akt/endothelial nitric oxide synthase pathway.* Circulation, 2001. **103**(16): p. 2102-7.
51. Morales-Ruiz, M., Fulton, D., Sowa, G., et al., *Vascular endothelial growth factor-stimulated actin reorganization and migration of endothelial cells is regulated via the serine/threonine kinase Akt.* Circ Res, 2000. **86**(8): p. 892-6.
52. Rouhl, R.P., van Oostenbrugge, R.J., Damoiseaux, J., et al., *Endothelial progenitor cell research in stroke: a potential shift in pathophysiological and therapeutical concepts.* Stroke, 2008. **39**(7): p. 2158-65.
53. Sen, S., McDonald, S.P., Coates, P.T., et al., *Endothelial progenitor cells: novel biomarker and promising cell therapy for cardiovascular disease.* Clin Sci (Lond), 2011. **120**(7): p. 263-83.
54. Dimmeler, S. and Vasa-Nicotera, M., *Aging of progenitor cells: limitation for regenerative capacity?* J Am Coll Cardiol, 2003. **42**(12): p. 2081-2.
55. Flores-Ramirez, R., Uribe-Longoria, A., Rangel-Fuentes, M.M., et al., *Intracoronary infusion of CD133+ endothelial progenitor cells improves heart function and quality of life in patients with chronic post-infarct heart insufficiency.* Cardiovasc Revasc Med, 2010. **11**(2): p. 72-8.
56. Dobert, N., Britten, M., Assmus, B., et al., *Transplantation of progenitor cells after reperfused acute myocardial infarction: evaluation of perfusion and myocardial viability with FDG-PET and thallium SPECT.* Eur J Nucl Med Mol Imaging, 2004. **31**(8): p. 1146-51.
57. Assmus, B., Honold, J., Schachinger, V., et al., *Transcoronary transplantation of progenitor cells after myocardial infarction.* N Engl J Med, 2006. **355**(12): p. 1222-32.
58. Losordo, D.W., Schatz, R.A., White, C.J., et al., *Intramyocardial transplantation of autologous CD34+ stem cells for intractable angina: a phase I/IIa double-blind, randomized controlled trial.* Circulation, 2007. **115**(25): p. 3165-72.
59. Bartunek, J., Vanderheyden, M., Vandekerckhove, B., et al., *Intracoronary injection of CD133-positive enriched bone marrow progenitor cells promotes cardiac recovery after recent myocardial infarction: feasibility and safety.* Circulation, 2005. **112**(9 Suppl): p. I178-83.
60. Erbs, S., Linke, A., Adams, V., et al., *Transplantation of blood-derived progenitor cells after recanalization of chronic coronary artery occlusion: first randomized and placebo-controlled study.* Circ Res, 2005. **97**(8): p. 756-62.
61. Hamano, K., Nishida, M., Hirata, K., et al., *Local implantation of autologous bone marrow cells for therapeutic angiogenesis in patients with ischemic heart disease: clinical trial and preliminary results.* Jpn Circ J, 2001. **65**(9): p. 845-7.

62. Strauer, B.E., Brehm, M., Zeus, T., et al., *Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans.* Circulation, 2002. **106**(15): p. 1913-8.
63. Meyer, G.P., Wollert, K.C., Lotz, J., et al., *Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial.* Circulation, 2006. **113**(10): p. 1287-94.
64. Janssens, S., Dubois, C., Bogaert, J., et al., *Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial.* Lancet, 2006. **367**(9505): p. 113-21.
65. Sprague, A.H. and Khalil, R.A., *Inflammatory cytokines in vascular dysfunction and vascular disease.* Biochem Pharmacol, 2009. **78**(6): p. 539-52.
66. Blanchet, X., Langer, M., Weber, C., et al., *Touch of chemokines.* Front Immunol, 2012. **3**: p. 175.
67. Ben-Baruch, A., Bengali, K.M., Biragyn, A., et al., *Interleukin-8 receptor beta. The role of the carboxyl terminus in signal transduction.* J Biol Chem, 1995. **270**(16): p. 9121-8.
68. Koenen, R.R. and Weber, C., *Chemokines: established and novel targets in atherosclerosis.* EMBO Mol Med, 2011. **3**(12): p. 713-25.
69. Farouk, S.S., Rader, D.J., Reilly, M.P., et al., *CXCL12: a new player in coronary disease identified through human genetics.* Trends Cardiovasc Med, 2010. **20**(6): p. 204-9.
70. Zernecke, A., Schober, A., Bot, I., et al., *SDF-1alpha/CXCR4 axis is instrumental in neointimal hyperplasia and recruitment of smooth muscle progenitor cells.* Circ Res, 2005. **96**(7): p. 784-91.
71. Abi-Younes, S., Sauty, A., Mach, F., et al., *The stromal cell-derived factor-1 chemokine is a potent platelet agonist highly expressed in atherosclerotic plaques.* Circ Res, 2000. **86**(2): p. 131-8.
72. Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., et al., *A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1).* J Exp Med, 1996. **184**(3): p. 1101-9.
73. Askari, A.T., Unzek, S., Popovic, Z.B., et al., *Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy.* Lancet, 2003. **362**(9385): p. 697-703.
74. Karin, N., *The multiple faces of CXCL12 (SDF-1alpha) in the regulation of immunity during health and disease.* J Leukoc Biol, 2010. **88**(3): p. 463-73.
75. Stellos, K., Bigalke, B., Langer, H., et al., *Expression of stromal-cell-derived factor-1 on circulating platelets is increased in patients with acute coronary syndrome and correlates with the number of CD34+ progenitor cells.* Eur Heart J, 2009. **30**(5): p. 584-93.
76. Gupta, S.K., Lysko, P.G., Pillarisetti, K., et al., *Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines.* J Biol Chem, 1998. **273**(7): p. 4282-7.
77. Damas, J.K., Waehre, T., Yndestad, A., et al., *Stromal cell-derived factor-1alpha in unstable angina: potential antiinflammatory and matrix-stabilizing effects.* Circulation, 2002. **106**(1): p. 36-42.
78. Ghasemzadeh, N., Hritani, A.W., De Staercke, C., et al., *Plasma stromal cell-derived factor 1alpha/CXCL12 level predicts long-term adverse cardiovascular outcomes in patients with coronary artery disease.* Atherosclerosis, 2015. **238**(1): p. 113-8.
79. Mehta, N.N., Li, M., William, D., et al., *The novel atherosclerosis locus at 10q11 regulates plasma CXCL12 levels.* Eur Heart J, 2011. **32**(8): p. 963-71.
80. Hu, X., Dai, S., Wu, W.J., et al., *Stromal cell derived factor-1 alpha confers protection against myocardial ischemia/reperfusion injury: role of the cardiac stromal cell derived factor-1 alpha CXCR4 axis.* Circulation, 2007. **116**(6): p. 654-63.
81. Doring, Y., Pawig, L., Weber, C., et al., *The CXCL12/CXCR4 chemokine ligand/receptor axis in cardiovascular disease.* Front Physiol, 2014. **5**: p. 212.

82. Wang, J.F., Liu, Z.Y., and Groopman, J.E., *The alpha-chemokine receptor CXCR4 is expressed on the megakaryocytic lineage from progenitor to platelets and modulates migration and adhesion.* Blood, 1998. **92**(3): p. 756-64.
83. De La Luz Sierra, M., Yang, F., Narazaki, M., et al., *Differential processing of stromal-derived factor-1alpha and stromal-derived factor-1beta explains functional diversity.* Blood, 2004. **103**(7): p. 2452-9.
84. Mazo, I.B., Massberg, S., and von Andrian, U.H., *Hematopoietic stem and progenitor cell trafficking.* Trends Immunol, 2011. **32**(10): p. 493-503.
85. Jones, D.L. and Wagers, A.J., *No place like home: anatomy and function of the stem cell niche.* Nat Rev Mol Cell Biol, 2008. **9**(1): p. 11-21.
86. Semerad, C.L., Christopher, M.J., Liu, F., et al., *G-CSF potently inhibits osteoblast activity and CXCL12 mRNA expression in the bone marrow.* Blood, 2005. **106**(9): p. 3020-7.
87. Winkler, I.G., Sims, N.A., Pettit, A.R., et al., *Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs.* Blood, 2010. **116**(23): p. 4815-28.
88. Lataillade, J.J., Clay, D., Dupuy, C., et al., *Chemokine SDF-1 enhances circulating CD34(+) cell proliferation in synergy with cytokines: possible role in progenitor survival.* Blood, 2000. **95**(3): p. 756-68.
89. Lee, Y., Gotoh, A., Kwon, H.J., et al., *Enhancement of intracellular signaling associated with hematopoietic progenitor cell survival in response to SDF-1/CXCL12 in synergy with other cytokines.* Blood, 2002. **99**(12): p. 4307-17.
90. Guo, Y., Hangoc, G., Bian, H., et al., *SDF-1/CXCL12 enhances survival and chemotaxis of murine embryonic stem cells and production of primitive and definitive hematopoietic progenitor cells.* Stem Cells, 2005. **23**(9): p. 1324-32.
91. Dar, A., Schajnovitz, A., Lapid, K., et al., *Rapid mobilization of hematopoietic progenitors by AMD3100 and catecholamines is mediated by CXCR4-dependent SDF-1 release from bone marrow stromal cells.* Leukemia, 2011. **25**(8): p. 1286-1296.
92. Collins, T., Williams, A., Johnston, G.I., et al., *Structure and chromosomal location of the gene for endothelial-leukocyte adhesion molecule 1.* J Biol Chem, 1991. **266**(4): p. 2466-73.
93. Leeuwenberg, J.F., Smeets, E.F., Neefjes, J.J., et al., *E-selectin and intercellular adhesion molecule-1 are released by activated human endothelial cells in vitro.* Immunology, 1992. **77**(4): p. 543-9.
94. Vajkoczy, P., Blum, S., Lamparter, M., et al., *Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis.* J Exp Med, 2003. **197**(12): p. 1755-65.
95. Nishiwaki, Y., Yoshida, M., Iwaguro, H., et al., *Endothelial E-selectin potentiates neovascularization via endothelial progenitor cell-dependent and -independent mechanisms.* Arterioscler Thromb Vasc Biol, 2007. **27**(3): p. 512-8.
96. Sharma, S.Y., M., *Gene silencing of E-selectin block recruitment of endothelial progenitor cell to vascular endothelium under flow. .* J. Biomedical Science and Engineering, 2010, 3, 550-555, 2010.
97. Oh, I.Y., Yoon, C.H., Hur, J., et al., *Involvement of E-selectin in recruitment of endothelial progenitor cells and angiogenesis in ischemic muscle.* Blood, 2007. **110**(12): p. 3891-9.
98. Liu, Z.J., Daftarian, P., Kovalski, L., et al., *Directing and Potentiating Stem Cell-Mediated Angiogenesis and Tissue Repair by Cell Surface E-Selectin Coating.* PLoS One, 2016. **11**(4): p. e0154053.
99. Liu, Z.J., Tian, R., Li, Y., et al., *SDF-1alpha-induced dual pairs of E-selectin/ligand mediate endothelial progenitor cell homing to critical ischemia.* Sci Rep, 2016. **6**: p. 34416.
100. Ingram, D.A., Caplice, N.M., and Yoder, M.C., *Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells.* Blood, 2005. **106**(5): p. 1525-31.
101. Shi, Q., Rafii, S., Wu, M.H., et al., *Evidence for circulating bone marrow-derived endothelial cells.* Blood, 1998. **92**(2): p. 362-7.
102. Lin, Y., Weisdorf, D.J., Solovey, A., et al., *Origins of circulating endothelial cells and endothelial outgrowth from blood.* J Clin Invest, 2000. **105**(1): p. 71-7.

103. Peichev, M., Naiyer, A.J., Pereira, D., et al., *Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors.* Blood, 2000. **95**(3): p. 952-8.
104. Hirschi, K.K., Ingram, D.A., and Yoder, M.C., *Assessing identity, phenotype, and fate of endothelial progenitor cells.* Arterioscler Thromb Vasc Biol, 2008. **28**(9): p. 1584-95.
105. Fadini, G.P., Losordo, D., and Dimmeler, S., *Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use.* Circ Res, 2012. **110**(4): p. 624-37.
106. Medina, R.J., Barber, C.L., Sabatier, F., et al., *Endothelial Progenitors: A Consensus Statement on Nomenclature.* Stem Cells Transl Med, 2017. **6**(5): p. 1316-1320.
107. Basile, D.P. and Yoder, M.C., *Circulating and tissue resident endothelial progenitor cells.* J Cell Physiol, 2014. **229**(1): p. 10-6.
108. Pearson, J.D., *Endothelial progenitor cells--an evolving story.* Microvasc Res, 2010. **79**(3): p. 162-8.
109. Sandhu, K., Mamas, M., and Butler, R., *Endothelial progenitor cells: Exploring the pleiotropic effects of statins.* World J Cardiol, 2017. **9**(1): p. 1-13.
110. Yoon, C.H., Hur, J., Park, K.W., et al., *Synergistic neovascularization by mixed transplantation of early endothelial progenitor cells and late outgrowth endothelial cells: the role of angiogenic cytokines and matrix metalloproteinases.* Circulation, 2005. **112**(11): p. 1618-27.
111. Barber, C.L. and Iruela-Arispe, M.L., *The ever-elusive endothelial progenitor cell: identities, functions and clinical implications.* Pediatr Res, 2006. **59**(4 Pt 2): p. 26R-32R.
112. Murasawa, S. and Asahara, T., *Endothelial progenitor cells for vasculogenesis.* Physiology (Bethesda), 2005. **20**: p. 36-42.
113. Medina, R.J., O'Neill, C.L., O'Doherty, T.M., et al., *Endothelial progenitors as tools to study vascular disease.* Stem Cells Int, 2012. **2012**: p. 346735.
114. Chan, J.K., Ng, C.S., and Hui, P.K., *A simple guide to the terminology and application of leucocyte monoclonal antibodies.* Histopathology, 1988. **12**(5): p. 461-80.
115. Ho, I.C., Tai, T.S., and Pai, S.Y., *GATA3 and the T-cell lineage: essential functions before and after T-helper-2-cell differentiation.* Nat Rev Immunol, 2009. **9**(2): p. 125-35.
116. Yin, A.H., Miraglia, S., Zanjani, E.D., et al., *AC133, a novel marker for human hematopoietic stem and progenitor cells.* Blood, 1997. **90**(12): p. 5002-12.
117. Quirici, N., Soligo, D., Caneva, L., et al., *Differentiation and expansion of endothelial cells from human bone marrow CD133(+) cells.* Br J Haematol, 2001. **115**(1): p. 186-94.
118. Timmermans, F., Van Hauwermeiren, F., De Smedt, M., et al., *Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors.* Arterioscler Thromb Vasc Biol, 2007. **27**(7): p. 1572-9.
119. Case, J., Mead, L.E., Bessler, W.K., et al., *Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors.* Exp Hematol, 2007. **35**(7): p. 1109-18.
120. Gill, M., Dias, S., Hattori, K., et al., *Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells.* Circ Res, 2001. **88**(2): p. 167-74.
121. Bertolini, F., Shaked, Y., Mancuso, P., et al., *The multifaceted circulating endothelial cell in cancer: towards marker and target identification.* Nat Rev Cancer, 2006. **6**(11): p. 835-45.
122. Madeddu, P., Emanueli, C., Pelosi, E., et al., *Transplantation of low dose CD34+KDR+ cells promotes vascular and muscular regeneration in ischemic limbs.* FASEB J, 2004. **18**(14): p. 1737-9.
123. Schmidt-Lucke, C., Fichtlscherer, S., Aicher, A., et al., *Quantification of circulating endothelial progenitor cells using the modified ISHAGE protocol.* PLoS One, 2010. **5**(11): p. e13790.
124. Farace, F., Gross-Goupil, M., Tournay, E., et al., *Levels of circulating CD45(dim)CD34(+)VEGFR2(+) progenitor cells correlate with outcome in metastatic renal cell carcinoma patients treated with tyrosine kinase inhibitors.* Br J Cancer, 2011. **104**(7): p. 1144-50.

125. Fadini, G.P., Baesso, I., Albiero, M., et al., *Technical notes on endothelial progenitor cells: ways to escape from the knowledge plateau.* Atherosclerosis, 2008. **197**(2): p. 496-503.
126. Hristov, M., Erl, W., and Weber, P.C., *Endothelial progenitor cells: mobilization, differentiation, and homing.* Arterioscler Thromb Vasc Biol, 2003. **23**(7): p. 1185-9.
127. Urbich, C. and Dimmeler, S., *Endothelial progenitor cells: characterization and role in vascular biology.* Circ Res, 2004. **95**(4): p. 343-53.
128. Wu, X., Lensch, M.W., Wylie-Sears, J., et al., *Hemogenic endothelial progenitor cells isolated from human umbilical cord blood.* Stem Cells, 2007. **25**(11): p. 2770-6.
129. Kaushal, S., Amiel, G.E., Guleserian, K.J., et al., *Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo.* Nat Med, 2001. **7**(9): p. 1035-40.
130. Brown, M. and Wittwer, C., *Flow cytometry: principles and clinical applications in hematology.* Clin Chem, 2000. **46**(8 Pt 2): p. 1221-9.
131. Prokopi, M., Pula, G., Mayr, U., et al., *Proteomic analysis reveals presence of platelet microparticles in endothelial progenitor cell cultures.* Blood, 2009. **114**(3): p. 723-32.
132. Kim, S.J., Kim, J.S., Papadopoulos, J., et al., *Circulating monocytes expressing CD31: implications for acute and chronic angiogenesis.* Am J Pathol, 2009. **174**(5): p. 1972-80.
133. Yoder, M.C., Mead, L.E., Prater, D., et al., *Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals.* Blood, 2007. **109**(5): p. 1801-9.
134. Sieveking, D.P., Buckle, A., Celermajer, D.S., et al., *Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay.* J Am Coll Cardiol, 2008. **51**(6): p. 660-8.
135. Werner, N., Kosiol, S., Schiegl, T., et al., *Circulating endothelial progenitor cells and cardiovascular outcomes.* N Engl J Med, 2005. **353**(10): p. 999-1007.
136. Zhao, J., Mitrofan, C.G., Appleby, S.L., et al., *Disrupted Endothelial Cell Layer and Exposed Extracellular Matrix Proteins Promote Capture of Late Outgrowth Endothelial Progenitor Cells.* Stem Cells Int, 2016. **2016**: p. 1406304.
137. Matsumura, M., Fukuda, N., Kobayashi, N., et al., *Effects of atorvastatin on angiogenesis in hindlimb ischemia and endothelial progenitor cell formation in rats.* J Atheroscler Thromb, 2009. **16**(4): p. 319-26.
138. Asahara, T., Kawamoto, A., and Masuda, H., *Concise review: Circulating endothelial progenitor cells for vascular medicine.* Stem Cells, 2011. **29**(11): p. 1650-5.
139. Rehman, J., Li, J., Orschell, C.M., et al., *Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors.* Circulation, 2003. **107**(8): p. 1164-9.
140. Zhang, S.J., Zhang, H., Wei, Y.J., et al., *Adult endothelial progenitor cells from human peripheral blood maintain monocyte/macrophage function throughout in vitro culture.* Cell Res, 2006. **16**(6): p. 577-84.
141. Asakage, M., Tsuno, N.H., Kitayama, J., et al., *Early-outgrowth of endothelial progenitor cells can function as antigen-presenting cells.* Cancer Immunol Immunother, 2006. **55**(6): p. 708-16.
142. Vaughan, E.E. and O'Brien, T., *Isolation of circulating angiogenic cells.* Methods Mol Biol, 2012. **916**: p. 351-6.
143. O'Neill, C.L., Guduric-Fuchs, J., Chambers, S.E., et al., *Endothelial cell-derived pentraxin 3 limits the vasoreparative therapeutic potential of circulating angiogenic cells.* Cardiovasc Res, 2016. **112**(3): p. 677-688.
144. Stitt, A.W., O'Neill, C.L., O'Doherty, M.T., et al., *Vascular stem cells and ischaemic retinopathies.* Prog Retin Eye Res, 2011. **30**(3): p. 149-66.
145. Chambers, S.E., O'Neill, C.L., O'Doherty, T.M., et al., *The role of immune-related myeloid cells in angiogenesis.* Immunobiology, 2013. **218**(11): p. 1370-5.
146. Kanayasu-Toyoda, T., Tanaka, T., Kikuchi, Y., et al., *Cell-Surface MMP-9 Protein Is a Novel Functional Marker to Identify and Separate Proangiogenic Cells from Early Endothelial Progenitor Cells Derived from CD133(+) Cells.* Stem Cells, 2016. **34**(5): p. 1251-62.

147. Urbich, C., Aicher, A., Heeschen, C., et al., *Soluble factors released by endothelial progenitor cells promote migration of endothelial cells and cardiac resident progenitor cells.* J Mol Cell Cardiol, 2005. **39**(5): p. 733-42.
148. Medina, R.J., O'Neill, C.L., O'Doherty, T.M., et al., *Myeloid angiogenic cells act as alternative M2 macrophages and modulate angiogenesis through interleukin-8.* Mol Med, 2011. **17**(9-10): p. 1045-55.
149. Ingram, D.A., Mead, L.E., Tanaka, H., et al., *Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood.* Blood, 2004. **104**(9): p. 2752-60.
150. Zhao, J., Bolton, E.M., Randle, L., et al., *Functional characterization of late outgrowth endothelial progenitor cells in patients with end-stage renal failure.* Transpl Int, 2014. **27**(5): p. 437-51.
151. Tasev, D., Koolwijk, P., and van Hinsbergh, V.W., *Therapeutic Potential of Human-Derived Endothelial Colony-Forming Cells in Animal Models.* Tissue Eng Part B Rev, 2016. **22**(5): p. 371-382.
152. Lin, R.Z., Moreno-Luna, R., Li, D., et al., *Human endothelial colony-forming cells serve as trophic mediators for mesenchymal stem cell engraftment via paracrine signaling.* Proc Natl Acad Sci U S A, 2014. **111**(28): p. 10137-42.
153. Stroncek, J.D., Grant, B.S., Brown, M.A., et al., *Comparison of endothelial cell phenotypic markers of late-outgrowth endothelial progenitor cells isolated from patients with coronary artery disease and healthy volunteers.* Tissue Eng Part A, 2009. **15**(11): p. 3473-86.
154. Medina, R.J., O'Neill, C.L., O'Doherty, T.M., et al., *Ex vivo expansion of human outgrowth endothelial cells leads to IL-8-mediated replicative senescence and impaired vasoreparative function.* Stem Cells, 2013. **31**(8): p. 1657-68.
155. Patel, J., Wong, H.Y., Wang, W., et al., *Self-Renewal and High Proliferative Colony Forming Capacity of Late-Outgrowth Endothelial Progenitors Is Regulated by Cyclin-Dependent Kinase Inhibitors Driven by Notch Signaling.* Stem Cells, 2016. **34**(4): p. 902-12.
156. Yamamoto, K., Takahashi, T., Asahara, T., et al., *Proliferation, differentiation, and tube formation by endothelial progenitor cells in response to shear stress.* J Appl Physiol (1985), 2003. **95**(5): p. 2081-8.
157. Obi, S., Yamamoto, K., Shimizu, N., et al., *Fluid shear stress induces arterial differentiation of endothelial progenitor cells.* J Appl Physiol (1985), 2009. **106**(1): p. 203-11.
158. Mazzolai, L., Bouzourene, K., Hayoz, D., et al., *Characterization of human late outgrowth endothelial progenitor-derived cells under various flow conditions.* J Vasc Res, 2011. **48**(5): p. 443-51.
159. Brown, M.A., Wallace, C.S., Angelos, M., et al., *Characterization of umbilical cord blood-derived late outgrowth endothelial progenitor cells exposed to laminar shear stress.* Tissue Eng Part A, 2009. **15**(11): p. 3575-87.
160. Prater, D.N., Case, J., Ingram, D.A., et al., *Working hypothesis to redefine endothelial progenitor cells.* Leukemia, 2007. **21**(6): p. 1141-9.
161. Prasain, N., Meador, J.L., and Yoder, M.C., *Phenotypic and functional characterization of endothelial colony forming cells derived from human umbilical cord blood.* J Vis Exp, 2012(62).
162. Sandhu K, N.W., Yang Y, Harper AGS, Butler R, *Endothelial Progenitor Cell Identification, Classification and Nomenclature: A Review.* J Blood Lymph, 2018. **8: 221.**
163. Werner, N. and Nickenig, G., *Influence of cardiovascular risk factors on endothelial progenitor cells: limitations for therapy?* Arterioscler Thromb Vasc Biol, 2006. **26**(2): p. 257-66.
164. Schmidt-Lucke, C., Rossig, L., Fichtlscherer, S., et al., *Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair.* Circulation, 2005. **111**(22): p. 2981-7.
165. Jie, K.E., Goossens, M.H., van Oostrom, O., et al., *Circulating endothelial progenitor cell levels are higher during childhood than in adult life.* Atherosclerosis, 2009. **202**(2): p. 345-7.

166. Hoetzer, G.L., Van Guilder, G.P., Irmiger, H.M., et al., *Aging, exercise, and endothelial progenitor cell clonogenic and migratory capacity in men.* J Appl Physiol (1985), 2007. **102**(3): p. 847-52.
167. Thum, T., Hoeber, S., Froese, S., et al., *Age-dependent impairment of endothelial progenitor cells is corrected by growth-hormone-mediated increase of insulin-like growth-factor-1.* Circ Res, 2007. **100**(3): p. 434-43.
168. Siddique, A., Shantsila, E., Lip, G.Y., et al., *Endothelial progenitor cells: what use for the cardiologist?* J Angiogenes Res, 2010. **2**: p. 6.
169. Chang, E.I., Loh, S.A., Ceradini, D.J., et al., *Age decreases endothelial progenitor cell recruitment through decreases in hypoxia-inducible factor 1alpha stabilization during ischemia.* Circulation, 2007. **116**(24): p. 2818-29.
170. Shaffer, R.G., Greene, S., Arshi, A., et al., *Effect of acute exercise on endothelial progenitor cells in patients with peripheral arterial disease.* Vasc Med, 2006. **11**(4): p. 219-26.
171. Pelliccia, F., Pasceri, V., Meoni, G., et al., *Numbers of endothelial progenitor cells in peripheral blood are similar in younger and older patients with coronary artery disease.* Int J Cardiol, 2009. **133**(2): p. 277-9.
172. Laufs, U., Werner, N., Link, A., et al., *Physical training increases endothelial progenitor cells, inhibits neointima formation, and enhances angiogenesis.* Circulation, 2004. **109**(2): p. 220-6.
173. Rauscher, F.M., Goldschmidt-Clermont, P.J., Davis, B.H., et al., *Aging, progenitor cell exhaustion, and atherosclerosis.* Circulation, 2003. **108**(4): p. 457-63.
174. Maisel, A.S., Knowlton, K.U., Fowler, P., et al., *Adrenergic control of circulating lymphocyte subpopulations. Effects of congestive heart failure, dynamic exercise, and terbutaline treatment.* J Clin Invest, 1990. **85**(2): p. 462-7.
175. Rehman, J., Li, J., Parvathaneni, L., et al., *Exercise acutely increases circulating endothelial progenitor cells and monocyte-/macrophage-derived angiogenic cells.* J Am Coll Cardiol, 2004. **43**(12): p. 2314-8.
176. Fukai, T., Siegfried, M.R., Ushio-Fukai, M., et al., *Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training.* J Clin Invest, 2000. **105**(11): p. 1631-9.
177. De Biase, C., De Rosa, R., Luciano, R., et al., *Effects of physical activity on endothelial progenitor cells (EPCs).* Front Physiol, 2013. **4**: p. 414.
178. Volaklis, K.A., Tokmakidis, S.P., and Halle, M., *Acute and chronic effects of exercise on circulating endothelial progenitor cells in healthy and diseased patients.* Clin Res Cardiol, 2013. **102**(4): p. 249-57.
179. Cesari, F., Marcucci, R., Gori, A.M., et al., *Impact of a cardiac rehabilitation program and inflammatory state on endothelial progenitor cells in acute coronary syndrome patients.* Int J Cardiol, 2013. **167**(5): p. 1854-9.
180. Ikeda, N., Yasu, T., Kubo, N., et al., *Daily exercise and bone marrow-derived CD34+/133+ cells after myocardial infarction treated by bare metal stent implantation.* Circ J, 2008. **72**(6): p. 897-901.
181. Antonio, N., Fernandes, R., Soares, A., et al., *Reduced levels of circulating endothelial progenitor cells in acute myocardial infarction patients with diabetes or pre-diabetes: accompanying the glycemic continuum.* Cardiovasc Diabetol, 2014. **13**: p. 101.
182. Wang, X., Zhu, J., Chen, J., et al., *Effects of nicotine on the number and activity of circulating endothelial progenitor cells.* J Clin Pharmacol, 2004. **44**(8): p. 881-9.
183. Kondo, T., Hayashi, M., Takeshita, K., et al., *Smoking cessation rapidly increases circulating progenitor cells in peripheral blood in chronic smokers.* Arterioscler Thromb Vasc Biol, 2004. **24**(8): p. 1442-7.
184. Whittaker, A., Moore, J.S., Vasa-Nicotera, M., et al., *Evidence for genetic regulation of endothelial progenitor cells and their role as biological markers of atherosclerotic susceptibility.* Eur Heart J, 2008. **29**(3): p. 332-8.

185. Chironi, G., Walch, L., Pernollet, M.G., et al., *Decreased number of circulating CD34+KDR+ cells in asymptomatic subjects with preclinical atherosclerosis.* Atherosclerosis, 2007. **191**(1): p. 115-20.
186. Kunz, G.A., Liang, G., Cuculi, F., et al., *Circulating endothelial progenitor cells predict coronary artery disease severity.* Am Heart J, 2006. **152**(1): p. 190-5.
187. Werner, N., Wassmann, S., Ahlers, P., et al., *Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease.* Basic Res Cardiol, 2007. **102**(6): p. 565-71.
188. Wojakowski, W., Tendera, M., Michalowska, A., et al., *Mobilization of CD34/CXCR4+, CD34/CD117+, c-met+ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction.* Circulation, 2004. **110**(20): p. 3213-20.
189. Massa, M., Rosti, V., Ferrario, M., et al., *Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction.* Blood, 2005. **105**(1): p. 199-206.
190. Leone, A.M., Rutella, S., Bonanno, G., et al., *Mobilization of bone marrow-derived stem cells after myocardial infarction and left ventricular function.* Eur Heart J, 2005. **26**(12): p. 1196-204.
191. Gaspardone, A., Menghini, F., Mazzuca, V., et al., *Progenitor cell mobilisation in patients with acute and chronic coronary artery disease.* Heart, 2006. **92**(2): p. 253-4.
192. Shintani, S., Murohara, T., Ikeda, H., et al., *Mobilization of endothelial progenitor cells in patients with acute myocardial infarction.* Circulation, 2001. **103**(23): p. 2776-9.
193. George, J., Goldstein, E., Abashidze, S., et al., *Circulating endothelial progenitor cells in patients with unstable angina: association with systemic inflammation.* Eur Heart J, 2004. **25**(12): p. 1003-8.
194. Regueiro, A., Cuadrado-Godia, E., Bueno-Beti, C., et al., *Mobilization of endothelial progenitor cells in acute cardiovascular events in the PROCELL study: time-course after acute myocardial infarction and stroke.* J Mol Cell Cardiol, 2015. **80**: p. 146-55.
195. Santas-Alvarez, M., Rodino-Janeiro, B.K., Paradela-Dobarro, B., et al., *Endothelial progenitor cells mobilisation after percutaneous coronary intervention: a pilot study.* Br J Biomed Sci, 2016. **73**(4): p. 194-200.
196. Banerjee, S., Brilakis, E., Zhang, S., et al., *Endothelial progenitor cell mobilization after percutaneous coronary intervention.* Atherosclerosis, 2006. **189**(1): p. 70-5.
197. Laufs, U. and Liao, J.K., *Targeting Rho in cardiovascular disease.* Circ Res, 2000. **87**(7): p. 526-8.
198. Kipshidze, N., Dangas, G., Tsapenko, M., et al., *Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions.* J Am Coll Cardiol, 2004. **44**(4): p. 733-9.
199. Wojakowski, W., Pyrlik, A., Krol, M., et al., *Circulating endothelial progenitor cells are inversely correlated with in-stent restenosis in patients with non-ST-segment elevation acute coronary syndromes treated with EPC-capture stents (JACK-EPC trial).* Minerva Cardioangiol, 2013. **61**(3): p. 301-11.
200. Schober, A., Hoffmann, R., Opree, N., et al., *Peripheral CD34+ cells and the risk of in-stent restenosis in patients with coronary heart disease.* Am J Cardiol, 2005. **96**(8): p. 1116-22.
201. Duckers, H.J., Soullie, T., den Heijer, P., et al., *Accelerated vascular repair following percutaneous coronary intervention by capture of endothelial progenitor cells promotes regression of neointimal growth at long term follow-up: final results of the Healing II trial using an endothelial progenitor cell capturing stent (Genous R stent).* EuroIntervention, 2007. **3**(3): p. 350-8.
202. Duckers, H.J., Silber, S., de Winter, R., et al., *Circulating endothelial progenitor cells predict angiographic and intravascular ultrasound outcome following percutaneous coronary interventions in the HEALING-II trial: evaluation of an endothelial progenitor cell capturing stent.* EuroIntervention, 2007. **3**(1): p. 67-75.

203. Beijk, M.A., Klomp, M., Verouden, N.J., et al., *Genous endothelial progenitor cell capturing stent vs. the Taxus Liberte stent in patients with de novo coronary lesions with a high-risk of coronary restenosis: a randomized, single-centre, pilot study.* Eur Heart J, 2010. **31**(9): p. 1055-64.
204. Schachinger, V., Erbs, S., Elsasser, A., et al., *Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial.* Eur Heart J, 2006. **27**(23): p. 2775-83.
205. Numaguchi, Y., Sone, T., Okumura, K., et al., *The impact of the capability of circulating progenitor cell to differentiate on myocardial salvage in patients with primary acute myocardial infarction.* Circulation, 2006. **114**(1 Suppl): p. I114-9.
206. Kawamoto, A., Tkebuchava, T., Yamaguchi, J., et al., *Intramyocardial transplantation of autologous endothelial progenitor cells for therapeutic neovascularization of myocardial ischemia.* Circulation, 2003. **107**(3): p. 461-8.
207. Kong, D., Melo, L.G., Mangi, A.A., et al., *Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells.* Circulation, 2004. **109**(14): p. 1769-75.
208. Assmus, B., Schachinger, V., Teupe, C., et al., *Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI).* Circulation, 2002. **106**(24): p. 3009-17.
209. Strauer, B.E., Brehm, M., Zeus, T., et al., *Regeneration of human infarcted heart muscle by intracoronary autologous bone marrow cell transplantation in chronic coronary artery disease: the IACT Study.* J Am Coll Cardiol, 2005. **46**(9): p. 1651-8.
210. Ince, H., Petzsch, M., Kleine, H.D., et al., *Prevention of left ventricular remodeling with granulocyte colony-stimulating factor after acute myocardial infarction: final 1-year results of the Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony-Stimulating Factor (FIRSTLINE-AMI) Trial.* Circulation, 2005. **112**(9 Suppl): p. I73-80.
211. Zohlnhofer, D., Kastrati, A., and Schomig, A., *Stem cell mobilization by granulocyte-colony-stimulating factor in acute myocardial infarction: lessons from the REVIVAL-2 trial.* Nat Clin Pract Cardiovasc Med, 2007. **4 Suppl 1**: p. S106-9.
212. Zohlnhofer, D., Ott, I., Mehilli, J., et al., *Stem cell mobilization by granulocyte colony-stimulating factor in patients with acute myocardial infarction: a randomized controlled trial.* JAMA, 2006. **295**(9): p. 1003-10.
213. Aoki, J., Serruys, P.W., van Beusekom, H., et al., *Endothelial progenitor cell capture by stents coated with antibody against CD34: the HEALING-FIM (Healthy Endothelial Accelerated Lining Inhibits Neointimal Growth-First In Man) Registry.* J Am Coll Cardiol, 2005. **45**(10): p. 1574-9.
214. Steinhoff, G., Nesteruk, J., Wolfien, M., et al., *Cardiac Function Improvement and Bone Marrow Response -: Outcome Analysis of the Randomized PERFECT Phase III Clinical Trial of Intramyocardial CD133(+) Application After Myocardial Infarction.* EBioMedicine, 2017. **22**: p. 208-224.
215. Lemcke, H., Voronina, N., Steinhoff, G., et al., *Recent Progress in Stem Cell Modification for Cardiac Regeneration.* Stem Cells Int, 2018. **2018**: p. 1909346.
216. Schwartz, G.G., Olsson, A.G., Ezekowitz, M.D., et al., *Effects of atorvastatin on early recurrent ischemic events in acute coronary syndromes: the MIRACL study: a randomized controlled trial.* JAMA, 2001. **285**(13): p. 1711-8.
217. Nissen, S.E., Tuzcu, E.M., Schoenhagen, P., et al., *Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis: a randomized controlled trial.* JAMA, 2004. **291**(9): p. 1071-80.
218. Ridker, P.M., Morrow, D.A., Rose, L.M., et al., *Relative efficacy of atorvastatin 80 mg and pravastatin 40 mg in achieving the dual goals of low-density lipoprotein cholesterol <70 mg/dl and C-reactive protein <2 mg/l: an analysis of the PROVE-IT TIMI-22 trial.* J Am Coll Cardiol, 2005. **45**(10): p. 1644-8.

219. Baigent, C., Keech, A., Kearney, P.M., et al., *Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins.* Lancet, 2005. **366**(9493): p. 1267-78.
220. Patti, G., Pasceri, V., Colonna, G., et al., *Atorvastatin pretreatment improves outcomes in patients with acute coronary syndromes undergoing early percutaneous coronary intervention: results of the ARMYDA-ACS randomized trial.* J Am Coll Cardiol, 2007. **49**(12): p. 1272-8.
221. Di Sciascio, G., Patti, G., Pasceri, V., et al., *Efficacy of atorvastatin reload in patients on chronic statin therapy undergoing percutaneous coronary intervention: results of the ARMYDA-RECAPTURE (Atorvastatin for Reduction of Myocardial Damage During Angioplasty) Randomized Trial.* J Am Coll Cardiol, 2009. **54**(6): p. 558-65.
222. Patti, G., Cannon, C.P., Murphy, S.A., et al., *Clinical benefit of statin pretreatment in patients undergoing percutaneous coronary intervention: a collaborative patient-level meta-analysis of 13 randomized studies.* Circulation, 2011. **123**(15): p. 1622-32.
223. Athyros, V.G., Tziomalos, K., Gossios, T.D., et al., *Safety and efficacy of long-term statin treatment for cardiovascular events in patients with coronary heart disease and abnormal liver tests in the Greek Atorvastatin and Coronary Heart Disease Evaluation (GREACE) Study: a post-hoc analysis.* Lancet, 2010. **376**(9756): p. 1916-22.
224. Baller, D., Notohamiprodjo, G., Gleichmann, U., et al., *Improvement in coronary flow reserve determined by positron emission tomography after 6 months of cholesterol-lowering therapy in patients with early stages of coronary atherosclerosis.* Circulation, 1999. **99**(22): p. 2871-5.
225. Vasa, M., Fichtlscherer, S., Adler, K., et al., *Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease.* Circulation, 2001. **103**(24): p. 2885-90.
226. *Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S).* Lancet, 1994. **344**(8934): p. 1383-9.
227. *Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group.* N Engl J Med, 1998. **339**(19): p. 1349-57.
228. Sacks, F.M., Pfeffer, M.A., Moye, L.A., et al., *The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators.* N Engl J Med, 1996. **335**(14): p. 1001-9.
229. LaRosa, J.C., Grundy, S.M., Waters, D.D., et al., *Intensive lipid lowering with atorvastatin in patients with stable coronary disease.* N Engl J Med, 2005. **352**(14): p. 1425-35.
230. Lacoste, L., Lam, J.Y., Hung, J., et al., *Hyperlipidemia and coronary disease. Correction of the increased thrombogenic potential with cholesterol reduction.* Circulation, 1995. **92**(11): p. 3172-7.
231. Maron, D.J., Fazio, S., and Linton, M.F., *Current perspectives on statins.* Circulation, 2000. **101**(2): p. 207-13.
232. Shepherd, J., Cobbe, S.M., Ford, I., et al., *Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group.* N Engl J Med, 1995. **333**(20): p. 1301-7.
233. Pedersen, T.R., Berg, K., Cook, T.J., et al., *Safety and tolerability of cholesterol lowering with simvastatin during 5 years in the Scandinavian Simvastatin Survival Study.* Arch Intern Med, 1996. **156**(18): p. 2085-92.
234. Amsterdam, E.A., Wenger, N.K., Brindis, R.G., et al., *2014 AHA/ACC Guideline for the Management of Patients with Non-ST-Elevation Acute Coronary Syndromes: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines.* J Am Coll Cardiol, 2014. **64**(24): p. e139-228.
235. O'Gara, P.T., Kushner, F.G., Ascheim, D.D., et al., *2013 ACCF/AHA guideline for the management of ST-elevation myocardial infarction: a report of the American College of*

Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. Circulation, 2013. **127**(4): p. e362-425.
236. *Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS).* Circulation, 1998. **97**(15): p. 1440-5.
237. Bustos, C., Hernandez-Presa, M.A., Ortego, M., et al., *HMG-CoA reductase inhibition by atorvastatin reduces neointimal inflammation in a rabbit model of atherosclerosis.* J Am Coll Cardiol, 1998. **32**(7): p. 2057-64.
238. Laufs, U., Gertz, K., Huang, P., et al., *Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice.* Stroke, 2000. **31**(10): p. 2442-9.
239. Dogra, G.K., Watts, G.F., Chan, D.C., et al., *Statin therapy improves brachial artery vasodilator function in patients with Type 1 diabetes and microalbuminuria.* Diabet Med, 2005. **22**(3): p. 239-42.
240. Dangas, G., Smith, D.A., Unger, A.H., et al., *Pravastatin: an antithrombotic effect independent of the cholesterol-lowering effect.* Thromb Haemost, 2000. **83**(5): p. 688-92.
241. Dimmeler, S., Aicher, A., Vasa, M., et al., *HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells via the PI 3-kinase/Akt pathway.* J Clin Invest, 2001. **108**(3): p. 391-7.
242. Laufs, U., La Fata, V., Plutzky, J., et al., *Upregulation of endothelial nitric oxide synthase by HMG CoA reductase inhibitors.* Circulation, 1998. **97**(12): p. 1129-35.
243. Kureishi, Y., Luo, Z., Shiojima, I., et al., *The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animals.* Nat Med, 2000. **6**(9): p. 1004-10.
244. Laufs, U. and Liao, J.K., *Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase.* J Biol Chem, 1998. **273**(37): p. 24266-71.
245. Knowles, R.G. and Moncada, S., *Nitric oxide synthases in mammals.* Biochem J, 1994. **298 (Pt 2)**: p. 249-58.
246. Moncada, S. and Higgs, A., *The L-arginine-nitric oxide pathway.* N Engl J Med, 1993. **329**(27): p. 2002-12.
247. Sessa, W.C., *The nitric oxide synthase family of proteins.* J Vasc Res, 1994. **31**(3): p. 131-43.
248. Moncada, S., Palmer, R.M., and Higgs, E.A., *Nitric oxide: physiology, pathophysiology, and pharmacology.* Pharmacol Rev, 1991. **43**(2): p. 109-42.
249. Wilcox, J.N., Subramanian, R.R., Sundell, C.L., et al., *Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels.* Arterioscler Thromb Vasc Biol, 1997. **17**(11): p. 2479-88.
250. Stuehr, D.J. and Griffith, O.W., *Mammalian nitric oxide synthases.* Adv Enzymol Relat Areas Mol Biol, 1992. **65**: p. 287-346.
251. Dimmeler, S., Fleming, I., Fisslthaler, B., et al., *Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation.* Nature, 1999. **399**(6736): p. 601-5.
252. Fleming, I., Fisslthaler, B., Dimmeler, S., et al., *Phosphorylation of Thr(495) regulates Ca(2+)/calmodulin-dependent endothelial nitric oxide synthase activity.* Circ Res, 2001. **88**(11): p. E68-75.
253. Rubanyi, G.M. and Vanhoutte, P.M., *Superoxide anions and hyperoxia inactivate endothelium-derived relaxing factor.* Am J Physiol, 1986. **250**(5 Pt 2): p. H822-7.
254. Carmeliet, P., *Mechanisms of angiogenesis and arteriogenesis.* Nat Med, 2000. **6**(4): p. 389-95.
255. Carmeliet, P. and Jain, R.K., *Angiogenesis in cancer and other diseases.* Nature, 2000. **407**(6801): p. 249-57.
256. Semenza, G.L., *Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling.* J Cell Biochem, 2007. **102**(4): p. 840-7.
257. Wahlberg, E., *Angiogenesis and arteriogenesis in limb ischemia.* J Vasc Surg, 2003. **38**(1): p. 198-203.
258. Landmesser, U., Engberding, N., Bahlmann, F.H., et al., *Statin-induced improvement of endothelial progenitor cell mobilization, myocardial neovascularization, left ventricular*

function, and survival after experimental myocardial infarction requires endothelial nitric oxide synthase. Circulation, 2004. **110**(14): p. 1933-9.
259. Matsuno, H., Takei, M., Hayashi, H., et al., *Simvastatin enhances the regeneration of endothelial cells via VEGF secretion in injured arteries.* J Cardiovasc Pharmacol, 2004. **43**(3): p. 333-40.
260. Liao, J.K., Shin, W.S., Lee, W.Y., et al., *Oxidized low-density lipoprotein decreases the expression of endothelial nitric oxide synthase.* J Biol Chem, 1995. **270**(1): p. 319-24.
261. Liao, J.K. and Clark, S.L., *Regulation of G-protein alpha i2 subunit expression by oxidized low-density lipoprotein.* J Clin Invest, 1995. **95**(4): p. 1457-63.
262. Tamai, O., Matsuoka, H., Itabe, H., et al., *Single LDL apheresis improves endothelium-dependent vasodilatation in hypercholesterolemic humans.* Circulation, 1997. **95**(1): p. 76-82.
263. Lopez-Farre, A., Caramelo, C., Esteban, A., et al., *Effects of aspirin on platelet-neutrophil interactions. Role of nitric oxide and endothelin-1.* Circulation, 1995. **91**(7): p. 2080-8.
264. Latini, R., Bianchi, M., Correale, E., et al., *Cytokines in acute myocardial infarction: selective increase in circulating tumor necrosis factor, its soluble receptor, and interleukin-1 receptor antagonist.* J Cardiovasc Pharmacol, 1994. **23**(1): p. 1-6.
265. Ma, X.L., Weyrich, A.S., Lefer, D.J., et al., *Diminished basal nitric oxide release after myocardial ischemia and reperfusion promotes neutrophil adherence to coronary endothelium.* Circ Res, 1993. **72**(2): p. 403-12.
266. Assmus, B., Urbich, C., Aicher, A., et al., *HMG-CoA reductase inhibitors reduce senescence and increase proliferation of endothelial progenitor cells via regulation of cell cycle regulatory genes.* Circ Res, 2003. **92**(9): p. 1049-55.
267. Kusuyama, T., Omura, T., Nishiya, D., et al., *The effects of HMG-CoA reductase inhibitor on vascular progenitor cells.* J Pharmacol Sci, 2006. **101**(4): p. 344-9.
268. Laufs, U., Fata, V.L., and Liao, J.K., *Inhibition of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase blocks hypoxia-mediated down-regulation of endothelial nitric oxide synthase.* J Biol Chem, 1997. **272**(50): p. 31725-9.
269. Davis, M.E., Cai, H., McCann, L., et al., *Role of c-Src in regulation of endothelial nitric oxide synthase expression during exercise training.* Am J Physiol Heart Circ Physiol, 2003. **284**(4): p. H1449-53.
270. Kosmidou, I., Moore, J.P., Weber, M., et al., *Statin treatment and 3' polyadenylation of eNOS mRNA.* Arterioscler Thromb Vasc Biol, 2007. **27**(12): p. 2642-9.
271. Minami, Y., Satoh, M., Maesawa, C., et al., *Effect of atorvastatin on microRNA 221 / 222 expression in endothelial progenitor cells obtained from patients with coronary artery disease.* Eur J Clin Invest, 2009. **39**(5): p. 359-67.
272. Petersen, C.P., Bordeleau, M.E., Pelletier, J., et al., *Short RNAs repress translation after initiation in mammalian cells.* Mol Cell, 2006. **21**(4): p. 533-42.
273. Felli, N., Fontana, L., Pelosi, E., et al., *MicroRNAs 221 and 222 inhibit normal erythropoiesis and erythroleukemic cell growth via kit receptor down-modulation.* Proc Natl Acad Sci U S A, 2005. **102**(50): p. 18081-6.
274. Cerda, A., Fajardo, C.M., Basso, R.G., et al., *Role of microRNAs 221/222 on Statin Induced Nitric Oxide Release in Human Endothelial Cells.* Arq Bras Cardiol, 2014. **0**: p. 0.
275. Kuehbacher, A., Urbich, C., and Dimmeler, S., *Targeting microRNA expression to regulate angiogenesis.* Trends Pharmacol Sci, 2008. **29**(1): p. 12-5.
276. Bader, A.G., Kang, S., Zhao, L., et al., *Oncogenic PI3K deregulates transcription and translation.* Nat Rev Cancer, 2005. **5**(12): p. 921-9.
277. Dimmeler, S. and Zeiher, A.M., *Akt takes center stage in angiogenesis signaling.* Circ Res, 2000. **86**(1): p. 4-5.
278. Whitman, M., Downes, C.P., Keeler, M., et al., *Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate.* Nature, 1988. **332**(6165): p. 644-6.
279. Toker, A. and Cantley, L.C., *Signalling through the lipid products of phosphoinositide-3-OH kinase.* Nature, 1997. **387**(6634): p. 673-6.

280. Karar, J. and Maity, A., *PI3K/AKT/mTOR Pathway in Angiogenesis.* Front Mol Neurosci, 2011. **4**: p. 51.
281. Kawasaki, K., Smith, R.S., Jr., Hsieh, C.M., et al., *Activation of the phosphatidylinositol 3-kinase/protein kinase Akt pathway mediates nitric oxide-induced endothelial cell migration and angiogenesis.* Mol Cell Biol, 2003. **23**(16): p. 5726-37.
282. Ellis, L.M. and Hicklin, D.J., *VEGF-targeted therapy: mechanisms of anti-tumour activity.* Nat Rev Cancer, 2008. **8**(8): p. 579-91.
283. Papapetropoulos, A., Garcia-Cardena, G., Madri, J.A., et al., *Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells.* J Clin Invest, 1997. **100**(12): p. 3131-9.
284. Fulton, D., Gratton, J.P., McCabe, T.J., et al., *Regulation of endothelium-derived nitric oxide production by the protein kinase Akt.* Nature, 1999. **399**(6736): p. 597-601.
285. Dimmeler, S., Dernbach, E., and Zeiher, A.M., *Phosphorylation of the endothelial nitric oxide synthase at ser-1177 is required for VEGF-induced endothelial cell migration.* FEBS Lett, 2000. **477**(3): p. 258-62.
286. Bahlmann, F.H., De Groot, K., Spandau, J.M., et al., *Erythropoietin regulates endothelial progenitor cells.* Blood, 2004. **103**(3): p. 921-6.
287. Aicher, A., Heeschen, C., Mildner-Rihm, C., et al., *Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells.* Nat Med, 2003. **9**(11): p. 1370-6.
288. Llevadot, J., Murasawa, S., Kureishi, Y., et al., *HMG-CoA reductase inhibitor mobilizes bone marrow--derived endothelial progenitor cells.* J Clin Invest, 2001. **108**(3): p. 399-405.
289. Rikitake, Y. and Liao, J.K., *Rho GTPases, statins, and nitric oxide.* Circ Res, 2005. **97**(12): p. 1232-5.
290. Lemoine, S., Zhu, L., Legallois, D., et al., *Atorvastatin-induced cardioprotection of human myocardium is mediated by the inhibition of mitochondrial permeability transition pore opening via tumor necrosis factor-alpha and Janus kinase/signal transducers and activators of transcription pathway.* Anesthesiology, 2013. **118**(6): p. 1373-84.
291. Sanada, S., Asanuma, H., Minamino, T., et al., *Optimal windows of statin use for immediate infarct limitation: 5'-nucleotidase as another downstream molecule of phosphatidylinositol 3-kinase.* Circulation, 2004. **110**(15): p. 2143-9.
292. Solenkova, N.V., Solodushko, V., Cohen, M.V., et al., *Endogenous adenosine protects preconditioned heart during early minutes of reperfusion by activating Akt.* Am J Physiol Heart Circ Physiol, 2006. **290**(1): p. H441-9.
293. Hausenloy, D.J. and Yellon, D.M., *New directions for protecting the heart against ischaemia-reperfusion injury: targeting the Reperfusion Injury Salvage Kinase (RISK)-pathway.* Cardiovasc Res, 2004. **61**(3): p. 448-60.
294. Efthymiou, C.A., Mocanu, M.M., and Yellon, D.M., *Atorvastatin and myocardial reperfusion injury: new pleiotropic effect implicating multiple prosurvival signaling.* J Cardiovasc Pharmacol, 2005. **45**(3): p. 247-52.
295. Hibbert, B., Ma, X., Pourdjabbar, A., et al., *Pre-procedural atorvastatin mobilizes endothelial progenitor cells: clues to the salutary effects of statins on healing of stented human arteries.* PLoS One, 2011. **6**(1): p. e16413.
296. Baran, C., Durdu, S., Dalva, K., et al., *Effects of preoperative short term use of atorvastatin on endothelial progenitor cells after coronary surgery: a randomized, controlled trial.* Stem Cell Rev, 2012. **8**(3): p. 963-71.
297. Schmidt-Lucke, C., Fichtlscherer, S., Rossig, L., et al., *Improvement of endothelial damage and regeneration indexes in patients with coronary artery disease after 4 weeks of statin therapy.* Atherosclerosis, 2010. **211**(1): p. 249-54.
298. Tousoulis, D., Andreou, I., Tsiatas, M., et al., *Effects of rosuvastatin and allopurinol on circulating endothelial progenitor cells in patients with congestive heart failure: the impact of inflammatory process and oxidative stress.* Atherosclerosis, 2011. **214**(1): p. 151-7.
299. Jialal, I., Devaraj, S., Singh, U., et al., *Decreased number and impaired functionality of endothelial progenitor cells in subjects with metabolic syndrome: implications for increased cardiovascular risk.* Atherosclerosis, 2010. **211**(1): p. 297-302.

300. Cohen, M.V., Yang, X.M., and Downey, J.M., *Nitric oxide is a preconditioning mimetic and cardioprotectant and is the basis of many available infarct-sparing strategies.* Cardiovasc Res, 2006. **70**(2): p. 231-9.
301. Scalia, R., Gooszen, M.E., Jones, S.P., et al., *Simvastatin exerts both anti-inflammatory and cardioprotective effects in apolipoprotein E-deficient mice.* Circulation, 2001. **103**(21): p. 2598-603.
302. Wassmann, S., Laufs, U., Muller, K., et al., *Cellular antioxidant effects of atorvastatin in vitro and in vivo.* Arterioscler Thromb Vasc Biol, 2002. **22**(2): p. 300-5.
303. Mensah, K., Mocanu, M.M., and Yellon, D.M., *Failure to protect the myocardium against ischemia/reperfusion injury after chronic atorvastatin treatment is recaptured by acute atorvastatin treatment: a potential role for phosphatase and tensin homolog deleted on chromosome ten?* J Am Coll Cardiol, 2005. **45**(8): p. 1287-91.
304. Mihos, C.G., Pineda, A.M., and Santana, O., *Cardiovascular effects of statins, beyond lipid-lowering properties.* Pharmacol Res, 2014. **88**: p. 12-9.
305. Ye, H., He, F., Fei, X., et al., *High-dose atorvastatin reloading before percutaneous coronary intervention increased circulating endothelial progenitor cells and reduced inflammatory cytokine expression during the perioperative period.* J Cardiovasc Pharmacol Ther, 2014. **19**(3): p. 290-5.
306. Ricottini E, M.R., Patti G, et al, *Benefit of atorvastatin reload on endothelial progenitor cells in patients on chronic statin treatment undergoing PCI.* J Am Coll Cardiol. , 2013. **61(10_S):. doi:10.1016/S0735-1097(13)61635-6**.
307. Tousoulis, D., Andreou, I., Antoniades, C., et al., *Role of inflammation and oxidative stress in endothelial progenitor cell function and mobilization: therapeutic implications for cardiovascular diseases.* Atherosclerosis, 2008. **201**(2): p. 236-47.
308. Yao, E.H., Yu, Y., and Fukuda, N., *Oxidative stress on progenitor and stem cells in cardiovascular diseases.* Curr Pharm Biotechnol, 2006. **7**(2): p. 101-8.
309. Morel, D.W., DiCorleto, P.E., and Chisolm, G.M., *Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation.* Arteriosclerosis, 1984. **4**(4): p. 357-64.
310. Lamb, D.J., Wilkins, G.M., and Leake, D.S., *The oxidative modification of low density lipoprotein by human lymphocytes.* Atherosclerosis, 1992. **92**(2-3): p. 187-92.
311. Lim, S. and Barter, P., *Antioxidant effects of statins in the management of cardiometabolic disorders.* J Atheroscler Thromb, 2014. **21**(10): p. 997-1010.
312. Carracedo, J., Merino, A., Briceno, C., et al., *Carbamylated low-density lipoprotein induces oxidative stress and accelerated senescence in human endothelial progenitor cells.* FASEB J, 2011. **25**(4): p. 1314-22.
313. Tso, C., Martinic, G., Fan, W.H., et al., *High-density lipoproteins enhance progenitor-mediated endothelium repair in mice.* Arterioscler Thromb Vasc Biol, 2006. **26**(5): p. 1144-9.
314. Griendling, K.K. and FitzGerald, G.A., *Oxidative stress and cardiovascular injury: Part II: animal and human studies.* Circulation, 2003. **108**(17): p. 2034-40.
315. Thum, T., Fraccarollo, D., Schultheiss, M., et al., *Endothelial nitric oxide synthase uncoupling impairs endothelial progenitor cell mobilization and function in diabetes.* Diabetes, 2007. **56**(3): p. 666-74.
316. Urbich, C., Knau, A., Fichtlscherer, S., et al., *FOXO-dependent expression of the proapoptotic protein Bim: pivotal role for apoptosis signaling in endothelial progenitor cells.* FASEB J, 2005. **19**(8): p. 974-6.
317. Fleissner, F. and Thum, T., *Critical role of the nitric oxide/reactive oxygen species balance in endothelial progenitor dysfunction.* Antioxid Redox Signal, 2011. **15**(4): p. 933-48.
318. Tao, J., Yang, Z., Wang, J.M., et al., *Shear stress increases Cu/Zn SOD activity and mRNA expression in human endothelial progenitor cells.* J Hum Hypertens, 2007. **21**(5): p. 353-8.
319. He, T., Peterson, T.E., Holmuhamedov, E.L., et al., *Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase.* Arterioscler Thromb Vasc Biol, 2004. **24**(11): p. 2021-7.

320. Dernbach, E., Urbich, C., Brandes, R.P., et al., *Antioxidative stress-associated genes in circulating progenitor cells: evidence for enhanced resistance against oxidative stress.* Blood, 2004. **104**(12): p. 3591-7.
321. Endres, M., *Statins: potential new indications in inflammatory conditions.* Atheroscler Suppl, 2006. **7**(1): p. 31-5.
322. Bauer, S.M., Goldstein, L.J., Bauer, R.J., et al., *The bone marrow-derived endothelial progenitor cell response is impaired in delayed wound healing from ischemia.* J Vasc Surg, 2006. **43**(1): p. 134-41.
323. Masuda, H., Kalka, C., Takahashi, T., et al., *Estrogen-mediated endothelial progenitor cell biology and kinetics for physiological postnatal vasculogenesis.* Circ Res, 2007. **101**(6): p. 598-606.
324. Ii, M., Nishimura, H., Iwakura, A., et al., *Endothelial progenitor cells are rapidly recruited to myocardium and mediate protective effect of ischemic preconditioning via "imported" nitric oxide synthase activity.* Circulation, 2005. **111**(9): p. 1114-20.
325. Zhang, Y., Devries, M.E., and Skolnick, J., *Structure modeling of all identified G protein-coupled receptors in the human genome.* PLoS Comput Biol, 2006. **2**(2): p. e13.
326. Bjarnadottir, T.K., Gloriam, D.E., Hellstrand, S.H., et al., *Comprehensive repertoire and phylogenetic analysis of the G protein-coupled receptors in human and mouse.* Genomics, 2006. **88**(3): p. 263-73.
327. Schoneberg, T., Hofreiter, M., Schulz, A., et al., *Learning from the past: evolution of GPCR functions.* Trends Pharmacol Sci, 2007. **28**(3): p. 117-21.
328. Pierce, K.L., Premont, R.T., and Lefkowitz, R.J., *Seven-transmembrane receptors.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 639-50.
329. Perez, D.M., *The evolutionarily triumphant G-protein-coupled receptor.* Mol Pharmacol, 2003. **63**(6): p. 1202-5.
330. Rosenbaum, D.M., Rasmussen, S.G., and Kobilka, B.K., *The structure and function of G-protein-coupled receptors.* Nature, 2009. **459**(7245): p. 356-63.
331. Venkatakrishnan, A.J., Deupi, X., Lebon, G., et al., *Molecular signatures of G-protein-coupled receptors.* Nature, 2013. **494**(7436): p. 185-94.
332. Callihan, P., Mumaw, J., Machacek, D.W., et al., *Regulation of stem cell pluripotency and differentiation by G protein coupled receptors.* Pharmacol Ther, 2011. **129**(3): p. 290-306.
333. Kobayashi, N.R., Hawes, S.M., Crook, J.M., et al., *G-protein coupled receptors in stem cell self-renewal and differentiation.* Stem Cell Rev, 2010. **6**(3): p. 351-66.
334. Nature Reviews Drug Discovery, G.Q.P., *The state of GPCR research in 2004.* Nat Rev Drug Discov, 2004. **3**(7): p. 575, 577-626.
335. Jacoby, E., Bouhelal, R., Gerspacher, M., et al., *The 7 TM G-protein-coupled receptor target family.* ChemMedChem, 2006. **1**(8): p. 761-82.
336. Kozasa, T., Jiang, X., Hart, M.J., et al., *p115 RhoGEF, a GTPase activating protein for Galpha12 and Galpha13.* Science, 1998. **280**(5372): p. 2109-11.
337. Noma, K., Oyama, N., and Liao, J.K., *Physiological role of ROCKs in the cardiovascular system.* Am J Physiol Cell Physiol, 2006. **290**(3): p. C661-8.
338. Taussig, R., Iniguez-Lluhi, J.A., and Gilman, A.G., *Inhibition of adenylyl cyclase by Gi alpha.* Science, 1993. **261**(5118): p. 218-21.
339. Morello, F., Perino, A., and Hirsch, E., *Phosphoinositide 3-kinase signalling in the vascular system.* Cardiovasc Res, 2009. **82**(2): p. 261-71.
340. Gilman, A.G., *G proteins: transducers of receptor-generated signals.* Annu Rev Biochem, 1987. **56**: p. 615-49.
341. Ghanemi, A., *Targeting G protein coupled receptor-related pathways as emerging molecular therapies.* Saudi Pharm J, 2015. **23**(2): p. 115-29.
342. Lin, S.H. and Civelli, O., *Orphan G protein-coupled receptors: targets for new therapeutic interventions.* Ann Med, 2004. **36**(3): p. 204-14.
343. Lynch, J.R. and Wang, J.Y., *G Protein-Coupled Receptor Signaling in Stem Cells and Cancer.* Int J Mol Sci, 2016. **17**(5).

344. Bush, L.R. and Shebuski, R.J., *In vivo models of arterial thrombosis and thrombolysis.* FASEB J, 1990. **4**(13): p. 3087-98.
345. Urbich, C., Dernbach, E., Zeiher, A.M., et al., *Double-edged role of statins in angiogenesis signaling.* Circ Res, 2002. **90**(6): p. 737-44.
346. Deschaseaux, F., Selmani, Z., Falcoz, P.E., et al., *Two types of circulating endothelial progenitor cells in patients receiving long term therapy by HMG-CoA reductase inhibitors.* Eur J Pharmacol, 2007. **562**(1-2): p. 111-8.
347. Treasure, C.B., Klein, J.L., Weintraub, W.S., et al., *Beneficial effects of cholesterol-lowering therapy on the coronary endothelium in patients with coronary artery disease.* N Engl J Med, 1995. **332**(8): p. 481-7.
348. Huang, B., Cheng, Y., Xie, Q., et al., *Effect of 40 mg versus 10 mg of atorvastatin on oxidized low-density lipoprotein, high-sensitivity C-reactive protein, circulating endothelial-derived microparticles, and endothelial progenitor cells in patients with ischemic cardiomyopathy.* Clin Cardiol, 2012. **35**(2): p. 125-30.
349. Hristov, M., Fach, C., Becker, C., et al., *Reduced numbers of circulating endothelial progenitor cells in patients with coronary artery disease associated with long-term statin treatment.* Atherosclerosis, 2007. **192**(2): p. 413-20.
350. Lin, L.Y., Huang, C.C., Chen, J.S., et al., *Effects of pitavastatin versus atorvastatin on the peripheral endothelial progenitor cells and vascular endothelial growth factor in high-risk patients: a pilot prospective, double-blind, randomized study.* Cardiovasc Diabetol, 2014. **13**: p. 111.
351. Eisen, A., Leshem-Lev, D., Yavin, H., et al., *Effect of High Dose Statin Pretreatment on Endothelial Progenitor Cells After Percutaneous Coronary Intervention (HIPOCRATES Study).* Cardiovasc Drugs Ther, 2015. **29**(2): p. 129-35.
352. Madonna, R., Renna, F.V., Lanuti, P., et al., *The acute impact of high-dose lipid-lowering treatment on endothelial progenitor cells in patients with coronary artery disease-The REMEDY-EPC early substudy.* PLoS One, 2017. **12**(4): p. e0172800.
353. Weis, M., Heeschen, C., Glassford, A.J., et al., *Statins have biphasic effects on angiogenesis.* Circulation, 2002. **105**(6): p. 739-45.
354. Banai, S., Shweiki, D., Pinson, A., et al., *Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis.* Cardiovasc Res, 1994. **28**(8): p. 1176-9.
355. Shao, H., Tan, Y., Eton, D., et al., *Statin and stromal cell-derived factor-1 additively promote angiogenesis by enhancement of progenitor cells incorporation into new vessels.* Stem Cells, 2008. **26**(5): p. 1376-84.
356. Grundmann, F., Scheid, C., Braun, D., et al., *Differential increase of CD34, KDR/CD34, CD133/CD34 and CD117/CD34 positive cells in peripheral blood of patients with acute myocardial infarction.* Clin Res Cardiol, 2007. **96**(9): p. 621-7.
357. Paczkowska, E., Larysz, B., Rzeuski, R., et al., *Human hematopoietic stem/progenitor-enriched CD34(+) cells are mobilized into peripheral blood during stress related to ischemic stroke or acute myocardial infarction.* Eur J Haematol, 2005. **75**(6): p. 461-7.
358. Wang, Y., Johnsen, H.E., Mortensen, S., et al., *Changes in circulating mesenchymal stem cells, stem cell homing factor, and vascular growth factors in patients with acute ST elevation myocardial infarction treated with primary percutaneous coronary intervention.* Heart, 2006. **92**(6): p. 768-74.
359. Povsic, T.J., Najjar, S.S., Prather, K., et al., *EPC mobilization after erythropoietin treatment in acute ST-elevation myocardial infarction: the REVEAL EPC substudy.* J Thromb Thrombolysis, 2013. **36**(4): p. 375-83.
360. Leone, A.M., Rutella, S., Giannico, M.B., et al., *Effect of intensive vs standard statin therapy on endothelial progenitor cells and left ventricular function in patients with acute myocardial infarction: Statins for regeneration after acute myocardial infarction and PCI (STRAP) trial.* Int J Cardiol, 2008. **130**(3): p. 457-62.

361. Kim, J.S., Kim, J., Choi, D., et al., *Efficacy of high-dose atorvastatin loading before primary percutaneous coronary intervention in ST-segment elevation myocardial infarction: the STATIN STEMI trial.* JACC Cardiovasc Interv, 2010. **3**(3): p. 332-9.
362. Kim, J.S., Kim, J.H., Shin, D.H., et al., *Effect of High-Dose Statin Therapy on Drug-Eluting Stent Strut Coverage.* Arterioscler Thromb Vasc Biol, 2015. **35**(11): p. 2460-7.
363. Ko, Y.G., Won, H., Shin, D.H., et al., *Efficacy of early intensive rosuvastatin therapy in patients with ST-segment elevation myocardial infarction undergoing primary percutaneous coronary intervention (ROSEMARY Study).* Am J Cardiol, 2014. **114**(1): p. 29-35.
364. Woywodt, A., Bahlmann, F.H., De Groot, K., et al., *Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer.* Nephrol Dial Transplant, 2002. **17**(10): p. 1728-30.
365. Khakoo, A.Y. and Finkel, T., *Endothelial progenitor cells.* Annu Rev Med, 2005. **56**: p. 79-101.
366. Istvan, E.S., *Structural mechanism for statin inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase.* Am Heart J, 2002. **144**(6 Suppl): p. S27-32.
367. Nemeno-Guanzon, J.G., Lee, S., Berg, J.R., et al., *Trends in tissue engineering for blood vessels.* J Biomed Biotechnol, 2012. **2012**: p. 956345.
368. Kim, B.S. and Mooney, D.J., *Development of biocompatible synthetic extracellular matrices for tissue engineering.* Trends Biotechnol, 1998. **16**(5): p. 224-30.
369. Demirbag, B., Huri, P.Y., Kose, G.T., et al., *Advanced cell therapies with and without scaffolds.* Biotechnol J, 2011. **6**(12): p. 1437-53.
370. Castells-Sala C, A.-R.M., Fernandez-Muiños T, Recha-Sancho L, Lopez-Chicon P et al., *Current Applications of Tissue Engineering in Biomedicine.* Journal of bioengineering and bioelectronics 2013. **S2**(004).
371. Ji, W., Sun, Y., Yang, F., et al., *Bioactive electrospun scaffolds delivering growth factors and genes for tissue engineering applications.* Pharm Res, 2011. **28**(6): p. 1259-72.
372. Langer, R. and Vacanti, J.P., *Tissue engineering.* Science, 1993. **260**(5110): p. 920-6.
373. van der Worp, H.B., Howells, D.W., Sena, E.S., et al., *Can animal models of disease reliably inform human studies?* PLoS Med, 2010. **7**(3): p. e1000245.
374. Musa, F.I., Harper, A.G., and Yang, Y., *A Real-Time Monitoring System to Assess the Platelet Aggregatory Capacity of Components of a Tissue-Engineered Blood Vessel Wall.* Tissue Eng Part C Methods, 2016. **22**(7): p. 691-9.
375. Yang, Y., Wimpenny, I., and Ahearne, M., *Portable nanofiber meshes dictate cell orientation throughout three-dimensional hydrogels.* Nanomedicine, 2011. **7**(2): p. 131-6.
376. Owida, H.A., De Las Heras Ruiz, T., Dhillon, A., et al., *Co-culture of chondrons and mesenchymal stromal cells reduces the loss of collagen VI and improves extracellular matrix production.* Histochem Cell Biol, 2017. **148**(6): p. 625-638.
377. Bjorkhem-Bergman, L., Lindh, J.D., and Bergman, P., *What is a relevant statin concentration in cell experiments claiming pleiotropic effects?* Br J Clin Pharmacol, 2011. **72**(1): p. 164-5.
378. Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/, 1997-2018.
379. http://rsbweb.nih.gov/ij/docs/index.html.
380. https://www.bio-rad-antibodies.com/flow-cytometry-optics-detection.html.
381. Hulspas, R., O'Gorman, M.R., Wood, B.L., et al., *Considerations for the control of background fluorescence in clinical flow cytometry.* Cytometry B Clin Cytom, 2009. **76**(6): p. 355-64.
382. Duda, D.G., Cohen, K.S., Scadden, D.T., et al., *A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood.* Nat Protoc, 2007. **2**(4): p. 805-10.
383. Estes, M.L., Mund, J.A., Mead, L.E., et al., *Application of polychromatic flow cytometry to identify novel subsets of circulating cells with angiogenic potential.* Cytometry A, 2010. **77**(9): p. 831-9.
384. Van Craenenbroeck, E.M., Conraads, V.M., Van Bockstaele, D.R., et al., *Quantification of circulating endothelial progenitor cells: a methodological comparison of six flow cytometric approaches.* J Immunol Methods, 2008. **332**(1-2): p. 31-40.

385. Heimbeck, I., Hofer, T.P., Eder, C., et al., *Standardized single-platform assay for human monocyte subpopulations: Lower CD14+CD16++ monocytes in females.* Cytometry A, 2010. **77**(9): p. 823-30.
386. Hristov, M. and Weber, C., *Differential role of monocyte subsets in atherosclerosis.* Thromb Haemost, 2011. **106**(5): p. 757-62.
387. Hristov, M., Schmitz, S., Schuhmann, C., et al., *An optimized flow cytometry protocol for analysis of angiogenic monocytes and endothelial progenitor cells in peripheral blood.* Cytometry A, 2009. **75**(10): p. 848-53.
388. Hristov, M., Leyendecker, T., Schuhmann, C., et al., *Circulating monocyte subsets and cardiovascular risk factors in coronary artery disease.* Thromb Haemost, 2010. **104**(2): p. 412-4.
389. Sutherland, D.R., Anderson, L., Keeney, M., et al., *The ISHAGE guidelines for CD34+ cell determination by flow cytometry. International Society of Hematotherapy and Graft Engineering.* J Hematother, 1996. **5**(3): p. 213-26.
390. Hristov, M., Schmitz, S., Nauwelaers, F., et al., *A flow cytometric protocol for enumeration of endothelial progenitor cells and monocyte subsets in human blood.* J Immunol Methods, 2012. **381**(1-2): p. 9-13.
391. Dahmen, U.M., Boettcher, M., Krawczyk, M., et al., *Flow cytometric "rare event analysis": a standardized approach to the analysis of donor cell chimerism.* J Immunol Methods, 2002. **262**(1-2): p. 53-69.
392. Maecker, H.T. and Trotter, J., *Flow cytometry controls, instrument setup, and the determination of positivity.* Cytometry A, 2006. **69**(9): p. 1037-42.
393. Maecker, H.T., Frey, T., Nomura, L.E., et al., *Selecting fluorochrome conjugates for maximum sensitivity.* Cytometry A, 2004. **62**(2): p. 169-73.
394. http://docs.flowjo.com/vx/graphs-and-gating/gw-transform-overview/gw-transform-benefits/.
395. World Medical, A., *World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects.* JAMA, 2013. **310**(20): p. 2191-4.
396. Roffi, M., Patrono, C., Collet, J.P., et al., *2015 ESC Guidelines for the management of acute coronary syndromes in patients presenting without persistent ST-segment elevation: Task Force for the Management of Acute Coronary Syndromes in Patients Presenting without Persistent ST-Segment Elevation of the European Society of Cardiology (ESC).* Eur Heart J, 2016. **37**(3): p. 267-315.
397. Ibanez, B., James, S., Agewall, S., et al., *2017 ESC Guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation.* Rev Esp Cardiol (Engl Ed), 2017. **70**(12): p. 1082.
398. Task Force, M., Montalescot, G., Sechtem, U., et al., *2013 ESC guidelines on the management of stable coronary artery disease: the Task Force on the management of stable coronary artery disease of the European Society of Cardiology.* Eur Heart J, 2013. **34**(38): p. 2949-3003.
399. Teicher, B.A. and Fricker, S.P., *CXCL12 (SDF-1)/CXCR4 pathway in cancer.* Clin Cancer Res, 2010. **16**(11): p. 2927-31.
400. Bachelerie, F., Ben-Baruch, A., Burkhardt, A.M., et al., *International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors.* Pharmacol Rev, 2014. **66**(1): p. 1-79.
401. Bourguignon, L.Y. and Singer, S.J., *Transmembrane interactions and the mechanism of capping of surface receptors by their specific ligands.* Proc Natl Acad Sci U S A, 1977. **74**(11): p. 5031-5.
402. O'Malley, P.G., Taylor, A.J., Jackson, J.L., et al., *Prognostic value of coronary electron-beam computed tomography for coronary heart disease events in asymptomatic populations.* Am J Cardiol, 2000. **85**(8): p. 945-8.

403. Greenland, P., LaBree, L., Azen, S.P., et al., *Coronary artery calcium score combined with Framingham score for risk prediction in asymptomatic individuals.* JAMA, 2004. **291**(2): p. 210-5.
404. Taylor, A.J., Bindeman, J., Feuerstein, I., et al., *Coronary calcium independently predicts incident premature coronary heart disease over measured cardiovascular risk factors: mean three-year outcomes in the Prospective Army Coronary Calcium (PACC) project.* J Am Coll Cardiol, 2005. **46**(5): p. 807-14.
405. LaMonte, M.J., FitzGerald, S.J., Church, T.S., et al., *Coronary artery calcium score and coronary heart disease events in a large cohort of asymptomatic men and women.* Am J Epidemiol, 2005. **162**(5): p. 421-9.
406. Arad, Y., Spadaro, L.A., Goodman, K., et al., *Prediction of coronary events with electron beam computed tomography.* J Am Coll Cardiol, 2000. **36**(4): p. 1253-60.
407. Vliegenthart, R., Oudkerk, M., Hofman, A., et al., *Coronary calcification improves cardiovascular risk prediction in the elderly.* Circulation, 2005. **112**(4): p. 572-7.
408. Rosen, B.D., Fernandes, V., McClelland, R.L., et al., *Relationship between baseline coronary calcium score and demonstration of coronary artery stenoses during follow-up MESA (Multi-Ethnic Study of Atherosclerosis).* JACC Cardiovasc Imaging, 2009. **2**(10): p. 1175-83.
409. Budoff, M.J., Diamond, G.A., Raggi, P., et al., *Continuous probabilistic prediction of angiographically significant coronary artery disease using electron beam tomography.* Circulation, 2002. **105**(15): p. 1791-6.
410. Haberl, R., Becker, A., Leber, A., et al., *Correlation of coronary calcification and angiographically documented stenoses in patients with suspected coronary artery disease: results of 1,764 patients.* J Am Coll Cardiol, 2001. **37**(2): p. 451-7.
411. Skinner, J.S., Smeeth, L., Kendall, J.M., et al., *NICE guidance. Chest pain of recent onset: assessment and diagnosis of recent onset chest pain or discomfort of suspected cardiac origin.* Heart, 2010. **96**(12): p. 974-8.
412. Nieman, K., Galema, T.W., Neefjes, L.A., et al., *Comparison of the value of coronary calcium detection to computed tomographic angiography and exercise testing in patients with chest pain.* Am J Cardiol, 2009. **104**(11): p. 1499-504.
413. *National Institute for Health and Clinical Excellence . Chest pain of recent onset: assessment and diagnosis of recent onset chest pain or discomfort of suspected cardiac origin. CG95. London: National Institute for Health and Clinical Excellence; 2010.*
414. *National Institute for Health and Care Excellence. Chest pain of recent onset: assessment and diagnosis. .* London: NICE. 2016 https://www.nice.org.uk/guidance/cg95.
415. Maddox, T.M., Stanislawski, M.A., Grunwald, G.K., et al., *Nonobstructive coronary artery disease and risk of myocardial infarction.* JAMA, 2014. **312**(17): p. 1754-63.
416. Lee AJX, M.M., Quaderi SA, Richardson JA, Aggarwal SK, Speehly-Dick ME, *Implementation of NICE clinical guideline 95 for assessment of stable chest pain in a rapid access chest pain clinical reduces the mean number of investigations and cost per patient.* Open Heart, 2015(2:e000151.).
417. Genders, T.S., Steyerberg, E.W., Alkadhi, H., et al., *A clinical prediction rule for the diagnosis of coronary artery disease: validation, updating, and extension.* Eur Heart J, 2011. **32**(11): p. 1316-30.
418. Shaw, L.J., Raggi, P., Schisterman, E., et al., *Prognostic value of cardiac risk factors and coronary artery calcium screening for all-cause mortality.* Radiology, 2003. **228**(3): p. 826-33.
419. Sekhri, N., Feder, G.S., Junghans, C., et al., *How effective are rapid access chest pain clinics? Prognosis of incident angina and non-cardiac chest pain in 8762 consecutive patients.* Heart, 2007. **93**(4): p. 458-63.
420. Moss, A.J. and Newby, D.E., *CT coronary angiographic evaluation of suspected anginal chest pain.* Heart, 2016. **102**(4): p. 263-8.
421. Gray, A.J., Roobottom, C., Smith, J.E., et al., *The RAPID-CTCA trial (Rapid Assessment of Potential Ischaemic Heart Disease with CTCA) - a multicentre parallel-group randomised trial to compare early computerised tomography coronary angiography versus standard*

care in patients presenting with suspected or confirmed acute coronary syndrome: study protocol for a randomised controlled trial. Trials, 2016. **17**(1): p. 579.
422. Douglas, P.S., Hoffmann, U., Patel, M.R., et al., *Outcomes of anatomical versus functional testing for coronary artery disease.* N Engl J Med, 2015. **372**(14): p. 1291-300.
423. Investigators, S.-H., *CT coronary angiography in patients with suspected angina due to coronary heart disease (SCOT-HEART): an open-label, parallel-group, multicentre trial.* Lancet, 2015. **385**(9985): p. 2383-2391.
424. Investigators, S.-H., Newby, D.E., Adamson, P.D., et al., *Coronary CT Angiography and 5-Year Risk of Myocardial Infarction.* N Engl J Med, 2018. **379**(10): p. 924-933.