Endothelial Progenitor Cells and Vascular Injury

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For my mother and father.

STATEMENT OF MY INVOLVEMENT WITH THE WORK PRESENTED

I designed all of the experiments that were performed. I gathered and prepared the samples used in each set of experiments. I developed and performed the quantitative PCR assays. I gathered and analysed all of the data presented.

Signed

Date..........31. 3. 2007..........

ABBREVIATIONS

A	adenosine
ANOVA	analysis of variance
APC	Allophycocyanin
BMMC	bone marrow mononuclear cell
cDNA	complementary deoxyribonucleic acid
C	cytidine
CEC	circulating endothelial cell
CFU	colony forming unit
CRP	C-reactive protein
cv	coefficient of variation
DEPC	diethyl pyrocarbonate
dNTP	deoxynucleotide triphosphates
ELISA	enzyme linked immunoassay
EPC	endothelial progenitor cell
Epo	erythropoietin
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G	guanosine
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte macrophage colony stimulating factor
HIF-1	hypoxia-inducible factor-1
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA

HSC	haematopoietic stem cell
i.m.	intramuscular
KDR	kinase insert domain receptor
KS	Kologorov-Smirnov
LDL	low-density lipoprotein
Mab	monoclonal antibody
MFI	mean fluorescence intensity
MI	myocardial infarction
MMP-9	matrix metalloproteinase-9
Ng	nanograms
NOS	nitric oxide synthase
PAGE	polyacrylamide gel electrophoresis
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PE	phycoerythrin
PerCP	peridin chlorophylla protein
RNA	ribonucleic acid
RQ	relative quantity
RT	reverse transcriptase
SDF-1	stromal cell derived factor-1
sKitL	soluble Kit ligand

T	thymidine
TCO_2	transcutaneous oxygen
VE-cadherin	vascular endothelial cell adhesion molecule
VEGF	vascular endothelial growth factor
VEGFR-2	vascular endothelial growth factor receptor-2
vWF	von-Willebrand factor

CONTENTS

Abstract **p11**

1. Introduction **p13**

1.1 General overview and aims of thesis

1.2 Stem cells and progenitors: general terms and definitions

1.3 Embryonic stem cells

1.4 Adult stem cells

1.5 Stem and progenitor cells and the development of new blood vessels

1.6 Embryonic vasculogenesis

1.7 Postnatal endothelial progenitor cells

1.8 Characteristics of EPCs

1.9 Mobilisation, release and homing of EPCs

1.10 Exogenous factors affecting the recruitment of EPCs to the periphery

1.11 EPCs and tumour angiogenesis

1.12 EPCs and endothelial maintenance and regeneration

1.13 Clinical applications of EPC mediated neovascularisation

2. Methods **p46**

2.1 RNA isolation

2.2 Reverse transcription to complementary DNA

2.3 Polymerase chain reaction

2.4 EPC colony assay

2.5 Flow cytometry

2.6 ELISA

2.7 Statistical methods

3. Preliminary studies- PCR p74

3.1 Stability of RNA in blood samples

3.2 Establishing the best endogenous control

3.3 Relative quantitation of gene expression- comparative C_T method

3.4 Reproducibility data

3.5 End-point PCR

3.6 Measuring PCR product from different source materials

4. Endothelial progenitor cells and ischaemic heart disease p110

4.1 Introduction

4.2 Methods

4.3 Results

4.4 Discussion

4.5 Conclusion

5. Endothelial progenitor cells and aortic aneurysm repair p162

5.1 Introduction

5.2 Methods

5.3 Results

5.4 Discussion

6. Endothelial progenitor cells and type 1 diabetes **p203**

6.1 Introduction

6.2 Methods

6.3 Results

6.4 Discussion

7. Endothelial progenitor cells and chronic renal failure **p218**

7.1 Introduction

7.2 Methods

7.3 Results

7.4 Discussion

8. Conclusion **p237**

9. Abstracts **p241**

10. References **p245**

Abstract

Endothelial progenitor cells (EPCs) are bone marrow derived stem cells that contribute towards neovascularisation. I have primarily used real time polymerase chain reaction (PCR), but also flow cytometry and cell culture techniques, to investigate the effect of vascular injury on the expression of the putative markers of EPCs (CD34, CD133, VEGFR-2 and VE-cadherin) and their number in peripheral blood.

Preliminary studies were performed in order to optimise the real-time PCR assay, which was performed using the ABI 7900™ Taqman PCR system. It was shown that the relative quantities (RQ) of messenger RNA (mRNA) for the markers of EPCs were stable over a 24-hour period, with no significant reduction in expression when blood samples were serially sampled under different conditions. In addition, ribosomal 18s was found to be the best endogenous control. Furthermore, the coefficient of variation for the real-time PCR assay was shown to be less than 10%. Finally, the RQ of EPC mRNA was shown to vary depending on the type of source material, but was generally greater in cord blood, mobilised peripheral blood and bone marrow compared with peripheral blood.

In the first study I investigated the effect of percutaneous coronary intervention (PCI) on EPCs in a group of patients with stable coronary disease. After PCI, EPC markers did not conclusively demonstrate a rise in expression, although the number of VEGFR-2+

cells did increase. However, the number of EPC colony forming units (CFUs) increased significantly.

In the next study, I investigated the effect of open aortic aneurysm repair on EPCs in a group of elective surgical patients. There were changes in the level of expression of EPC markers, using both real-time PCR and flow cytometry, but statistical significance was not reached. However, there were significant increases in the mean fluorescent intensities (MFI) of VEGFR-2 and VE-cadherin expression. EPC-CFUs did not change significantly.

The next study investigated the effect of type 1 diabetes on EPC levels. The percentage of CD34+ cells, the RQ of VE-cadherin mRNA and the number of EPC-CFUs were significantly reduced in the diabetic cohort compared with control groups.

Finally, the effect of chronic renal impairment and administration of human recombinant erythropoietin (Epo) on EPC levels was investigated. The RQs of CD34, VEGFR-2 and VE-cadherin mRNA species increased over the period analysed, but this increase did not correspond with an increase in VEGF expression.

This thesis provides further insight into the effect of endogenous and exogenous causes of vascular injury on EPCs. It also highlights the difficulty in accurately defining and measuring EPCs.

INTRODUCTION

1.1. General overview and aims of thesis

An intact vascular network is a prerequisite for normal cellular function. As cells and organs grow this network must adapt to the changing environment. In the embryo, new blood vessels develop from primitive stem cells, which differentiate into mature endothelial cells by a process called vasculogenesis. In adults, new blood vessels are not only required for tissue growth, but also for revascularisation of damaged or ischaemic tissue. This was originally thought to occur by angiogenesis, which is vascular sprouting and intussusception, followed by myogenesis. However, recent studies have focussed on the ability of bone marrow derived stem cells in adults to participate in new vessel formation, both in physiological and pathological settings, by a process called postnatal vasculogenesis.

The introduction will begin by defining a stem cell, and distinguishing between embryonic and adult stem cells. A description of embryonic and adult vasculogenesis will be made, followed by a phenotypic characterisation of the cell thought to be critical in the process in the adult, the bone marrow derived endothelial progenitor cell (EPC). A section will follow this on mobilisation and homing of EPCs to sites to neovascularisation, including exogenous factors that can affect their release from the bone marrow. In addition, there will be a summary of the role of EPCs in physiological and pathological adult vascular development. Finally, there will be a discussion on the clinical implications of EPCs for therapeutic vasculogenesis.

The aim of this thesis is to investigate the effect vascular injury has on EPC marker expression and EPC numbers. Peripheral blood samples, taken from human subjects, will be analysed using primarily real-time PCR, but also flow cytometry and cell culture techniques. The subjects will undergo elective percutaneous coronary intervention (PCI) or aneurysm repair (as examples of exogenous vascular injury), or be patients with diabetes or chronic renal failure (as examples of endogenous vascular injury). The specific aims of this thesis are as follows:

1. To design novel assays for quantitation of mRNA for the putative markers of EPCs CD34, CD133, VEGFR-2 and VE-cadherin, as well as the marker for mature endothelial cells (vWF) and monocytes (CD14).
2. To use these assays in combination with flow cytometric and cell culture techniques to measure EPCs in patients undergoing PCI or elective aortic aneurysm repair to see it vascular trauma affects these cells in the post operative period.
3. To measure EPCs, using the above techniques, in a group of patients with type 1 diabetes and compare the results with a control group.
4. To measure EPCs using real-time PCR in patients with chronic renal failure commencing recombinant human erythropoietin (Epo) to determine whether the hormone can affect mRNA levels of EPC markers.
5. To compare and contrast the different assays employed in the measurement of EPCs.

1.2. Stem cells and progenitors: general terms and definitions

Terminology surrounding stem cells and progenitor cells is confusing. Essentially there are two kinds of stem cell in the body, originating from embryonic or adult tissues. A working definition of stem cells is primitive entities that are clonal, self-renewing, pluripotent, and have the ability to repopulate a tissue in vivo. The clonality and self-renewing capacity of a stem cell refers to its ability to produce more identical stem cells for the lifetime of the organism; potency refers to the ability of stem cells to produce multiple differentiated cell types. Tissue repopulation requires a stem cell to home to a particular site, where it must differentiate into tissue specific cells. This definition can be readily applied to embryonic stems cells, but it is thought to be too broad for adult stem cells. These cells have, up until recently, been thought to have a more limited potential for differentiation, and have been termed multipotent. Furthermore, unipotent adult stem cells, better defined as progenitor cells, have been shown to be descendents of multipotent stem cells, with a reduced ability for self-renewal and a reduced potency.

1.3. Embryonic stem cells

Embryonic stem cells are derived from a blastocyst that is developed from an in vitro fertilised egg. They were first established in the 1980s from murine embryos, and were derived fro the inner cell mass of an expanded blastocyst at 3.5 days post-coitum under strict culture conditions. Human embryonic stem cells were first isolated from in vitro fertilised blastocysts in 1998. They were shown to have a high nucleo-cytoplasmic ratio,

a high transcriptional activity and to express markers that were down regulated upon differentiation. The interest surrounding embryonic stem cells arises from the potential to use them as a therapeutic agent in tissue regeneration. The advantage of these cells is that they are pluripotent and large numbers of them can be relatively easily obtained in culture. However, with the ethical and legal concerns surrounding embryonic stem cells, adult stem cells have become a focus of intense interest, because they can potentially be expanded in culture and reintroduced into the patient without the risk of immune rejection.

1.4. Adult stem cells

The origin of adult stem cells is less clear than for embryonic stem cells. Their existence has been known about since the 1960s, when it was shown that blood or bone marrow contained cells that could rescue humans and animals from bone marrow failure. Since then other tissue-specific stem cells have been defined, including neural stem cells found in the postnatal brain, and mesenchymal stem cells found in the bone marrow and adipose tissue. They are thought to be responsible for regenerating damaged tissue and maintaining tissue homeostasis. Until very recently, stem cells from adult tissue were thought to be restricted to producing cells specific to that particular tissue. However, recent evidence suggests that adult stem cells, rather than being multipotent in a particular organ, are "plastic". This means that they have the capacity to differentiate into cells of unrelated tissue in a similar way to embryonic stem cells. The traditional paradigm on adult stem cells being lineage restricted and derived from pluripotent stem

cells very early during development has now been put into question. There are a number of mechanisms that could underlie plasticity seen in adult stem cells. Firstly, there is evidence that stem cells for a given organ may exist in a distant organ. For example, it is known that haematopoietic stem cells can also be harvested from organs such as muscle. Second, plasticity may result from fusion between donor cells and recipient cells. For example, it was shown that co culture of embryonic stem cells with either bone marrow cells or neural stem cells could lead to fused cells with the functional capacity of the former cell type, whilst maintaining expression of some genes from the latter cell types. Furthermore, it was shown in an animal model of cirrhosis that bone marrow transplantation resulted in hepatic regeneration by fusion between haematopoietic stem cells and hepatocytes [1]. Lastly, different stem cell populations may be more heterogenous than previously thought. For example, it is now known that haematopoietic stem cells contain a number of subpopulations of cells involved in various aspects of haematopoietic repopulation. It is likely that earlier stem cells committed to a haemangioblast or mesodermal fate are also present, with the capacity to differentiate into non-haematopoietic cells if placed in the appropriate microenvironment. For example, Grant et al demonstrated both endothelial and blood reconstitution following single-cell transplant of haematopoietic stem cells (Lin^{neg} $Sca\text{-}1^{pos}$ $c\text{-}kit^{pos}$) from mice expressing green fluorescent protein into lethally irradiated recipients [2]. One month after the mice were haematopoietically reconstituted, a retinal injury was induced with a laser. In every mouse that had haematopoietic reconstitution, the regenerating blood vessels expressed green fluorescent protein.

Adult stem cells may therefore be an alternative to embryonic stem cells as a source for tissue regeneration. The caveat, however, is that adult stem cells appear more difficult to grow in culture, so that obtaining clinically significant amount for therapeutic purposes may prove problematic.

1.5. Stem and progenitor cells and the development of new blood vessels

Embryonic development and tissue regeneration in adults rely on new blood vessel growth, termed neovascularisation. The traditional model of neovascularisation distinguished between the process in the embryo and in the adult. Embryonic neovascularisation was thought to occur by maturation of endothelial progenitor cells, termed embryonic vasculogenesis. In adults neovascularisation happened solely by sprouting of vessels from the division of differentiated endothelial cells. However, with the knowledge that tissue specific adult stem cells exist evidence has emerged which suggests that endothelial progenitor cells can also contribute to neovascularisation in the adult under various physiological and pathological conditions by a process called postnatal vasculogenesis. Blood vessel formation in the embryonic and adult will be summarised in the following sections.

1.6. Embryonic vasculogenesis

The formation of blood vessels occurs early in the embryo, at approximately day 7.5 in the mouse. It occurs at distinct locations in the yolk sac and within the embryo itself. In

the yolk sac mesodermal derived cells called haemangioblasts aggregate to form blood islands. Haemangioblasts are thought to be the common precursor of both haematopoietic stem cells and endothelial progenitor cells (EPCs), as both cell types develop in close association within the blood island. Cells from the outer rim develop into EPCs, whereas inner cells develop into erythrocytes, which are the first primitive blood cells. EPCs, also termed angioblasts, have the capacity to proliferate, migrate and differentiate into mature endothelial cells but have not yet acquired a mature phenotype. Within the embryo, blood vessels develop in a similar fashion from precursors within different areas of the splanchnic and somatic mesoderm. These blood islands fuse and grow to produce a primitive vascular plexus (vasculogenesis). Additional mechanisms for vessel development include "bridging" and "intussusception", termed angiogenesis. Vessel maturation then involves the endothelial-lined vessels acquiring a basement membrane and a smooth muscle layer (large vessels) and pericyte layer (small vessels) by a process called arteriogenesis. The acquisition of a muscular layer is determined by blood flow and pressure, with low-pressure areas having delayed smooth muscle differentiation. The vascular cells then acquire specialised characteristics to suit a particular environment. For example, endothelial cells in the brain are closely associated to produce an effective blood-brain barrier, whereas endothelial cells in endocrine glands are loosely associated to facilitate the passage of hormones. The molecular mechanisms underlying these processes are not completely understood. However, several ligand-receptor interactions have been identified that are likely to be important. In mice the interaction between the receptor tyrosine kinase flk-1 and its high affinity ligand vascular endothelial growth factor (VEGF) is thought to play a key role in

haematopoietic and endothelial development. Examination of the expression of flk-1 and VEGF in mouse embryo has demonstrated that both are present in the blood islands of the yolk sac of day 8.5-10.5 embryos It has also been shown that mice die in utero at days 8.5-9.5 if they are made homozygous for deficiency of the flk-1 gene. This has been shown to be because of a defect in the development of haematopoietic and endothelial cells. Yolk-sac blood islands are not present, organised blood vessels are not observed at any stage, and haematopoietic progenitors are markedly reduced.

1.7. Postnatal endothelial progenitor cells

Adult bone marrow contains a sub-population of cells with properties similar to those of embryonic angioblasts. These cells have the potential to proliferate and differentiate into mature endothelial cells and have, therefore, been called endothelial progenitor cells (EPCs). A landmark paper was published by Asahara et al in 1997, when EPCs were first isolated from peripheral blood [3]. Exploiting the knowledge that there are antigens shared by angioblasts and HSCs, they used magnetic beads coated with CD34 and Flk-1 to separate out EPCs from the mononuclear cell fraction of human peripheral blood. These cells were then plated on fibronectin-coated surfaces. It was observed that they quickly attached, became spindle shaped within three days, with the number of attaching cells increasing with time. Using a fluorescent dye DiI they demonstrated that these spindle shaped cells were derived from CD34-positive cells with the ability to form cellular networks and tube-like structures. After 7 days in culture the attached CD34-positive cells were seen to express endothelial cell markers by flow cytometry, and at the

molecular level by qualitative polymerase chain reaction (PCR). Finally, it was shown in animal models of hind limb ischaemia that these cells could contribute to postnatal vasculogenesis in-vivo. Circulating EPCs were also identified by other groups using similar methodologies [4-7]. In addition, it was discovered that these cells were likely to originate from the bone marrow. Asahara et al used established two murine models of bone marrow transplantation (BMT) [5]. In each case, immunodeficient mice underwent BMT from transgenic mice constitutively expressing β-galactosidase (lacZ) under transcriptional regulation of an endothelial cell-specific promoter, FLK-1 or TIE-2. Reconstitution of the transplanted bone marrow yielded Flk-1/lacZ/BMT or TIE-2/lacZ/BMT mice, in which lacZ was restricted to bone marrow derived cells expressing Flk-1 or TIE-2. LacZ expression was not observed in other somatic cells. Localisation of EPCs, indicated by flk-1/LacZ or TIE-2/LacZ fusion transcripts, was identified in corpus luteal and endometrial neovasculature after induction ovulation. In addition, injected mouse syngeneic colon cancer cells, cutaneous wounds and induced hind limb ischaemia, in the same mouse model, derived new vessel growth from the bone marrow derived LacZ expressing cell population.

1.8. Characterisation of EPCs

The phenotypic characterisation of EPCs has been difficult to achieve for a number of reasons. Firstly, it has been confounded by the presence of circulating endothelial cells (CECs) in the peripheral circulation [8]. CECs are mature cells that are thought to

originate from sloughing off the vessel wall following some sort of pathological insult. Elevated numbers of CECs occur in patients with a variety of conditions associated with vascular injury, including sickle cell anaemia [9], septic shock [10], and lupus [11]. The features that are thought to separate CECs from EPCs are summarised in **table 1.1.** In the peripheral circulation, bone marrow-derived CECs are thought to represent a small fraction of total CECs [7]. In a series of in-vitro experiments on peripheral blood from patients who had previously received gender-mismatched bone marrow transplants, it was found that endothelial cell colonies that appeared in culture within nine days were predominantly of recipient phenotype [7]. This suggested that these cells might represent CECs from the vascular endothelium of the recipient. In contrast, endothelial colonies that appeared later in culture were of donor origin, suggesting that these cells derived from bone marrow EPCs. These late outgrowth cells showed a high proliferative capacity compared to early-outgrowth cells. Quantification of EPCs is often done using colony-forming assays; these findings suggest that functional assays should include a pre-plating step to eliminate the background of CECs, as has been done elsewhere and in the series of experiments in this thesis [4, 12].

	CECs	EPCs
Origin	Blood vessel wall	Bone marrow
Phenotype	CD133 -ve	CD133 +ve
Morphology	Mature cells of diameter 20-50μM	Immature cells of diameter less than 20μM
Capacity to form colonies with high proliferative potential	No	Yes
Pathophysiology	Reflective of damage	Neovascularisation
CECs= circulating endothelial cells, EPCs= endothelial progenitor cells		

Table 1.1. Differences between CECs and EPCs [8].

Attempts to characterise EPCs are further confounded by the fact that they are not one type of cell but probably represent a heterogenous group of progenitor cells, each at a different stage of maturation [13]. In addition there is considerable overlap between proteins expressed on EPCs and those expressed on cells of haematopoietic lineages, including CD31, CD34, and vascular endothelial growth factor receptor-2 (VEGFR-2), also known as kinase insert domain receptor (KDR) [14, 15]. Moreover, the characteristic feature of EPCs in culture is their ability to take up acetylated low-density lipoprotein (LDL) and to bind to specific lectins, but this feature is also shared by non-endothelial cells [16, 17]. Despite this, however, the earliest EPCs are thought to express CD133, CD34, and VEGFR-2 [6, 18]. CD34+/CD133+ cells have been shown to have a high proliferative capacity and to give rise to endothelial colonies in culture [6, 18, 19].

CD133 (also known as AC133) is an early haematopoietic stem cell marker of unknown function. It is a 120-kDa transmembrane polypeptide, and is expressed on haematopoietic stem and progenitor cells from human bone marrow, fetal liver, and peripheral blood [20]. The number of CD133+ EPCs as a fraction of peripheral blood mononuclear cells is small, so this immature cell type is more likely to be present in the bone marrow [19]. In the peripheral blood EPCs appear to lose expression of CD133 as they mature, but continue to express VEGFR-2 and CD34. Further maturation of EPCs results in loss of CD34 expression and gain of markers such as vascular endothelial cadherin (VE-cadherin- CD144) and von-Willebrand factor. However, no clear definition exists for when an immature EPC changes into a mature endothelial cell. In summary, it is possible that CD133+/CD34+/VEGFR-2+ cells represent a more primitive EPC with high proliferative potential, which then gives rise to a more mature endothelial cell with the phenotype CD133-/CD34+/VEGFR-2+/ vWF+/VE-cadherin+ with a more limited proliferative capacity. Two separate EPC populations in human peripheral blood have been described, based on their differing proliferative potentials [21].

It has recently been demonstrated that cells of monocytic lineage, expressing CD14, are a source of EPCs. Monocytes have been shown to co express endothelial lineage markers such as VEGFR-2 and CD133 and to differentiate into adherent mature endothelial cells and to form cord like structures in Matrigel [22, 23]. Rehman et al has also reported that peripheral-blood endothelial-like cells are derived from monocytes/macrophages and secrete angiogenic growth factors [24]. These cells have

the ability to form spindle-shaped cells after several days in culture, take up LDL, and bind a specific lectin. Furthermore, it has been shown that a specific subset of monocytes, expressing CD14 and VEGFR-2, have endothelial-like functional capacity, and exhibit functional competence to improve re-endothelialisation in an animal model of arterial injury [25].

In summary, it can be seen that at least two cell populations can qualify as EPCs, each group containing a heterogenous sub-population of cells. A further cell group, with endothelial-like functional capacity, known as multipotent adult progenitor cells (MAPCs), may also represent a further type of EPC [26], although this population is less well defined. Furthermore, how each of these cell populations relates to the other is uncertain.

1.9. Mobilisation, release and homing of EPCs

In order that EPCs can contribute effectively towards neovascularisation they must first be recruited from the "stem cell niche", where stem cells are kept in an undifferentiated and quiescent state, to the "vascular niche", where progenitor cells are prepared for release into the circulation. At some point they must commit to the EPC differentiation pathway, be mobilised from the bone marrow into the circulation, and then delivered to the appropriate site. This process is complex and involves a number of triggers, enzymes, growth factors and cell surface receptors. An early event in mobilisation is the activation of matrix metalloproteinase-9 (MMP-9), which transforms membrane bound

Kit ligand to a soluble Kit ligand (sKitL). This allows the stem cells and early progenitor cells to detach from the local environment and to leave the bone marrow via transendothelial migration. In MMP-9 -/- mice levels of sKitL are low resulting in impaired progenitor cell motility, while administration of sKitL corrects this [27]. The bone marrow is also the place where the haemangioblast will commit to either the HSC or EPC pathways. How this occurs is largely not understood. However, it is likely that the local cytokine environment plays a role. Certain triggers, such as tissue ischaemia, are known to increase the levels of endogenous vascular endothelial growth factor (VEGF), mainly due to the effects of hypoxia-inducible factor-1 (HIF-1) on VEGF transcription [28-30]. VEGF binds to VEGFR-2, which is expressed on the haemangioblast. In addition, VEGF has been shown to activate MMP-9 [27]. Exogenous administration of VEGF has been shown to mobilise bone marrow derived EPCs in animal models [31]. The mobilisation is rapid; EPC levels in the peripheral blood rise within 24 hours after VEGF administration. Furthermore, studies in humans using plasmids containing the gene for VEGF show augmentation of circulating EPCs [32]. In patients with vascular trauma due to severe burn injury or coronary artery bypass grafting, EPC numbers rise by about 50 times at 12 hours post-injury, and return to baseline by 48-72 hours [33]. Similarly, in patients with acute myocardial infarction, levels of CD34+ cells increase one week after MI [34]. The kinetics of EPC levels seen in these patient groups closely mirrors the levels of VEGF detected in the peripheral circulation. Stromal cell derived factor-1 (SDF-1) is a member of the chemokine CXC subfamily and binds to CXCR4 positive stem cells. SDF-1/CXCR4 interaction has been shown to be important in stem cell mobilisation in vivo [35]. Other factors may also play

a role in EPC mobilization, including angiopoietins, endogenous erythropoietin (Epo) and endothelial nitric oxide synthase (eNOS) [35-40]. The intracellular pathways by which these factors mobilise EPCs have not been fully elucidated. However, it has been demonstrated that VEGF, Epo and eNOS signal through Akt, which is a tyrosine kinase belonging to the family of serine/threonine protein kinases [41]. Once mobilised and released into the peripheral circulation EPCs must home to areas of neovascularisation. This involves a multistep process beginning with (a) arrest of the EPCs at areas of neovasculogenesis; (b) adhesion to the endothelial cells lining blood vessels; (c) and transmigration across this surface into the interstitial space; (d) formation of cellular clusters; (e) formation of cellular networks; and (f) incorporation into the microvasculature [42]. Using a tumour model and mouse embryonic progenitor cells (eEPCs) Vajkoczy et al showed that initial cell arrest of eEPC homing was mediated by E- and P-selectin and P-selectin glycoprotein ligand 1 [42, 43]. In addition, it has been demonstrated that cultured EPCs express L-selectin, and that this adhesion receptor can interact with its ligand on endothelial cells to promote homing of the EPCs [44]. This mechanism of homing is similar to that of leukocytes with microvascular endothelium, where integrins are known to be important [45, 46]. In particular β2-integrins, found on haematopoietic cells, and α4β1-integrins, on endothelial cells, are important in cell-cell interactions. β2 integrins have been shown to be important in adhesion and transmigration of haematopoietic stem/progenitor cells [47, 48]. Chavakis et al have also shown that ex vivo expanded and murine Sca-1+/Lin- progenitor cells express β2-integrins, which mediate the adhesion of EPCs to endothelial cell monolayers and

transendothelial migration in vitro [49]. They have also shown that in a mouse model of hind limb ischaemia, progenitor cells from β2-integrin-deficient mice are less capable of homing to sites of ischaemia and promoting neovascularisation. Attraction of the circulating EPCs to the site of neoangiogenesis is mediate by chemokines. In particular SDF-1, which is known to be important in homing of stem cells to bone marrow, has been shown to stimulate recruitment of progenitor cells to sites of tissue ischaemia [50-52]. In addition, VEGF has also been shown to be important as a chemoattractant for progenitor cells, with increased levels during an ischaemic episode [34]. Less is known, however, about EPC migration and tissue invasion. Recently the protease cathepsin-L has been found in EPCs, and has been shown to be important in EPC invasion and matrix degradation in vitro [53]. In addition, this group also showed that mice made deficient in cathepsin-L had impaired functional recovery following hindlimb ischaemia, supporting the idea that this protease has an important in-vivo role. The final step is the differentiation of EPCs to mature endothelial cells, with integration of these cells into vessels. Although this is not fully understood, it is thought that VEGF plays a crucial role. This has been demonstrated at the embryonic stage, as well as in ex-vivo culture systems[54, 55].

1.10. Exogenous factors affecting the recruitment of EPCs to the periphery

The level of circulating EPCs has been shown to vary secondary to various physiological, pathological and pharmacological factors (**table 1.2**). For example, age

and sex hormones are important determinants that need to be taken into account if EPC numbers and function are being compared between groups in a study. It has been shown in experimental models that revascularization is impaired in senescent animals. In addition, older human subjects have lower numbers and impaired mobilization of EPCs [56]. Oestrogen is thought to mediate re-endothelialisation after arterial injury by its effect on EPC mobilisation. Re-endothelialisation after carotid injury occurred faster in ovariectomised mice treated with 17β-oestradiol than in controls [57, 58]. The oestrogen-treated animals had increased levels of circulating EPCs and a significant increase in bone marrow-derived cells participating in endothelial repair at the site of injury. Of note is the observation that oestrogen did not increase EPC levels or effect re-endothelialisation in mice deficient in endothelial nitric oxide synthase (eNOS), a finding consistent with observations that eNOS expressed in bone marrow plays an important role in the regulation of EPC release [57, 59]. EPC numbers have also been shown to increase in patients following acute myocardial infarction [34]. The number of CD34+ mononuclear cells increased by day 7 compared to control subjects, who had no evidence of myocardial ischaemia. Furthermore, patients with vascular trauma from coronary bypass grafting or burn injury had a transient rise in EPC numbers [33]. Congestive cardiac failure can also affect the number of circulating EPCs [60]. Interestingly there appears to be a biphasic pattern, with elevation in the early (class I and II) and reduction in the advanced (class III and IV) stages. Patients with diffuse in-stent restenosis had lower numbers of EPCs, which had an impaired adhesive capacity in-vitro [61]. In patients with stable coronary artery disease it has been shown that the

number and proliferative capacity of EPCs are reduced [62, 63]. In addition, EPC numbers are reduced in patients with risk factors for coronary artery disease, such as genetic predisposition or smoking [64]. Hill et al showed that in patients with risk factors for coronary artery disease the ability of EPCs to form colony forming units (CFUs) was inversely related to the Framingham risk score [12]. In addition, they found a direct correlation between numbers of EPCs and their function, with cells from high-risk patients showing increased senescence compared to lower risk patients. They concluded that EPC number and function could be used as an independent risk factor for coronary artery disease. Drug therapy can also affect EPC numbers. The best-characterised group of drugs in this respect are the HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase inhibitors ("statins"). This class of drugs may have a beneficial effect on vascular health independent of their ability to lower blood cholesterol [65]. Simvastatin has been shown to increase EPC CFUs from cells taken from treated animals, and to have migratory effect on mononuclear cells in an in vitro chemotaxis assay [66]. In addition, HMG-CoA reductase inhibitors have been shown to mobilize EPCs which can help to repair damaged coronary arteries [67]. This mechanism of action is via activation of the PI3-kinase/Akt pathway in EPCs, and requires the presence of endothelial nitric oxide [68, 69]. Cytokines have been used to mobilize EPCs and increase their number in the circulation. Granulocyte colony stimulating factor (G-CSF) administered to humans has been shown to increase the number of circulating EPCs to 5- to 10-fold [18]. In addition, G-CSF has been used to increase the number of EPCs in patients with coronary artery disease, where baseline levels are lower than in control subjects [70]. In animal studies G-CSF has mobilized

EPCs sufficiently to accelerate vascular repair following injury and to increase endothelialisation of prosthetic vessels [71, 72]. The use of G-CSF for therapeutic neovascularisation of ischaemic tissue and for ex-vivo expansion of EPCs in patients has been performed, and will be discussed later [73]. Increased numbers of EPCs can also be seen in mice and rabbits treated with granulocyte macrophage colony stimulating factor (GM-CSF), and enhanced neovascularisation with bone marrow-derived cells is seen in mice treated with GM-CSF in a corneal neovascularisation model [74]

Condition or factor	Changes in number/function of EPCs	Reference
Physiological		
Age	Decreased number	**[56]**
Gender (eg oestrogens)	Increased number	**[58]**
Physical training	Increased number	**[75, 76]**
Pathological		
Coronary artery disease	Decreased number and function	**[62-64]**
Smoking	Decreased number	**[64, 77]**
Family history of coronary artery disease	Decreased number	**[64]**
Hypertension	Decreased function	**[64, 78]**
Cumulative cardiovascular risk factor score	Decreased number	**[12]**
Myocardial infarction	Increased number	**[34]**
Vascular injury	Increased number	**[33]**
Congestive cardiac failure (class I-II)	Increased number	**[60]**
Congestive cardiac failure (class III-IV)	Decreased number	**[60]**
In-stent restenosis	Decreased number and function	**[61]**
Diabetes	Decreased number and function	**[64, 79, 80]**
Uraemia	Reduced number	**[81, 82]**
Hypercholesterolaemia	Decreased number and function	**[83]**
Systemic inflammatory conditions	Increased numbers	**[84-86]**
Surgery	Increased number	**[87]**
Drugs and cytokines		
HMG-CoA reductase inhibitors	Increased number and function	**[66-69, 88, 89]**
G-CSF	Increased number	**[70, 71]**
Erythropoietin	Increased number	**[40, 90]**

Table 1.2. Exogenous factors affecting the recruitment of EPCs to the periphery.

1.11. EPCs and tumour angiogenesis

There is a growing amount of evidence from animal models indicating that bone EPCs contribute to tumour neovascularisation, and that in the peripheral blood of patients with cancer there is an increased number of circulating CECs that may also participate in vessel formation [91-94]. How EPCs contribute to tumour expansion is uncertain, but it could be by directly incorporating into the vascular endothelium of the tumour, or by secretion of proangiogenic growth factors in perivascular sites within the tumour. In one study, murine colon cells were subcutaneously injected into mice that underwent bone marrow transplantation from transgenic mice constitutively expressing β-galactosidase gene regulated by an endothelial cell-specific promoter [5]. Three weeks after tumour implantation, histological examination of the tumour revealed multiple LacZ-positive cells in the tumour stroma and in the endothelial layer of tumour blood vessels. Factors known to mobilise EPCs from the bone marrow have also been shown to alter tumour growth, probably by regulating tumour angiogenesis. G-CSF has been shown to increase tumour growth in mice, and this has been attributed to increased tumour neovascularisation with increased bone marrow-derived cells being observed within tumour vasculature [95]. A further study has used a transgenic mouse model of multistep carcinogenesis to look at incorporation of bone marrow-derived cells into the neovasculature of a tumour [96]. Integration of bone marrow cells into the tumour vasculature was demonstrated, which correlated with VEGF release by the tumour and mobilisation of circulating EPCs in the periphery. Further evidence supporting the

existence of a bone marrow-derived component of tumour neovascularisation has come from studies of mice with mutations in the Id family of proteins. There are four members (Id1-Id4) that interact with basic-loop-helix transcription factors to regulate differentiation and cell cycle progression [97]. Two members of the Id family, Id1 and Id3, are co expressed in embryonic vasculature. Knocking out both copies of Id1 and Id3 is incompatible with life. Mice lacking Id3 and with one functional copy of Id1 (Id+/-Id3-/-) do not die in development but show defects in postnatal angiogenic sprouting and suppressed tumour growth [92]. Overall survival of these mice is increased after tumour cell implantation compared to wild-type mice. However, tumours grow normally in Id mutant mice transplanted with bone marrow from wild-type mice. Through genetic marking, it was determined that 90% of tumour blood vessels of these animals originate from the bone marrow. Conversely, wild-type mice that receive bone marrow transplants from Id1+/-Id3-/- animals abnormalities in their ability to support tumour angiogenesis and growth. Furthermore, Id1+/-Id3-/- mice have lower numbers of circulating EPCs than wild type mice at baseline, and a decreased EPC response to VEGF or to tumour implantation. These observations strongly suggest that the failure of bone marrow-derived EPCs to incorporate into tumour vasculature explains the defect in tumour angiogenesis seen in Id1+/+Id3-/- mice, and highlights the importance of bone marrow-derived EPCs in supporting tumour angiogenesis. The importance of Id genes in tumour angiogenesis has also been observed in PTEN+/- mice, which exhibit spontaneous lymphomas, uterine carcinomas, prostate intraepithelial neoplasias and pheochromocytomas [98]. When PTEN+/- mice were crossed with Id mutants tumour angiogenesis and growth was suppressed. In addition, when PTEN+/-Id wild-type mice

received bone marrow transplants from LacZ+ bone marrow, 17% of tumour vessels in uterine tumours were LacZ+. These findings suggest that EPCs make a significant contribution to tumour neovascularisation in spontaneously arising tumours. However, contrary to the findings of most studies, some reports have found that the contribution of bone marrow-derived EPCs to tumour vascular growth was minimal [99, 100]. This lack of consensus may be explained in part by different experimental settings, such as type of cells used to transplant animals (whole bone marrow vs. purified primitive stem cells), tumour type, and the time frame of the experiment and method of endothelial cell identification.

1.12. EPCs and endothelial maintenance and regeneration

Damage to the endothelium plays an important part in the development of atherosclerotic disease. Whereas, previously the endothelium was thought of as merely a static barrier, the current opinion is that it is a dynamic organ. Furthermore, there is a continual damage and repair process occurring, with the balance ultimately determining the rate of atherosclerotic disease progression. EPCs are now thought to be integral in repairing the damaged endothelium. This potential has been demonstrated in animal models of mechanical vascular injury [4, 67, 101]. Insights on the role of EPCs in atherosclerosis have also been obtained from experiments in ApoE-/- mice. Rauscher et al investigated the effect of aging on EPCs in ApoE deficient mice [102]. They found that injection of bone marrow-derived cells in the mice maintained on high-fat diets significantly reduced the atherosclerotic burden in these animals compared to controls.

In addition, they found that old ApoE deficient mouse bone marrow injections did not prevent atherosclerosis in the high fat diet fed ApoE-/- mice, but young bone marrow infusions did. Analysis of the young and old bone marrows found reduced vascular progenitor cells markers in the old marrow with no change in the number of haematopoietic stem cell markers or generalised murine stem cell markers. This result suggests an important role for EPCs in endothelial repair and attenuation of atherosclerosis. It also suggests that older animals may be more prone to atherosclerosis due to impaired EPC function. Studies in patients with atherosclerosis or with risk factors for atherosclerosis also suggest an important role for EPCs in maintaining vascular integrity. Studies have shown that the presence of disease or risk factors for disease correlate with decreased numbers and diminished functional capacity of circulating EPCs [12, 62, 64, 79, 81]. Hill et al described a reduction in EPC colony forming units in healthy individuals with risk factors for cardiovascular disease [12]. Furthermore, they showed that circulating levels of EPCs were better predictors of preserved vascular reactivity than the presence or absence of traditional cardiovascular risk factors. In addition, EPCs from patients with multiple cardiovascular risk factors had higher rates of in vitro senescence than those with few risk factors. Taken together with the data from the ApoE-/- model, one explanation for this could be that subjects exposed to continuous endothelial damage exhaust a presumably limited supply of functional bone marrow-derived EPCs, further increasing their predisposition to atherosclerosis owing to lower numbers of poorly functioning EPCs that remain in the bone marrow. It would also explain why an inverse correlation between age and number of EPCs has been observed, where an increase in cardiovascular mortality is associated

with normal aging. However, this explanation is undermined by the fact that patients with coronary artery disease can have their EPCs mobilised by G-CSF, statins or by exercise with or without ischaemia [75, 103-105]. An alternative explanation, therefore, is that the pathway controlling EPC release is down regulated by exposure to risk factors rather than EPCs themselves being consumed. Further evidence for the protective effect that EPCs have on vascular integrity comes from a mouse vein graft model of atherosclerosis [106]. Using this model in transgenic mice carrying LacZ genes driven by an endothelial TIE2 promoter, and hence expressing β-galactosidase only in endothelial cells, it was shown that endothelial cells of vein grafts were regenerated from circulating progenitor cells and not from migration of neighbouring mature endothelial cells [107]. EPCs have also been shown to play a role in arterial injury in a murine aortic allograft model, where recipient-derived endothelial cells repopulated the graft, although only a small percentage of the cells seemed to come from the bone marrow [108, 109]. Finally, EPCs may be important in maintaining intracoronary stent patency following coronary angioplasty [61]. In summary, EPCs may be important in endothelial repair in a variety of contexts, and levels of circulating EPCs may correlate with the overall vitality of the vascular repair mechanism.

1.13. Clinical applications of EPC mediated neovascularisation

The potential of EPCs to contribute towards neovascularisation in vivo was initially investigated using animal models of tissue ischaemia. One animal model that has been

extensively used is the hind limb ischaemia model. Essentially, in a mouse all flow through the femoral artery to the limb is diverted by ligating and then transecting the artery below the ligature. This significantly reduces, but does not stop, blood to the limb. Another animal model used is the myocardial ischaemia model. Myocardial ischaemia is induced in rodents by ligating the left anterior descending coronary artery. EPCs can be obtained from the mononuclear cells fraction of healthy human adult whole blood, for example, and cultured in appropriate medium before being harvested. The ex-vivo expanded EPCs are normally labelled with a fluorescent dye (carbocyanine 1,1'-dioctadecyl-1 to 3,3,3',3'-tetramethylindocarbocyanine percholate, also know as DiI dye) prior to being injected into the animal model. Certain parameters can then be measured, for example change in blood flow through the ischaemic tissue or serial echocardiography, to see if the EPCs have had a beneficial effect on tissue neovascularisation and function. New blood vessel growth can also be looked at using immunohistochemistry to determine the fate of the labelled EPCs. When Asahara et al isolated endothelial cells they also investigated their in vivo ability to form new blood vessels using the hind limb ischaemia model [3]. They injected human DiI labelled CD34+ cells into mice two days after creating unilateral hind limb ischaemia. Histological examination of the ischaemic limb 1 to 6 weeks later showed a number of DiI-labelled cells in the limb, with nearly all labelled cells integrating into capillary walls. By 6 weeks DiI-labelled cells were arranged into capillaries among preserved muscle structures. The ability of these cells to have an impact on tissue viability was demonstrated by Kalka et al [54]. They cultured human peripheral blood mononuclear cells. Differentiating EPCs were identified after 7 days culture by using two fluorescent

markers (FITC-labelled Ulex europaeus agglutinin and DiI-labelled acetylated low density lipoprotein). A hind limb ischaemia model was used, and EPCs were injected into the animal 1 day after femoral ligation. Using serial laser Doppler perfusion imaging it was observed that there was improvement in blood flow recovery in the ischaemic limb. More importantly, limb necrosis and auto amputation were reduced by 50% in comparison with control animals, which either received differentiated ECs or culture media alone. Similar work has been done by Kawamoto using a rat myocardial ischaemia model [110]. DiI labelled EPCs were injected intravenously 3 hours after induction of myocardial ischaemia. Seven days later, fluorescence-conjugated Bandeiraea simplicifolia lectin I (which is a murine-specific EC marker) was given intravenously, and the rats killed immediately. Microscopy showed that the transplanted ECs gathered in the ischaemic area and incorporated into foci of neovascularisation. Furthermore, compared to rats injected with culture media only, when heart function was assessed by echocardiography rats that received EPCs developed ventricular dimensions that were significantly smaller and fractional shortening that was significantly greater. In addition, regional wall motion was significantly greater in the EPC group. The overall interpretation of these animal studies and others has been complicated, however, by the fact that many use unfractionated bone marrow as the therapeutic agent, whereas others use different types of purified cell populations that represent cells with varying degrees of EPC-like properties.

With this background in mind research has now turned towards the therapeutic use of EPCs in humans with ischaemic disease (**table 1.3**). Most of these small studies have

looked at patients with acute myocardial infarction or chronic myocardial ischaemia with no option for revascularisation by traditional means. Although many report positive findings with few adverse effects, the follow-up period for most trials has been less than one year, and few trials have been done in a randomised, controlled fashion. Many of these studies report increases in myocardial perfusion, consistent with animal data that suggest that angiogenesis or vasculogenesis may be augmented after cell transplantation. Moreover, cells injected are generally heterologous, therefore the mechanism of benefit is unclear, and the relative contribution of EPCs to the observed benefit is difficult to establish [111]. Another problem with using EPCs in the clinical setting is that they are found in low numbers in the peripheral blood. There are a number of ways that this can be overcome, including: (i) the local infusion of autologous bone marrow cell suspensions without pre-selection, (ii) the mobilisation of autologous EPCs in vivo, or (iii) the ex vivo transfection with different genes. Infusion of bone marrow cells was performed on humans with chronic limb ischaemia [112]. Twenty-two patients with bilateral leg ischaemia were recruited. For each patient one leg was randomly injected with autologous bone marrow mononuclear cells (BMMCs) and the other leg was randomly injected with peripheral blood mononuclear cells (PBMCs) as a control. Patients were assessed for changes in ankle-brachial index (ABI), transcutaneous oxygen pressure (TcO_2), rest pain and pain-free walking time. At 4 weeks ABI was significantly improved in legs injected with BMMCs compared with those injected with PBMCs. Similar improvements were seen for $TcO_{2,}$ rest pain and pain-free walking and these effects were sustained at 24 weeks. Further studies have shown the beneficial effect of BMMCs or ex vivo expanded autologous EPCs for repair of ischaemic and infarcted

myocardium in humans. Of particular attention is the MAGIC trial in which 27 patients with myocardial infarction and requiring coronary stenting were randomized to three groups; (i) infusion of mobilized PBMCs using G-CSF, (ii) administration of G-CSF alone and (iii) control group [113]. Exercise capacity myocardial perfusion and systolic function were significantly improved in the cell infusion group at 6 months follow up. Importantly, however, an unexpectedly high rate of in-stent restenosis was noted in patients who received G-CSF, so the trial was stopped prematurely. Similarly suggested a potential increase in adverse effects in patients with chronic myocardial ischaemia treated with G-CSF [114]. This has, therefore, raised concerns about the safety of such procedures, and prompted a search for other agents that can promote EPC mobilisation without the risk of augmenting a generalised inflammatory response. Another potential complication of cell therapy for subjects with myocardial ischaemia is intracoronary administration of the cells. A study in which mesenchymal stromal cells were injected into the coronary circulation of healthy dogs precipitated ECG changes compatible with acute myocardial ischaemia 7 days later, with raised cardiac troponin I and histological evidence of myocardial fibrosis [115].

In the treatment of cancer, inhibition of EPC mobilisation from the bone marrow has tremendous therapeutic potential, as evidenced by the ability of tumours to grow in animals that lack functional EPCs [92, 116]. Preliminary work in animal models suggests that agents that inhibit EPC mobilisation may be effective cancer therapeutics [117]. Further work will be required, however, before this strategy can be applied to human neoplasms.

Reference	Controls	Pathology	Patient numbers	Cells used	Mode of injection	Result	Adverse effects	Follow-up period
[118]	None	Chronic myocardial ischaemia	5	BMMCs	Intramyocardial injection while performing CABG	Improved perfusion by cardiac radionuclide imaging in 3/5 patients	None	1 year
[112]	Each patient acting as own control	Bilateral limb ischaemia	22	BMMCs (n=22, injected into one leg) PBMCs (n=22, injected into the other leg)	i.m. (gastrocnemius)	Improved ABI, $Tc0_2$, rest pain and pain free walking in legs injected with BMMCs	None	4 weeks and 24 weeks
[119]	10 patients refusing cell therapy	Acute MI	10	BMMCs	Intracoronary	Fewer dysfunctional myocardial segments at 3 months	None	3 months
[120]	Matched reference group	Acute MI	20	BMMCs (n=11) Ex vivo expanded cells (n=9)	Intracoronary	Improved EF compared to controls; no difference among cell types	Stent restenosis in 5/19 patients	4 months

Reference	Controls	Pathology	Patient numbers	Cells used	Mode of injection	Result	Adverse effects	Follow-up period
[121]	None	Chronic MI	6	CD133+ BMMCs	Intramyocardial during CABG	Improved EF, perfusion to peri-infarct zone	Pericardial effusion in 2/6 patients	9-16 months
[122]	None	Chronic myocardial ischaemic heart disease	8	BMMCs	Percutaneous transendocardial	Decreased angina; increased myocardial perfusion	None	3 months
[111]	7 patients- no procedure performed	Chronic myocardial ischaemia	14	BMMCs	Percutaneous transendocardial	Reduction in reversible defect; decreased ESV	None	2-4 months
[113]	7 patients having coronary stenting only	Acute MI	20	Mobilised PBMCs (n=10) G-CSF alone (n=10)	Intracoronary	Exercise capacity, myocardial perfusion and systolic function increased in patients after PBMC infusion	Increased restenosis in 5/7 patients in cell infusion group and 2/3 patients in G-CSF group	6 months

Reference	Controls	Pathology	Patient numbers	Cells used	Mode of injection	Result	Adverse effects	Follow-up period
[123]	30 patients having coronary stenting only	Acute MI	30	BMMCs	Intracoronary	Increased global LVEF in cell infusion group	None	6 months
[124]	None	Chronic myocardial infarction	14	BMMCs	Injection into myocardial scar while performing CAGB	Improved myocardial performance in segments receiving bypass and cells	None	10 months
[125]	None	Ischaemic cardiomyopathy	6	PBMCs after G-CSF mobilisation	Intramyocardial and intracoronary injection while performing CABG	Improved EF, improved myocardial perfusion	Death from sepsis (1/6), AF with RVR (1/6)	4-10 months

Table 1.3. Current trials involving the use of EPCs as a therapeutic intervention in patients with vascular disease.

2. METHODS

2.1. RNA isolation

Introduction

RNA isolation from whole human blood was performed using the QIAamp® RNA Blood Mini Kit (Qiagen, Crawley, West Sussex, UK), following the protocol supplied with the kit. The main reasons for using a kit method as opposed to the acid-guanidium-thiocyanate method or CsCl step-gradient ultracentrifugation was because of ease of use and the ability to process multiple blood samples simultaneously in a shorter time period. The kit contained:

- Spin columns- contained in 2ml collection tubes. For separating out total RNA using a silica-gel-based membrane.
- Shredder columns- contained in 2ml collection tubes. For homogenization of cell lysates.
- EL buffer- a hypotonic buffer that facilitates selective lysis of erythrocytes.
- RLT buffer- for cell disruption. β-mercaptoethanol was added prior to use (10μl to 1ml of buffer).
- RW1 buffer- wash buffer.
- RPE buffer- wash buffer. 4 volumes of 100% ethanol was added prior to use to obtain a working solution.
- RNase free water- for elution of total RNA from silica-gel-based membrane.
- 2ml collection tubes.
- 1.5ml collection tubes.

Method

1. 1ml of EDTA anticoagulated whole blood was mixed with 5ml of EL buffer in a 15ml centrifuge tube.
2. The mixture was then incubated for 15 minutes on ice, and vortexed twice during incubation. Translucency of the suspension was looked for, indicating that lysis of erythrocytes had occurred.
3. The mixture was centrifuged at 400xg for 10 minutes at 4°C, and the supernatant containing lysed erythrocytes was completely removed and discarded.
4. 2ml of EL buffer was then added to the remaining cell pellet, which was resuspended by a brief vortex.
5. The mixture was then centrifuged again at 400xg for 10 minutes at 4°C, and the supernatant was completely removed and discarded to prevent interference with cell disruption and RNA binding to the spin column.
6. 600μl of RLT buffer was then added to the pelleted leukocytes, and vortexed to mix. It was ensured that no cell clumps could be seen prior to commencing the homogenization step.
7. The lysate was then transferred into a shredder column and centrifuged in a micro centrifuge for 2 minutes at 13,000 rpm to homogenize. The column was then discarded and the homogenized lysate saved.
8. 600μl of 70% ethanol was then added to the lysate, in order to optimise binding conditions for the RNA, and mixed by pipetting.

9. The sample, including any precipitate that may have formed, was then transferred to a spin column and centrifuged in a micro centrifuge for 15 seconds at 10,000rpm. As the maximum loading volume of the column is 700µl this step was performed on successive aliquots of the same sample through the same column. Following centrifugation the flow-through and collection tube were discarded and the column saved.
10. The spin column was then transferred to a new 2ml collection tube, and 700µl of RW1 buffer was applied to the column, which was centrifuged at 10,000rpm for 15 seconds to wash. The flow-through and collection tube were then discarded and the column saved
11. The spin column was transferred to a new 2ml collection tube, and 500µl of RPE buffer was applied to the column, which was centrifuged at 10,000rpm for 15 seconds. The flow-through and collection tube were then discarded and the column saved.
12. The spin column was transferred to a new 2ml collection tube, and a further 500µl of RPE buffer was added to the column, which was centrifuged at 13,000rpm for 3 minutes.
13. To eliminate any chance of RPE buffer carryover, the spin column was transferred to a new 2ml collection tube and centrifuged at 13,000rpm for 1 minute.
14. The spin column was then transferred to a 1.5ml collection tube, and 50µl of RNase-free water was pipetted directly onto the membrane. The column was spun in a micro

centrifuge at 10,000rpm for 1 minute to elute the RNA. Step 14 was repeated to obtain a total volume of 100μl containing eluted RNA.

15. To determine the concentration of RNA present 4μl of the eluted RNA solution was added to 996μl of distilled water and the optical density was read using UV light at wavelengths of 260nm and 280nm*.

* The 260nm reading in Absorbance units (A) (calibrated to 0.0A for distilled water) multiplied by 10^4 gives an approximate concentration of RNA in nanograms per micro litre. For example, 0.018A at 260nm = 180ng/μl. This also allows estimation of purity of the sample from protein contamination. A ratio for the optical density at 260nm: 280nm of around 2:1 suggests a pure sample with little contamination.

2.2. Reverse transcription to complementary DNA (cDNA)

Introduction

In order to be able to quantify mRNA in the real-time PCR it first has to be converted into cDNA. This depends on the enzyme reverse transcriptase (RT) to create a double stranded DNA copy from the single stranded RNA strand.

Method

All reagents were obtained from Promega, Southampton, UK.

1. 1μl (0.5μg/μl) of random hexamers was added to 10μl of RNA (1μg of RNA made up to 10μl with DEPC distilled water) in a sterile RNase-free micro centrifuge tube.
2. The tube was heated to 70°C for 5 minutes to melt secondary structure within the template, then cooled on immediately on ice for 5 minutes to prevent secondary structure from reforming.
3. On ice the following components were added to the annealed random hexamers/template: 1μl (200units) of M-MLV RT, 0.625μl (40units/μl) of RNase inhibitor, 5μl of dNTP mix (containing dATP, dCTP, dGTP and dTTP, each at a concentration of 10nM in water) and 5μl of 5X RT reaction buffer (diluted 1:5, containing 50mM Tris-HCL-pH 8.3 @ 25°C, 75mM KCL, 3mM $MgCl_2$ and 10mM DTT).

4. The tube was then incubated for 10 minutes at 25°C followed by a 60 minute incubation at 37°C

2.3. Polymerase chain reaction (PCR)

Introduction

Reverse transcriptase PCR (RT-PCR) is used to amplify RNA. The principle underlying PCR is well known. Traditionally PCR has relied on the detection of product at the end of the reaction by using agarose gels (end-point PCR) (**figure 3.5**). Now, with the advancement in PCR technology, it is possible to detect product while the reaction is occurring (real-time PCR). This process is summarised in **figure 2.1**. Measuring the kinetics of the reaction in the exponential phase of PCR provides distinct advantages over end-point PCR. The main one is the ability to accurately quantitate an amplicon. Quantitation of mRNA relies on the use of TaqMan® probes. These probes are oligonucleotides, which are designed to anneal to a specific sequence of template between the forward and reverse primers. The probe sits in the path of the DNA polymerase as it starts to copy cDNA. When the enzyme reaches the annealed probe the 5' exonuclease activity of the enzyme cleaves the probe. The TaqMan® probe is designed with a high-energy dye termed a reporter at the 5' end, and a low-energy molecule termed a quencher at the 3' end. When this probe is intact and excited by a light source, the reporter dye's emission is suppressed by the quencher dye as a result of the close proximity of the dyes. When the probe is cleaved by the 5' nuclease activity of the enzyme, the distance between the reporter and the quencher increases causing the transfer of energy to stop. The fluorescent emissions of the reporter increase and the quencher decrease. The increase in reporter signal is captured by the sequence detection

instrument and displayed by the software in the form of an amplification plot. The amount of reporter signal increase is proportional to the amount of product being produced for a given sample. The amplification plot displays the threshold line and the cycle threshold (Ct), both of which are important for the quantitation of RNA. The threshold line is the level of detection at which a reaction reaches a fluorescent intensity above background, and is set in the exponential phase of the amplification for the most accurate reading. The Ct is the cycle at which the sample reaches this level.

There are two main ways of quantifying RNA by real-time PCR. The first is by absolute quantitation. This is unnecessary in most cases, however, and requires standards whose concentrations are known absolutely. The second approach is by relative quantitation. This method compares target signals in different samples to a reference sample. To correct for differences in the amount of cDNA added to a reaction or to compensate for different levels of PCR inhibition each target signal is normalised to an endogenous control. This is a "housekeeping gene" the amount of which will not vary greatly between samples. The endogenous control used here was ribosomal 18s RNA (see **section 3.2**). The relative difference between samples is then calculated. If the efficiencies of the target and the endogenous control reactions are equivalent then a standard curve is not needed and the comparative Ct method can be used (see **section 3.3**). Real time quantitative PCR was performed using the ABI Prism® 7900 system (Applied Biosystems, Warrington, Cheshire, UK). All reagents were supplied by Applied Biosystems.

Primers and probes

All primer and probe sets were ordered through Applied Biosystems Assays-on-Demand™ service. Each Assay-on-Demand™ gene expression product consisted of a 20X mix of unlabelled PCR primers and TaqMan® MGB probe (FAM™ dye-labelled). These assays have been specifically designed for the detection and quantitation of specific human genetic sequences in RNA samples converted to cDNA. Each assay has been optimized and validated to ensure that there are comparable efficiencies between target and endogenous control reactions if performed in separate wells (singleplex assay). Although the exact primer and probe sequences are not supplied by the company, each set of assays goes through an 8 point manufacturing process, as shown in **table 2.1**. The conditions which must be adhered to for primer/probe design for use of the ABI 7900 quantitative PCR system are shown in **table 2.2**. The gene expression products purchased were for quantitation of mRNA of CD34, VEGFR-2, CD133, CD14, VE-cadherin, vWF and 18s. In addition, each gene expression product has been optimised to work with pre-developed TaqMan® Universal PCR master mix.

Step 1	Benefit
• Obtain transcript sequence, mask repeats, and map to genome • Mask sequence discrepancies between public and private databases • BLAST transcript sequence versus Celera SNP database and mask SNP locations	Assay designs avoid regions of ambiguity, resulting in assays with high fidelity
Step 2	**Benefit**
• Choose assay target position where possible over an exon-exon junction	Designing assay over an exon-exon junction helps to prevent the detection of contaminating genomic DNA
Step 3	**Benefit**
• Apply optimal design parameters for good 5' nuclease assay	High quality 5' nuclease assays
Step 4	**Benefit**
• BLAST probe and primer designs versus transcript databases to	Chosen assays detect only transcript(s) from the gene of interest

ensure specificity	
Step 5 • BLAST probe and primer designs versus genome databases	**Benefit** Avoid assays that detect pseudo-genes or genomic DNA
Step 6 • Apply penalty to assays that span a small intron	**Benefit** Gene-specific assays will not amplify contaminating genomic DNA
Step 7 • Synthesise and QC probes and primers: mass spec and yield	**Benefit** Proper probe and primers are manufactured at the correct concentrations
Step 8 • Formulate probe and primers into single tube at 20X concentration	**Benefit** Easy-to-use, convenient, no-optimisation required

Table 2.1. The TaqMan® gene expression assay design schematic. Taken from Applied Biosystems website (www.appliedbiosystems.com).

Primer
• Tm (melting temperature) 58-60°C • 20-80% of nucleotides GC • Length 9-40 bases • <2°C difference in Tm between the two primers • Maximum of 2/5 G or C at 3' end
Probe
• Tm 10°C higher than primer Tm • 20-80% of nucleotides GC • Length 9-40 bases • No G on the 5' end • <4 contiguous Gs • Must not have more Gs than Cs
Amplicon
• 50-150bp in length • 3' end of primer as close to the probe as possible without overlapping

Table 2.2. Conditions, which must be adhered to for design of primers and probes using the ABI 7900™ quantitative PCR system.

Method for real-time PCR

1. RNA was reverse transcribed into cDNA as described in **section 2.2**. Reverse transcription was performed on the Peltier Thermal Cycler (PTC-225, MJ research).
2. The reaction components were prepared according to **table 2.3**. Different reaction mixes were prepared depending on the primer/probe set used and, therefore, the gene expression of interest. The pre-developed master mix contained TaqMan buffer, $MgCl_2$, dNTPs and Taq polymerase.
3. 16μl of reaction mix (without the cDNA) was then added to each well of a 96 well reaction plate. Specific reaction mixes, containing different primer/probe sets, were added to separate wells. Each plate had wells containing reaction mix with 18s primers/probes.
4. 4μl of cDNA was then added to each well (each cDNA sample was added in triplicate) using a separate pipette tip each time, and mixed by pipetting gently up and down to avoid aerosol formation.
5. The plate was sealed with an optical adhesive cover and PCR was performed on the ABI 7900. The following thermal cycler conditions were used: Initial setup-hold at 95°C for 10 minutes; Cycle- denature at 95°C for 15 seconds, anneal/extend at 60°C for 1 minute. A total of 50 cycles was preformed for each reaction.

Reaction component	Volume/Well (20μl)	Final concentration
TaqMan® Universal PCR master mix (2X)	10	1X
20X Assays-on-demand™ gene expression assay mix	1	1X
cDNA	4	-
RNase-free water	5	-
Total	20	

Table 2.3. Singleplex PCR reaction.

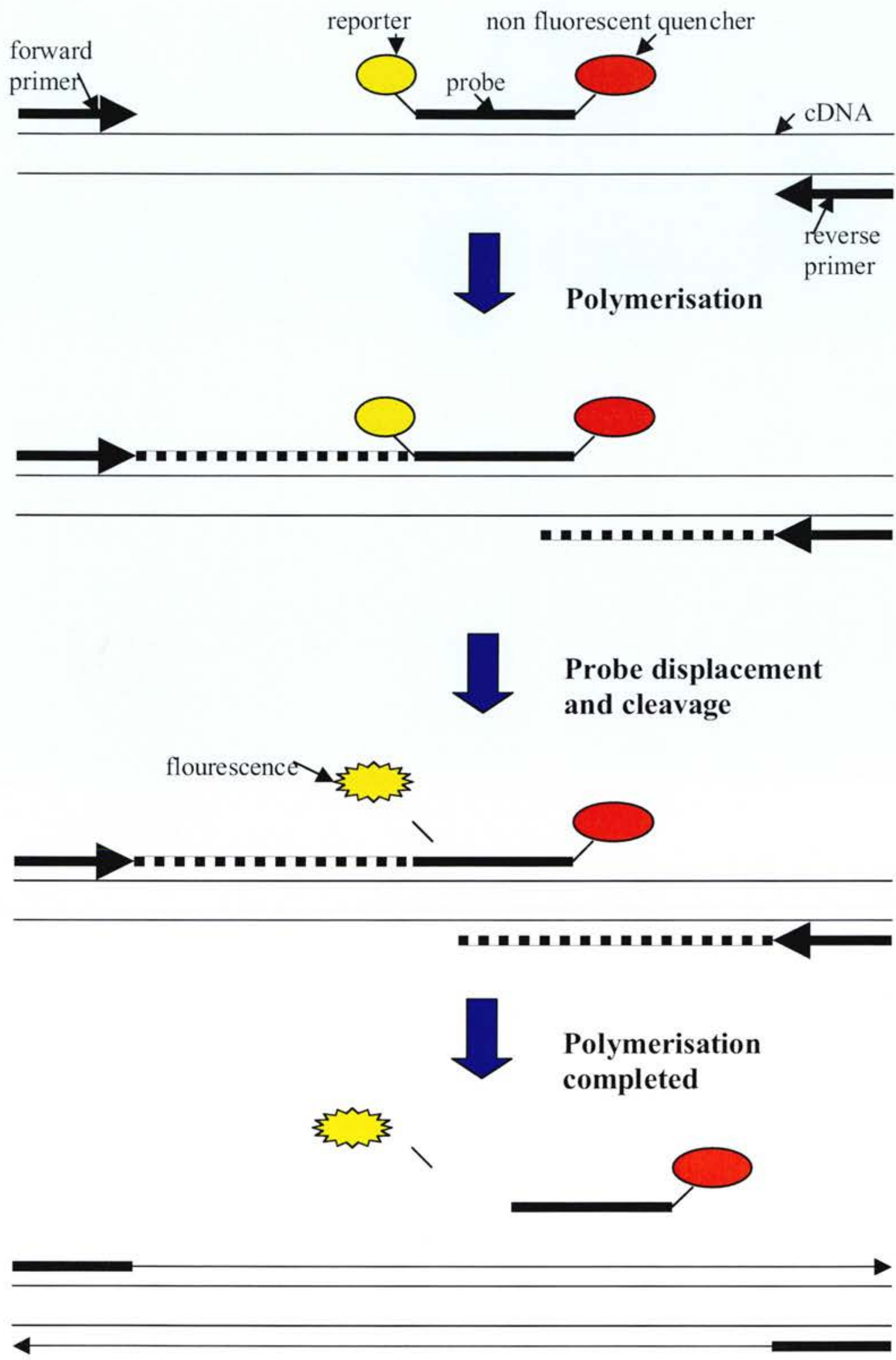

reporter
non fluorescent quencher
forward primer
probe
cDNA
reverse primer
Polymerisation
Probe displacement and cleavage
flourescence
Polymerisation completed

Figure 2.1. TaqMan® probes and quantitative PCR. The TaqMan® probe anneals to the corresponding CDNA. When both the fluorophore and quencher are attached there is no fluorescence. During the subsequent PCR reaction, when the 5' nuclease activity of the DNA polymerase separates the fluorophore from the quencher it can then fluoresce and this can be quantified.

2.4 EPC colony assay

Introduction

Studies to purify and characterize EPCs have been difficult due to the lack of cell surface antigens or markers that distinguish these cells from mature vessel wall-derived endothelial cells and from subsets of haematopoietic cells. Alternatively, EPCs can be defined based on their different growth properties following in vitro culture of peripheral blood. Colonies that form after 3-7 days in culture have a low proliferative potential (6 fold expansion) and are referred to as early outgrowth colony forming unit- endothelial cell or CFU-EC. Colonies that form after 2-4 weeks exhibit a higher proliferative potential (100 fold expansion) and are referred to as late outgrowth CFU-EC. The EndoCult™ Liquid Medium Kit (StemCell technologies) was used to support the growth of human EPCs from peripheral blood, and based on the established assay used by Hill et al.

Reagents

All reagents were supplied by StemCell Technologies, unless otherwise stated.

- The EndoCult™ Liquid medium Kit included the following- basal medium and supplements. Both are used to make EndoCult™ Liquid medium.
- Phosphate buffered saline (PBS)
- 2% fetal bovine serum
- 3% acetic acid

- 6-well and 24-well fibronectin-coated plates (Becton-Dickinson, Oxford, UK).

Preparation of EndoCult™ Liquid Medium

1. EndoCult™ supplements were thawed at room temperature overnight.
2. 20mls of the supplements was added to 80mls of Endocult™ basal medium, to make a 1/5 dilution.

Method

Day0

1 EDTA anticoagulated blood was added to a 50ml tube. A mononuclear cell suspension was prepared by adding an equal volume of PBS to the 50ml tube. 15ml of Ficoll-Paque® PLUS was added to a new 50ml tube. The diluted blood sample was then carefully layered over the Ficoll. The tube was centrifuged at 1200rpm for 25 minutes, and then the buffy coat was collected into a new 50ml tube. The mononuclear cells were resuspended in PBS and 2% FBS up to a final volume of 40mls, followed by a further spin at 1200rpm for 7 minutes in order to pellet the cell suspension.

2 The supernatant was decanted and 6mls of PBS/2% FBS was added to the tube. The pellet was resuspended and transferred to a new 15ml tube. It was then spun at 1200rpm for 7 minutes to pellet the cell suspension.

3 The supernatant was decanted and the pellet resuspended in 1-3mls of EndoCult™ Liquid Medium. Nucleated cells were counted using 3% acetic acid

in a 1/20 dilution with the sample (eg. 10μl of cells and 190μl of acetic acid) and counting using a haemocytometer.

4 2mls of medium was added to a 6-well fibronectin-coated dish. Each experiment was performed in duplicate using 2 wells of a 6-well dish per sample.

5 $5x10^6$ mononuclear cells were plated per well in the 6-well fibronectin-coated dish in duplicate and incubated for 2 days at 37°C, 5% CO_2 with ≥95% humidity. This step removed monocytes and mature endothelial cells.

Day2

After 2 days the mature endothelial cells and monocytes adhered to the bottom of the well. The non-adherent cells contained the EPCs for harvesting at day2 and for further culturing for 3 days to allow formation of endothelial colonies.

6 The non-adherent cells were collected by pipetting the medium in each well up and down 3-4 times using a 2ml pipette.

7 These cells were then transferred into individual 5ml tubes and the volume from each well measured using a 2ml pipette. Nucleated cells were counted as previously described, but this time a 1/10 dilution was used (10μl of cells and 90μl of acetic acid).

8 $1x10^6$ cells/well, from each well of the 6-well dish, were plated in duplicate in a 24-well fibronectin-coated dish. The dish was incubated at 37°C, 5% CO_2 with ≥ 95% humidity for three days.

Day5

9 At day 5 of the assay the number of colonies per well for each sample was counted. Colonies were defined as a central core of "round" cells with elongated "sprouting" cells at the periphery and were classified as early outgrowth colony forming unit- CFU-EC.

2.5. Flow cytometry

Introduction and method

Whole blood cells were phenotyped by flow cytometry using FACS-Calibur flow cytometer (Becton-Dickinson, Oxford, UK). Cells were directly stained and analysed for phenotypic expression of surface proteins with monoclonal antibodies (MAbs) conjugated to phycoerythrin (PE), fluorescein isothiocynate (FITC), Peridin Chlorophylla protein (PERCP) or Allophycocyanin (APC). The MAbs included anti-CD34-FITC, PerCP-conjugated anti-human CD45 (Becton Dickinson, Oxford, UK). Anti-VEGFR2-PE from (R&D systems), anti-VE-cadherin-PE from Santa Cruz Biotechnology, and anti-CD133-APC from Myltenyi Biotec, UK. Appropriate negative controls (Isotype and/or no antibody) were used to establish positive stain boundaries. 100ul of the sample was stained with the appropriate antibodies for 30 minutes in the dark; the erythrocytes were lysed with lysing solution (Becton Dickinson, Oxford, UK) for 15 minutes in the dark. Afterwards the samples were centrifuged and washed with PBS at 200g and finally the cells were fixed with Cell Fix solution (Becton Dickinson, Oxford, UK). 50.000 events were counted and measured by BD FACSCalibur System.

2.6. ELISA

Introduction

The principle of the ELISA is described in **figure 2.2**. A commercial kit was used to perform the assay to determine VEGF levels in patients having angiography ± angioplasty (see **chapter 4**) and in patients wit chronic renal failure receiving human recombinant erythropoietin (see **chapter 7**) (R&D Systems, MN, USA).

Reagents

- VEGF microplate- 96 well plate coated with a mouse monoclonal antibody against VEGF.
- VEGF conjugate- polyclonal antibody against VEGF conjugated to horseradish peroxidase.
- VEGF standard- recombinant human $VEGF_{165}$ in a buffered protein base (2000pg/vial).
- Assay diluent RD1W- a buffered protein base.
- Calibrator diluent RD6U- animal serum.
- Wash buffer concentrate- 25-fold concentrated solution of buffered surfactant.
- Colour reagent A- stabilised hydrogen peroxide.
- Colour reagent B- stabilised chromogen (tetramethylbenzidine).
- Stop solution

Method

1. 2.5ml of EDTA anticoagulated whole blood was collected from each patient/at each time point and centrifuged for 15 minutes at 1000xg within 30 minutes of collection. The plasma fraction was then pipetted off and aliquoted and stored at –20°C.
2. All reagents and samples were brought to room temperature before use. All samples and standards were assayed in duplicate.
3. 100µl of diluent RD1W was added to each well.
4. 100µl of standard or sample was add to each well. The plate was then incubated for two hours at room temperature.
5. Each well was aspirated then washed for a total of three times, using 400µl of wash buffer. The plate was then inverted and blotted against clean paper towels.
6. 200µl of VEGF conjugate was added to each well and the plate was incubated for a further two hours.
7. The plate was then aspirated and washed as in step 5.
8. 200µl of substrate solution was then added to each well and the plate was incubated for 25 minutes.
9. Then 50µl of stop solution was added to each well.
10. The optical density of each well was then determined using a microplate reader set at 450nm.

11 The average of the duplicate readings for each standard and sample was calculated and the average zero standard optical density subtracted.

12 A standard curve was then constructed by plotting the average absorbance for each standard against the concentration. Sample concentrations were then read off the graph.

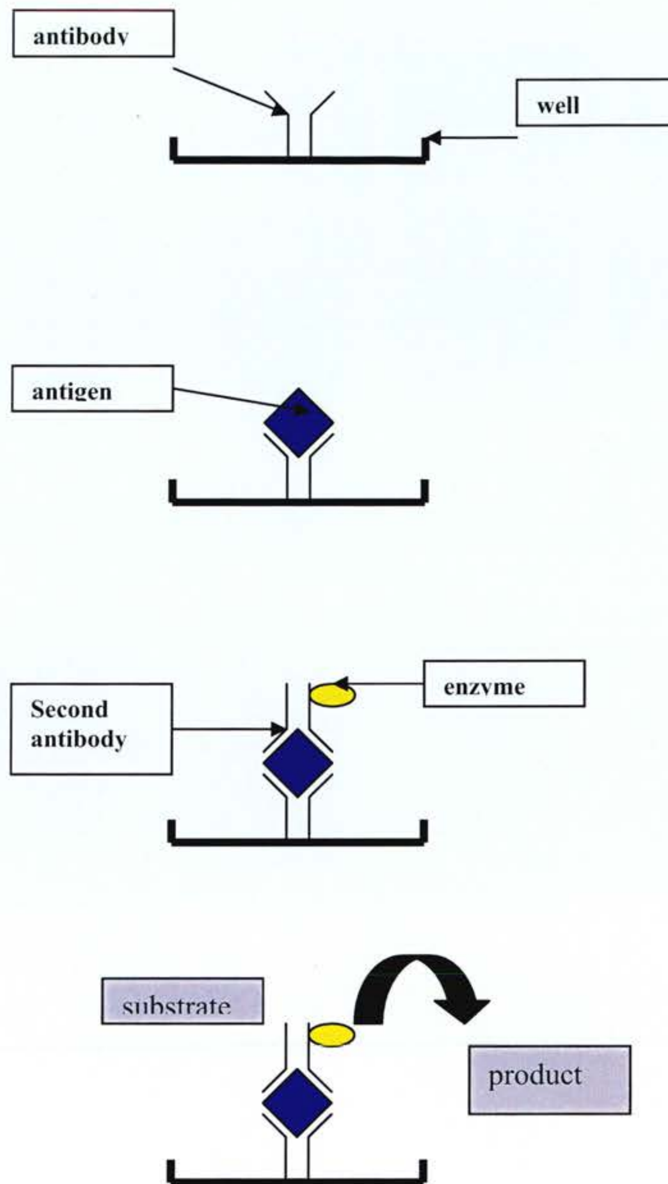


Figure 2.2. Principle of the ELISA assay. The assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for VEGF has been pre-coated onto a microplate. Standards and samples are pipetted into the wells and any VEGF present is bound by the immobilised antibody. After washing away any unbound

substances, an enzyme-linked polyclonal anibody specific for VEGF is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of VEGF bound in the initial step. The colour development is stopped and the intensity of the colour is measured.

2.7. Statistical Methods

Age groups, whole blood counts and differential counts passed the Kologorov- Smirnov (KS) test of normality, and were therefore analysed using parametric tests (unpaired t-test, paired t-test and repeated measures ANOVA with Tukey post-test analysis). All other data, including PCR, flow cytometric, cell culture, ELISA and CRP data failed the KS test and were analysed using non-parametric tests (Mann-Whitney U test, Wilcoxen matched pairs test and repeated measures ANOVA with Dunn post-test analysis). The strength of association between two quantitative variables was analysed using Spearman's correlation coefficient (**chapter 5**). Where multiple paired data points were analysed over a time course type 1 errors were a possibility. To reduce this a p value of <0.01 was considered to be significant. Where one set of paired data was being analysed, or when ANOVA was performed a p value of <0.05 was considered to be significant. Results were analysed using GraphPad Instat 3 software (San Diego, CA, USA).

3. PRELIMINARY STUDIES- PCR

3.1. Stability of RNA in blood samples

Introduction

Each series of experiments to be described in the subsequent chapters involved the isolation of cellular RNA from whole blood samples and reverse transcription to cDNA, prior to performing real-time PCR. The initial isolation of RNA is critical if real-time PCR is to be accurate. However, RNA is relatively labile and is susceptible to degradation by ribonucleases present in blood. For optimal results, therefore, blood samples should be processed soon after collection. To investigate this further various pre-analytical factors were employed to assess the stability of RNA extracted from whole blood samples.

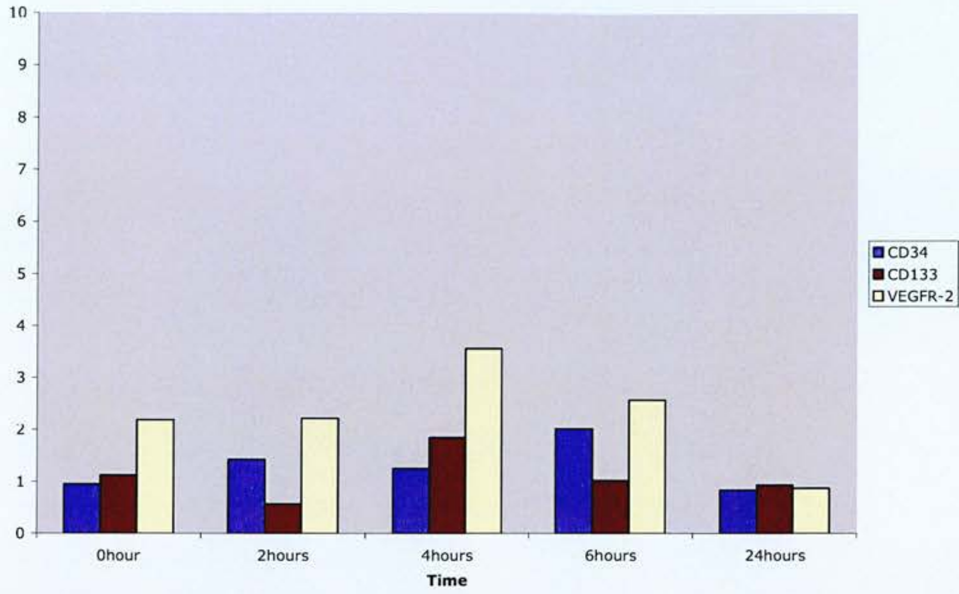
Methods

1. 15mls (3x5mls) of EDTA anticoagulated blood were taken from each of 6 control subjects.
2. For each control subject 5mls of blood were kept at room temperature on an agitator, 5mls of blood were kept on the laboratory bench at room temperature, and the remaining 5mls of blood were put in the fridge at -4°C.
3. 1ml of blood was taken from each 5ml sample at the following time points: immediately after venesection (0 hours), at 2 hours, 4 hours, 6 hours and 24 hours after venesection.
4. Total cellular RNA was then immediately extracted from each 1 ml sample, according to the method described in **section 2.1**.

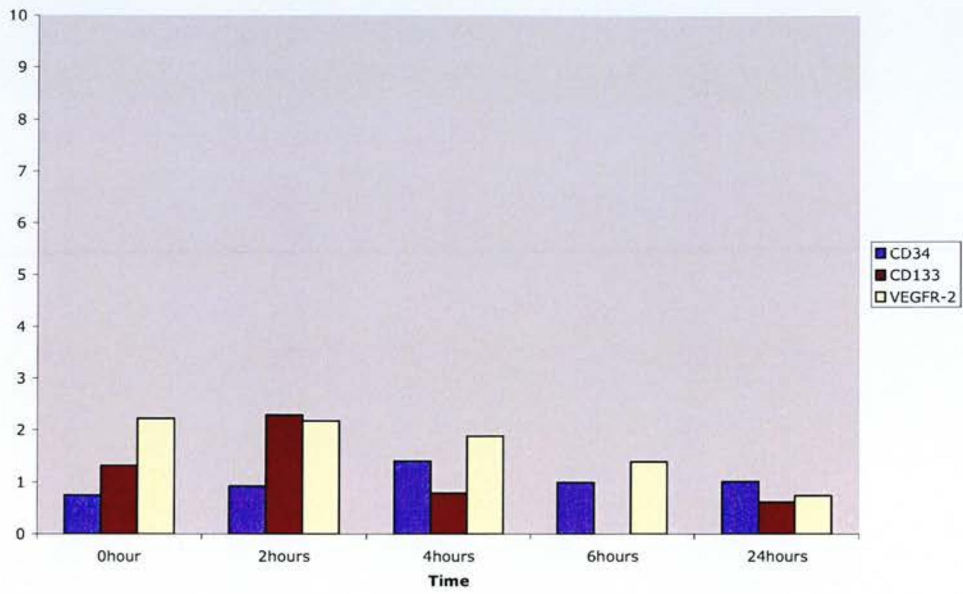
5. 1μg of RNA extracted from each 1ml sample was reverse transcribed using the method in **section 2.2**.
6. Real-time PCR was then performed on the cDNA using Applied Biosystems 'assay-on-demand' primer/probe sets to amplify CD34, CD133 and VEGFR-2.

Results

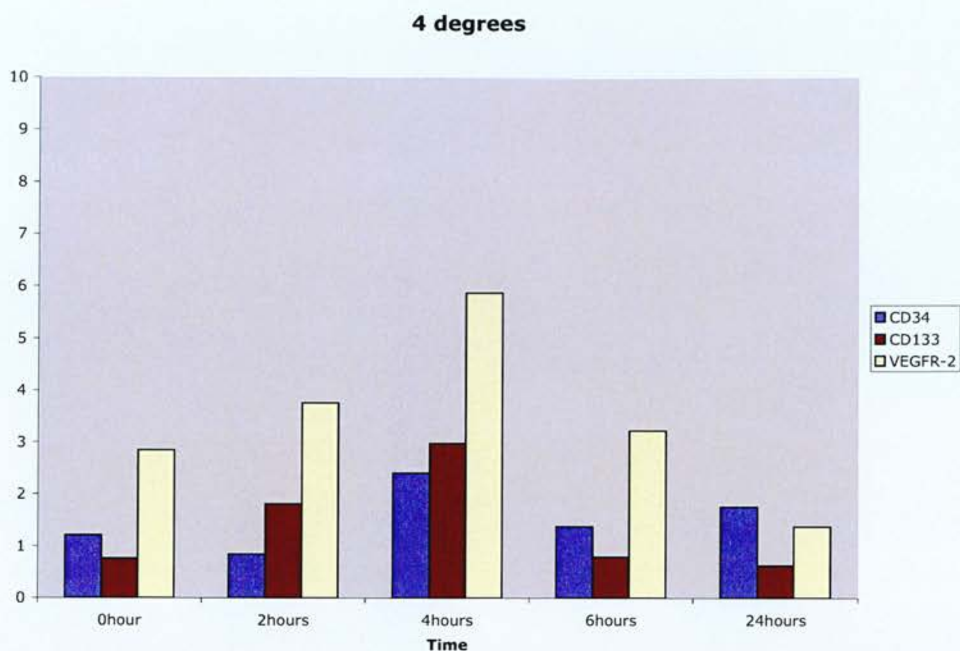
The results for each condition/marker are shown in **figure 3.1**. There was no statistically significant change in the median RQs of CD34 mRNA from 0 hour to 24 hours in the roller samples (p=0.56), in the room temperature samples (p=0.31) and in the 4°C samples (p=0.81). Furthermore, there was no statistically significant change in the median RQs of CD133 mRNA from 0 hour to 24 hours in the roller samples (p=0.12), from 0 hour to 6 hours in the room temperature samples (p=0.81), and from 0 hour to 24 hours in the 4°C samples (p=0.12). Finally, there was no statistically significant change in the mean RQ values of VEGFR-2 mRNA from 0 hour to 24 hours in the roller samples (p=0.25), in the room temperature samples (p=0.50), and in the 4°C samples (p=0.50).



a.



b.



c.

Figure 3.1. The median RQ values of CD34, CD133 and VEGFR-2 mRNA species from 6 control peripheral blood samples each kept at room temperature on the bench **(a)**, at room temperature on a roller device **(b)** and at 4oC **(c)**. Each sample was tested at serial time points, as described in the methods section.

Discussion

The results presented here suggest that cellular RNA is stable over the period measured. The most reliable data was that for the RQs of CD34 mRNA. Amplification of this mRNA species was better in the 6 control subjects studied, compared to that of CD133 and VEGFR-2. This is reflected in the observation that not all time points in the graphs are represented for CD133, and there is highly variable amplification of VEGR-2. Poor and variable amplification is likely to be due to the relative insensitivity of the technique in detecting the markers of EPCs in control subjects, where EPCs represent a very small proportion of the mononuclear cell population. In spite of this, however, the results highlight an important point, which is that is any changes in RQs of mRNA in the patient groups tested are likely to be genuine, rather than artefactual and related to degradation of the mRNA being measured.

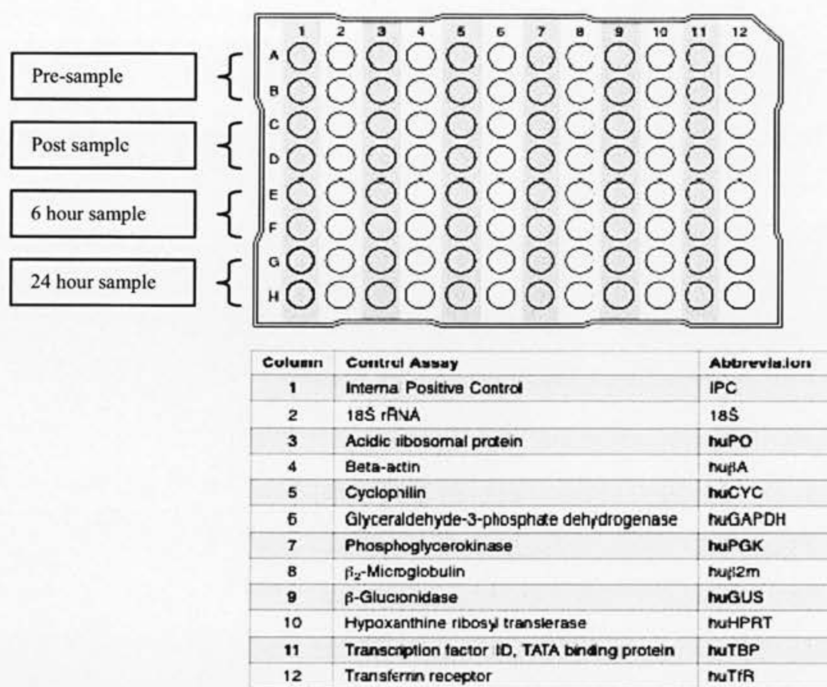
There is very little published on the stability of RNA extracted from whole blood samples. There is some data, however, on the stability of circulating RNA extracted from plasma. Using real-time PCR, Tsui et al reported the effect of time delay in blood processing after venesection on plasma GAPDH mRNA concentrations [126]. 5 EDTA samples were left at room temperature for 0, 6, and 24 hours, then the plasma was subjected to RNA extraction. Similarly, 5 EDTA samples were left at 4°C for 0, 6 and 24 hours, following which the plasma was collected and the RNA extracted. From their results they concluded that plasma RNA was stable over the time period studied, but that storing samples at 4°C resulted in better RNA stability. All of the whole blood samples

used in this thesis where RNA extraction was performed were stored at 4°C and processed within 2 hours of collection.

3.2. Establishing the best endogenous control

Introduction

An endogenous control is RNA or DNA that is present in each experimental sample. By using an endogenous control as an active reference, one can normalise quantification of an mRNA target of interest for differences in the amount of total RNA added to each reaction. This is important when comparing the relative quantities of an mRNA target in serial blood samples following a particular intervention. However, several studies have indicated that the expression of traditional endogenous controls, such as GAPDH and beta-actin, varies among tissues and developmental stages. To ensure that the most appropriate endogenous control would be used for our studies, an ABI TaqMan® human endogenous control plate was used. This plate is designed to simplify the selection of endogenous controls for gene expression studies. The plate evaluates the expression of eleven select housekeeping genes in total RNA samples using the two-step, reverse transcription–polymerase chain reaction (RT-PCR). The plate also features a unique internal positive control (IPC) designed to detect the presence of PCR inhibitors in test samples. The plate itself is an optical 96-well reaction plate divided into 12 columns, one for every control assay. Each column consists of 8 identical wells containing TaqMan primers and probes for the detection of one target gene (**figure 3.2**).



Column	Control Assay	Abbreviation
1	Internal Positive Control	IPC
2	18S rRNA	18S
3	Acidic ribosomal protein	huPO
4	Beta-actin	huβA
5	Cyclophilin	huCYC
6	Glyceraldehyde-3-phosphate dehydrogenase	huGAPDH
7	Phosphoglycerokinase	huPGK
8	β_2-Microglobulin	huβ2m
9	β-Glucuronidase	huGUS
10	Hypoxanthine ribosyl transferase	huHPRT
11	Transcription factor IID, TATA binding protein	huTBP
12	Transferrin receptor	huTfR

Figure 3.2. Assay configurations of the endogenous control plate.

Methods

1. The series of samples taken from patient 25 in the coronary angiography study (see **chapter 4**) were chosen to run on the endogenous control plate. The samples had been taken before coronary angiography, immediately after, and at 6 hours and at 24 hours after the procedure. Total cellular RNA had previously been extracted from the samples and stored at –80°C, according to the protocol previously described (see **section 2.1**).
2. 6μg of RNA from each sample was used for reverse transcription, according to the protocol in **section 2.2**, which meant that each well would receive 250ng of RNA per sample.
3. 650μl of TaqMan Universal PCR master mix (2X) was pipetted into 4 micro centrifuge tubes (for the 4 test samples).
4. The 4 samples were then diluted to a volume of 650μl with RNase-free water. Each sample tube contained 150μl of cDNA.
5. The samples were then pipetted separately into the micro centrifuge tubes containing the master mix making a total volume of 1.3mls, and the tubes were mixed by gentle inversion.
6. The samples plus master mix were pipetted into the wells of the control plate in 50μl aliquots.
7. The wells were sealed over with an optical adhesive cover, and the plate spun to eliminate any air bubbles.

8. Quantitative PCR was then performed using the ABI 7900™ sequence detection system.

Results

The pre-sample was designated the calibrator. Derivation of ΔCT values from the average CT values of the calibrator and samples is the final step in comparative gene expression analysis. The following equation describes the ΔCT calculation.

$$\Delta CT_{(sample)} = \text{Average } CT_{(calibrator)} - \text{Average } CT_{(sample)}$$

The equation above uses the average CT of the calibrator as a baseline for evaluating target gene expression in each sample. Samples with initial template concentrations higher than the calibrator have lower average CT values and yield positive numbers. Samples with lower initial template concentrations have higher average CT values and yield negative numbers. The results of the Endogenous Control Plate are expressed in ΔCT, greater than or less than the calibrator ΔCT. Thus, the calibrator serves as a baseline for the assays and is shown as zero on the graph. One ΔCT is equal to a twofold difference in initial template concentration. The best control is the one with the least variation in ΔCT levels. Ideally, it is expressed at a constant level in all samples regardless of cell cycle, cell type, or tissue. Because the ΔCT indicates the level of gene expression relative to the calibrator, the ΔCT values of a good control do not vary much from zero.

Results for the samples from patient 25 are shown in **figure 3.3**. From the ΔCT profile shown, the 18S ribosomal RNA (18S) phosphglycerokinase (huPGK) and β_2-microglobulin (huβ2m) were shown to be good candidate control genes because their expression remained relatively consistent across the test samples. All three assays produced ΔCT values that deviated little from zero, indicating a fairly stable level of gene expression relative to the other candidate controls. 18s was chosen as the endogenous control, however, because of its relative abundance and ease of amplification at relatively low cycle thresholds.

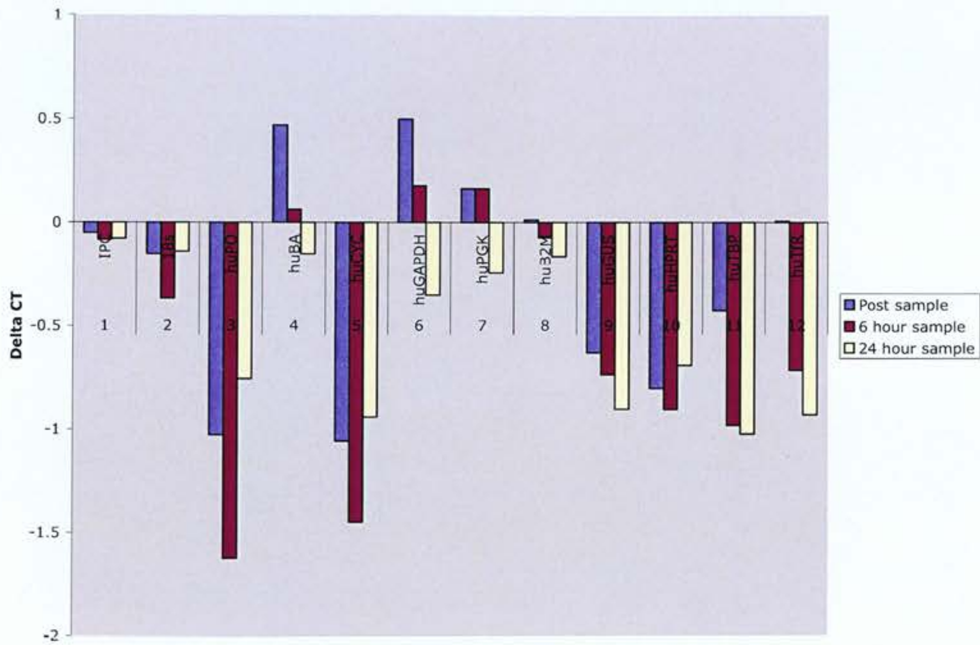


Figure 3.3. Gene expression profile of patient 25. No bars shown for the calibrator (ΔCT= 0). Blue bars represent the post-sample, brown bars represent the 6-hour sample, and white bars represent the 24-hour sample.

3.3. Relative quantitation of gene expression- Comparative C_T method

Introduction

Relative quantitation of RNA can be performed using the standard curve method or the comparative method. It is relatively straightforward to prepare standard curves because quantity is expressed relative to some basis sample, such as the calibrator. For all experimental targets, target quantity is determined from the standard curve and divided by the target quantity of the calibrator. Thus, the calibrator becomes the 1X sample, and all other quantities are expressed as an n-fold difference relative to the calibrator. Because the sample quantity is divided by the calibrator quantity, the unit from the standard curve drops out. Thus, all that is required of the standards is that their relative dilutions are known. Any stock DNA containing the appropriate target can be used to prepare standards. There are, however, some important points to note when performing relative quantitation using the standard curve method.

- It is important that stock DNA is accurately diluted.
- For quantitation normalised to an endogenous control, standard curves need to be prepared for both the target and the endogenous reference. For each experimental sample, the amount of target and endogenous control is determined from the appropriate standard curve. Then, the target amount is divided by the endogenous control amount to obtain the normalised target value. Then the relative expression levels of the target are derived using a calibrator sample, as previously described.

To overcome the relatively labour intensive approach of the standard curve method, relative quantitation of RNA can be achieved using the comparative C_T method. This uses the arithmetic formula $2^{-\Delta\Delta CT}$ to obtain RQ values. The exact derivation of the equation is explained in the Applied Biosystems user bulletin [168]. Essentially it is the amount of target, normalised to an endogenous control and relative to a calibrator, where $\Delta\Delta C_T$ is the ΔC_T of the target minus the ΔC_T of the calibrator. The software used by the ABI 7900™ analyser automatically calculates these parameters. For the comparative C_T method to be valid, however, the efficiency of the target amplification and the efficiency of the endogenous control amplification must be approximately equal. Therefore, when using custom designed probe and primer sets it is recommended that validation experiments be performed to demonstrate this. Validation can be done by assessing how the ΔC_T of a target varies with dilution of the RNA used (see below). Fortunately, the Applied Biosystems TaqMan gene expression assays used in our experiments have been extensively tested and validated and found to have very similar efficiencies (close to 100%), so it is not routinely necessary to measure efficiency when using them. However, to prove that this is the case a validation experiment was performed, using the 'assay-on-demand' primer/probe sets for CD34 and ribosomal 18s.

Methods

1. RNA was extracted from a sample of bone marrow aspirate according to the method described in **section 2.1**. The concentration of the RNA was 170ng/μl, diluted to a working concentration of 100ng/μl.
2. Serial dilutions of the RNA were made (**table 3.1**) and each dilution was reverse transcribed according to the method in **section 2.2**.
3. Primer/probe sets for amplification CD34 cDNA (as the target) and ribosomal 18s (as the endogenous control) were purchased from the Applied Biosystems "assay-on-demand" service.
4. Quantitative PCR was performed using the ABI 7900™ analyser.

Results

1. The average C_T values for CD34 and 18s were obtained and the average ΔCT values calculated (**table 3.1**).
2. The average ΔCT values were then plotted against the log (input RNA) to generate a linear graph (**figure 3.4**).
3. The slope of the graph was calculated.

RNA conc. (ng)	Ave. C_T (CD34)	Ave. C_T (18s)	ΔC_T
1000	27.81	14.35	13.46
800	28.39	14.58	13.81
600	28.14	15.31	12.83
400	29.50	15.85	13.65
200	30.92	17.51	13.41
100	31.77	18.32	13.45

Table 3.1. Average C_T value for CD34 and 18s at different RNA input amounts.

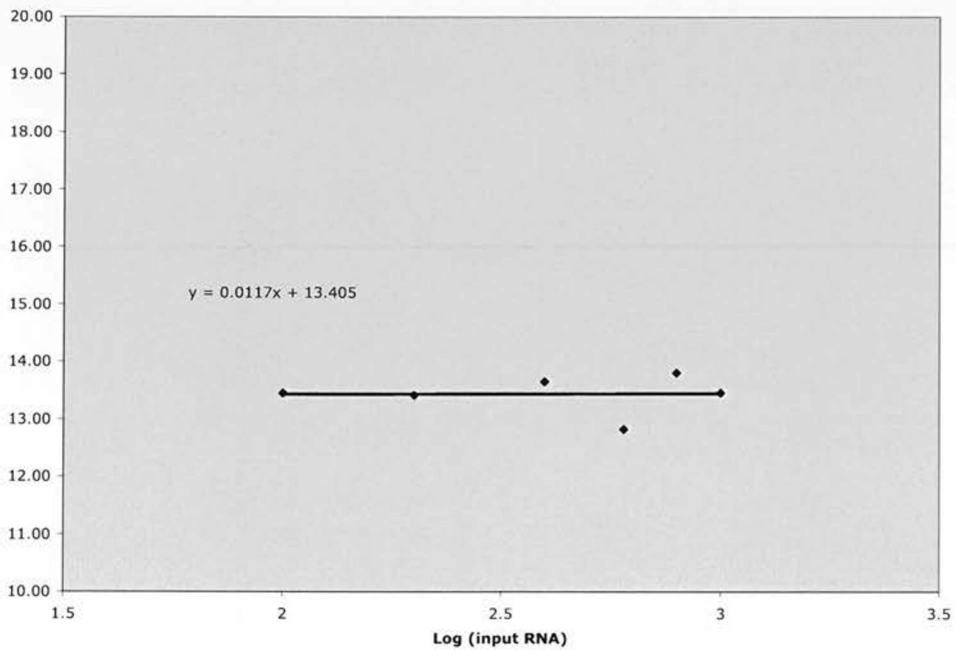


Figure 3.4. Plot of log input RNA versus ΔC_T.

Conclusion

For the efficiencies of the target and endogenous control amplifications to be similar the slope of the graph should be less than 0.1. The slope of the graph in **figure 3.4** is 0.01, confirming that the efficiencies are very similar. The $\Delta\Delta C_T$ method of calculation of the RQ values is the method of choice used in this thesis.

3.4 Reproducibility Data

Introduction

The coefficient of variation (cv) is a measure of dispersion of a probability distribution, and is defined as the ratio of the standard deviation to the mean multiplied by 100 to give a percentage value. It can be used to determine the intra- and inter-variability of a particular assay, and can be seen as a way of assessing assay reproducibility.

Methods

Ct values for the endogenous control, ribosomal 18s, from a cohort of normal blood samples were used to calculate the cv for the real-time PCR assay. Ribosomal 18s was chosen because it has been shown that levels vary little between samples (**section 3.2**), implying that any marked variability will be due to methodology.

1. 2.7mls of EDTA anticoagulated blood was drawn from each of 36 control subjects.
2. Total cellular RNA was extracted from each sample, according to the method described in **section 2.1**.
3. 1μg of RNA extracted from each sample was reverse transcribed using the method in **section 2.2**.

4. Real-time PCR was then performed on the cDNA using the Applied Biosystems 'assay-on-demand' primer/probe set to amplify 18s. Each sample was run in triplicate.
5. Ct values were determined for each well/sample and intra- and inter assay cvs were calculated.

Results and discussion

See **Table 3.2**. The mean intra-assay cv was 2.74%. The inter-assay cv was 6.68%. Both cv values are below 10%, which is regarded as the cut-off for acceptable assay reproducibility. The data from real-time PCR assay used in this thesis can, therefore, be interpreted with confidence.

Control	Ct	Mean intra Ct	Intra sd	Intra cv
c1	8.914182			
c1	8.468726			
c1	8.485065	8.62265767	0.25259962	2.929486831
c2	8.323936			
c2	8.500703			
c2	8.431259	8.41863267	0.08905735	1.05786001
c3	7.559304			
c3	7.832469			
c3	7.897216	7.76299633	0.17934873	2.310302963
c4	7.115218			
c4	6.975356			
c4	7.419798	7.170124	0.22725135	3.169420033
c5	8.081259			
c5	7.54479			
c5	7.990533	7.872194	0.28714602	3.647598356
c6	8.643373			
c6	8.631191			
c6	8.796629	8.69039767	0.09220045	1.060946236
c7	9.939116			
c7	9.842138			
c7	9.376766	9.71934	0.30061419	3.092948612
c8	9.186432			
c8	9.173496			
c8	8.976012	9.11198	0.11792925	1.294221992
c9	8.790575			
c9	8.842152			
c9	8.923827	8.85218467	0.06719014	0.759023233
c10	8.939893			
c10	8.804809			
c10	8.046533	8.59707833	0.48154651	5.601280981
c11	7.813177			
c11	8.423806			
c11	8.299778	8.17892033	0.32275668	3.946201559
c12	9.273266			
c12	9.116021			
c12	8.959337	9.116208	0.15696458	1.721818804
c13	8.226588			
c13	8.387432			
c13	8.795276	8.46976533	0.29314773	3.461108094

Control	Ct	Mean intra Ct	Intra sd	Intra cv
c14	7.549772			
c14	7.611612			
c14	8.025742	7.729042	0.25880343	3.348454144
c15	8.847471			
c15	8.508533			
c15	8.663116	8.67304	0.16968679	1.956485717
c16	8.481077			
c16	8.501577			
c16	8.635908	8.53952067	0.08410084	0.984842612
c17	7.74364			
c17	7.876286			
c17	8.107315	7.90908033	0.18404205	2.32697161
c18	8.183282			
c18	7.865273			
c18	8.668533	8.23902933	0.4045213	4.90981743
c19	8.375304			
c19	8.83628			
c19	7.853721	8.35510167	0.49159094	5.883721767
c20	8.414792			
c20	7.811428			
c20	7.83649	8.02090333	0.34134768	4.255726117
c21	7.354102			
c21	7.266655			
c21	7.34779	7.322849	0.04876766	0.665965651
c22	7.423119			
c22	7.246335			
c22	7.434873	7.368109	0.10562301	1.433515793
c23	8.927596			
c23	8.886479			
c23	8.860986	8.891687	0.03360901	0.377982365
c24	8.895221			
c24	8.864418			
c24	8.691408	8.81701567	0.10986433	1.246048919
c25	8.569964			
c25	8.693617			
c25	8.120214	8.461265	0.30176043	3.566374836
c26	8.803024			
c26	8.730175			
c26	8.017135	8.516778	0.43423391	5.098570274

Control	Ct	Mean intra Ct	Intra sd	Intra cv
c27	7.901772			
c27	7.700288			
c27	7.702748	7.76826933	0.11562324	1.488404153
c28	8.449559			
c28	8.120722			
c28	8.53469	8.36832367	0.21861327	2.612390203
c29	8.427888			
c29	8.180111			
c29	8.260093	8.289364	0.12645534	1.525513223
c30	8.463441			
c30	8.441451			
c30	8.468628	8.45784	0.0144283	0.170590792
c31	9.3928			
c31	9.523085			
c31	9.496444	9.47077633	0.06883071	0.726769498
c32	8.626861			
c32	8.63723			
c32	8.966708	8.74359967	0.19328703	2.210611599
c33	8.010114			
c33	8.231137			
c33	8.73736	8.32620367	0.37282696	4.477754465
c34	9.025117			
c34	9.233336			
c34	8.556451	8.93830133	0.34669301	3.878734905
c35	8.112525			
c35	8.605731			
c35	7.840089	8.186115	0.3880896	4.740827553
c36	7.944379			
c36	8.044102			
c36	8.991658	8.326713	0.57801389	6.941681449

Table 3.2. Triplicate Ct values for ribosomal 18s from 36 control samples. The intra-assay co was calculated for each triplicate result. The inter-assay cv was calculated from the mean standard deviations (sd) of the Ct values divided by their mean, then multiplied by 100 to give a percentage.

3.5. End-point PCR

Introduction

In order to demonstrate that the PCR products from a real-time reaction were amplicons of CD34, CD133 and VEGFR-2 cDNA, the proposed markers of EPCs, a polyacrylamide gel electrophoresis (PAGE) was performed on amplified cDNA derived from a control peripheral blood sample (**figure 3.5**).

Methods

RNA was extracted from the sample using the protocol described in **section 2.1**. 1μg of RNA extracted from the sample was then reverse transcribed using the protocol described in **section 2.2**. 4μl of cDNA was then used in a real-time reaction using the protocol and reaction conditions and appropriate primer/probe combinations described in **section2.3**. 5μl of the final product was then run on 8% acrylamide gel for 45 minutes at 90 volts. The gel was then stained with ethidium bromide. A high molecular weight marker was also run on the gel to confirm that the amplicons were of the appropriate size.

Results

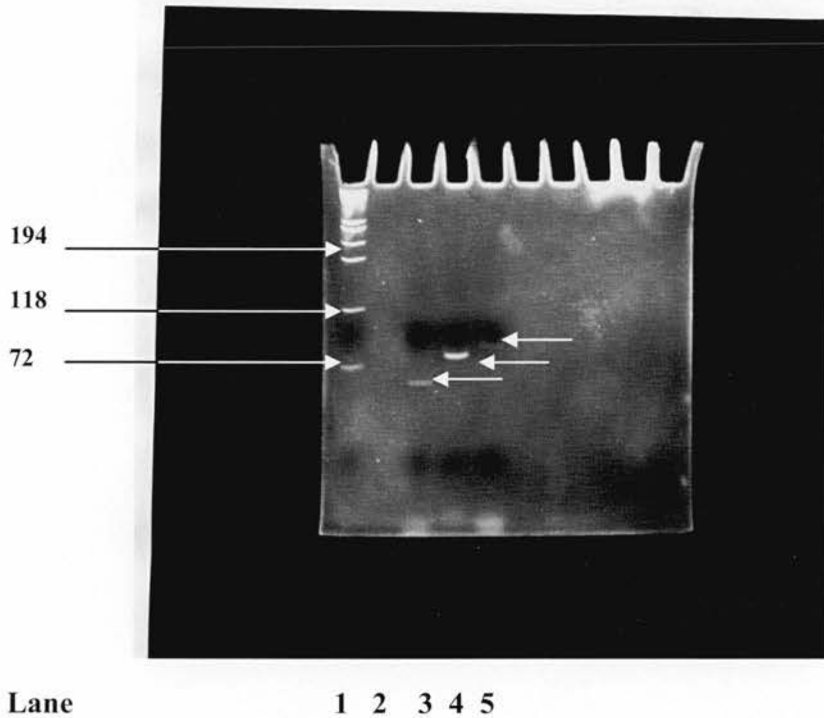


Figure 3.5. PCR product run on 8% acrylamide gel. Lane 1- molecular weight marker. Lane 2- negative control. Lane 3- CD34 band. Lane 4- CD133 band. Lane 5- VEGFR-2 band.

Conclusions

The real-time system used in this thesis produces pure products that can be detected by PAGE. This is reassuring and means that the PCR results in subsequent sections can be interpreted with confidence.

3.6. Measuring PCR product from different source materials

Introduction

There is growing interest in clinical use of locally injected autologous endothelial precursor cells (EPC) to promote revascularisation of ischemic tissue. EPCs appear to share many properties with haematopoietic stem cells (HSC), and sources of HSC such as bone marrow, or HSC mobilised to peripheral blood following G-CSF administration are becoming regarded as sources of autologous EPC for clinical use. However while haematopoietic potential is now assessed by numbers of HSC expressing CD34, there is no generally recognised unambiguous equivalent determinant for assessment of vasculogenic potential.

The identification of suitable EPC sources is of the highest importance as a prerequisite for any clinical EPC use. Freshly isolated bone marrow mononuclear cells, HSC-mobilised peripheral blood mononuclear cells, and the CD34+ enriched or CD133+ enriched subpopulations of these cells have been injected locally in infarcted myocardium or ischaemic limbs with, in most cases, some recorded clinical benefit [121, 127, 128]. Shi *et al* proposed G-CSF administration as a therapy to mobilize and increase EPCs in circulation [4], and infusion of peripheral blood stem cells after G-CSF administration was shown to increase vascularisation in patients after myocardial infarction [113] and in limb ischaemia [129]. In a comparative clinical trial of peripheral blood derived EPC versus bone marrow derived EPC, implantation of both cell preparations into infarcted myocardium gave similar positive effects showing an

improvement in regional myocardial contractility and viability [120]. While it cannot be used for autologous EPC transplant, cord blood also has high numbers of HSCs and these cells have been differentiated to ECs [18, 130]. Different research groups preferred different sources of EPCs, and reports do not demonstrate any obvious advantage or disadvantage of any source tested.

Functional assays are of utmost importance to ultimately define a real EPC phenotype. Hill *et al* in 2003 have described a colony assay based on mononuclear cell colony outgrowth on fibronectin coated plates in simple medium [12]. This colony assay is available in a standardized commercial kit which should allow inter-laboratory comparison of results from in increasing number of studies of different clinical conditions employing this technique [129, 131-137].

In summary, different research groups prefer different sources of EPCs, and reports do not demonstrate any obvious advantage or disadvantage of any source tested. In this section I have attempted to examine the different potential sources of autologous EPCs, using real-time PCR to measure their markers, in an effort to determine which source would provide the most plentiful supply of EPCs.

Methods

Venous blood samples (10ml) were collected from patients immediately following cell-separator leukapheresis collection of G-CSF mobilised PBSC for autologous transplant and from healthy donors for allogeneic transplant. Cord blood products (20-50 ml) were

aspirated from the umbilical placental veins from normal cesarean deliveries. Bone marrow samples (3ml) were obtained by aspiration from the posterior iliac crest of haematologically normal donors. Peripheral blood samples were drawn form normal controls. Two different mobilised stem cell samples, three different cord blood samples, one bone marrow sample and two peripheral blood samples were analysed.

RNA was isolated from 1ml of each patient sample using the technique described **section 2.1**. 10μl of the RNA was then reverse transcribed into complementary DNA using the method in **section 2.2**. 4μl of cDNA was then added to each well of a 96 well reaction plate, which then underwent RT-PCR using reaction conditions described in **section 2.3**. Each sample/ primer/probe was run in triplicate and ribosomal 18s was used as the endogenous control **(figure 3.6)**. The primer/probe sets used were supplied off-the-shelf from Applied Biosystems (Warrington, UK), and used to detect the following amplicons: CD34, CD133, VEGFR-2 (plate 1), CD14, VE-cadherin and vWF (plate 2). The relative quantities of these mRNA species in each sample were calculated by the ABI software using the ΔΔCt method described in **section 3.3**. Results obtained were not statistically analysed due to the small number of samples involved in this study.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PB1 34	PB1 34	PB1 34	PB2 34	PB2 34	PB2 34	CB1 34	CB1 34	CB1 34	CB2 34	CB2 34	CB2 34
B	CB3 34	CB3 34	CB3 34	MB1 34	MB1 34	MB1 34	MB2 34	MB2 34	MB2 34	BM 34	BM 34	BM 34
C	PB1 133	PB1 133	PB1 133	PB2 133	PB2 133	PB2 133	CB1 133	CB1 133	CB1 133	CB2 133	CB2 133	CB2 133
D	CB3 133	CB3 133	CB3 133	MB1 133	MB1 133	MB1 133	MB2 133	MB2 133	MB2 133	BM 133	BM 133	BM 133
E	PB1 V2	PB1 V2	PB1 V2	PB2 V2	PB2 V2	PB2 V2	CB1 V2	CB1 V2	CB1 V2	CB2 V2	CB2 V2	CB2 V2
F	CB3 V2	CB3 V2	CB3 V2	MB1 V2	MB1 V2	MB1 V2	MB2 V2	MB2 V2	MB2 V2	BM V2	BM V2	BM V2
G	PB1 18s	PB1 18s	PB1 18s	PB2 18s	PB2 18s	PB2 18s	CB1 18s	CB1 18s	CB1 18s	CB2 18s	CB2 18s	CB2 18s
H	CB3 18s	CB3 18s	CB3 18s	MB1 18s	MB1 18s	MB1 18s	MB2 18s	MB2 18s	MB2 18s	BM 18s	BM 18s	BM 18s

Figure 3.6. The 96 well plate layout used. Two plates were constructed, the first containing primers for CD34, CD133 and VEGFR-2, and the second containing primers for VE-cadherin, vWF and CD14. Key- **PB1**: peripheral blood-1, **PB2**: peripheral blood-2, **CB1**: cord blood-1, **CB2**: cord blood-2, **CB3**: cord blood-3, **MB1**: mobilised peripheral blood-1, **MB2**: mobilised peripheral blood-2, **BM**: bone marrow, **34**: CD34, **133**: CD133, **V2**: VEGFR-2, **18s**: ribosomal 18s.

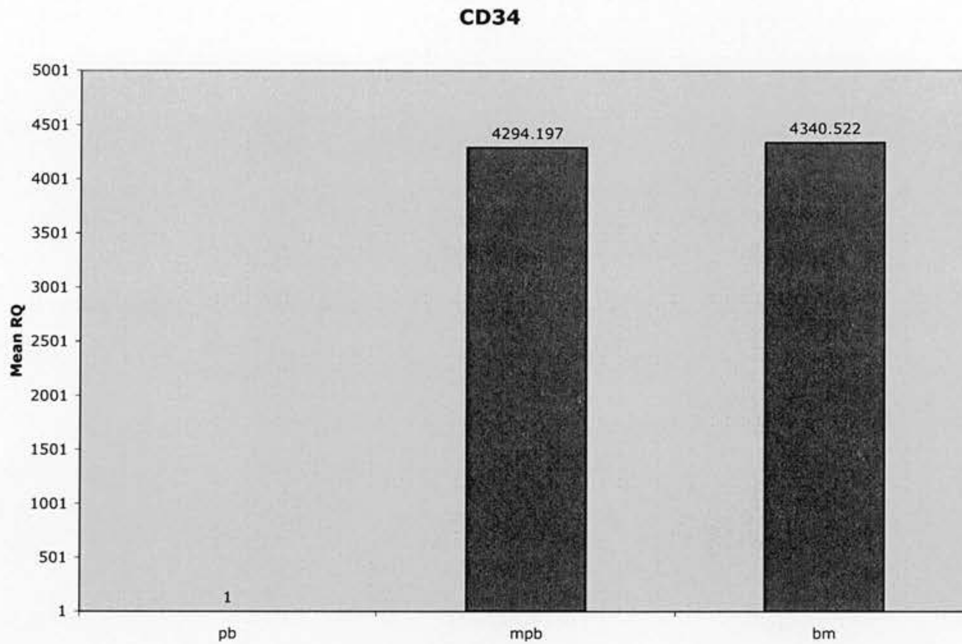


Figure 3.7. The mean RQ of CD34 mRNA in peripheral blood (pb), mobilised peripheral blood (mpb) and bone marrow (bm). There is a far greater amount of CD34 mRNA in mpb and bm compared with pb. pb= 2 samples, cb= 3 samples, mpb= 2 samples, bm= 1 sample.

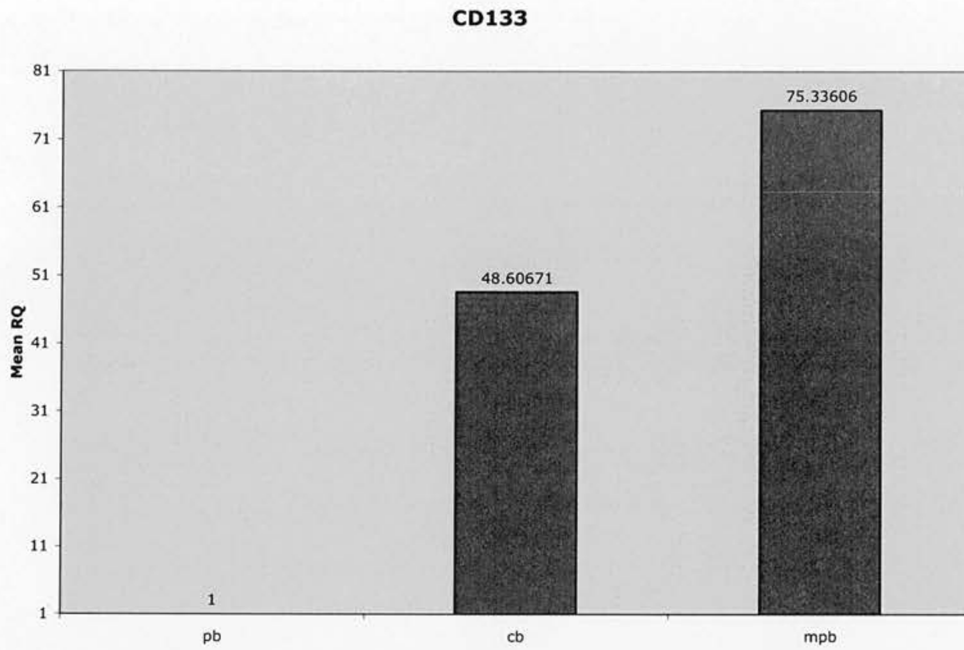


Figure 3.8. The mean RQ of CD133 mRNA in peripheral blood (pb), cord blood (cb) and mobilised peripheral blood (mpb). There is a far greater amount of CD133 mRNA in cb and mpb compared with pb. pb= 2 samples, cb= 3 samples, mpb= 2 samples, bm= 1 sample.

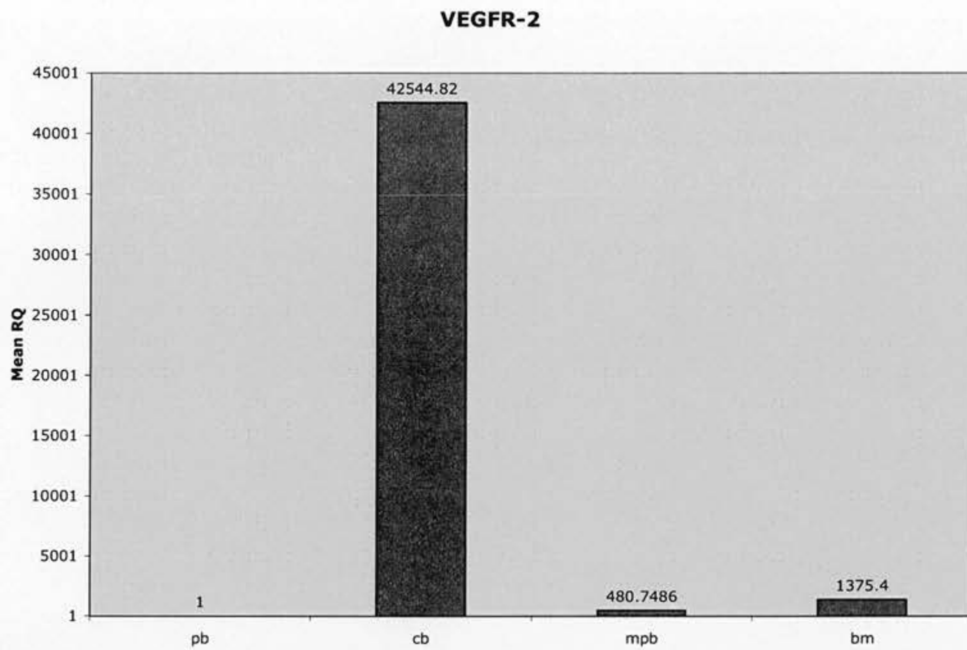


Figure 3.9. The mean RQ of VEGFR-2 mRNA in peripheral blood (pb), cord blood (cb), mobilised peripheral blood (mpb) and bone marrow (bm). The greatest amount of VEGFR-2 mRNA appears to be in cb, followed by bm then mpb. Peripheral blood has the lowest relative amount of VEGFR-2 mRNA. pb= 2 samples, cb= 3 samples, mpb= 2 samples, bm= 1 sample.

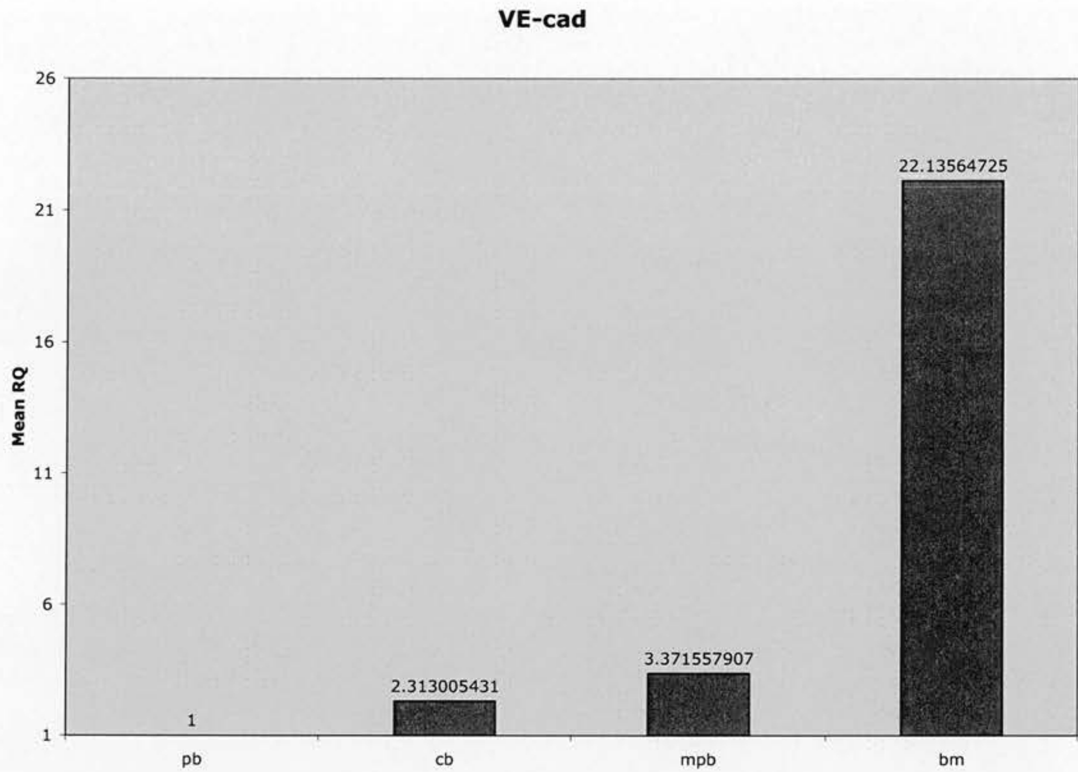


Figure 3.10. The mean RQ of VE-cadherin mRNA in peripheral blood (pb), cord blood (cb), mobilised peripheral blood (mpb) and bone marrow (bm). The greatest amount of VE-cadherin mRNA appears to be in bm, followed by mpb then cb. Peripheral blood has the lowest relative amount of VE-cadherin mRNA. pb= 2 samples, cb= 3 samples, mpb= 2 samples, bm= 1 sample.

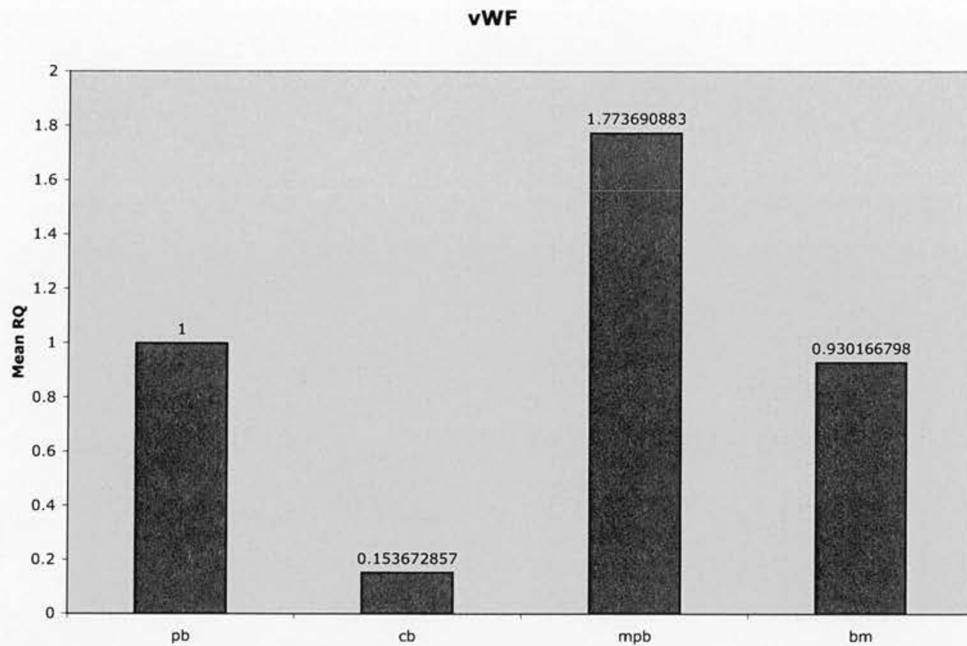


Figure 3.11. The mean RQ of vWF mRNA in peripheral blood (pb), cord blood (cb), mobilised peripheral blood (mpb) and bone marrow (bm). The greatest amount of vWF mRNA appears to be in mpb, followed by pb then bm. Cord blood has the lowest relative amount of vWF mRNA. pb= 2 samples, cb= 3 samples, mpb= 2 samples, bm= 1 sample.

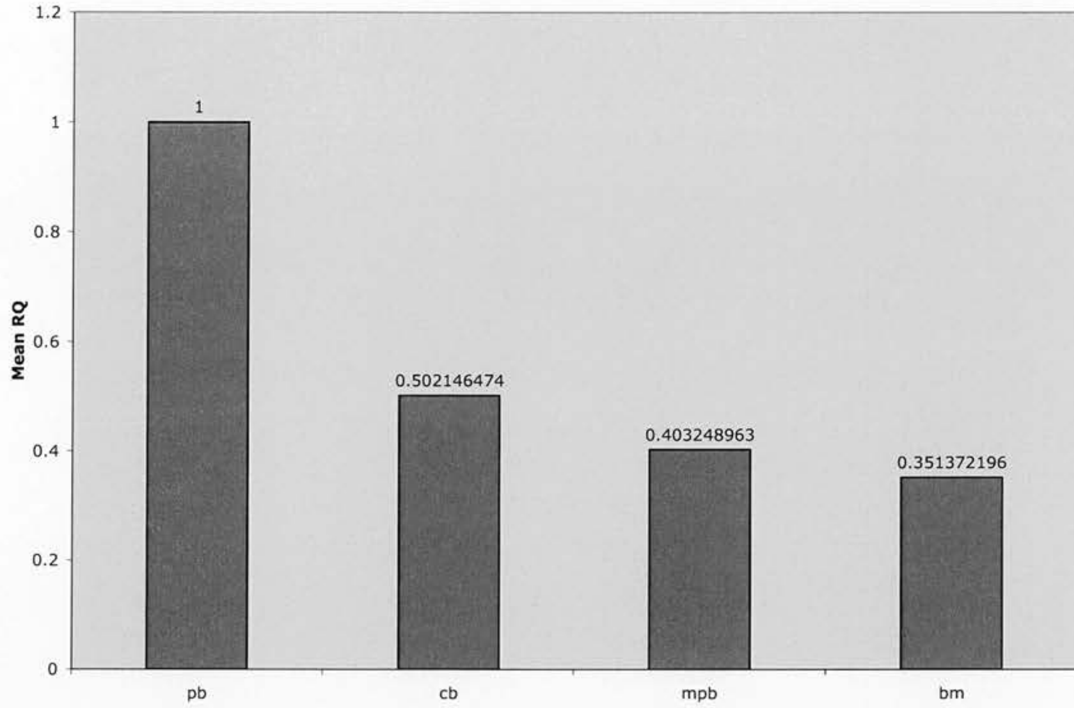


Figure 3.12. The mean RQ of CD14 mRNA in peripheral blood (pb), cord blood (cb), mobilised peripheral blood (mpb) and bone marrow (bm). The greatest amount of CD14 mRNA appears to be in pb, followed by cb then mpb. Bone marrow has the lowest relative amount of CD14 mRNA. pb= 2 samples, cb= 3 samples, mb= 2 samples, bm= 1 sample.

Discussion

Identification and enumeration of angiogenic cells present in the adult remains difficult and non-standardised. No single markers truly specific for endothelial cells have been found, therefore it is necessary to utilise marker combinations to best identify CEC and EPCs before clinical application of such cells is routine. I have attempted to measure the mRNA levels of the markers of EPCs from the most common potential EPC sources. It should be remembered that the number of samples studied here was small and the results not subjected to statistical analysis. Despite this, however, all sources except normal peripheral blood had high RQ values for CD34, CD133, VEGFR-2 and VE-cadherin mRNA species, the putative markers of EPCs. In contrast, the RQs of vWF and CD14 mRNA species were greater in the peripheral blood samples. These results suggest that a source other than peripheral blood would be more appropriate as a potential source of EPCs. My observations are similar to those of unpublished results from a collaborative group (personal communication). They showed, using flow cytometric techniques, that mobilised peripheral blood contained higher levels of CD34+, CD133+ and double positive cells. In addition, and in keeping with my results, they showed that cord blood contained higher numbers of VEGFR-2+ cells. Most of the samples used in this thesis were peripheral blood samples. The advantage of using such material is the relative easy with which it can be obtained compared with cord blood or mobilised peripheral blood. The disadvantage, however, is the relative paucity of EPC mRNA in such material. This may explain, in part, the difficulty I have had with reproducibility of triplicate RQ values for a sample, particularly when CD133 probe/primer sets were used.

4. ENDOTHELIAL PROGENITOR CELLS AND ISCHAEMIC HEART DISEASE

4.1. Introduction

Ischaemic heart disease is a major cause of morbidity and mortality worldwide. Despite advances in percutaneous coronary intervention (PCI), major adverse cardiac events occur in 30% of patients following balloon angioplasty and 20% following stenting [138]. Vascular trauma, induced by percutaneous intervention, initiates a sequence of events in which the release of cytokines and growth factors result in the proliferation of smooth muscle and deposition of platelets and leucocytes at the site of injury, accelerating vascular repair. Endothelialisation is necessary to prevent mural thrombus formation and neointimal hyperplasia, which may result in peri-procedural ischaemic complications or restenosis.

The discovery by Asahara and colleagues that mononuclear cells in peripheral blood have the potential to differentiate into endothelial cells has launched a new field of cardiovascular research [3]. Endothelial progenitor cells (EPCs) have been characterised by their expression of both haematopoietic (CD34) and endothelial cell antigens (CD133 and VEGFR-2), and by their ability to proliferate, migrate and differentiate into mature cell types. These putative EPCs form vascular structures in vitro and are incorporated into the vessel wall in experimental models of neovascularisation [130]. The traditional paradigm of vascular repair is based on the proliferation and migration of pre-existing mature endothelial cells from adjacent vasculature [139]. The discovery of circulating progenitors has altered our view of this process, with progenitor cells potentially playing

an important role in the maintenance and repair of the vascular endothelium and in the pathogenesis of atherosclerotic plaque formation and its consequences.

EPCs can be isolated and cultured from a variety of cell populations in peripheral blood and bone marrow, but as yet no definitive phenotype has been ascribed to EPCs. Comparisons between clinical studies have been limited by the use of a variety of phenotypic markers to discriminate EPCs and by the lack of comparable functional assays. In the face of an uncertain phenotype, the EPC colony forming unit assay (CFU-EPC) has emerged as an alternative specific enumeration system for EPCs [12]. Increasingly, groups quantify phenotypic EPCs or functional CFU-EPCs, however few clinical studies report both or comment on the relationship between phenotype and function.

EPCs are infrequent in peripheral blood, but numbers have been shown to increase rapidly in response to myocardial ischaemia and acute myocardial infarction (**table 1.2**) [105, 140]. Reduced numbers of EPCs have been demonstrated in cigarette smokers [64], patients with diabetes mellitus [141], and in those with evidence of endothelial dysfunction [142]. These patients are at high risk of complications following PCI. Furthermore, patients with diffuse in-stent re-stenosis have reduced EPC number and function in comparison with matched controls at the time of presentation [61]. Inadequate EPC number and function prior to angioplasty, as well as inadequate early and sustained EPC recruitment may favour a maladaptive response to arterial injury and result in an increased incidence of in-stent thrombosis, re-stenosis and ischaemic

complications. The immediate effects of local vascular injury during angioplasty and stenting on the mobilization of EPCs are not known. The aim of this part of the thesis was to measure circulating EPCs (using PCR and flow cytometry) and functional CFU-EPCs following PCI in patients with stable coronary disease.

4.2 Methods

Forty patients undergoing elective coronary angiography participated in this study, which was performed with the approval of the local research ethics committee. Written informed consent was obtained from all volunteers. All patients were recruited from the Medical Day Case Unit at the Royal Infirmary, Edinburgh following referral for diagnostic angiography to investigate symptoms suggestive of stable angina. Patients with a recent acute coronary syndrome or coronary intervention (<3 months), renal or hepatic failure, or a systemic inflammatory disorder or malignancy were excluded from the study. Twenty patients underwent diagnostic coronary angiography alone, and 20 required balloon angioplasty and stenting because of flow limiting coronary stenosis (**table 4.1**).

All patients were treated for two weeks with 75 mg clopidogrel prior to angiography or PCI. Coronary angiography was performed via right femoral or radial artery approach with 6F arterial catheters. Elective PCI was performed in all patients after 7,500 IU intravenous heparin administration and in one patient after intravenous glycoprotein IIb/IIIa inhibitor. Coronary stents (Liberté, Boston Scientific) were implanted in all patients after balloon predilatation of the lesion without apparent procedural complications.

	Angiography	**PCI**
	n=20	n=20
Age (years)	62 ± 2	59 ± 3
Sex (male/female)	11/9	14/6
Cigarette smokers	4	3
Diabetes mellitus	7	2
Hypertension	12	12
Previous MI	2	3
Previous PCI	1	7
Prior CABG	0	1
Total cholesterol (mg/dL)	90±7	81±9
LDL-cholesterol (mg/dL)	47±18	43±9
HDL-cholesterol (mg/dL)	22±9	22±2
Triglycerides (mg/dL)	45±5	40±5
Fasting glucose (mg/dL)	151±18	99±40
C-reactive protein	n.a.	n.a.
Number of diseased vessels		
Minor plaque only	12	0
1 vessel disease	0	9
2 vessel disease	4	8
3 vessel disease	4	3
Stent implantation		
De novo lesion	0	17
Restenosis	0	3

Table 4.1. Clinical characteristics and angiographic findings of patients undergoing diagnostic angiography or percutaneous coronary intervention (values are presented as number or mean ± SEM).

A venous cannula (17-gauge) was inserted into a large subcutaneous vein of the ante-cubital fossae for blood sampling before, immediately after and at 6 and 24 hours following angiography. EDTA anti-coagulated blood (Sarstedt-Monovette, Germany) was collected for real-time PCR, flow cytometry and for preparation of plasma for assay of VEGF by ELISA. In 24 subjects (12 diagnostic angiograms and 12 PCI), 20 mL of whole blood was drawn at baseline and 24 hours for mononuclear cell preparation and cell culture. Whole blood was analysed for total cells, differential count and platelets using an autoanalyzer (Sysmex, UK). Plasma Troponin I concentrations were measured using an automated immunometric assay (method not described) (Ortho-clinical Diagnostics, High Wycombe, UK). Serum was prepared for measurement of C-reactive protein (CRP) concentrations using an immunonephelometric assay (method not described) (Behring BN II nephelometer, Marburg, Germany).

RNA isolation

Total leukocyte RNA extraction from 1 mL of whole blood was performed using Qiagen's RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) (**section 2.1**). RNA concentration and purity were estimated by UV absorbance at 260 and 280 nm. The 260:280 nm ratios were all greater than 1.8 indicating that little protein contamination was present. One microgram of total RNA was transcribed into cDNA (**section 2.2**) in each reverse transcription reaction with 200 units of M-MLV reverse transcriptase for 60 minutes at 37°C in 20 μL reactions containing 1 μL (0.5μg/μl) of random hexamer primers, with 0.625 μL (40 units/μL) of RNase inhibitor, 5 μL of dNTP mix (containing

dATP, dCTP, dGTP and dTTP, each at a concentration of 10 nM in water) and 5 μL of 5X RT reaction buffer (containing 50 mM Tris-HCL-pH 8.3 at 25°C, 75 mM KCL, 3 mM $MgCl_2$ and 10 mM DTT).

Real-time PCR

Real-time PCR was carried out using the ABI Prism ®7900HT system (Applied Biosystems, Warrington, UK) to determine the relative quantity (RQ) of mRNA for selected genes (**section 2.3**). RQ can be defined as a comparison of a target signal in different samples to a reference sample and normalised to an endogenous control. PCR primers and probes for amplification of cDNA derived from CD34, CD14, VE-cadherin and vWF transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Each assay contained forward and reverse PCR primers (final concentration of 900 nM each) and one TaqMan MGB probe (6-FAM dye-labelled, to a final concentration of 250 nM). All primer-probe sets had been quality control checked and validated. The ribosomal 18s gene was used as an internal control. 4 μL of the reverse transcription reaction was analysed in each PCR reaction. The PCR reactions were run in triplicate in 20 μL assays, each containing cDNA, 1 μL of primer/probe, 10 μL of universal PCR master mix and distilled water. The cycling program was as follows: (a) initial activation for 10 minutes at 95°C; (b) 50 amplification cycles with a 15 second denaturing step at 95°C, and a 1 minute combined annealing and extension step at 60°C. Analysis was performed using ABI 7900HT SDS software (version 2.1), in order to obtain the relative quantities (RQ) of mRNA compared to a calibrator.

Flow cytometry

Whole blood cells were phenotyped by flow cytometry, according to the protocol in **section 2.5**. Cells were directly stained and analysed for phenotypic expression of surface proteins using anti-human monoclonal antibodies (MAbs) conjugated to phycoerythrin (PE), fluorescein isothiocynate (FITC), or Peridin Chlorophylla protein (PerCP). The MAbs used were anti-CD34-FITC, anti-CD133-APC (Myltenyi Biotec, UK), anti-CD45-PerCP, (Becton Dickinson, Oxford, UK), anti-VEGFR2-PE (R&D systems, Minneapolis, USA) and anti-VE-cadherin-PE (Santa Cruz Biotechnology). Appropriate negative controls (isotype and/or no antibody) were used to establish positive stain boundaries. Undiluted samples (100μL) were stained with antibodies for 30 minutes in the dark. Erythrocytes were lysed (lysing solution, Becton Dickinson), and samples were centrifuged at 200 g for 10 minutes, washed with phosphate buffered saline, and fixed (Cell Fix solution, Becton Dickinson). For each sample, 50,000 events were acquired in the leucocyte region (as determined by characteristic forward and side scatter profile), using a FACS Calibur flow cytometer (Becton Dickinson) and data were analysed using FCS Express (DeNovo Software). EPCs were quantified from the total white cell count at a given time point and based on the percentage of cells expressing the above markers, either singly or in combination, and expressed as number of cells per mL of blood.

Cell culture

Mononuclear cells were isolated by Ficoll density gradient separation, washed twice with PBS and resuspended at 2.5×10^6/mL in Complete Endothelial Culture Medium (CECM) (comprising EndoCult Basal Medium supplemented with 1/5 dilution of EndoCult supplements, Stem Cell Technologies, UK). 2 mL of cell suspension was placed into each of 6-well Fibronectin-coated plates (Becton Dickinson) and incubated at 37°C, 5% CO_2 with 95% humidity (see **section 2.4**). After two days, mature endothelial cells and monocytes remain adhered to the bottom of the well and non-adherent cells, containing EPCs, were transferred to a fibronectin-coated 24-well plate (Becton Dickinson) at 1×10^6/well for three days at 37°C, 5% CO_2 with 95% humidity. Colonies (CFU-EPC, early outgrowth colony forming unit endothelial progenitor cell; Stem Cell Technologies) were defined as a central core of "round" cells with elongated "sprouting" cells at the periphery and were counted on day 5 in a minimum of four wells by observers unaware of the subjects' clinical profiles. In order to confirm endothelial-cell lineage, direct staining was performed on colonies using (Fluorochrome-) acetylated LDL and co-stained with (Fluorochrome-) Lectin (*Ulex europaeus I agglutinin*).

ELISA

Plasma was extracted from EDTA anticoagulated blood samples at baseline and 6 hours after the procedure in patients undergoing angiography alone and those having both angiography and angioplasty. The concentration of VEGF in each sample was then determined by ELISA according to the protocol in **section 2.6**.

4.3. Results

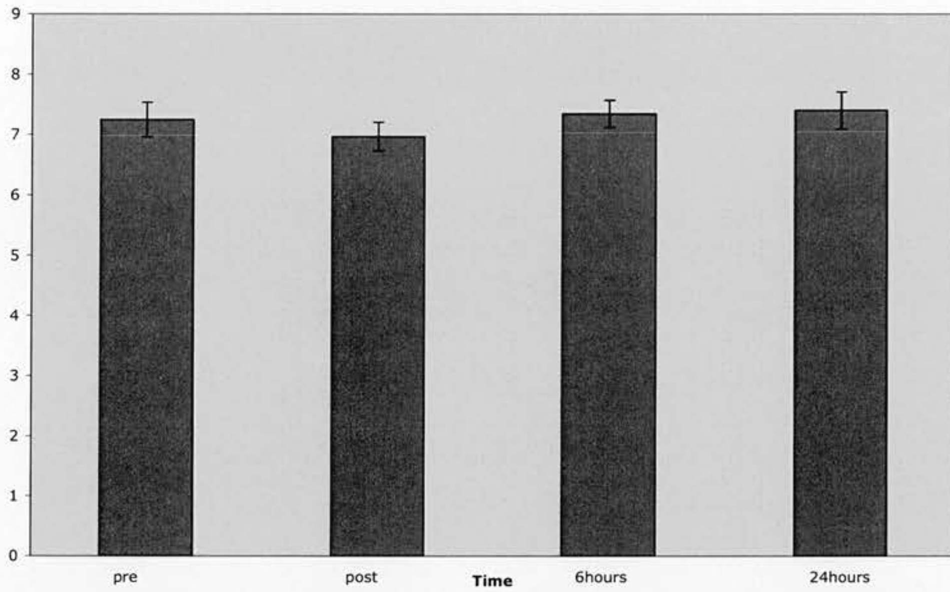
There were no complications arising from angiography or PCI and all patients (**Table 4.1**) were discharged home 24 hours after procedure.

Systemic inflammatory response

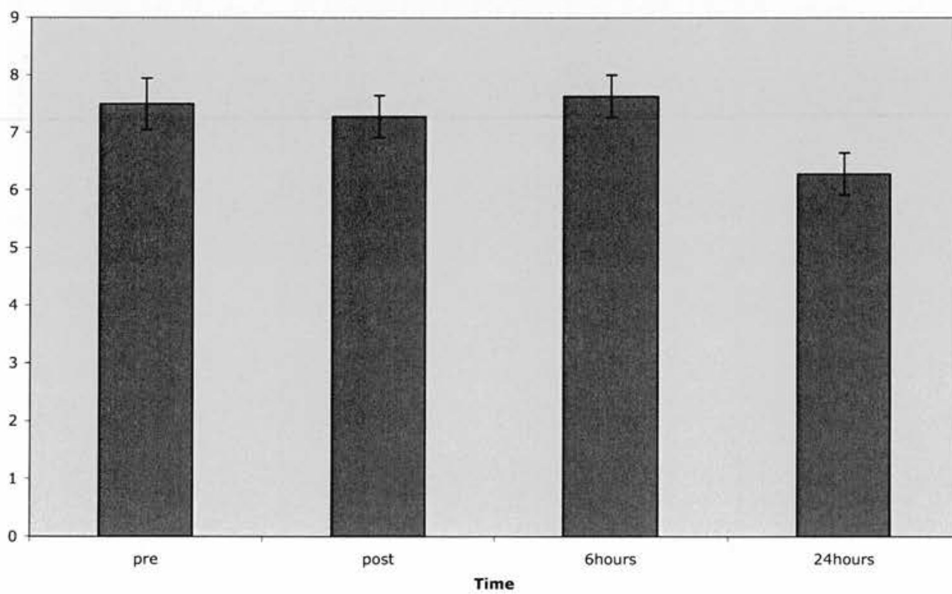
Results are shown in **table 4.2** and depicted graphically in **figures 4.1-4.5**. Diagnostic angiography alone did not increase the mean peripheral blood leucocyte count (**figure 4.1b**), neutrophil count (**figure 4.2b**), lymphocyte count (**figure 4.3b**) monocyte count (**figure 4.4b**) or the median serum C-reactive protein concentration (**figure 4.5b**). Coronary intervention increased the mean total leucocyte count ($7.0\pm0.36 \times 10^9$/l to $8.2\pm0.34 \times 10^9$/l, $p<0.005$, **figure 4.1c**), the mean neutrophil count ($4.36\pm0.31 \times 10^9$/l to $5.73\pm0.31 \times 10^9$/l, $p<0.005$, **figure 4.2c**), and median serum C-reactive protein concentration (1.1mg/l to 3.1mg/l, $p<0.005$, **figure 4.3c**) at 24-hours. There was an apparent transient reduction in the mean monocyte count immediately following coronary intervention ($0.57\pm0.03 \times 10^9$/l to $0.48\pm0.04 \times 10^9$/l, $p<0.01$, **figure 4.4c**), with a return to pre-procedure levels by 24 hours. Finally, there was no evidence of significant myocyte necrosis 24 hours following diagnostic angiography or PCI (**table 4.2**).

	Pre-procedure	Post-procedure	6 hours	24 hours
Angiography				
WBC (x10^9/L)	7.94±0.45	7.28±0.37	7.63±0.37	6.28±0.37
Neutrophils (x10^9/L)	4.54±0.34	4.72±0.32	4.59±0.26	3.72±0.25
Lymphocytes (x10^9/L)	2.07±0.14	1.84±0.13	2.19±0.17	1.75±0.16
Monocytes (x10^9/L)	0.61±0.04	0.49±0.03	0.63±0.06	0.59 ± 0.09
C-reactive protein (mg/l)	3.62	-	-	4.66
Troponin I (g/L)	0.20 ± 0.01	-	-	0.20 ± 0.01
PCI				
WBC (x10^9/L)	7.0 ± 0.36	6.66 ± 0.3	7.07 ± 0.2	8.2 ± 0.34*
Neutrophils (x10^9/L)	4.36 ± 0.31	3.97 ± 0.29	4.39±0.23	5.73 ± 0.31*
Lymphocytes (x10^9/L)	1.81 ± 0.1	1.73 ± 0.12	1.89 ± 0.14	1.71 ± 0.11
Monocytes (x10^9/L)	0.57 ± 0.03	0.48 ± 0.04**	0.57 ± 0.03	0.56 ± 0.03
C-reactive protein (mg/l)	1.14	-	-	3.14*
Troponin I (g/L)	0.20 ± 0.01	-	-	0.40 ± 0.11
Values are presented as mean ± SEM, except CRP where values are presented as median; pre-procedure versus time point, *P<0.005, **P<0.01.				

Table 4.2. Markers of inflammation and myocyte necrosis following diagnostic angiography or percutaneous coronary intervention.



a.



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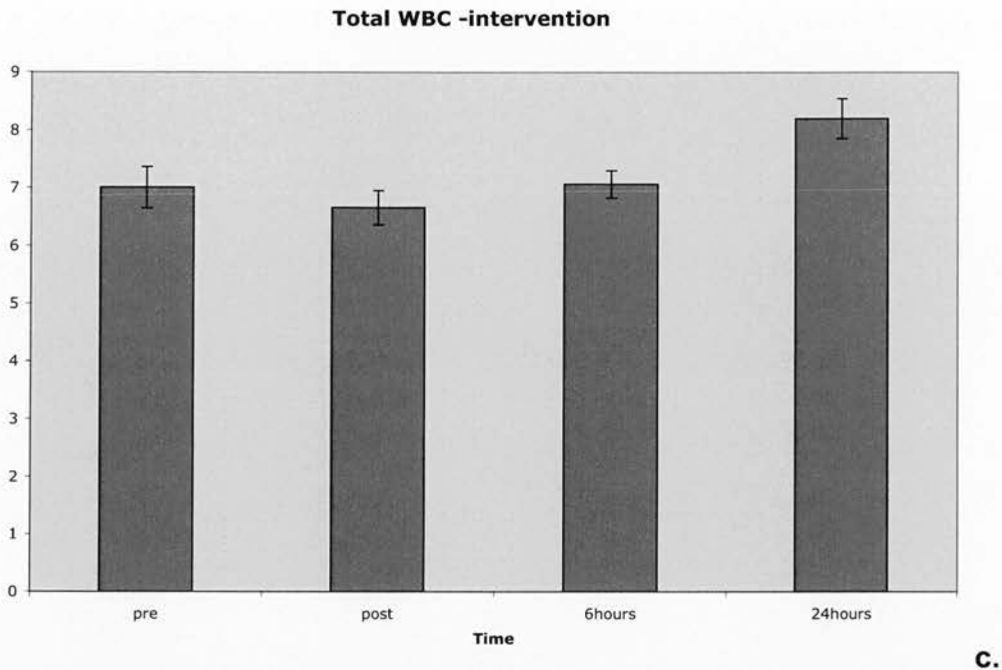
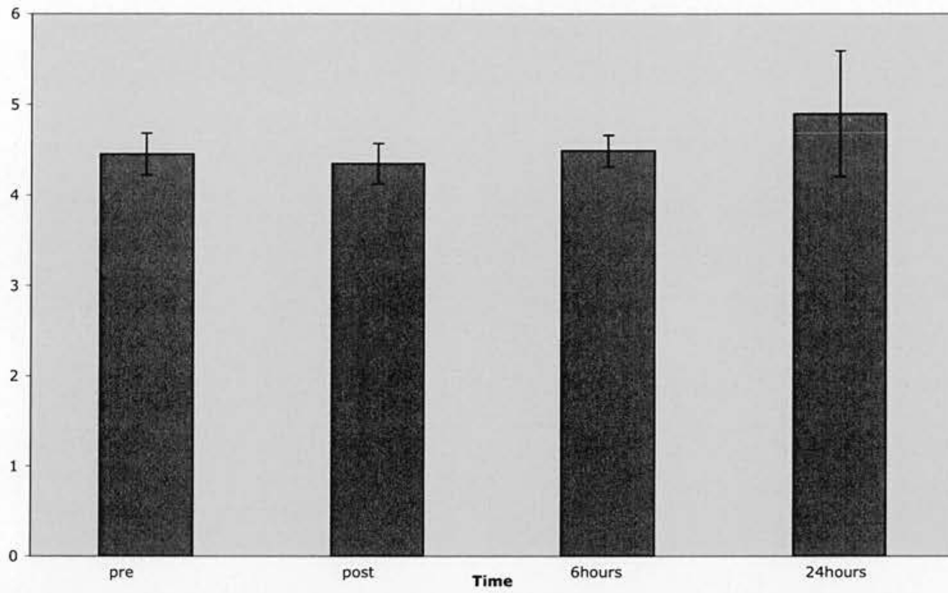
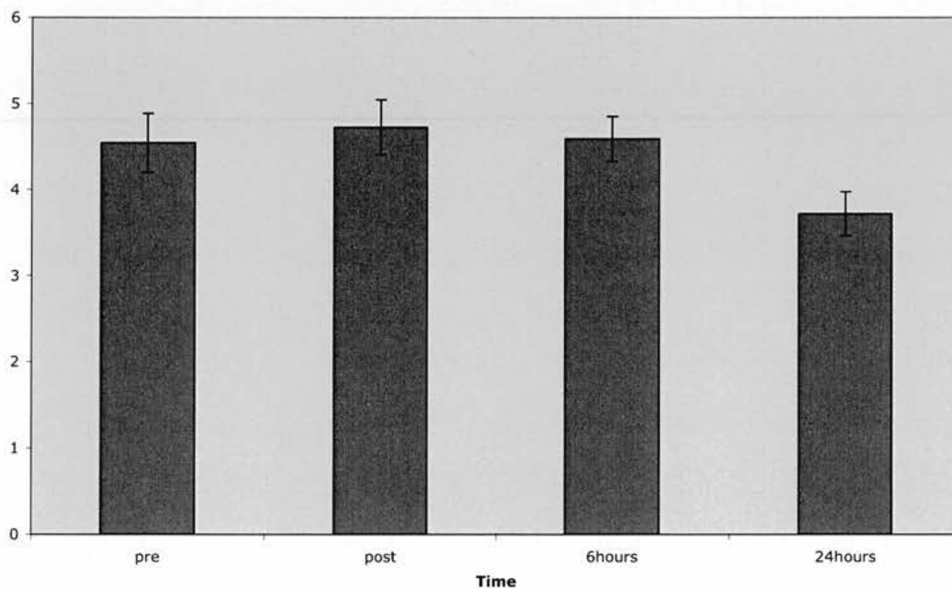


Figure 4.1. Change in the mean total white cell count (WBC±sem) in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant increase in the total WBC in all patients by 24 hours after the procedure (7.25 ± 0.28 to $7.41 \pm 0.3 \times 10^9/l$, $p<0.05$ by ANOVA) **(a)**. This increase was confined to those patients undergoing angiography plus angioplasty (7.00 ± 0.36 to $8.20 \pm 0.34 \times 10^9/l$, $p<0.001$ by ANOVA) **(c)**.



a.



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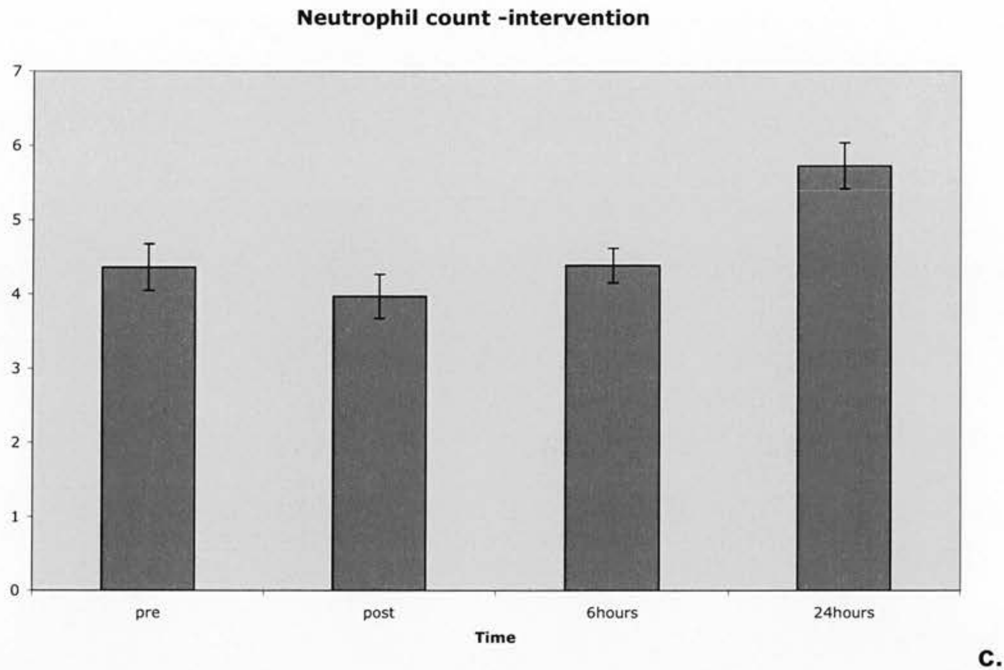
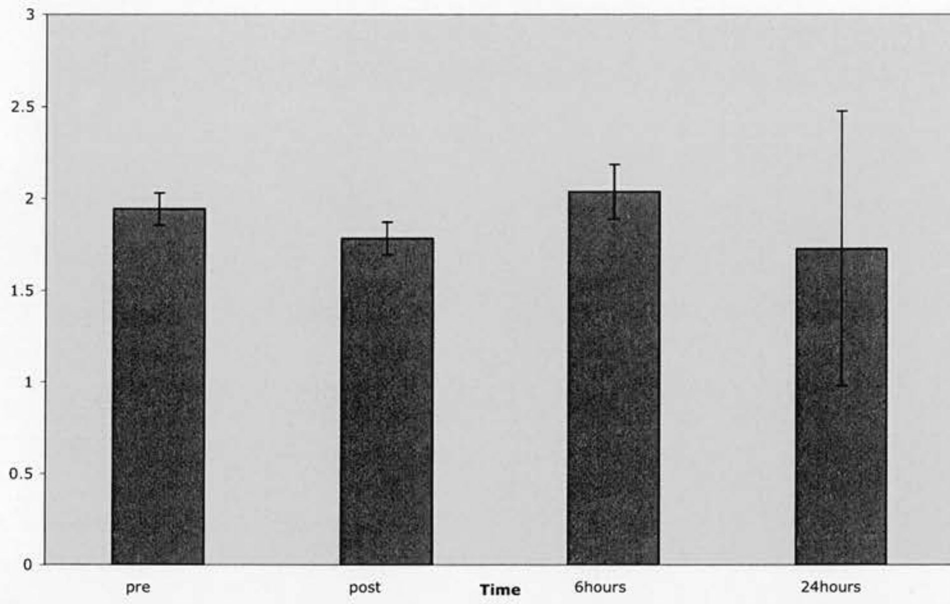
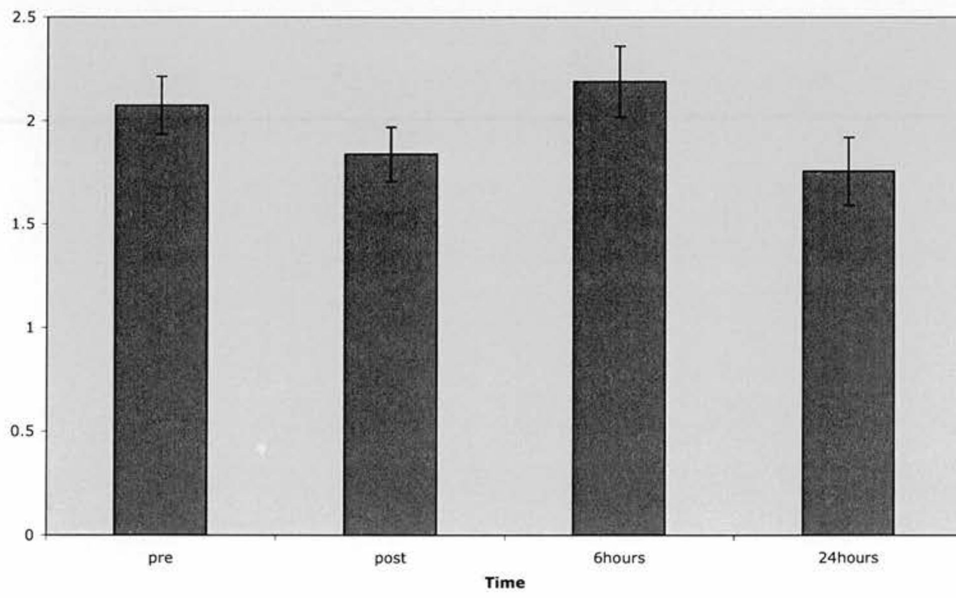


Figure 4.2. Change in the mean neutrophil count in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant increase in the neutrophil count in all patients by 24 hours after the procedure (4.45±0.23 to $4.90 \pm 0.69 \times 10^9$/l, $p<0.05$ by ANOVA) **(a)**. This increase was confined to those patients undergoing angiography plus angioplasty (4.36±0.39 to $5.73 \pm 0.31 \times 10^9$/l, $p<0.001$ by ANOVA) **(c)**.



a.



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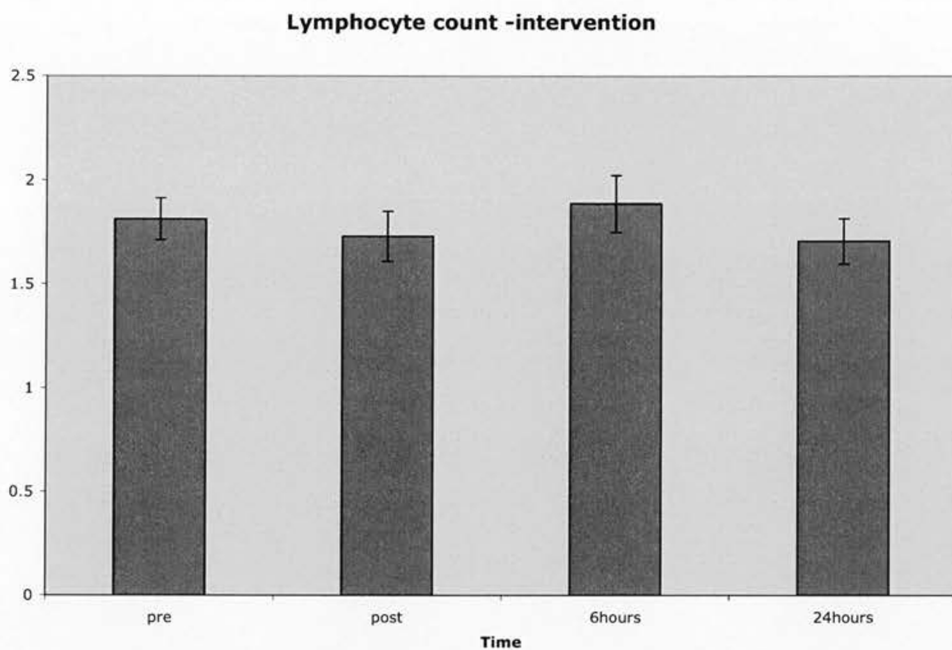
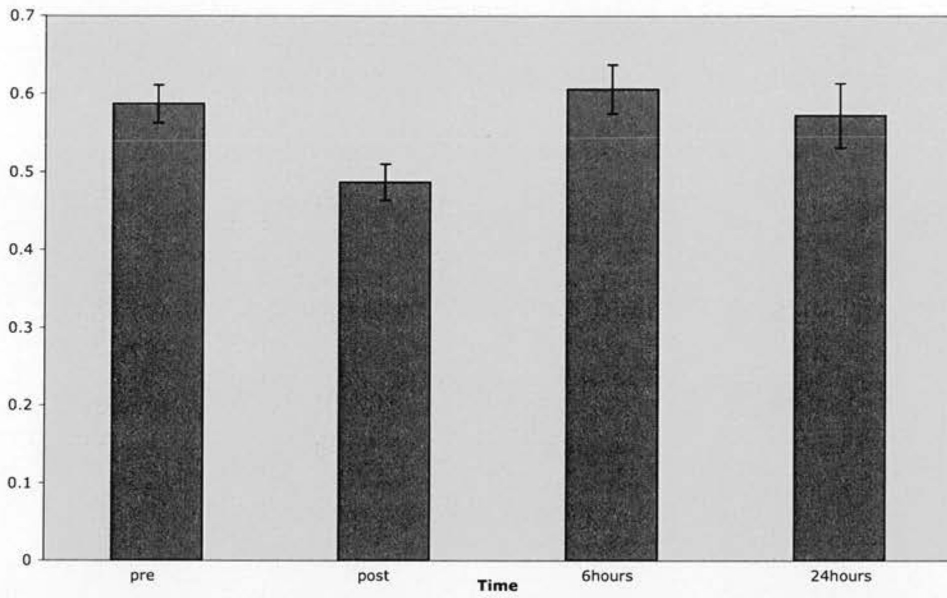
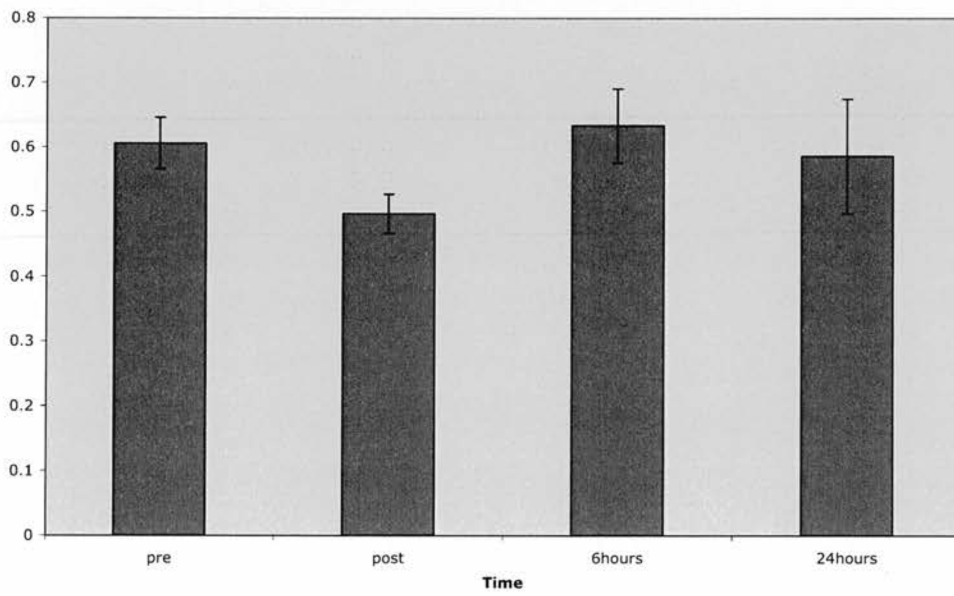


Figure 4.3. Change in the mean lymphocyte count in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant decrease in the lymphocyte count in all patients immediately after the procedure followed by a recovery in the count by 6 hours and then a decrease again by 24 hours after the procedure (1.94 ± 0.08 to 1.78 ± 0.08 to2.03 ± 0.14 to $1.72\pm0.74x10^9/l$, $p<0.05$ by ANOVA) **(a)**. There was no significant change in the mean lymphocyte count when control and intervention groups were analysed **(a & b)**.



a.



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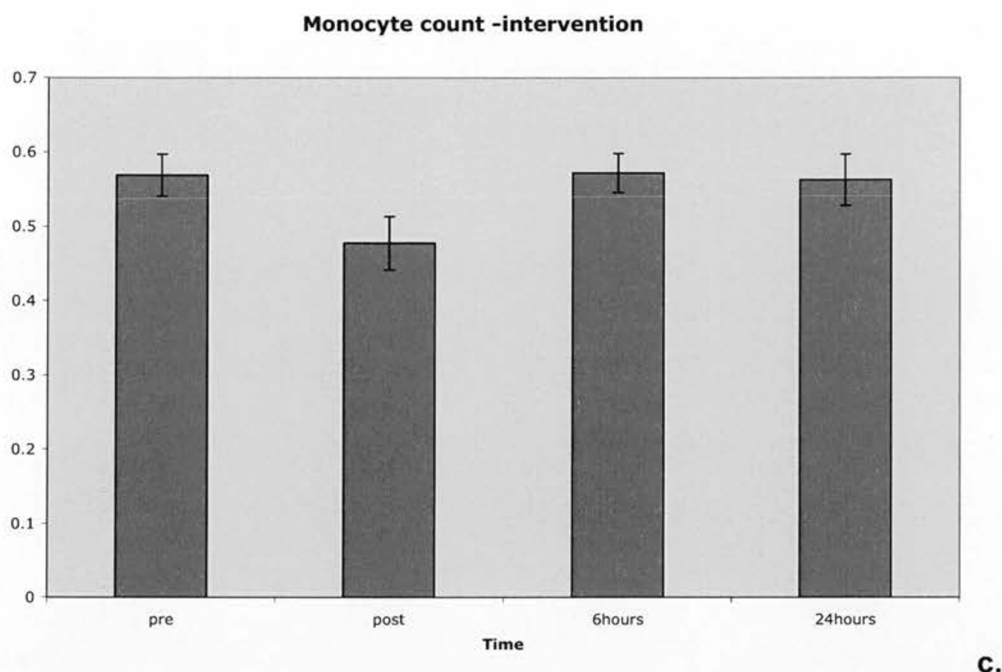
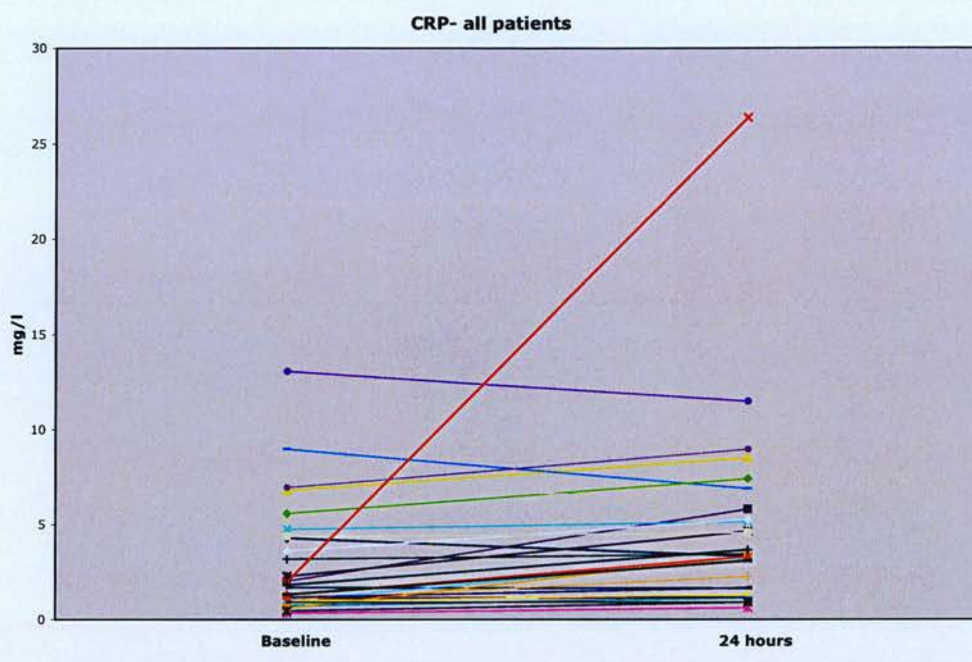
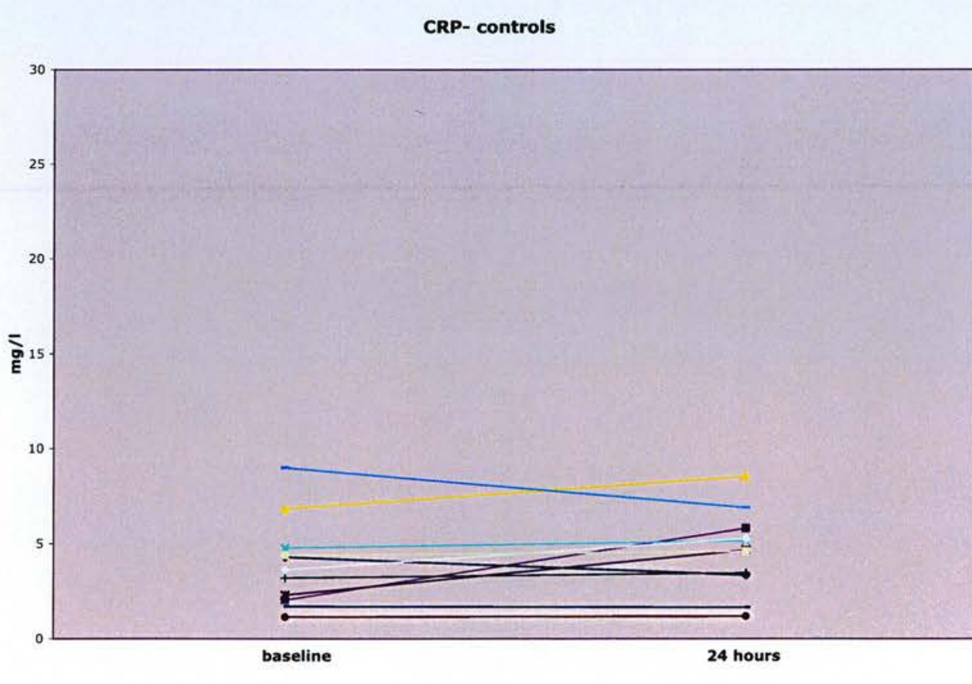


Figure 4.4. Change in the mean monocyte count in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant decrease in the monocyte count in all patients immediately after the procedure followed by a recovery in the count by 6 hours (0.59 ± 0.02 to 0.49 ± 0.02 to $0.61 \pm 0.03 x 10^9/l$, $p<0.005$ by ANOVA) **(a)**. This change was confined to those patients undergoing angiography plus angioplasty (0.57 ± 0.03 to 0.48 ± 0.04 to $0.57 \pm 0.03 x 10^9/l$, $p<0.005$ by ANOVA) **(c)**.



a.



b.

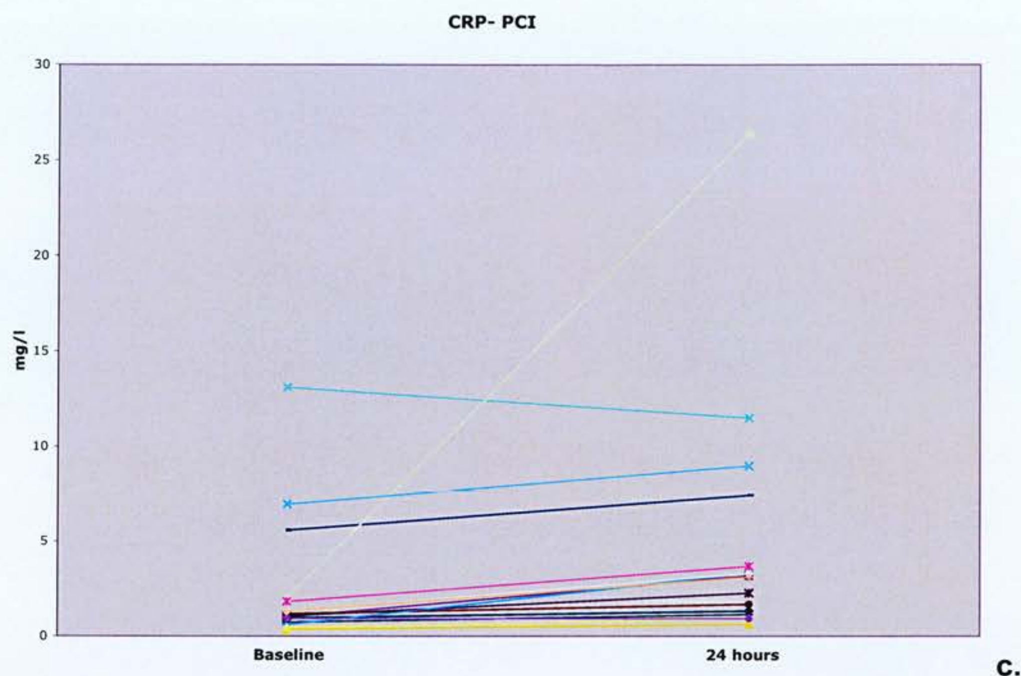


Figure 4.5. Change in the median CRP level in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant increase in the CRP level in all patients immediately by 24 hours after the procedure (1.83 to 3.35mg/l, $p<0.001$) **(a)**. This increase was confined to those patients undergoing angiography plus angioplasty (1.14 to 3.14mg/l, $p<0.005$) **(c)**.

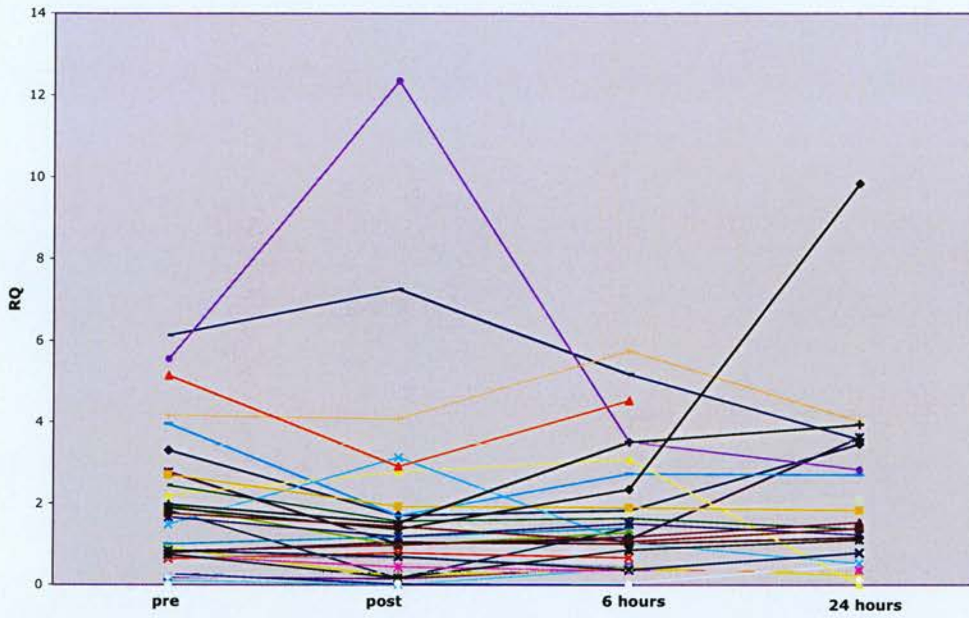
PCR

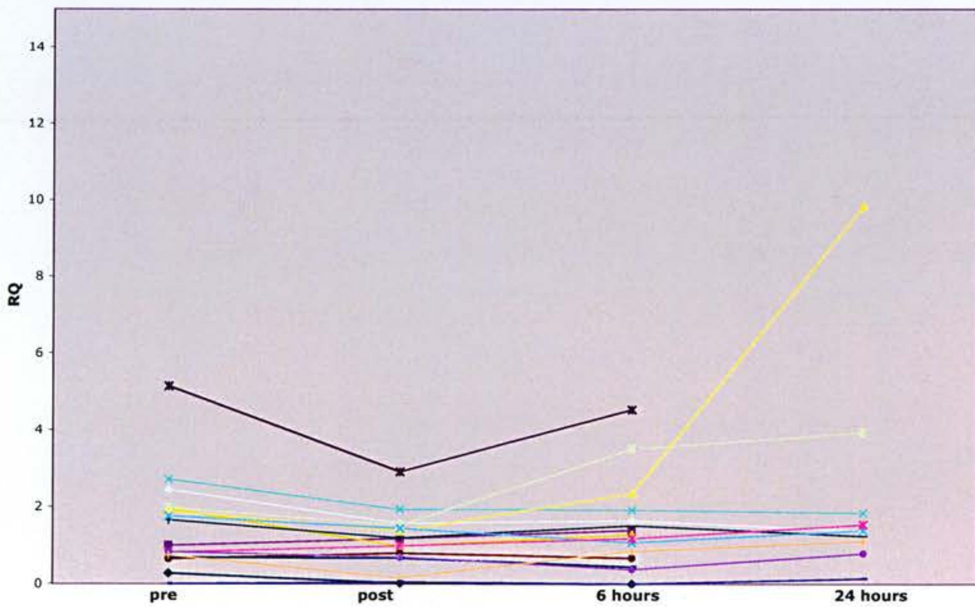
The results of the effect of angiography ± PCI on messenger RNA levels are presented in **table 4.3**, and depicted graphically in **figures 4.6-4.11**. There was a significant reduction in the median RQ of CD34 mRNA immediately following procedure in those patients undergoing diagnostic angiography only, with a recovery to baseline levels by 6 hours after the procedure (1.71 to 1.17, $p<0.005$, **figure 4.6b**). In addition, there was a significant reduction in the median RQ of VEGFR-2 mRNA by 6 hours following the procedure in the group undergoing angiography only (9.93 to 8.82, $p<0.005$, **figure 4.8b**) (b). Furthermore, there was a significant reduction in the median RQ of VE-cadherin mRNA immediately following the procedure, followed by a return to baseline levels by 6 hours in those patients undergoing angiography plus angioplasty (1.18 to 0.77, $p<0.001$, **figure 4.9c**). Finally, there was a significant reduction in the median RQ of CD14 mRNA immediately following the procedure in the group undergoing angiography only (2.94 to 2.05, $p<0.01$, **figure 4.11b**). There was also a significant reduction in the median RQ of CD14 mRNA immediately following the procedure, with a return to baseline then an increase by 24 hours in the group undergoing angiography plus angioplasty (2.82 to 1.90 immediately post to 3.13 by 24 hours, $p<0.05$ by ANOVA, **figure 4.11c**).

	Pre-procedure		Post-procedure		6 hours		24 hours	
	Median Value	Inter-quartile	Median Value	Inter-quartile	Median Value	Inter-quartile	Median Value	Inter-quartile
Angiography								
CD34	1.71	0.80-1.97	1.17*	0.69-1.52	1.34	0.80-2.02	1.38	1.17-2.88
CD133	2.35	1.37-3.40	1.08	0.91-2.00	2.06	1.41-2.94	1.44	0.75-2.08
VEGFR-2	9.93	6.58-26.71	10.24	1.59-28.76	8.82*	6.67-14.79	13.71	8.40-25.16
VE-cadherin	1.61	0.89-2.62	1.02	0.68-1.39	0.97	0.74-1.30	1.13	0.63-2.61
VWF	2.15	1.64-3.21	1.57	1.16-2.13	1.46	1.01-2.17	2.17	1.20-3.25
CD14	2.94	2.40-4.33	2.05**	1.28-3.08	1.98	1.81-2.73	2.84	2.07-3.64
PCI								
CD34	1.78	0.77-3.47	1.14	0.19-2.87	1.26	0.44-2.82	2.40	0.52-3.49
CD133	2.26	1.54-3.91	2.02	1.09-5.11	1.85	0.90-3.51	2.94	1.69-6.17
VEGFR-2	7.59	4.35-13.00	17.85	1.91-28.48	2.76	0.38-22.14	7.97	1.74-28.89
VE-cadherin	1.18	0.90-2.26	0.77***	0.56-1.09	0.89	0.61-1.67	0.98	0.69-1.77
VWF	2.06	1.30-2.47	1.49	1.04-3.43	1.76	1.12-2.10	2.34	1.24-2.93
CD14	2.82	1.57-4.45	1.90	1.22-3.20	1.99	1.57-2.62	3.13	2.50-6.28

Values relative quantities (median ±interquartile ranges); pre-procedure versus time point, $*P<0.005$, $**P<0.01$, $***P<0.001$

Table 4.3. Leucocyte mRNA levels following diagnostic angiography or percutaneous coronary intervention


CD34 mRNA- all patients
RQ
14
12
10
8
6
4
2
0
pre
post
6 hours
24 hours
a.


CD34 mRNA- control patients
RQ
14
12
10
8
6
4
2
0
pre
post
6 hours
24 hours
b.

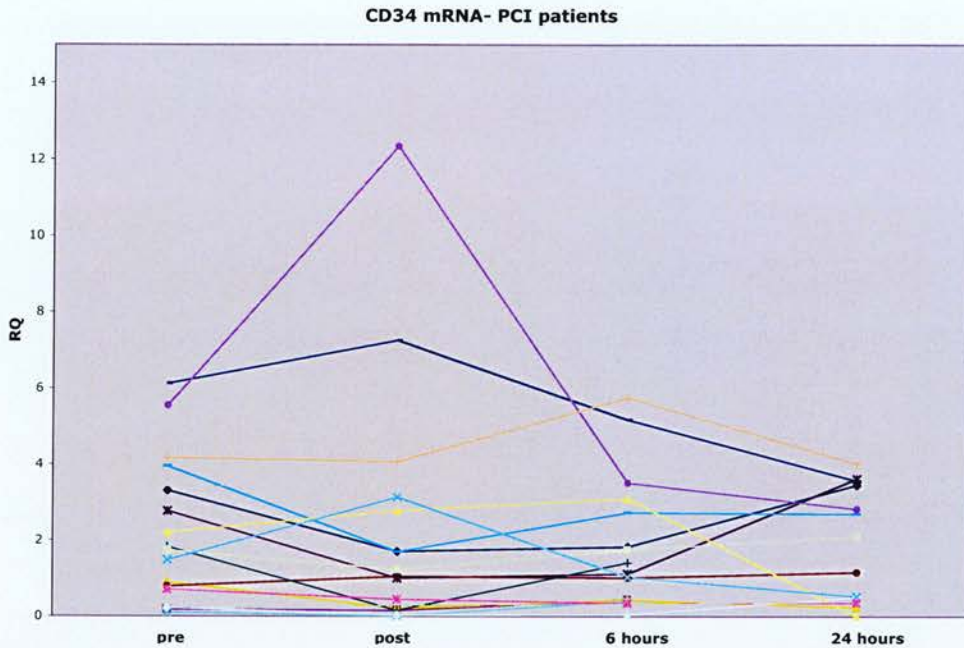
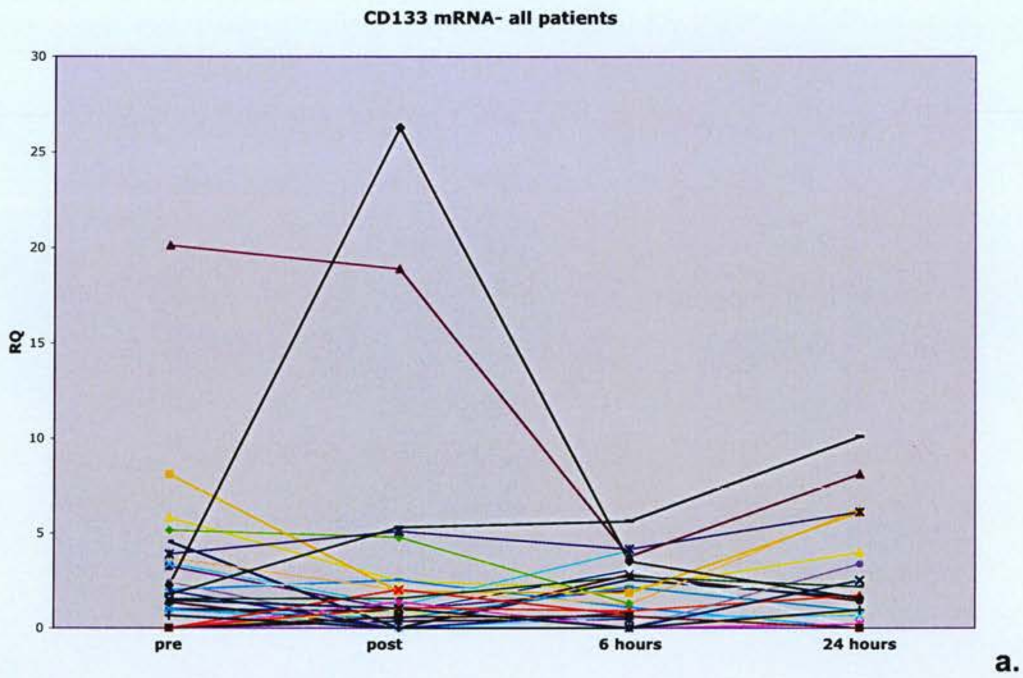
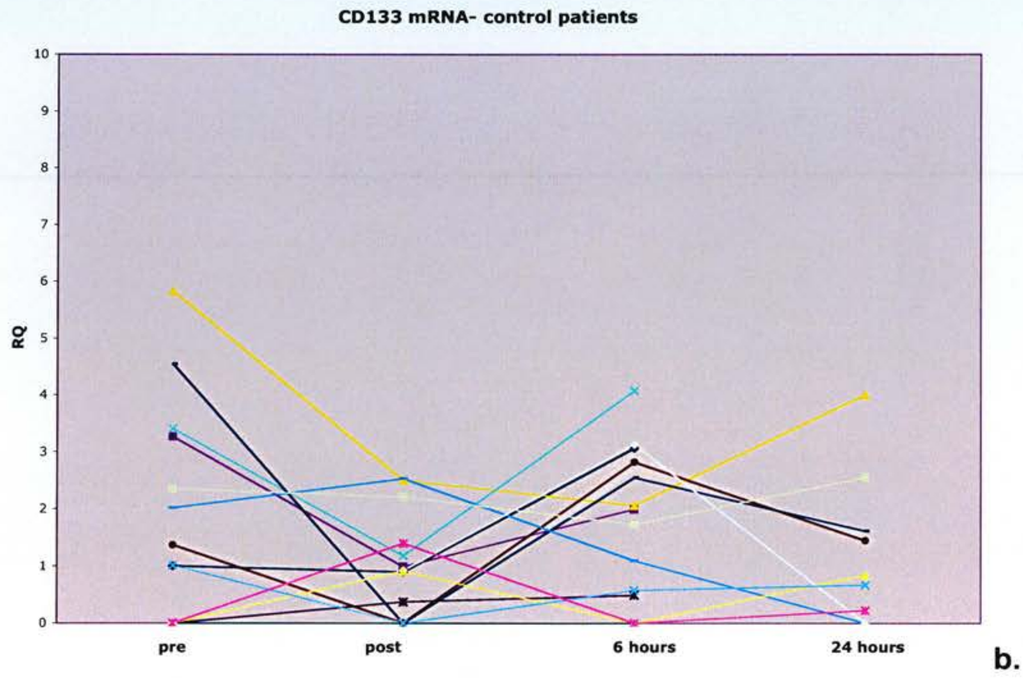


Figure 4.6. Changes in the relative quantity (RQ) of CD34 mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was a decrease in the median RQ of CD34 mRNA immediately following the procedure when all patients were analysed **(a)**. This decrease was confined to patients undergoing angiography only ($P<0.005$) **(b)**.



a.



b.

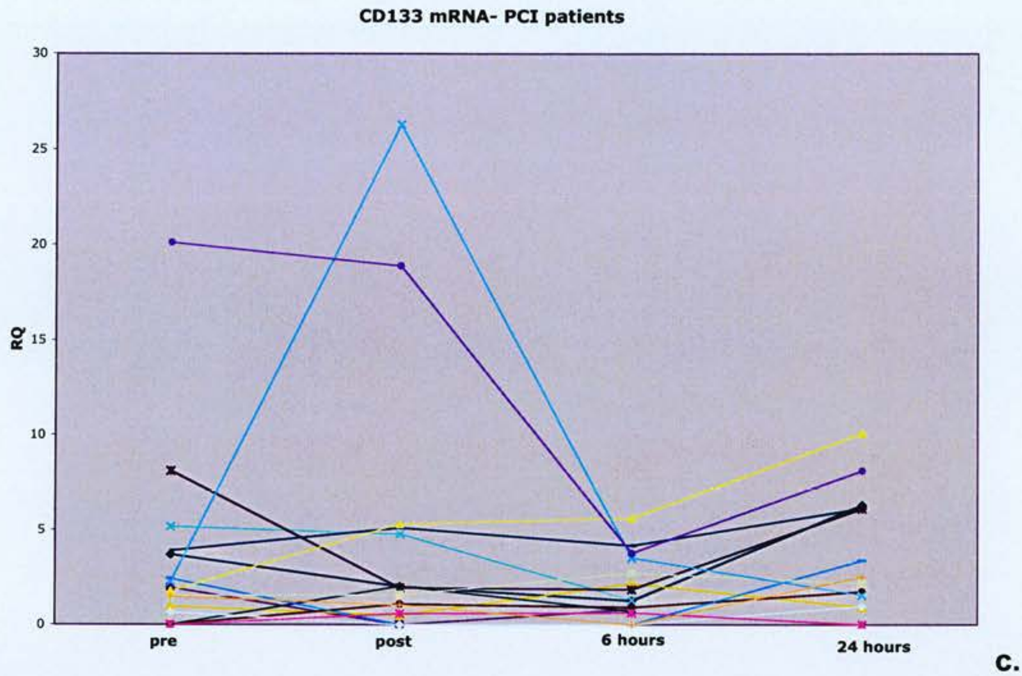
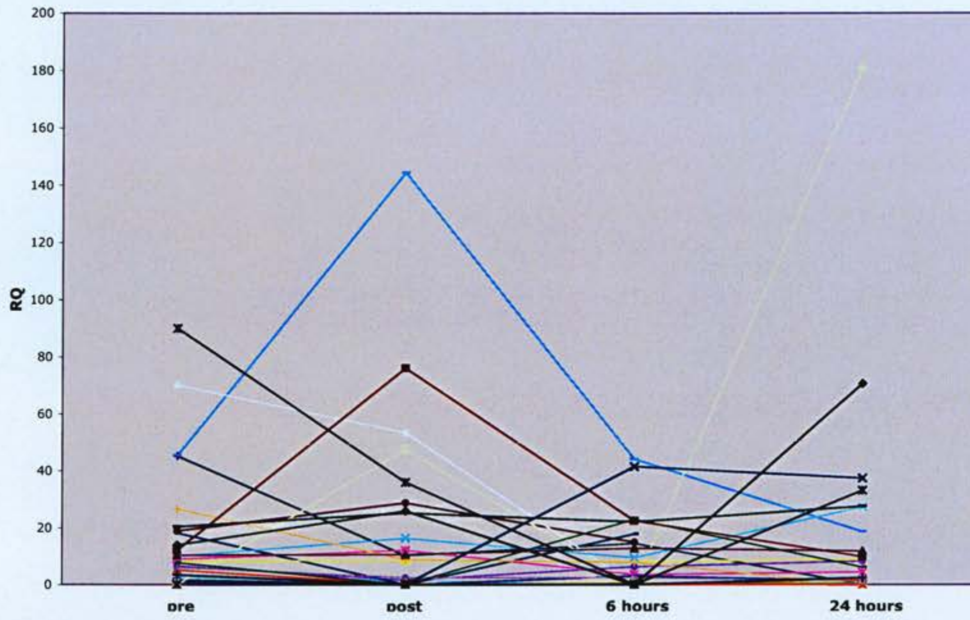
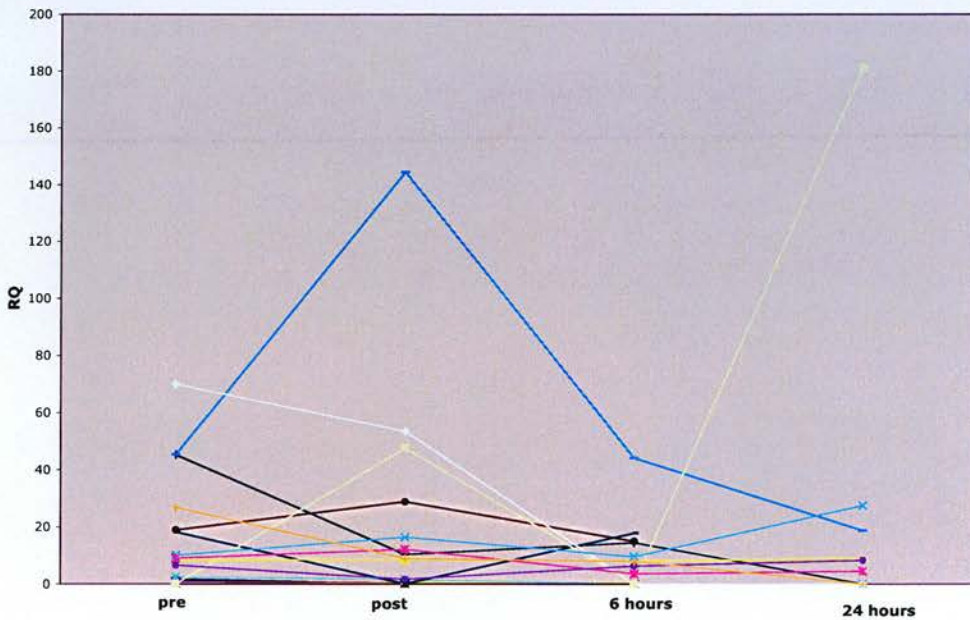


Figure 4.7. Changes in the relative quantity (RQ) of CD133 mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was no significant change in the median RQ of CD133 mRNA following angiography and angioplasty.



a.



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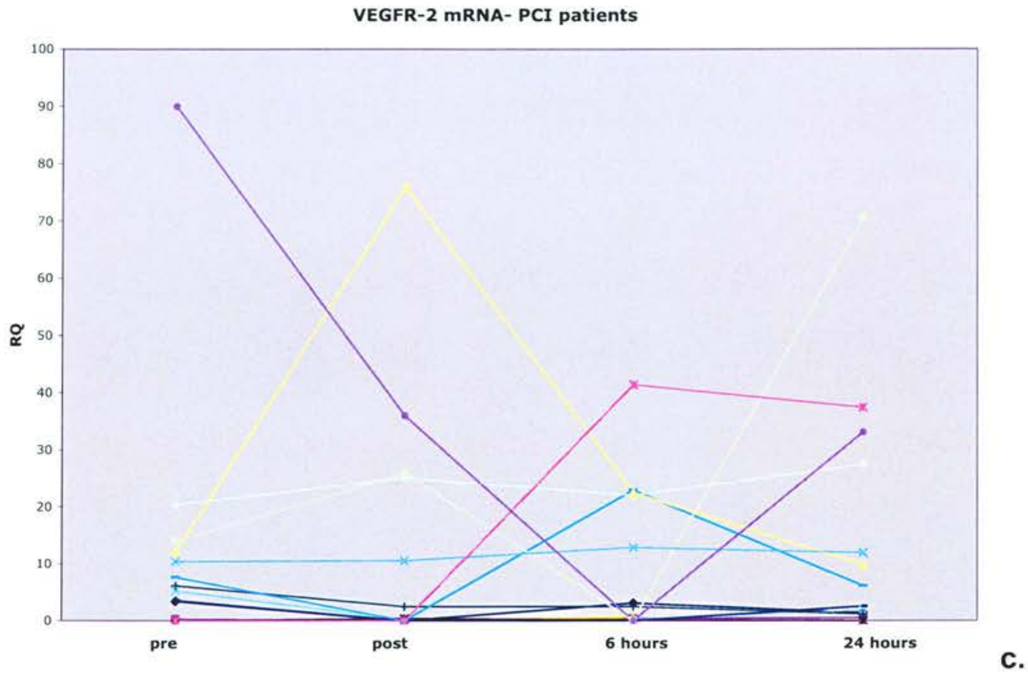
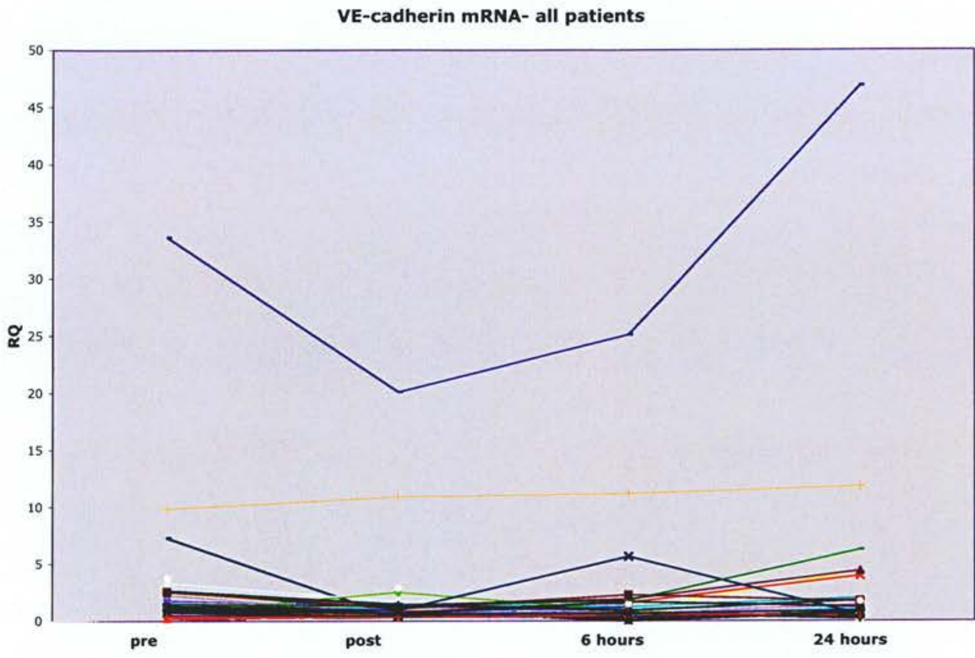
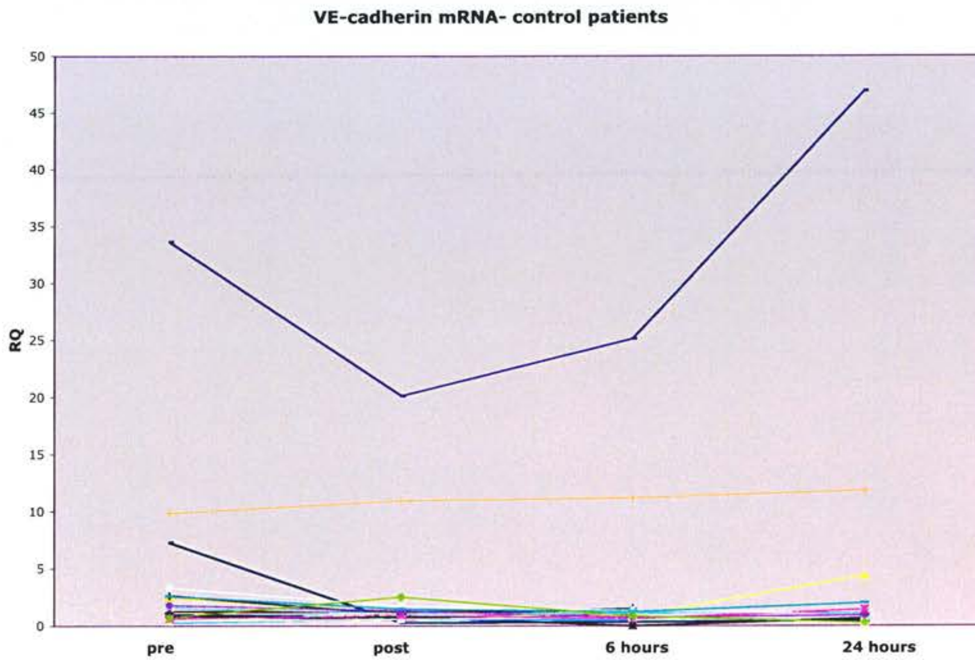


Figure 4.8. Changes in the relative quantity (RQ) of VEGFR-2 mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was a significant reduction in the RQ of VEGFR-2 mRNA immediately following the procedure in the group undergoing angiography only ($p<0.005$) **(b)**.


VE-cadherin mRNA- all patients
RQ
50
45
40
35
30
25
20
15
10
5
0
pre
post
6 hours
24 hours
a.


VE-cadherin mRNA- control patients
RQ
50
45
40
35
30
25
20
15
10
5
0
pre
post
6 hours
24 hours
b.

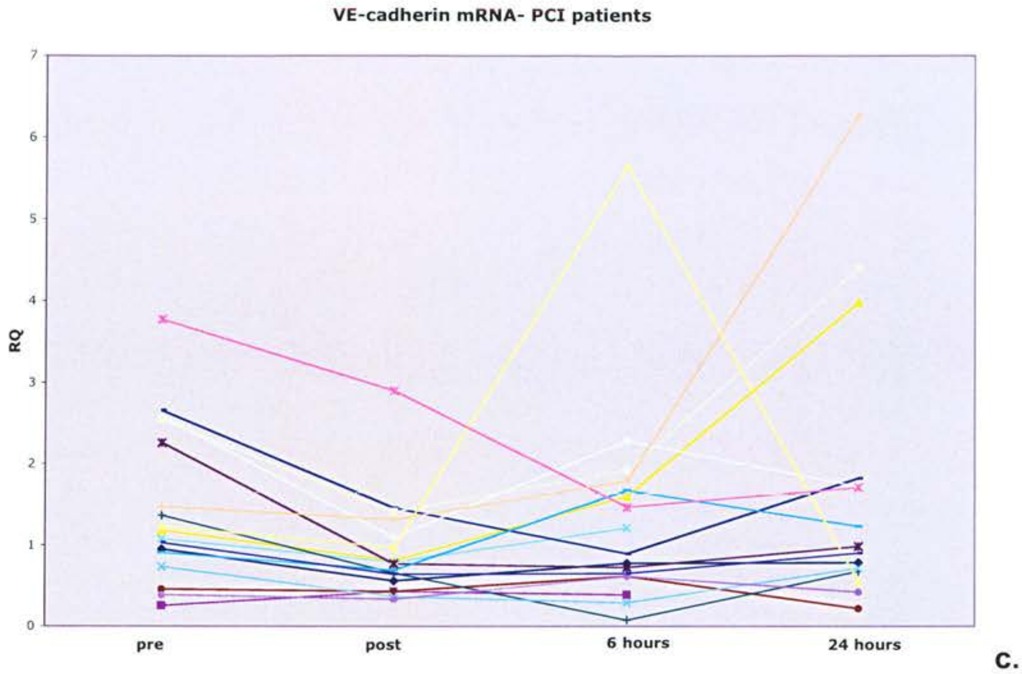
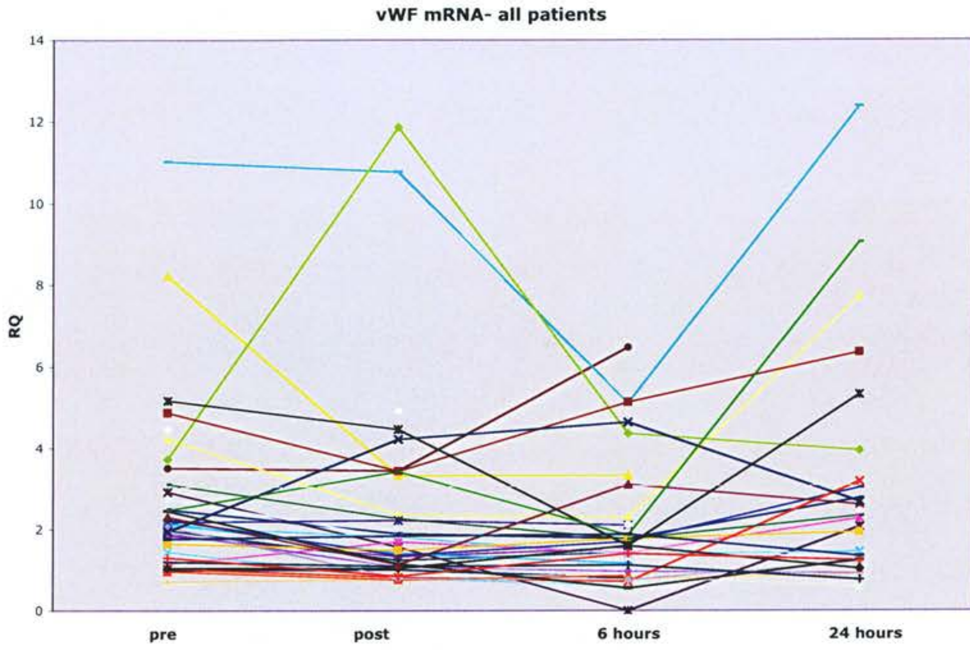
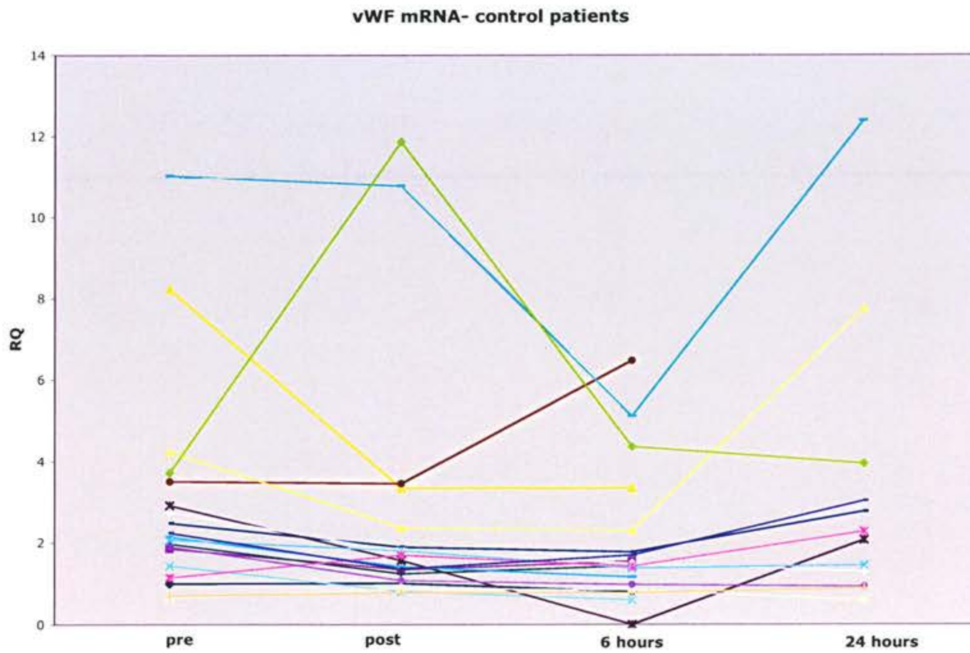


Figure 4.9. Changes in the relative quantity (RQ) of VE-cadherin mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was a significant reduction in the RQ of VE-cadherin mRNA immediately following the procedure with a return to baseline levels by 24 hours when all patients were analysed ($p<0.005$, by ANOVA) **(a)**. This change was confined to the group undergoing angiography plus angioplasty ($p<0.005$) **(c)**.



a.



b.

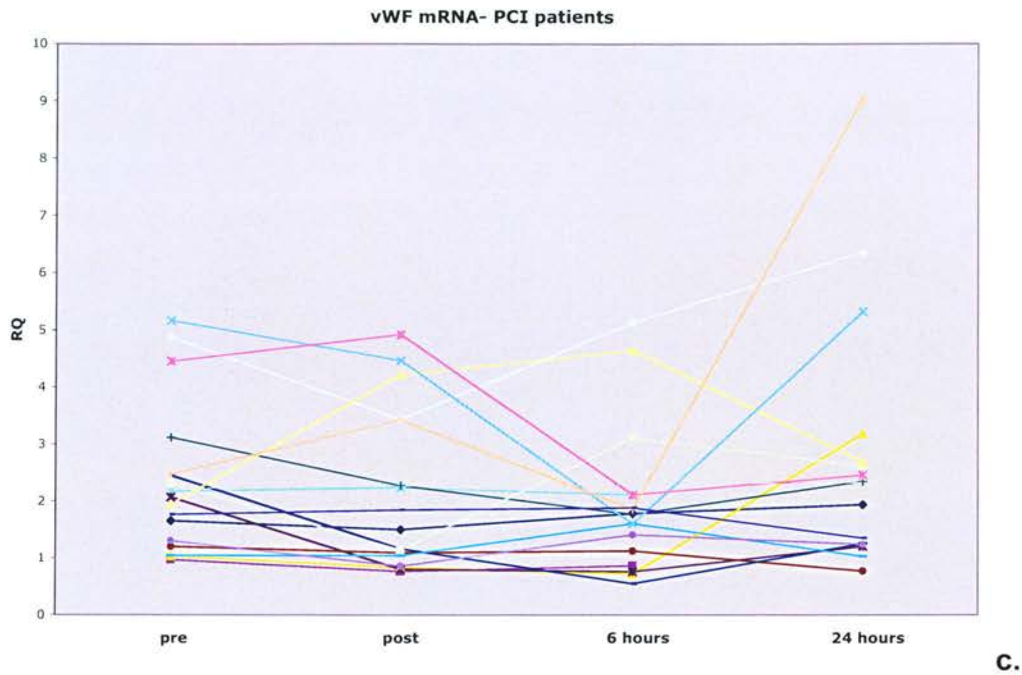
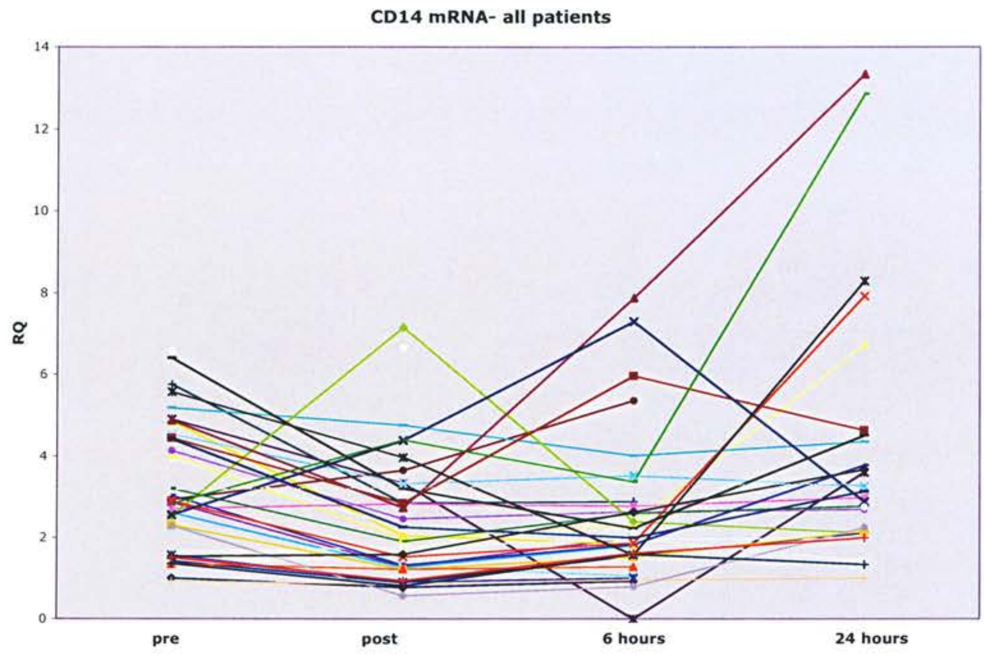
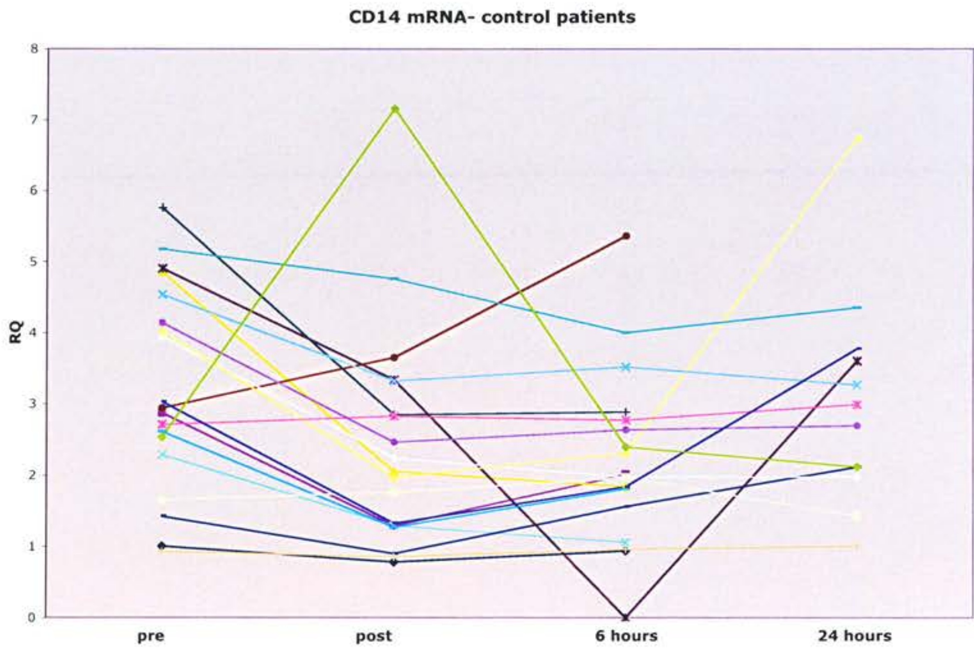


Figure 4.10. Changes in the relative quantity (RQ) of vWF mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was a significant reduction in the RQ of vWF mRNA immediately following the procedure and by 6 hours when all patients were analysed ($p<0.01$) **(a)**. However, no significant changes were found when the data was analysed according to intervention.



a.



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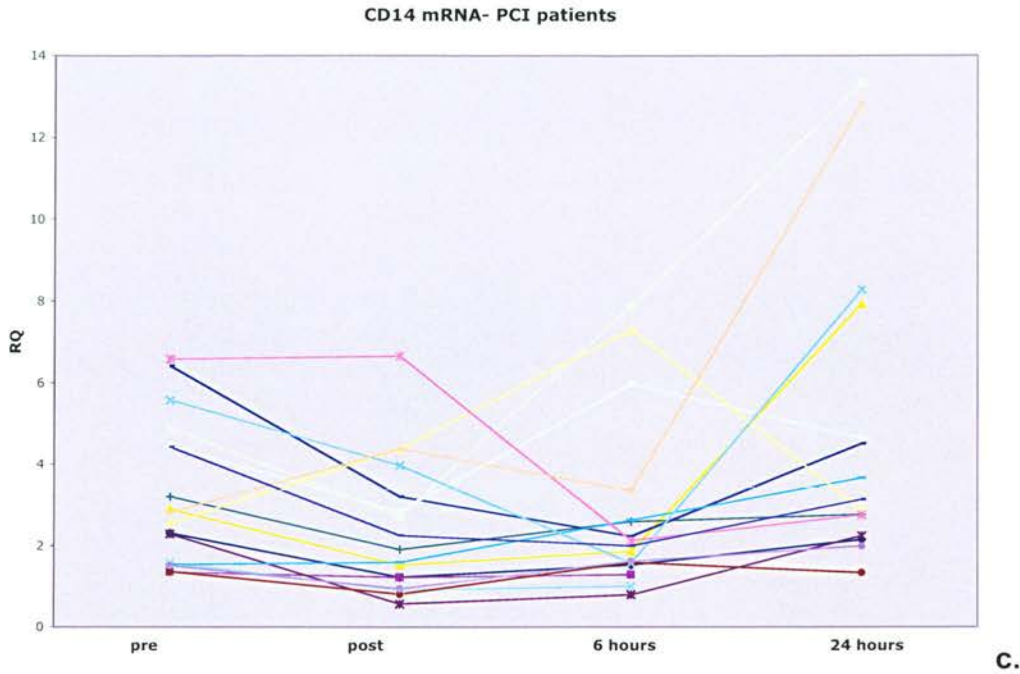


Figure 4.11. Changes in the relative quantity (RQ) of CD14 mRNA in **(a)** all patients undergoing angiography ± angioplasty, in **(b)** patients undergoing angiography only and in **(c)** patients undergoing both angiography and angioplasty. There was a significant reduction in the median RQ of CD14 mRNA immediately following the procedure followed by an increase and return to baseline by 24 hours when all patients were analysed ($p<0.01$ by ANOVA) **(a)**. There was a significant reduction in the median RQ of CD14 mRNA immediately following the procedure in the group undergoing angiography only ($p<0.01$) **(b)**. There was a significant reduction in the median RQ of CD14 mRNA immediately following the procedure, with a return to baseline then an increase by 24 hours in the group undergoing angiography and angioplasty ($p<0.05$, by ANOVA) **(c)**.

Flow cytometry

Results are shown in **table 4.4**. No increase was observed in the number of CD34+VEGFR-2+ cells following the procedure in patients undergoing angiography alone or with angioplasty. In addition no correlation was found between EPCs identified by phenotype and the number of functional EPCs quantified using cell culture (CFU-EPCs) either before (r=-0.15, p=0.58) or 24 hours (r=0.02, p=0.94) after angiography.

	Pre-procedure	Post-procedure	6 hours	24 hours
Angiography				
CD34+ cells (% leucocytes)	0.100±0.019	0.082±0.015	0.087±0.016	0.102±0.028
CD34+VEGFR-2+ cells (% leucocytes)	0.030±0.010	0.020±0.005	0.021±0.004	0.026±0.007
CD34+ cells (x10^3/ml blood)	6.54±0.92	5.43±0.80	5.98±0.81	5.67±1.15
CD34+VEGFR-2+ cells (x10^3/ml)	2.11±0.69	1.45±0.41	1.47±0.26	1.52±0.38
PCI				
CD34+ cells (% leucocytes)	0.083±0.011	0.105±0.011	0.091±0.012	0.083±0.010
CD34+VEGFR-2+ cells (% leucocytes)	0.019±0.003	0.029±0.006	0.020±0.004	0.021±0.004
CD34+ cells (x10^3/ml blood)	5.40±0.61	7.00±0.82	6.33±0.84	6.55±0.76
CD34+VEGFR-2+ cells (x10^3/ml)	1.15±0.19	1.91±0.47	1.28±0.29	1.65±0.36

Table 4.4. Circulating CD34+ and CD34+VEGFR-2+ cells following angiography or angiography plus percutaneous coronary intervention (PCI). Values expressed as mean ± SEM.

Cell Culture

Results are shown in **figures 4.20-4.22**. Mononuclear cells plated on fibronectin formed typical colony forming units (CFU-EPCs), characterized previously as a central cluster of rounded cells surrounded by radiating thin, flat cells [12]. Although unaffected by diagnostic angiography, the number and cellularity of CFU-EPCs were increased 24 hours after PCI (from 0.02 to 0.08 $x10^4$ cells plated, $p<0.01$, **figures 4.20&4.22**). Direct staining confirmed that CFU-EPCs, like mature endothelial cells, bind lectin and integrate acetylated-LDL. The majority of both small round cells and spindle shaped cells expressed CD14 (**figure 4.21**).

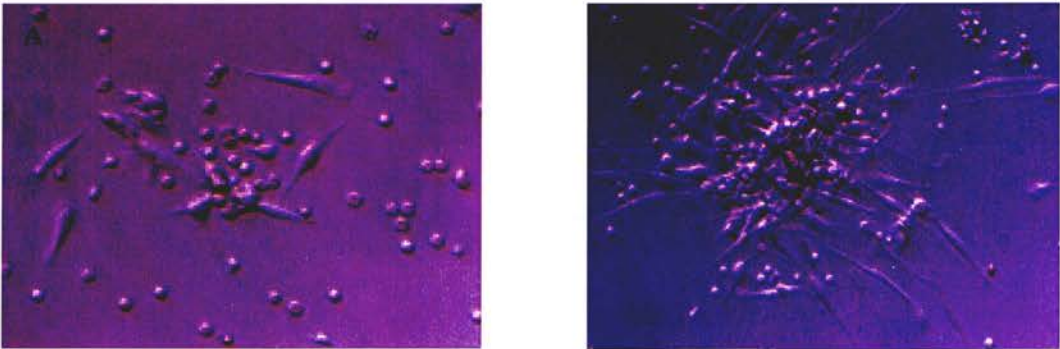


Figure 4.20. Phase contrast pictures illustrate a typical CFU-EPC before **(a)** and 24 hours after coronary angioplasty and stenting **(b)**.

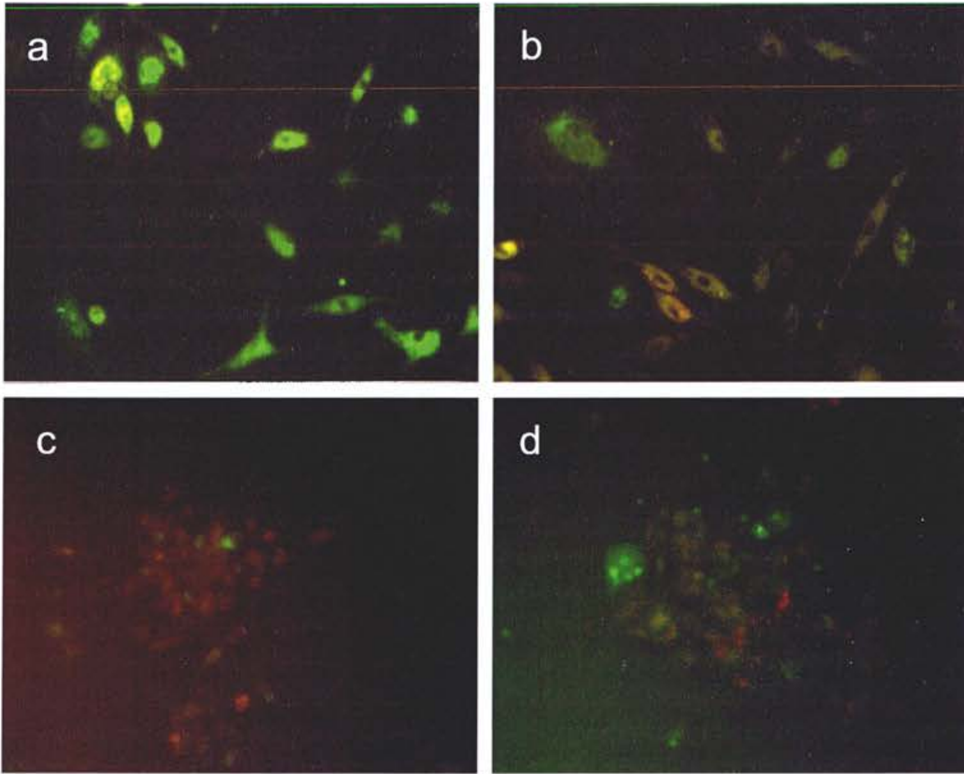
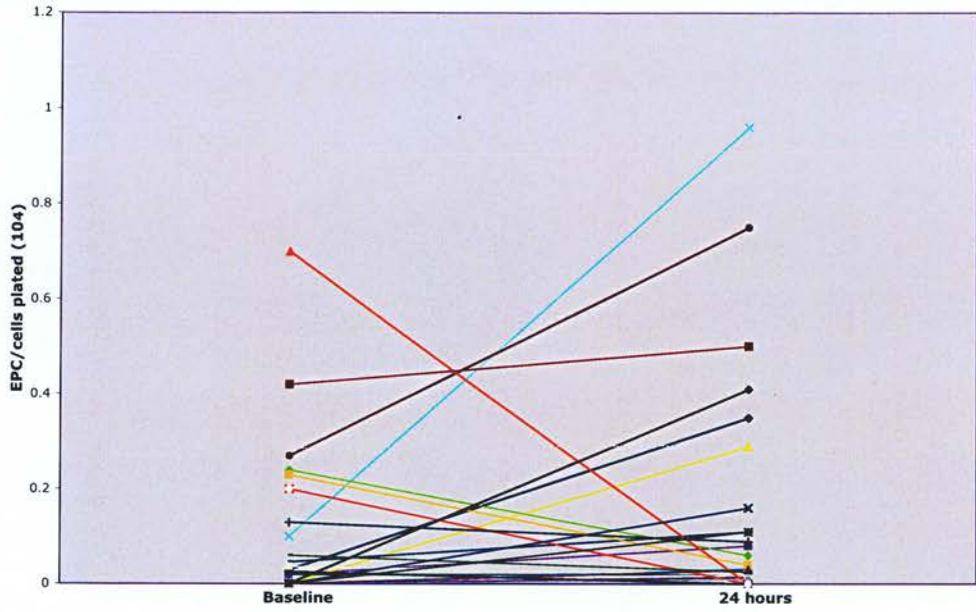
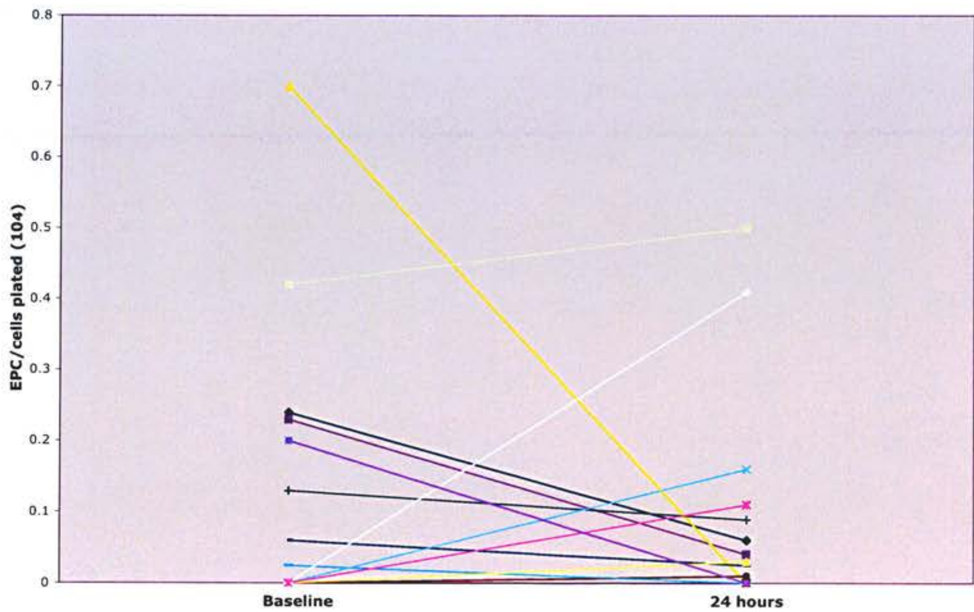


Figure 4.21. Micrographs of CFU-EPC with direct staining; solitary spindle-shaped cells from CFU-EPC assay stained for ac-LDL (green) **(a)** solitary spindle-shaped cells **(b)** and CFU-EPC colonies **(c)** stained for ac-LDL (green) and CD14 (orange); CFU-EPC colony stained for lectin (green) and VE-cadherin (orange) **(d)**.



a.



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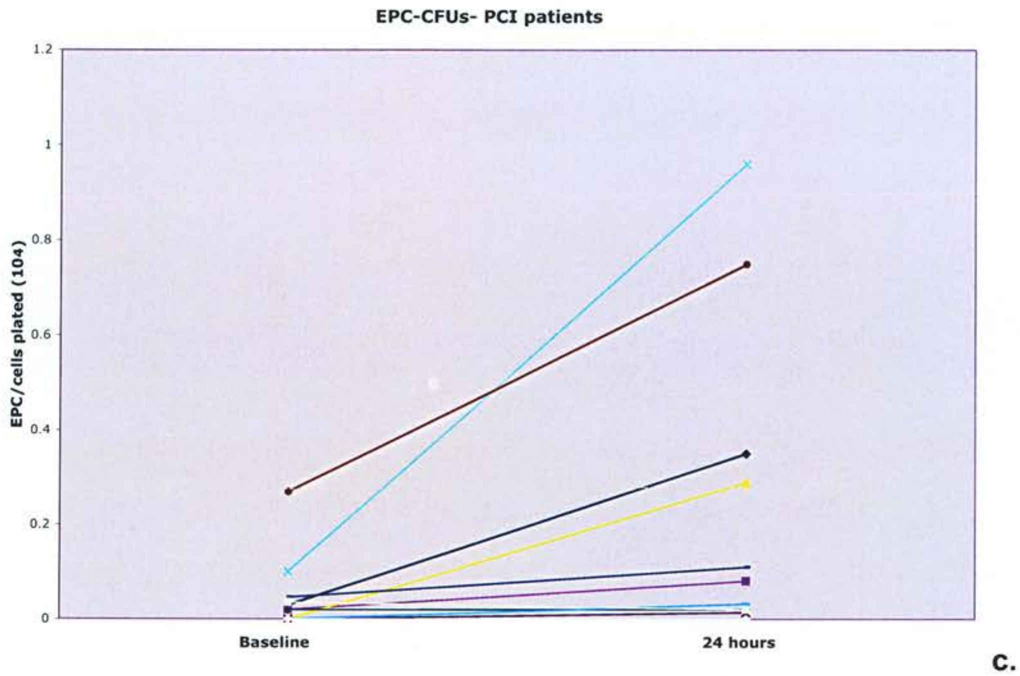
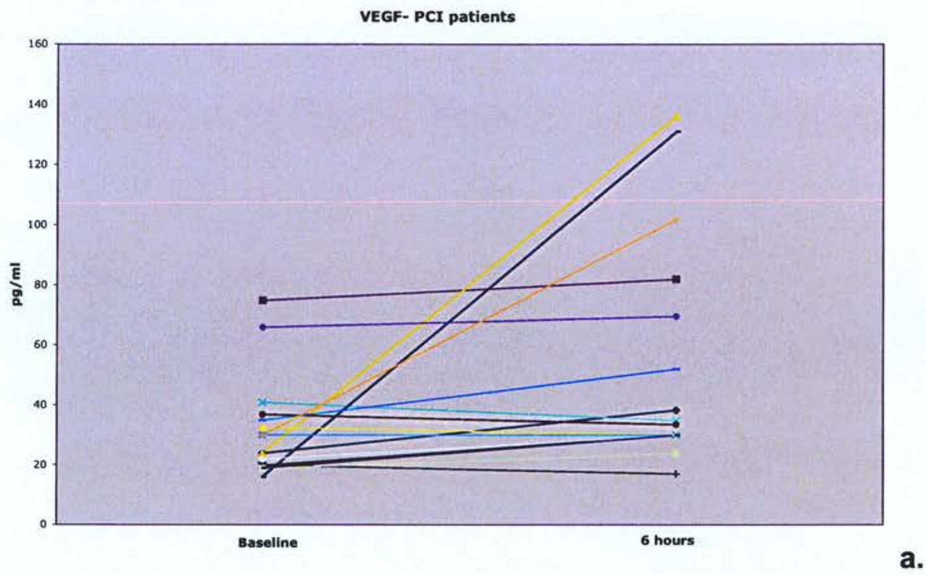


Figure 4.22. Number of CFU-EPCs before and 24 hours after the procedure in **(a)** all patients undergoing angiography ± angioplasty, **(b)** in patients undergoing angiography only, and **(c)** in patient undergoing angiography plus angioplasty. There was a significant increase in the number of CFU-EPCs in those patients undergoing angiography plus angioplasty (0.02 to 0.08 per 10^4 cells, $p<0.01$) **(c)**.

ELISA

Results are shown in **figure 4.23**. The median concentration of VEGF was significantly higher by 6 hours after the procedure in patients undergoing angiography plus angioplasty (27 to 34.25pg/ml, $p<0.005$, **figure 4.23a**). However, there was no significant change in the median concentration of VEGF in patients undergoing angiography alone (40 to 38.9pg/ml, $p=0.41$, **figure 4.23b**).



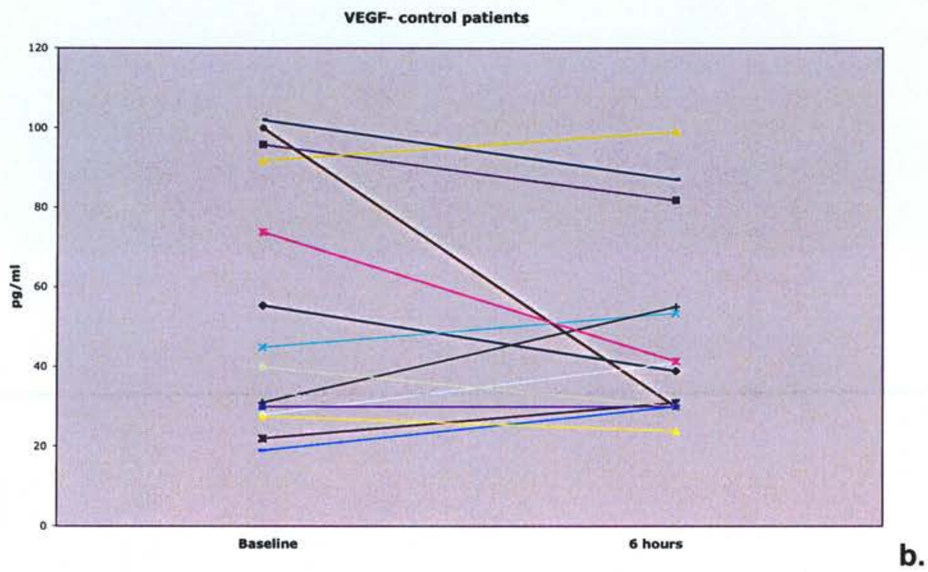


Figure 4.23. VEGF levels at baseline and 6 hours after the procedure in patients having angiography and angioplasty **(a)**, and in patients having angiography only **(b)**.

4.4 Discussion

It has been demonstrated in this study that PCI, but not diagnostic angiography, is associated with a mild systemic inflammatory response and an increase in functional CFU-EPCs in the peripheral blood. However, there was no associated increase in CD34, CD133 or VEGFR-2 mRNA species and no increase in the number of CD34+ or CD34+VEGFR-2+ cells. In this clinical model of acute vascular injury, it is possible that the acute response to injury is mediated by more mature EPCs (CFU-EPC) rather than the primitive CD34+, CD133+ VEGFR-2+ cells that have hitherto been considered as the major endothelial progenitor cell source in blood and bone marrow. Circulating CD34+, CD133+, VEGFR-2+ cells may ultimately be capable of endothelial cell differentiation, but are rare in peripheral blood. Moreover, the data presented here does not support the idea that these cells are mobilised rapidly in response to acute vascular injury and are responsible for CFU-EPC.

CFU-EPC are reduced in those with cardiovascular risk factors and evidence of vascular impairment, raising the possibility that a limited EPC reserve may contribute to a maladaptive response to vascular injury and predispose to atheroma formation [12]. Previous studies have demonstrated early mobilisation of EPCs following vascular injury in patients with acute myocardial infarction [140], major burns and following CABG surgery [33]. These clinical events involve extensive damage to a number of tissues in addition to the vasculature, which may contribute to the mobilisation of progenitors. This study extends this by showing that local, selective vascular injury can

influence the number of circulating progenitor cells, and provides a rational for the therapeutic mobilisation of EPCs at the time of PCI to influence outcome.

Current strategies to reduce the incidence of complications following percutaneous intervention are based on suppressing cellular proliferation rather than enhancing vascular repair. Vessel injury during PCI exposes underlying collagen and tissue factor, activating platelets and the coagulation cascade, and may result in acute or sub-acute stent thrombosis [143]. In the absence of an intact endothelium, local platelet/platelet and platelet/leukocyte complexes form, and persistent inflammation encourages smooth muscle hypertrophy and in-stent restenosis. Drug eluting stents have dramatically reduced the incidence of early in-stent restenosis [144], but local anti-proliferative therapy may interfere with vascular healing and prevent formation of a functional endothelial layer [145]. In experimental studies, transfusion of EPCs following vascular injury prevents both thrombus formation and neointimal proliferation [146]. Indeed, patients with diffuse in-stent restenosis have reduced numbers of circulating EPC in comparison with matched controls [61]. Stents coated with antibody to CD34 may encourage seeding of the stent and early endothelium formation and in feasibility studies have been safely deployed in man [147].

Coronary intervention increased the number of CFU-EPCs, but was not associated with a mobilisation of cells expressing CD34 and VEGFR-2, either at the molecular or phenotypic levels. These findings initially appear discordant and require further discussion. The original description of the putative endothelial progenitor cell was based

on cell culture and adhesion techniques. Asahara et al. described a population of adult human circulating $CD34^{+}$ cells that could differentiate into cells with endothelial-like characteristics in vitro [3]. The exact origin and phenotype of these progenitors remains a matter of debate in part because the purity of $CD34^{+}$ cells used in this initial study was only 15% [3]. Subsequently the co-expression of transmembrane glycoproteins CD34, CD133 and VEGFR-2 has been used in an increasing number of clinical studies to phenotype and quantify circulating endothelial progenitor cells.

As the field has developed, an increasing number of methods have emerged to define vascular progenitors and quantify regenerative capacity. The CFU-EPC assay used to quantify the number of circulating functional endothelial progenitors in this study has been used widely since it was described by Hill et al. [148]. Whilst quantification of CFU-EPC provides an accurate measure of the capacity of circulating mononuclear cells to form endothelial cells, it is doubtful whether these colonies primarily arise from the $CD34^{+}$ stem cells. No correlation between the number of peripheral blood $CD34^{+}$ cells and the number of CFU-EPCs was found in this study (see **results section**). Studies addressing the origin of endothelial progenitor lineage in adult peripheral blood have demonstrated that monocytes also express endothelial lineage markers such as VEGFR-2 and can differentiate into mature endothelial cells [23]. Rehman et al. found that the majority of CFU-EPCs expressed monocyte markers such as CD14, Mac-1, and CD11c, suggesting that peripheral-blood endothelial-like cells are derived from monocytes/macrophages [24]. The concept that functional endothelial cells may originate from a $CD14^{+}$ expressing progenitor is supported by reports that mature

endothelial cells isolated from human umbilical vein express CD14 [149]. Furthermore, Urbich et al. demonstrate that isolated $CD14^+$ cells also have the capacity to improve neovascularisation after hind limb ischemia [150].

Direct staining of our colonies demonstrates that CFU-EPCs behave like mature endothelial cells and incorporate acetylated LDL, but also confirmed that these cells strongly express CD14. In keeping with recent reports unpublished observations show that isolated $CD34^+$ cells do not give rise to spontaneous CFU-EPC but instead die in this assay: in contrast, all CFU-EPC activity is found in $CD14^+$ enriched, but not in $CD14^+$ depleted, fractions of peripheral blood mononuclear cells [25, 151]. This population of circulating $CD14^+$ monocytes appear more functionally mature than $CD34^+$ cells, are ready to home to sites of vascular injury, and are more abundant in normal peripheral blood than in bone marrow. In contrast, while CD34+, CD133+, VEGFR-2+ cells may ultimately give rise to endothelial cells, they are much less prevalent in peripheral blood than in bone marrow, and may only be the precursors of the more functionally mature CFU-EPC-capable cells that appear more crucial for an immediate response to vascular injury.

Whilst measurement of CD14+ monocytes by flow cytometry was not performed, total leucocyte CD14 mRNA and monocyte numbers were quantified. It was found that there was a significant and reproducible decrease in both immediately after catheterisation in the patient group having angiography plus PCI. It is possible that these cells immediately localise to the site of vessel damage – both at the site of femoral arterial

puncture and at the site of coronary angioplasty and stenting. This may also explain the reduction in other molecular and phenotypic markers immediately following the procedure (see **results section**). The number of peripheral blood monocytes was restored to pre-procedural levels by 24 hours, and CD14 mRNA levels significantly increased by 24 hours after PCI. Mobilisation of $CD14^+$ monocyte-like cells may explain the increase in CFU-EPC in peripheral blood observed 24 hours after arterial injury. These cells may contribute to vascular repair either through formation of mature endothelial cells and incorporation into the vessel wall, or through the release of angiogenic growth factors at the site of vessel injury. Further studies are required to confirm these observations and to explore specifically the role of $CD14^+$ monocytes in vascular injury and repair.

In considering the therapeutic potential of mobilised stem cells and stents with cell specific antibodies, it should be acknowledged that the process of vessel injury and repair is a complex one. It has been demonstrated that there is an early increase in circulating neutrophils at 24 hours, and others have observed a later rise in monocyte numbers 48 hours following PCI [152]. These clinical observations mirror the findings of Wilcox et al. who demonstrated in a porcine angioplasty model an early influx of neutrophils into the adventitia of the site of vessel injury at 6-24 hours, and the later incorporation of monocytes at 72 hours [153]. Interestingly, Fukuda et al., found a strong correlation between circulating monocyte numbers and in stent restenosis [152]. It is likely that circulating monocytic cells contains sub-populations of bone marrow derived progenitor cells that may contribute to both re-endothelialisation and neointimal formation. Autologous cell therapy with unselected mononuclear cells has the potential

to affect outcome adversely, with a recent study by George et al. suggesting that repeated intravenous treatment with bone marrow derived mononuclear cells may actually increase atheroma formation and reduce plaque stability [154].

It is likely that a variety of host factors and persistent localised inflammation will dictate whether endothelialisation or neointimal expansion will ensue following PCI. Previous studies have reported a systemic inflammatory response to the vascular injury associated with percutaneous coronary intervention, with serum IL-6, CRP and SAA levels elevated following intervention [155]. Elevated pre-procedural CRP-levels predict adverse outcome after coronary stent implantation and are associated with a greater degree of angiographic restenosis, suggesting systemic inflammation is associated with a proliferative response [156]. Mechanical injury of the atherosclerotic plaque by balloon inflation triggers local inflammatory mechanisms with leucocyte adhesion, activation and cytokine release [157]. The mobilisation of neutrophils and increase in CRP concentrations observed following PCI, are presumably systemic manifestations of this local inflammatory response to arterial injury. Finally the increased VEGF expression only in patients with local vascular injury following PCI (**figure 4.23a**) is consistent with other studies showing that EPC mobilization is cytokine driven [31].

4.5. Conclusion

Local vascular injury following angioplasty and stenting results in a mild systemic inflammatory response and increases the number of CFU-EPCs. Circulating

CD34+VEGFR-2+ cells are rare in peripheral blood, and are not mobilised rapidly in response to vascular injury. Therefore, the acute response to injury may be mediated by more mature EPCs (CFU-EPC) rather than the primitive CD34+VEGFR-2+ cells that have hitherto been considered as the major endothelial progenitor cell source in blood and bone marrow. A better understanding of the cellular response to vascular injury is necessary to allow a more sophisticated approach to reducing the complications of percutaneous coronary intervention with new strategies designed to enhance vessel repair.

5. ENDOTHELIAL PROGENITOR CELLS AND AORTIC ANEURYSM REPAIR

5.1 Introduction

The recent identification of circulating stem cells capable of differentiating into endothelial cells has lead to a re-evaluation of our understanding of how the vascular endothelium is repaired [3]. The long held theory that endothelium could only be replaced by the proliferation of existing mature endothelium has been extended to include the process of vasculogenesis from a stem cell population. These stem cells (endothelial progenitor cells, EPCs) have been identified in a variety of tissues, including endothelium, and can be isolated from peripheral blood and bone marrow [158]. In animal models of tissue ischaemia, heterologous and autologous, EPCs derived from blood and bone marrow have been shown to incorporate into sites of vascular damage and neovascularisation [3, 5, 6]. Furthermore, autologous transplantation of mononuclear cells containing EPCs have been used therapeutically to good effect in patients with ischaemic myocardium and peripheral ischaemia [110, 112]. It is known that EPCs are mobilised from the bone marrow in response to vascular injury and tissue ischemia and that this is cytokine driven [4, 74, 159]. Canine models of graft endothelialisation have demonstrated that endothelial cells, originating from the blood stream, contribute towards healing of the inner wall of Dacron grafts made impervious to transmural capillary ingrowths [160, 161]. It has also been shown, in animal models of carotid artery injury, that autologous EPCs can be harvested from blood and transplanted into denuded vessels, resulting in preservation of vascular integrity [162, 163]. EPCs are therefore considered an important part of the natural repair mechanism, helping to maintain vessel wall integrity and blood supply following organ ischaemia.

EPCs have been identified and characterised using a number of different methodologies, and these reflect the difficulty in precisely defining this diverse group of cells [4, 6, 7, 18, 164]. They may express CD34, vascular endothelial growth factor receptor-2 (VEGFR-2), VE-cadherin, a junctional molecule, and AC133, a receptor of unknown function which is lost from EPCs as they mature [18]. VE-cadherin (CD144) is thought to be a specific marker of endothelial cells and a marker of more mature EPCs [165-167]. EPCs are also defined functionally by their high proliferative capacity and ability to form colonies in-vitro [7, 12, 19].

In spite of our understanding that ischemic insults and blood vessel damage are major stimuli to EPC mobilisation, and the deposition at the site of tissue injury of mature, functional endothelial cells [33, 74], there is uncertainty over where this maturation event takes place or how dynamic a process it is. Knowledge of this process is hampered by the lack of a simple universally agreed marker for EPCs. The objective of this study was to investigate the dynamics of EPC mobilisation, using elective abdominal aortic aneurysm repair in patients as a model of tissue ischemia and blood vessel damage. This patient group undergo a relatively severe ischaemic event, in a controlled situation, making them a very suitable model. In pre-operative and serial post-operative blood samples we quantitated EPCs by a variety of techniques. Cell culture assays of endothelial progenitors were used in combination with measurement of CD34, VEGFR-2, CD133, VE-cadherin and von Willebrand factor (vWF) expression by flow cytometry, and real-time PCR assays of their specific mRNAs.

5.2 Methods

Study patients

15 consecutive patients, who were undergoing elective abdominal aortic aneurysm repair, were recruited. There were 11 males and 4 females. Their ages ranged from 54 to 85 years (median 72 years). Each patient underwent a laparotomy followed by insertion of a Dacron graft (Scientific Medi-tech, NJ, USA) at the level of the infra-renal aorta. 5ml of blood was taken into EDTA coated tubes pre-operatively and at the following times post-operatively: 24 hours, 48 hours and 5 days. A limit of a 5-day follow up was chosen because patients were discharged on the following day. Furthermore, because the operations were performed at a regional centre, patients had necessarily travelled long distances, making it difficult to obtain blood samples after discharge. There were two control groups used because the study was performed in two different centres. Group A was the control group for the PCR studies, and group B was the control group for flow cytometry and colony assay studies. Single samples for control group A were obtained from patients attending an orthopaedic pre-operative clinic, who were found to have normal routine full blood counts and serum biochemistry (6 males and 6 females, median 71 years). Single samples for control group B were obtained from healthy volunteers in the laboratory (8 males and 7 females, median 51 years). The study was approved by the local ethics committee, and all patients gave written informed consent.

Full blood counts

Full blood counts and differential counts, including monocyte counts, were obtained from each patient, and at each time point, by collecting 2.7ml of EDTA anticoagulated blood and processing the samples through a Sysmex analyser.

RNA extraction and real-time PCR

Protocols were followed as described in **sections 2.1** to **2.3**. 1ml of whole blood was subject to total leukocyte RNA extraction using Qiagen's "RNeasy mini-kit". RNA concentration and purity were estimated by UV absorbance at 260 and 280 nm. The 260:280 nm ratios were all greater than 1.8 indicating that little protein contamination was present. One microgram of total RNA was transcribed into cDNA in each reverse transcription reaction with 200 units of M-MLV reverse transcriptase for 60 minutes at 37°C in 20µl reactions containing 1µl (0.5µg/µl) of random hexamer primers, with 0.625µl (40units/µl) of RNase inhibitor, 5µl of dNTP mix (containing dATP, dCTP, dGTP and dTTP, each at a concentration of 10nM in water) and 5µl of 5X RT reaction buffer (containing 50mM Tris-HCL-pH 8.3 @ 25°C, 75mM KCL, 3mM $MgCl_2$ and 10mM DTT).

Real-time PCR was carried out using the ABI Prism ®7900HT system (Applied Biosystems, Warrington, Cheshire, UK) to determine the relative quantity (RQ) of mRNA for selected genes. RQ can be defined as a comparison of a target signal in different samples to a reference sample and normalised to an endogenous control [168].

PCR primers and probes for amplification of cDNA derived from CD34, VEGFR2, VE-cadherin and vWF transcripts were obtained from Applied Biosystems (Foster City, CA, USA). Each assay contained forward and reverse PCR primers (final concentration of 900nM each) and one TaqMan MGB probe (6-FAM dye-labelled, to a final concentration of 250nM). All primer-probe sets had been quality control checked and validated. The ribosomal 18s gene was used as the internal control. 4μl of the reverse transcription reaction was analysed in each PCR reaction. The PCR reactions were run in triplicate in 20μl assays, each containing cDNA, 1μl of primer/probe, 10μl of universal PCR master mix and distilled water. The cycling program was as follows: (a) initial activation for 10 minutes at 95°C; (b) 50 amplification cycles with a 15 second denaturing step at 95°C, and a 1 minute combined annealing and extension step at 60°C. Analysis was performed using ABI 7900HT SDS software (version 2.1), in order to obtain the relative quantities (RQ) of mRNA compared to a calibrator.

Flow cytometry

The protocol in **section 2.5** was used. Whole blood cells were phenotyped by flow cytometry using FACS-Calibur flow cytometer (Becton-Dickinson, Oxford, UK). Cells were directly stained and analysed for phenotypic expression of surface proteins with monoclonal antibodies (MAbs) conjugated to phycoerythrin (PE), fluorescein isothiocynate (FITC), Peridin Chlorophylla protein (PERCP) or Allophycocyanin (APC). The MAbs included anti-CD34-FITC, PerCP-conjugated anti-human CD45, anti-VEGFR2-PE, anti--cadherin-PE, and anti-CD133-APC. Appropriate negative controls

(Isotype and/or no antibody) were used to establish positive stain boundaries. 100ul of the sample was stained with the appropriate antibodies for 30 minutes in the dark; the erythrocytes were lysed with lysing solution for 15 minutes in the dark. Afterwards the samples were centrifuged and washed with PBS at 200g and finally the cells were fixed with Cell Fix solution. 50.000 events were counted and measured using the Becton Dickinson FACSCalibur System. Percentage positivity for a particular marker was obtained based on the total number of events. An absolute number was obtained using the total white cell count. The mean fluorescent intensity (MFI) was obtained and analysed for each marker for the total number of events and for the monocyte gate only.

Endothelial progenitor cell (EPC) colony assay

The protocol in **section 2.6** was followed. The EPC colony assay was performed according to the method specified by the kit manufacturer (Stem cell Technologies) after the method of Hill et al [12]. 3ml of venous blood was used to isolate endothelial progenitor cells. The peripheral blood mononuclear cell fraction was isolated by Ficoll density-gradient centrifugation. Recovered cells were then resuspended in Endocult. $5x10^6$ mononuclear cells in 2ml of Endocult were plated in a fibronectin-coated 6-well plate and incubated for two days at 37C, 5% CO2 with 95% humidity. After two days the non-adherent cells were transferred into fibronectin-coated 24-well plates at approximately $1x10^6$ cells/well for three days more at 37C, 5% CO2 with 95% humidity. 2 days culture resulted in the emergence of characteristic endothelial progenitor colonies. At day 5 of the assay the colonies per well were counted and the number of EPCs per 10^6 cells plated in the fibronectin 24-well plate calculated. The colonies are

defined following Stem Cell Technologies technical manual as a central core of "round" cells with elongated "sprouting" cells at the periphery and are classified as early outgrowth colony forming unit - endothelial cell or CFU-EPC.

5.3 Results

Comparison between patients and control subjects prior to aortic aneurysm repair

Prior to surgery, there was no significant difference between the patient group and control group A in the relative quantity of mRNA for CD34, VEGFR-2, VE-cadherin and vWF (**table 5.1**). Furthermore, there were no significant differences between the patient group and control group B in the number of EPCs by the colony assay and flow cytometric measurements of CD34, CD133, VEGFR-2 and VE-cadherin (**table 5.2**).

Target	Control group A (n=12)	Patient group (n=15)
Age		
Mean	71	70.3
P value	0.82	
CD34		
Median	2.538	0.984
P value	0.237	
VEGFR2		
Median	2.089	1.599
P value	0.6454	
VE-cadherin		
Median	3.373	2.498
P value	0.863	
VWF		
Median	2.363	2.435
P value	0.99	

Table 5.1. Comparison between median RQ values in the control group and pre-treatment patient group. The control group was cohort matched for age and gender with the patient group. There were no statistically significant differences found between the two groups in the RQ values for CD34 mRNA (p=0.237), VEGFR2 (p=0.6454), VE-cadherin (p=0.863) and vWF (p=0.999).

	Control group B (n=15)	Patients (n=15)
Age		
Mean	50.5	70.3
P value	0.003	
EPC/cells plated (10^4)		
Median	0.2350	0.0175
P value	0.0052	
%CD34+ cells		
Median	0.065	0.06
P value	0.7	
%CD133+ cells		
Median	0.07	0.08
P value	0.93	
%VEGFR2+ cell		
Median	1.66	4.24
P value	0.18	
% VE-cadherin+ cells		
Median	8.415	13.07
P value	0.08	

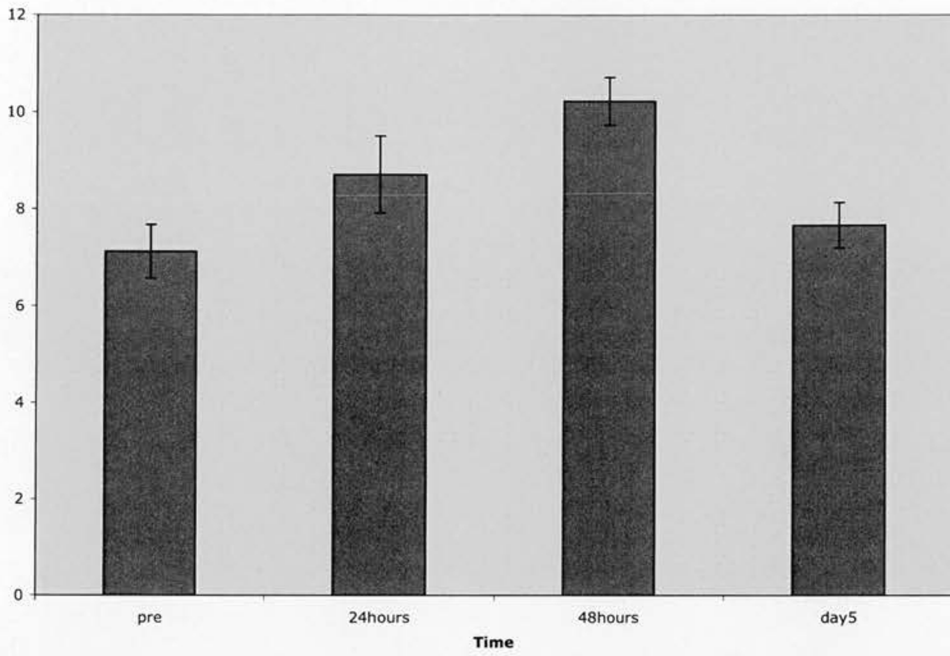
Table 5.2. Comparison of median colony numbers and median percentage positivity of EPCs in controls versus pre-treatment patient group. The control group was not cohort matched with the patient group. The control group had a higher median number of cell colonies ($0.23 \times 10^4 \pm 0.06 \times 10^4$) compared with the patient group ($0.017 \times 10^4 \pm 0.03 \times 10^4$). This difference was statistically significant ($p=0.005$). There were no statistically

significant differences between the groups in the median percentage of cells expressing CD34 ($p=0.7$), CD133 ($p=0.93$), VEGFR2 ($p=0.18$) and VE-cadherin ($p=0.08$).

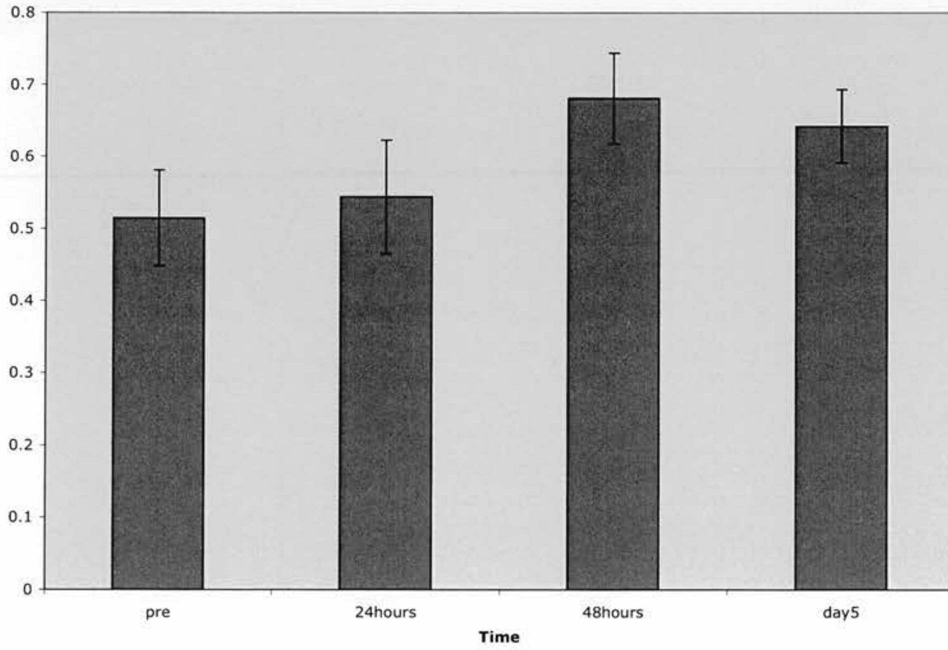
Leukocyte and EPC quantitation following surgery

Total white cell count and monocyte count. CD14 expression by PCR:

Patients demonstrated an increase in the mean total white cell count by 24 and 48 hours compared to pre-operatively (**figure 5.1a**). This was followed by a reduction to pre-operative levels by day 5. There was also an increase in the mean monocyte count by 48 hours post-operatively, but this did not reach statistical significance (**figure 5.1b**). Samples from 14 patients out of 15 patients were assessed for the RQ of CD14 mRNA. There was an increase in the mean RQ by 24 and 48 hours, followed by a reduction in the mean RQ by day 5 post-operatively (**figure 5.1c**).



a.



b.

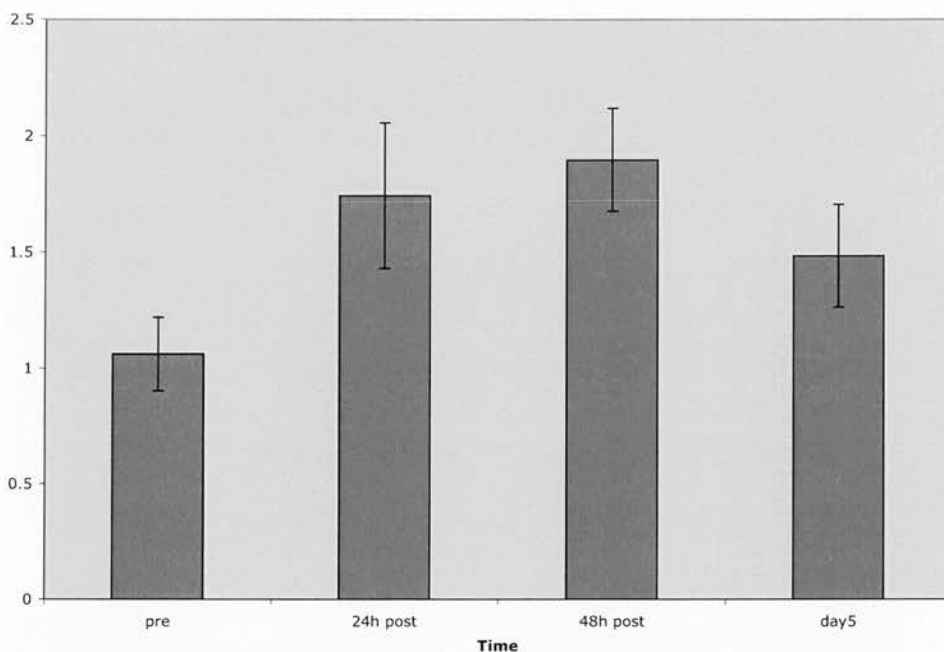


Figure 5.1. Effect of aortic aneurysm repair on the total white cell count (WBC), the monocyte count and the RQ of CD14 mRNA. **(a).** Mean (±SEM) WBC increases from a baseline of $7.13 \times 10^9/l$ (±0.55) to $8.71 \times 10^9/l$ (±0.79) by 24 hours, to a peak of $10.23 \times 10^9/l$ (±0.49) by 48 hours. The WBC then decreases by day 5 to $7.67 \times 10^9/l$ (±0.5) ($p=0.0001$). **(b).** Mean (±SEM) monocyte count increases from a baseline of $0.51 \times 10^9/l$ (±0.07) to $0.68 \times 10^9/l$ (±0.06) by 48 hours. The monocyte count is $0.64 \times 10^9/l$ (±0.05) by day 5. The change in monocyte count is not significant ($p=0.1$). **(c).** The mean (±SEM) RQ of CD14 mRNA mirrors the change in the mean WBC count. There is an increase from a pre-operative value of 1.06 (±0.16) to 1.74 (±0.31) by 24 hours. It increases further by 48 hours, before falling slightly to 1.48 (±0.22) by day 5 following the operation ($p=0.001$).

CD34 expression by PCR and flow cytometry:

Complete data was obtained on 9 out of 15 patients for the RQ of CD34 mRNA (**figure 5.2a**). There was a reduction in the median RQ by 24 hours post-operatively, with a recovery to the median pre-operative level by 48 hours. In addition, there was a reduction in the median number of CD34+ cells by 24 hours post-operatively, before a recovery to the pre-operative level by 48 hours (**figure 5.2b**). There was no significant change in the MFI of CD34 in the total cell population or in the monocyte population over the postoperative period (**figure 5.3a&b**).

CD133 expression by PCR and flow cytometry:

Complete data was obtained on 6 out of 15 patients for the RQ of CD133 mRNA. There was an increase in the median RQ by day 5 following the operation, but this change did not reach statistical significance (**figure 5.2c**). Samples from 8 patients were assessed for surface expression of CD133. There was a reduction in the median number of CD133+ cells by 24 hours, before a return to baseline levels by day 5 post operatively (**figure 5.2d**). There was no significant change in the MFI of CD133 post-operatively in the total cell population or in the monocyte population (**figure 5.3c&d**).

VEGFR-2 expression by PCR and flow cytometry:

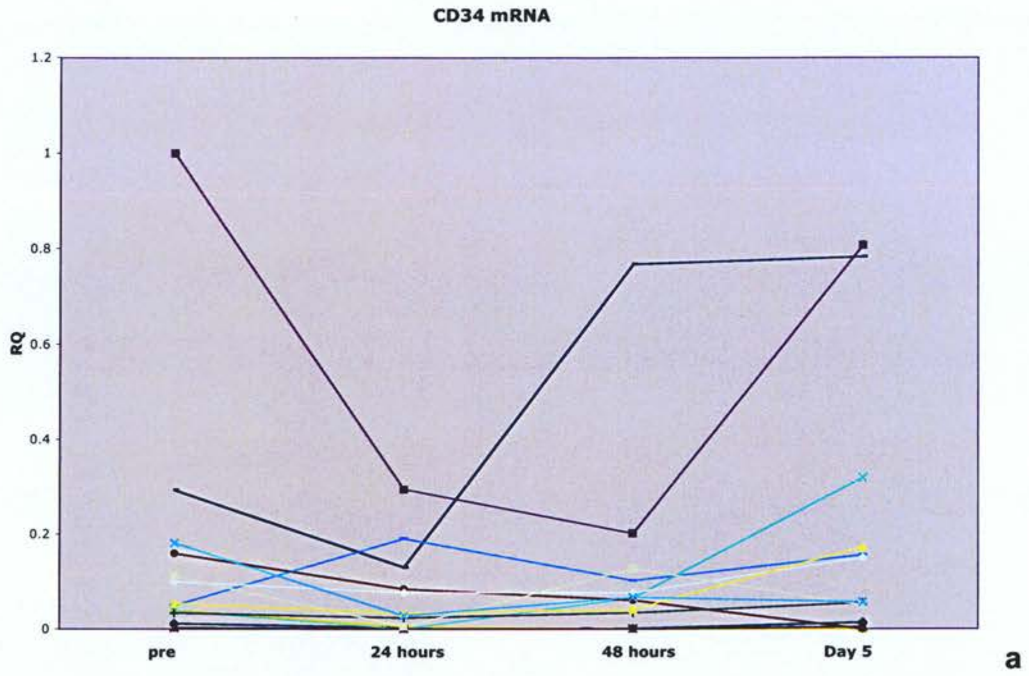
Complete data was obtained on 4 out of 15 patients for the RQ of VEGFR-2 mRNA. There was a reduction in the median RQ by 24 hours, but this change did not reach statistical significance (**figure 5.2e**). Samples from 9 patients were assessed for surface expression of VEGFR-2. There was an increase in the median number of VEGFR-2+ cells by 48 hours post-operatively, before a return to the pre-operative level by day 5 after the operation, but this change did not reach statistical significance (**figure 5.2f**). There was a statistically significant increase in the MFI of VEGFR-2 by 48 hours post-operatively in the total cell population and in the monocyte population, before a return to baseline levels by day 5 (**figure 5.3e&f**).

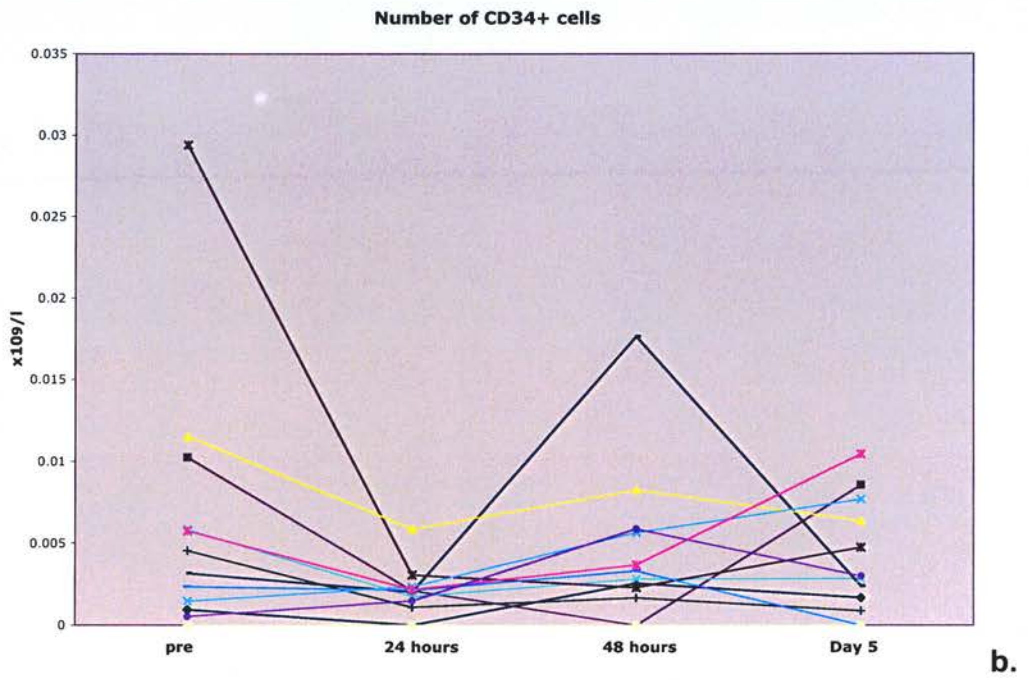
VE-cadherin expression by PCR and flow cytometry:

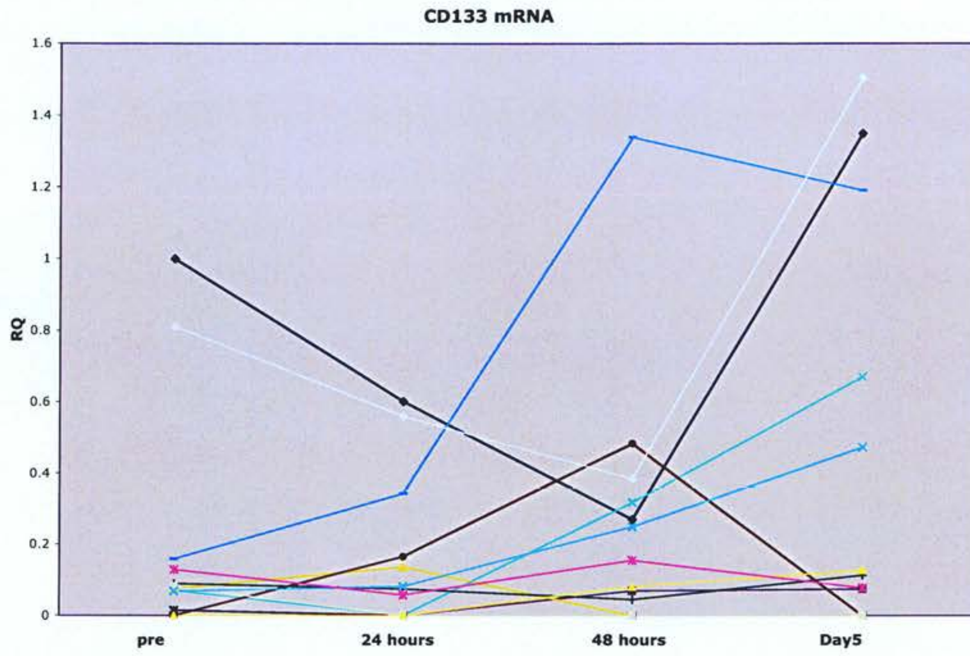
Complete data was obtained on 14 out of 15 patients for the RQ of VE-cadherin mRNA. There was a significant increase in the median RQ by 24 and 48 hours following surgery, with a return to the pre-operative level by day 5 post-operatively (**figure 5.2g**). Samples from 8 patients were assessed for surface expression of VE-cadherin. There was no statistically significant change in the median number of VE-cadherin+ cells over the specified time period (**figure 5.2h**). The MFI of VE-cadherin increased significantly by 24 and 48 hours after the operation, when the total events were analysed, and when the monocyte gate was analysed (**figure 5.3g&h**).

VWF expression by PCR:

Complete data was obtained on 14 out of 15 patients for the RQ of vWF mRNA. There was a significant reduction in the median RQ by 24 hours, followed by a recovery by 48 hours and a significant increase in the median RQ by day 5 following the operation (**figure 5.2i**).


CD34 mRNA
RQ
1.2
1
0.8
0.6
0.4
0.2
0
pre
24 hours
48 hours
Day 5
a


Number of CD34+ cells
x109/l
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
pre
24 hours
48 hours
Day 5
b.



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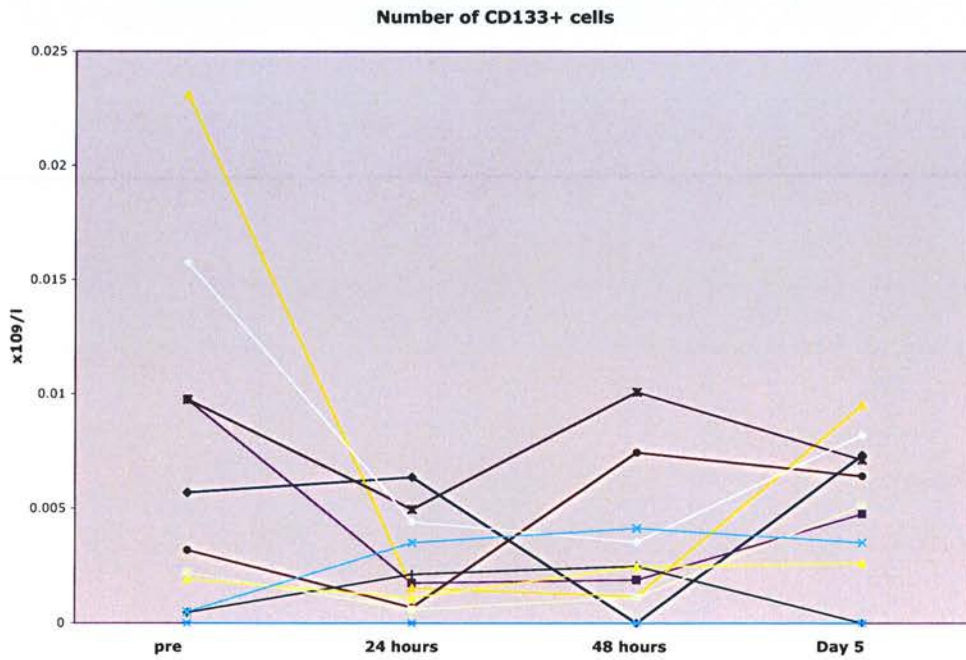

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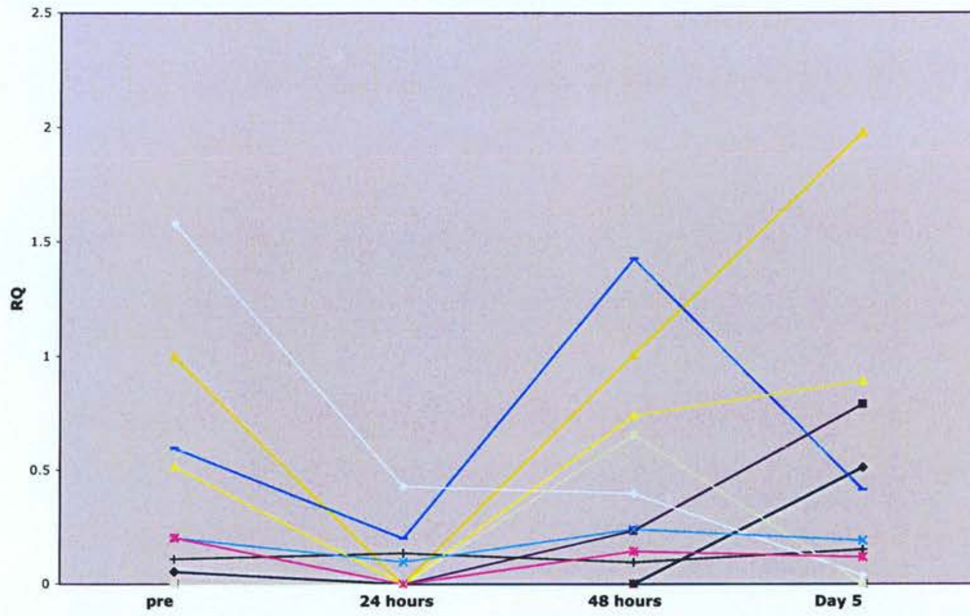
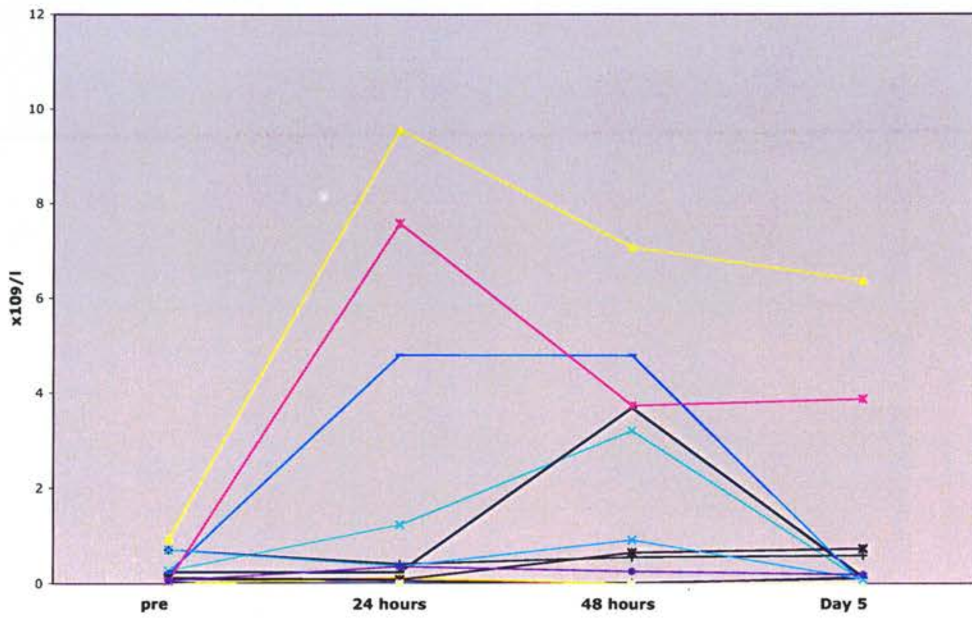


e.



f.

VE-cadherin mRNA

RQ

0 1 2 3 4 5 6 7 8 9 10

pre 24 hours 48 hours Day 5

g.

Number of VE-cadherin+ cells

x109/l

0 0.5 1 1.5 2 2.5 3

pre 24 hours 48 hours Day 5

h.

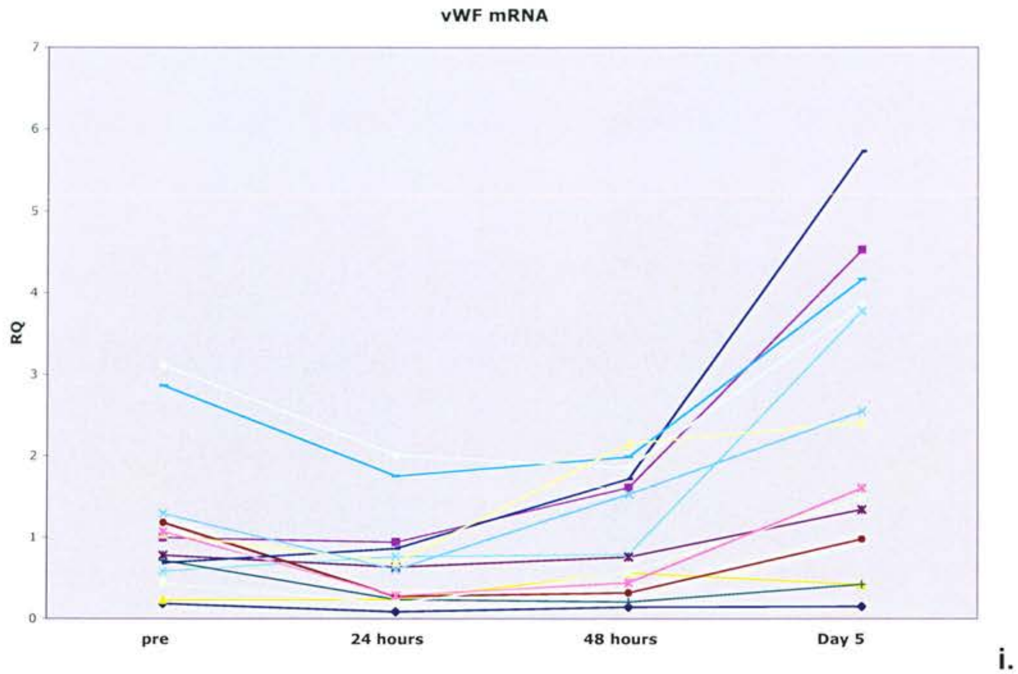
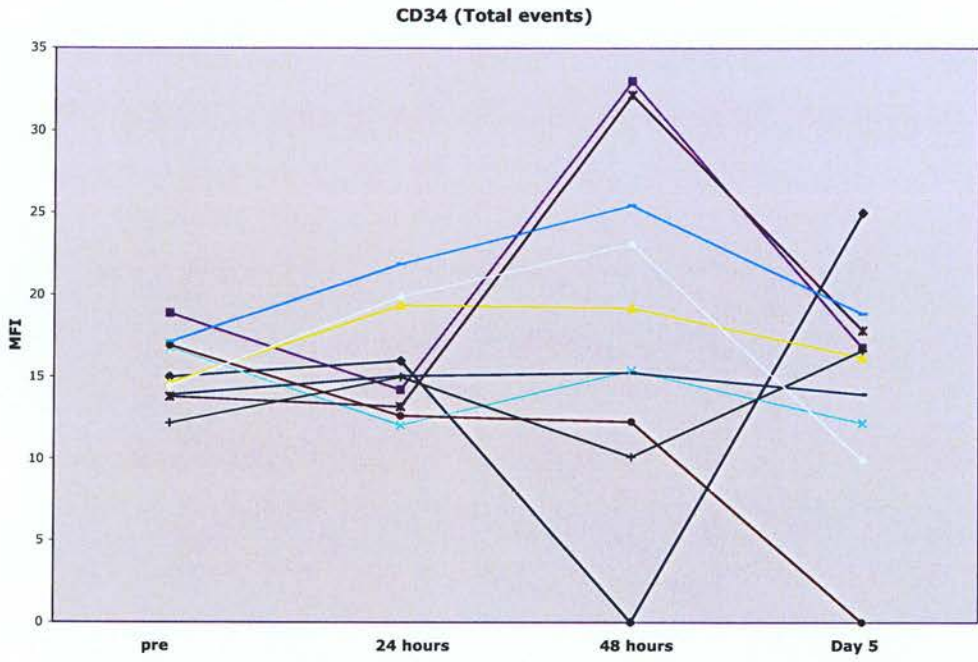


Figure 5.2. Effect of aortic aneurysm repair on markers of EPCs by PCR and flow cytometry. **(a).** The median RQ of CD34 mRNA transiently decreases by 24 hours, before recovering to baseline by 48 hours, but this does not reach statistical significance ($p=0.03$). **(b).** There is a reduction in the median number of CD34+ cells by 24 hours, with recovery by 48 hours, but this does not reach statistical significance ($p=0.04$) **(c).** The median RQ of CD133 increases by day 5 following the operation, but this does not reach statistical significance ($p=0.15$). **(d).** The median number of CD133+ cells increases from 24 hours to day 5 after the operation, but this does not reach statistical significance ($p=0.05$). **(e).** The median RQ of VEGFR-2 mRNA increases by 48 hours after the operation, but this does not reach statistical significance ($p=0.75$). **(f).** There is an increase in the median number of VEGFR-2+ cells by 48 hours, but this does not

reach statistical significance (p=0.5). **(g).** The median RQ of VE-cadherin mRNA increases by 24 hours from baseline, and further increases by 48 hours, and by day5. There is then a reduction in the median RQ of VE-cadherin mRNA from 48 hours to day 5 following the operation (p<0.0001). **(h).** There is an increase in the median number of VE-cadherin+ cells by 48 hours after the operation, but this does not reach statistical significance (p=0.41). **(i).** The median RQ of vWF mRNA falls significantly by 24 hours from baseline. It recovers by 48 hours, and increases significantly by day 5 after the operation (p<0.0001). **Note:** RQ values for the mRNA species studied were adjusted to take into account changes in the total white cell count over the post-operative period.



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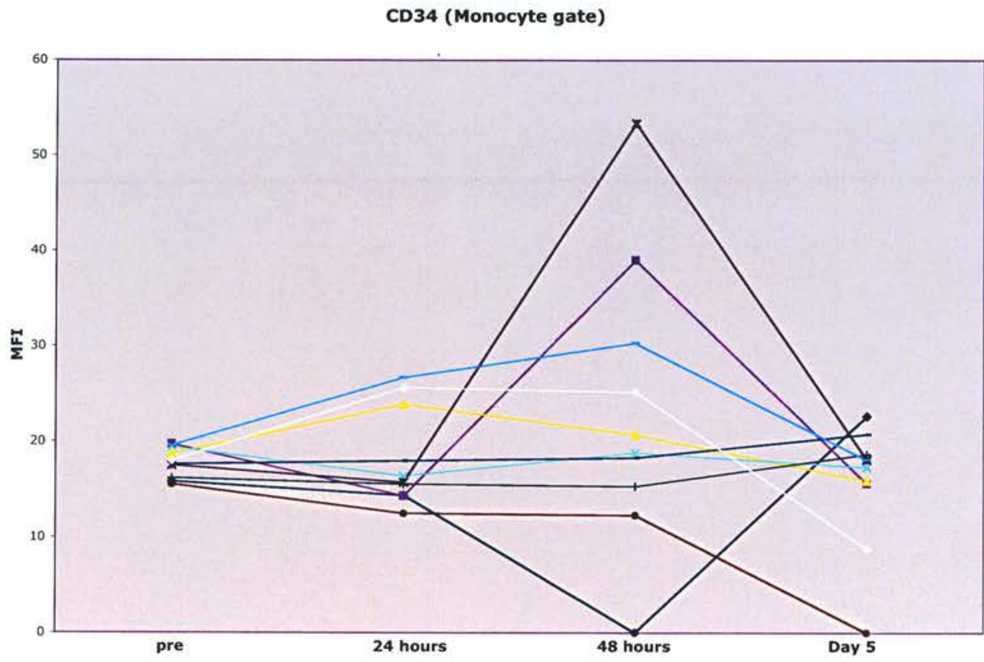

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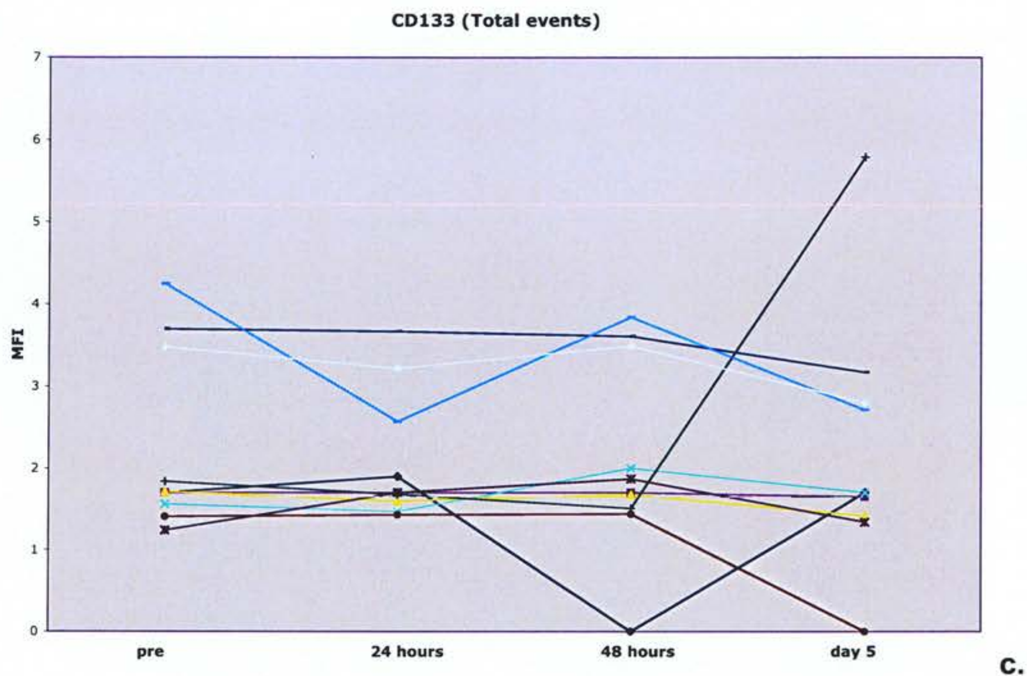
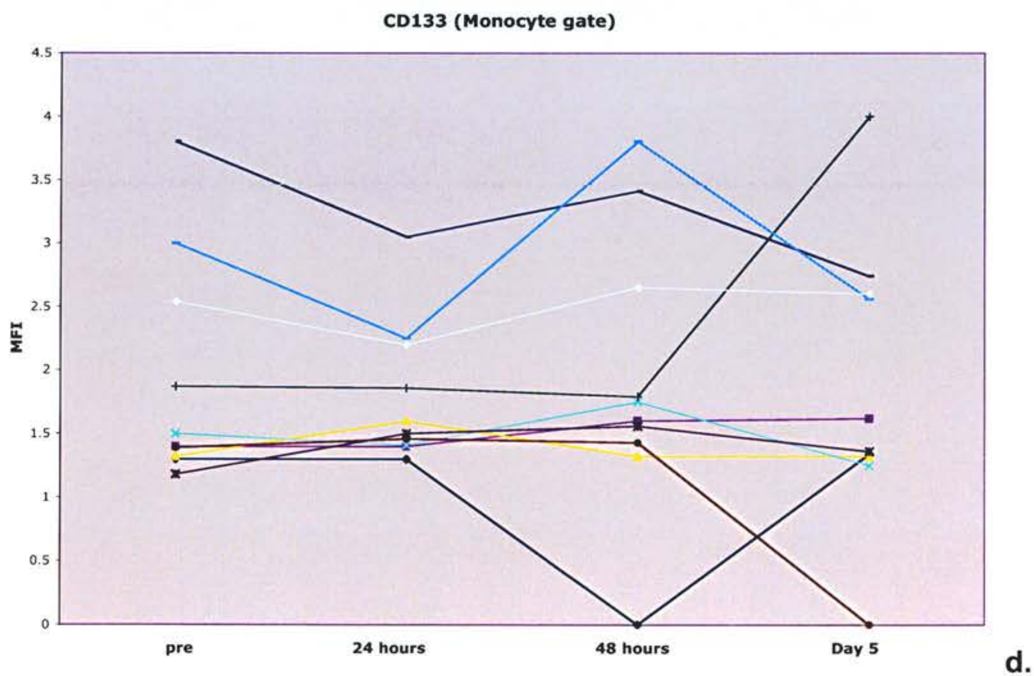
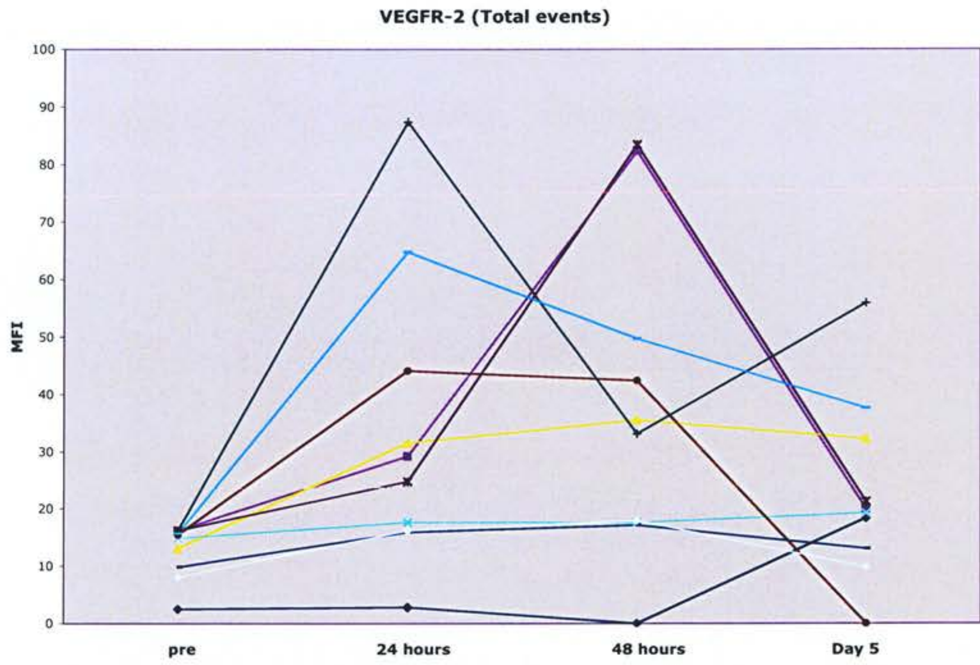
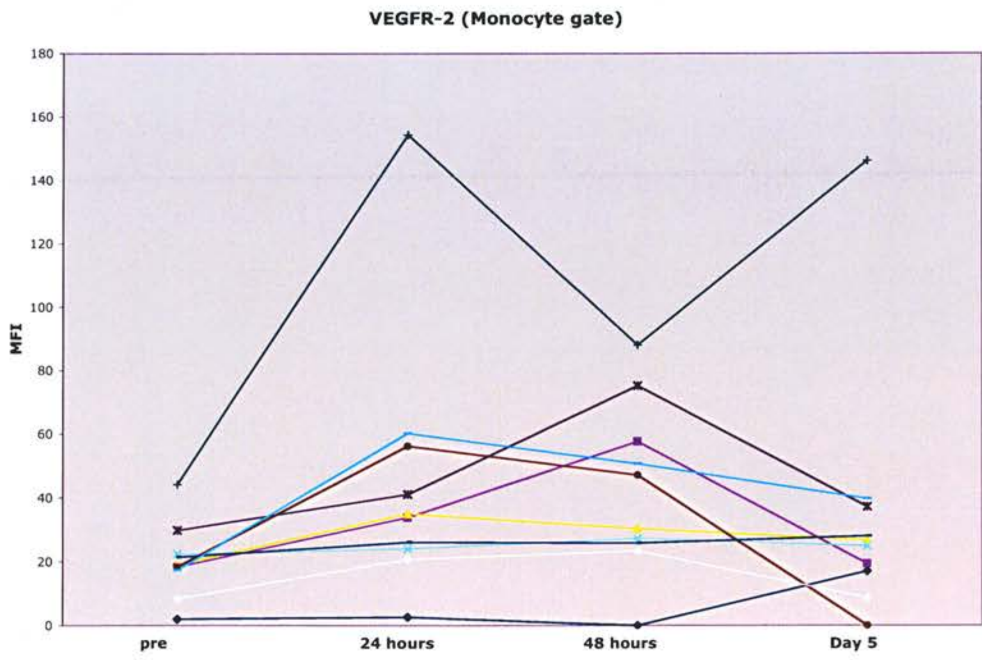

CD133 (Total events)
MFI
7
6
5
4
3
2
1
0
pre
24 hours
48 hours
day 5
c.

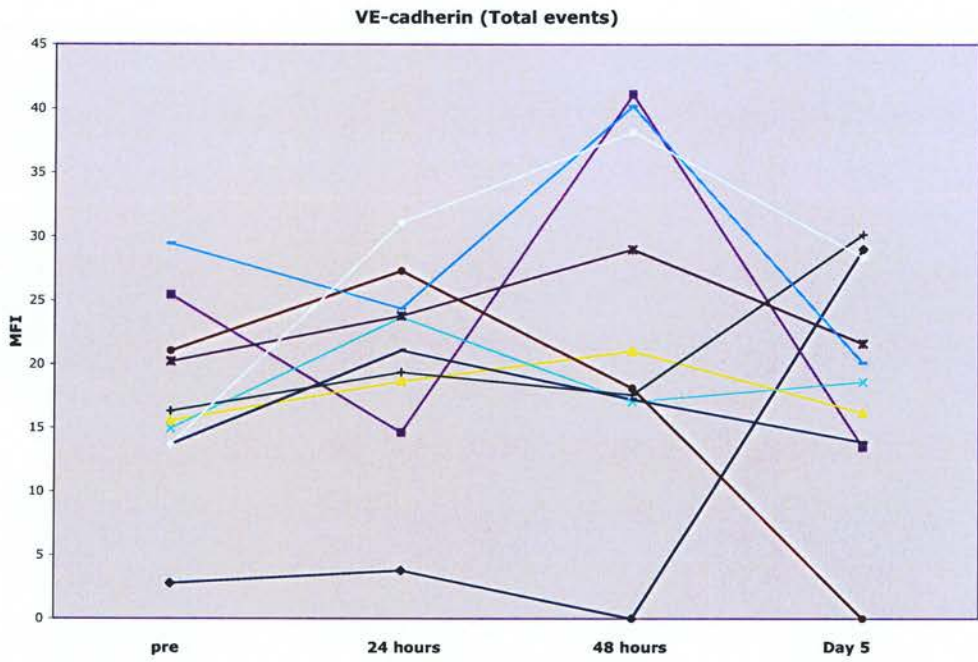

CD133 (Monocyte gate)
MFI
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
pre
24 hours
48 hours
Day 5
d.



e.



f.



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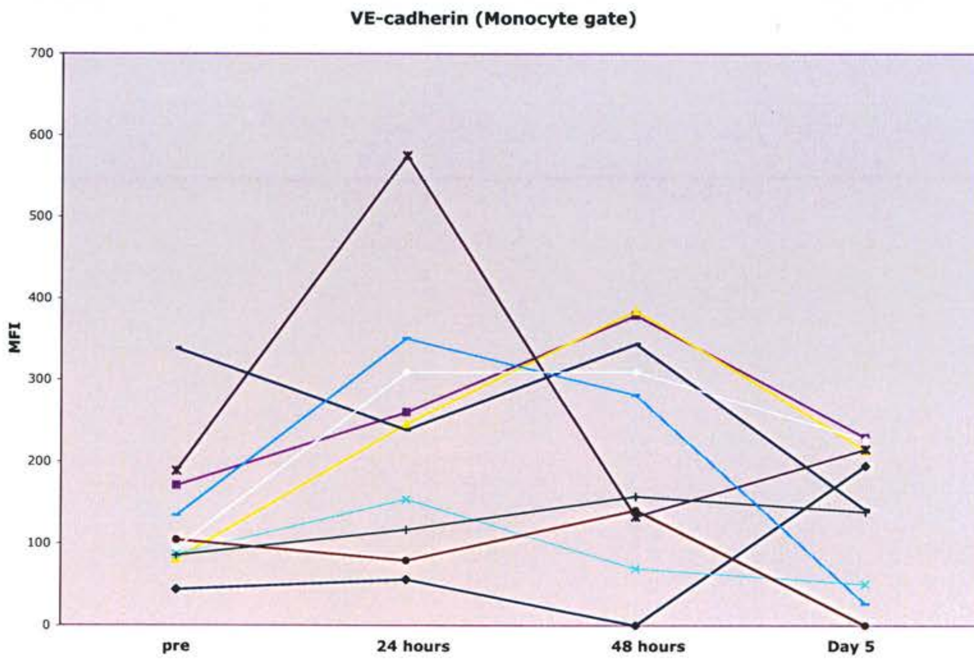
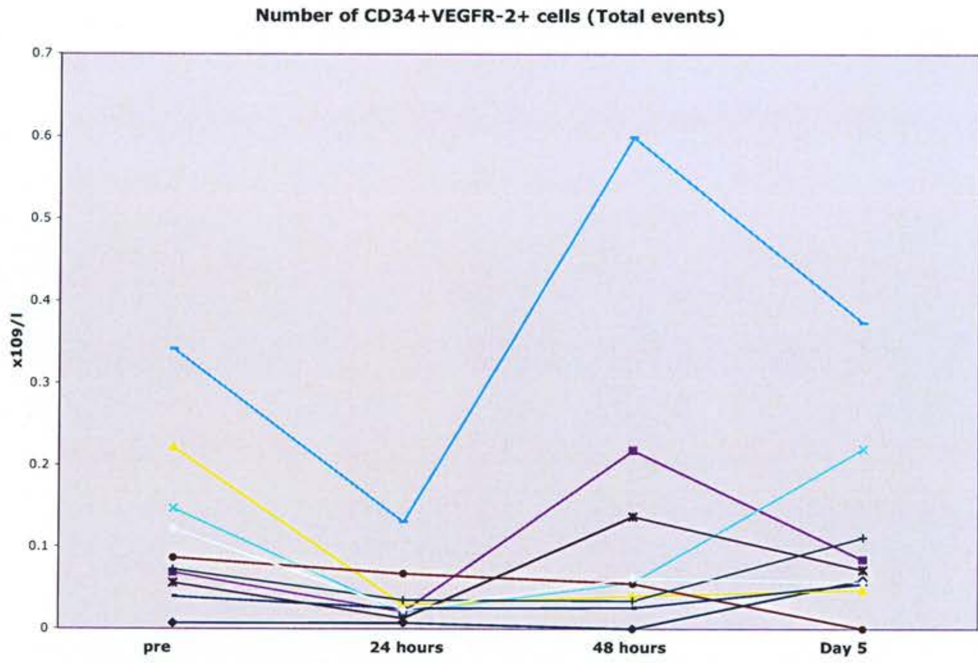


h.

Figure 5.3. Effect of aortic aneurysm repair on the mean fluorescent intensity (MFI), by flow cytometry, of the markers of EPCs. **(a).** There is no significant change in the MFI of CD34 over the postoperative period when all cells are analysed (p=0.15). **(b).** There is no change in the MFI of CD34 in the monocyte gate (p=0.46). **(c).** There is no significant change in the MFI of CD133 post operatively when all cells are analysed (p=0.15). **(d).** There is no change in the MFI of CD133 in the monocyte gate (p=0.37). **(e).** By analysing all cells, the MFI of VEGFR-2 increases significantly by 48 hours post operatively, before reducing back by day 5 (p=0.001). **(f).** By analysing the monocyte gate, the MFI of VEGFR-2 increases significantly by 48 hours post operatively, before reducing back by day 5 (p=0.001). **(g).** By analysing all cells, the MFI of VE-cadherin increases significantly by 24 hours post operatively, before reducing back by day 5 (p=0.01). **(h).** By analysing the monocyte gate, the MFI of VE-cadherin increases by 48 hours post operatively, before reducing back by day 5, but this does not reach statistical significance (p=0.02).

Dual CD34 and VEGFR-2 expression by flow cytometry:

Complete data was obtained on 8 out of 15 patients for assessment of surface expression of CD34 and VEGFR-2. There was a transient reduction in the number of CD34+, VEGFR-2+ cells by 24 hours after the operation when all cells were analysed (**figure 5.4a**). When the total cell population was analysed, there was found to be an increase in the number of CD34-, VEGFR-2+ cells by 24 hours post operatively (**figure 5.4b**). This increase was sustained over the course of the study. When the monocyte population was analysed it was also found that the number of CD34-, VEGFR-2+ cells increased by 48 hours following the procedure, but this change did not reach statistical significance (**figure 5.4c**). There was a change in the number of CD133+, VEGFR-2+ cells post operatively in the total cell population, with a doubling by day 5 (**figure 5.4d**).



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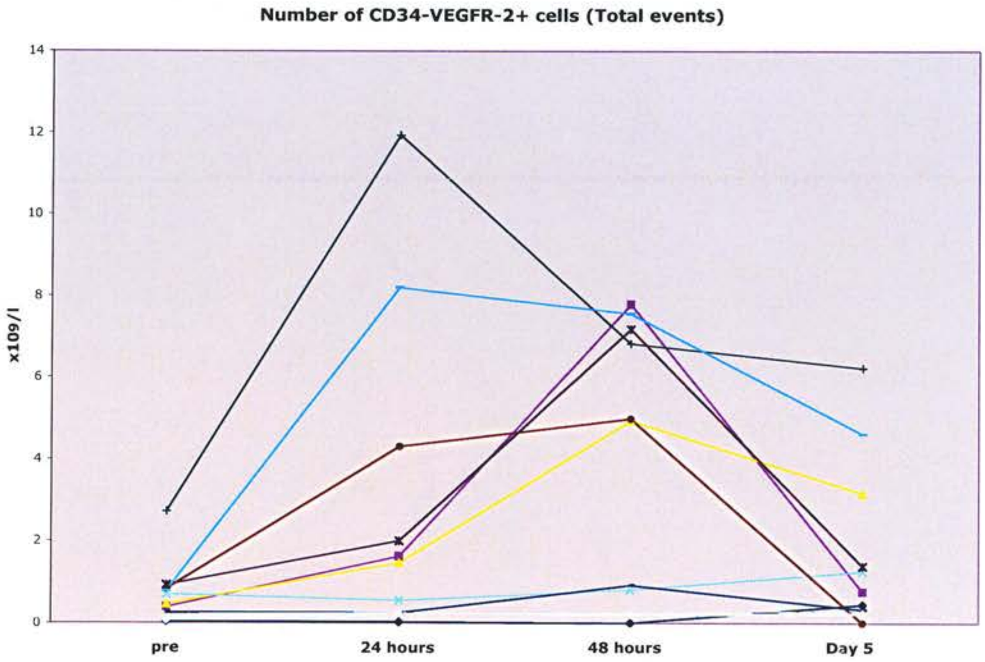

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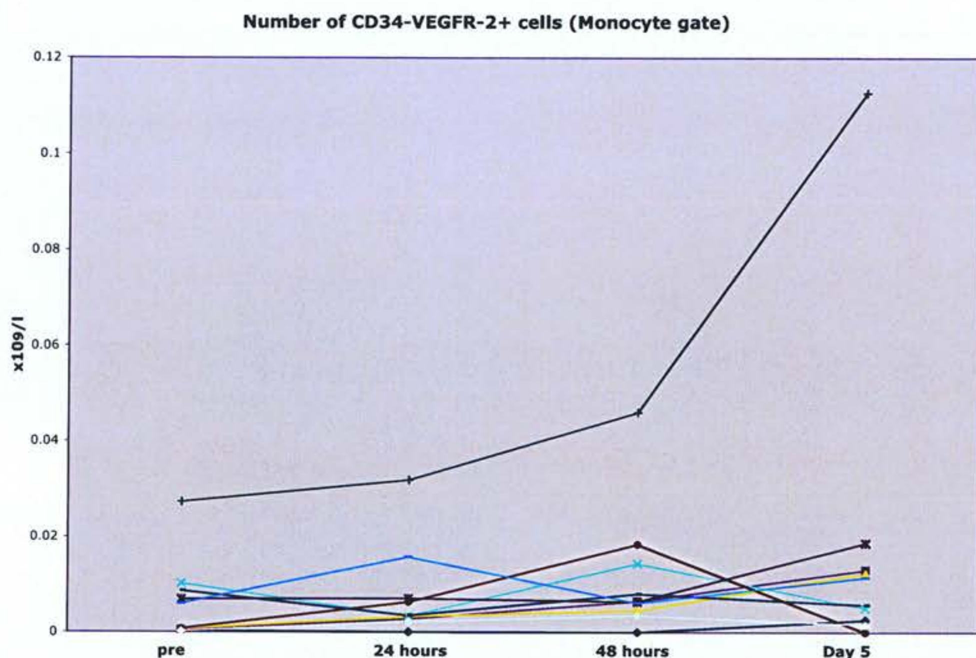
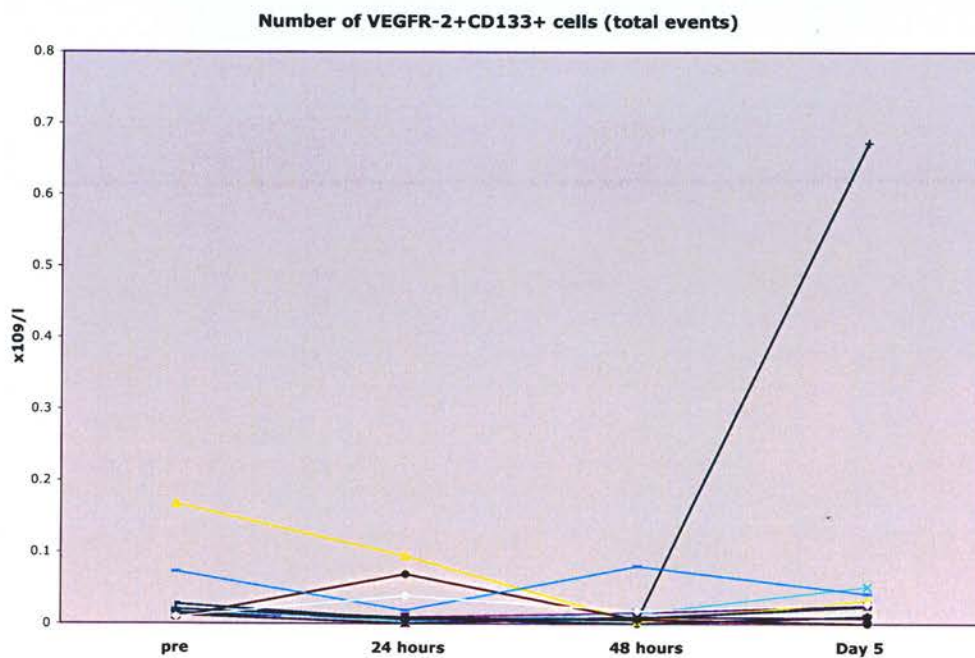


c.



d.

Figure 5.4. Effect of aortic aneurysm repair on EPCs, using dual staining of markers by flow cytometry. **(a).** When gated on all cells, the number of CD34+, VEGFR-2+ cells falls by 24 hours post operatively, before recovering to baseline by 48 hours, but this does not reach statistical significance ($p=0.02$). **(b).** When gated on all cells, the number of CD34-, VEGFR-2+ cells increases by 48 hours post operatively, before reducing back by day 5, but this does not reach statistical significance ($p=0.03$). **(c).** When gated on monocytes only, the number of CD34-, VEGFR-2+ cells does not vary significantly over the post operative period ($p=0.08$). **(d).** There is an increase in the number of CD133+, VEGFR-2+ cells by day 5 post operatively, when all cells are analysed, but this does not reach statistical significance ($p=0.03$).

EPC colony assay:

Samples from 10 patients were assessed in the colony assay. There was a transient reduction in the mean number of EPC colonies by 24 hours after the operation, but the median change in values did not reach statistical significance over the measured time period (**figure 5.5**).

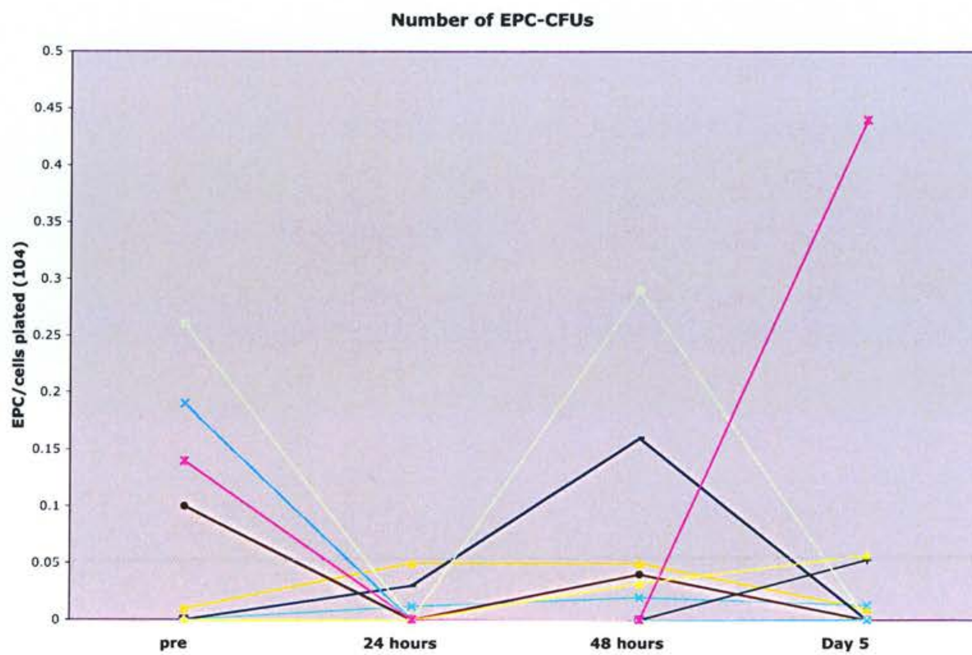


Figure 5.5. The median number of EPCs in culture is reduced by 24 hours after the operation, but this does not reach statistical significance (p=0.41).

5.4 Discussion

Endothelialisation of vascular grafts is important in order to provide a non-thrombogenic surface. Circulating EPCs are thought to play an important part in the vascular repair process in adults. Therefore, endogenous mobilisation of EPCs after aneurysm repair in humans is likely to play an important role in preventing potential graft induced thrombo-embolic events. The aim of this study was to investigate mobilisation of these cells, using patients undergoing elective abdominal aortic aneurysm repair with Dacron grafts as a model of vascular repair. Using three different techniques to investigate EPCs, we demonstrated that circulating EPC numbers did not differ significantly in patients pre-operatively compared to control subjects (**tables 5.1&2**). However, the circulating level of these progenitor cells was altered following elective abdominal aortic aneurysm repair.

The observation that there was no statistically significant difference in the level of circulating EPCs in pre-operative samples compared with control samples is perhaps surprising, knowing that reduced levels of circulating EPCs have been shown to be an independent risk factor for the progression of atherosclerotic disease [169, 170]. The lack of a difference in our study is likely to be due to a number of reasons. The patient and control groups were not age and sex matched. Both variables are known to affect EPC numbers, with older people having fewer circulating EPCs [56, 58, 171]. Despite our results showing a reduction in the number of EPC colonies in the patient group, the median age of this group was significantly higher than that of control group B, and this

difference is likely to have had an influence on the number of CFU-ECs counted in each group (**table 5.1**). In addition, we investigated a different patient group at a time when their vascular disease was clinically apparent. This is in contrast to the previous studies investigating cardiovascular outcome events in healthy patients at risk of coronary artery disease [169, 170].

The increase in the mean total white cell and monocyte counts observed following aortic aneurysm repair suggests that the bone marrow is being stimulated to produce cells, probably as part of an acute phase response (**figure 5.1a&b**). The data presented in **figures 5.2, 5.3 and 5.4** suggests that EPCs are being mobilised as part of this response. EPC mobilisation has been demonstrated previously in this patient group, reported by Eizawa et al. [172]. In this latter study, CD34 expression was noted to have doubled by day 7 following the operation, which is in contrast to our findings (**figure 5.2b**). One possible reason for this is that our study did not include this time point and, therefore, the subsequent increase may have been missed.

Cytokine mobilised EPCs are thought to comprise less that 0.1% of the total circulating mononuclear cell pool [18]. Therefore, even mobilised EPCs are few in number, resulting in low levels of detectable mRNA, and consequently difficulty in amplification of EPC mRNA as the PCR system reaches its limits of detection. This may explain why there were incomplete data sets for the RQs of the EPC mRNA species studied. This is particularly relevant to CD133 and VEGFR-2, where complete data sets were only achieved in 6 and 4 patients respectively. In contrast, only one patient failed to provide a

complete data set for CD14 mRNA, where CD14 is expressed on a wider population of cells and is known to be a marker of mature monocytes. Despite this, however, the increase seen in the RQs of VE-cadherin mRNA, and in the number of cells expressing surface CD133, suggests that EPCs are being mobilised as part of the overall bone marrow response to tissue injury (**figures 5.2d&g**). In order to investigate phenotypic expression on EPCs further we looked at the mean fluorescent intensity of the markers studied, both in the total cell population and in the monocyte population only (**figure 5.3**). The reason that the monocyte population was studied is that there is emerging evidence that monocytes are a major source of EPCs [24, 150, 173]. The MFIs of VEGFR-2 and Cadherin-cadherin were found to increase following the operation, both in the total cell population and in the monocyte population, adding further evidence in favour of EPC mobilisation following aortic aneurysm repair (**figures 5.3e, f, g, h**). We also looked at cells by flow cytometry that were CD34-, VEGFR-2+ and CD34+, VEGFR-2+ (**figure 5.4**). It was found that the number of CD34-, VEGFR-2+ cells increased following the operation, when the total cell population was analysed (**figure 5.4b**). In contrast, the number of CD34+, VEGFR-2+ cells decreased transiently after the procedure (**figure 5.4a**). Unfortunately, we did not perform dual staining using CD14 and VEGFR-2, which is thought to be the specific subset of monocytes responsible for endothelial-like functional capacity [25]. However, taken together this suggests that EPCs are being mobilised from a CD34- pool of cells, which may be monocytic in origin. The observation that there is a significant correlation between the monocyte count and the number of VEGFR-2 + cells in the patient group would seem to agree with this (**table 5.3**).

If CD34 is a marker of EPCs, it is difficult to reconcile the reduction seen in the RQ of CD34 mRNA and the surface expression of CD34+, VEGFR-2+ cells with mobilisation of EPCs, where one would expect to see an increase (**figures 5.2a&b, figure 5.4a**). One reason for this could be that circulating EPCs are being sequestered in the immediate post-operative period to the site of vascular injury, followed by a return to baseline levels as further cells are mobilised and released from the bone marrow (or, just possibly, as the sequestered cells are released back into the circulation). This would also explain the transient trend in reduction seen in the number of EPC colonies (**figure 5.5**). It is important to note, however, that CD34 is not a marker that is specific for EPCs, but is also expressed by haematopoietic stem cells and by mature endothelial cells. This was a limiting factor in the study by Eizawa et al, where CD34 alone was used as a marker of EPCs [172]. It could, therefore, be that there is sequestration of CD34+ cells and that these cells are not EPCs but mature endothelial cells. This would explain the transient reduction in the RQ of vWF mRNA observed in the immediate post-operative period (**figure 5.2i**). In addition, it would explain why there is a statistically significant correlation between the RQs of vWF and CD34 mRNA species (**table 5.3**).

As previously discussed, it has been discovered that the monocyte population in blood is the main source of EPCs, rather than CD34+ progenitors as previously thought. An interesting observation of the flow cytometry data was that in the patient group approximately 90% of monocytes were positive for VE-cadherin (**figure 5.6**). In addition, there were significant correlations found between the monocyte counts and VE-cadherin levels (by PCR and flow cytometry), and between CD14 and VE-cadherin

mRNA RQs, but not between CD14 and CD34 mRNA RQs (**figure 5.7, table 5.3**). This suggests that Cadherin-cadherin is not specific to endothelial cells as previously thought, but can also be expressed on monocytes. Furthermore, it could further demonstrate the monocytic origin of EPCs.

In summary, we have demonstrated that a major vascular insult, such as aortic aneurysm repair, results in the mobilisation of EPCs. Furthermore, these EPCs are likely to be derived from the monocyte pool. Previous work has focussed on the time to mobilisation of EPCs following a particular stimulus, or in-vitro maturation of EPCs. Our data gives a more dynamic picture of how EPCs respond to a major vascular insult. It also highlights the difficulty in measuring these cells using different techniques. This is presumably due to a combination of small patient numbers, sensitivity of the techniques used to measure this small population of cells, and the lack of consensus on an appropriate definition of an EPC. Further work will involve recruiting more patients to the study in order to extend our preliminary data, and to investigate whether some of the trends observed can be better characterised. For example, the changes seen in the numbers of EPCs in the colony assay, and the change in the RQ of VEGFR-2 over the period of the study.

Correlation	**R^2 value**	**P value**
Monocyte count *vs* CD14 mRNA RQ	0.1329	0.0057
Monocyte count *vs* VE-cadherin mRNA RQ	0.0848	0.0295
Monocyte count *vs* number of VE-cadherin+ cells	0.214	0.0035
Monocyte count *vs* number of VEGFR-2+ cells	0.2656	0.0007
CD14 mRNA RQ *vs* VE-cadherin mRNA RQ	0.3579	<0.0001
CD14 mRNA RQ *vs* CD34 mRNA RQ	$4x10^{-6}$	0.9892
VWF mRNA RQ *vs* CD34 mRNA RQ	0.1835	0.003

Table 5.3. Correlation studies.

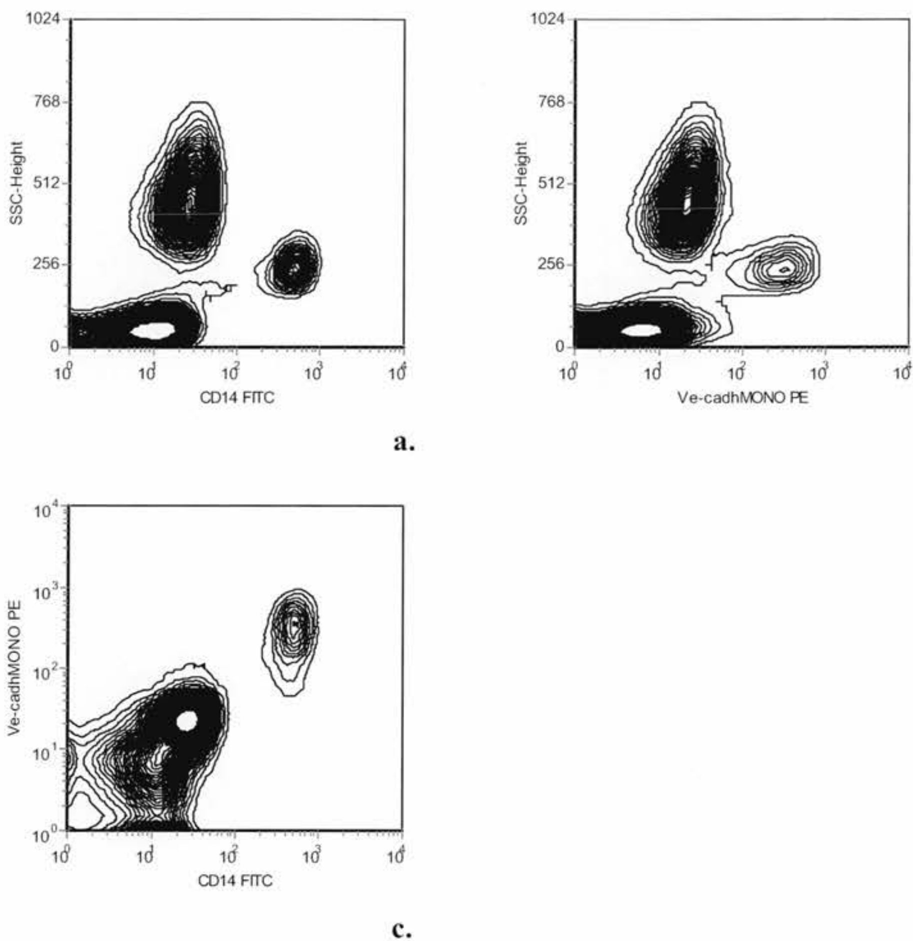


Figure 5.6. The leukocyte fraction was isolated from a healthy blood donor buffy coat. Cells were gated on all leukocytes (CD45+). **(a).** Side-scatter versus CD14. **(b).** Side-scatter versus VE-cadherin. **(c).** CD14 versus VE-cadherin. Most of the CD14+ monocytes are VE-cadherin positive.

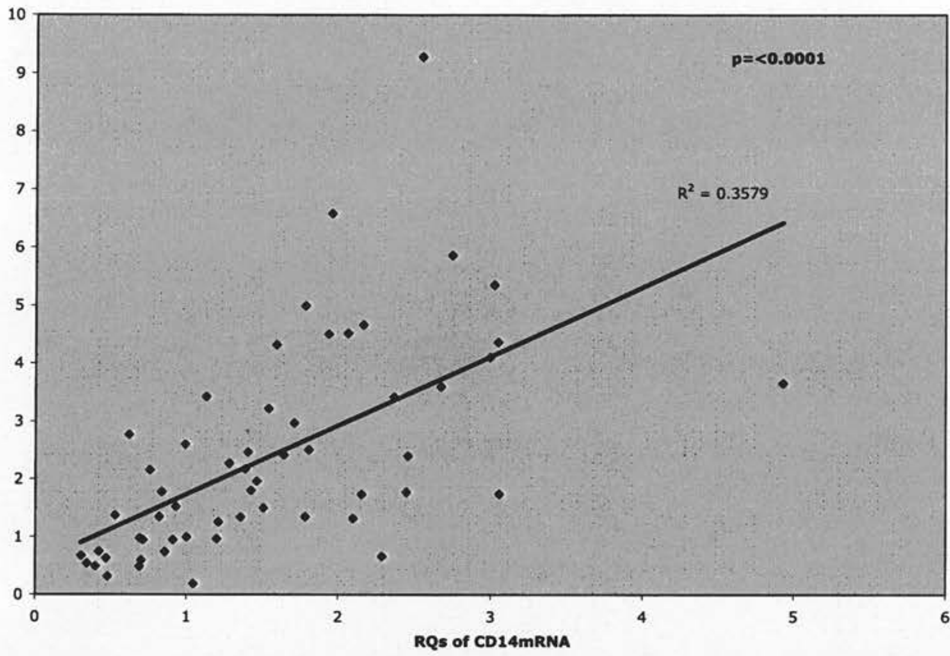


Figure 5.7. Graph showing a statistically significant correlation between CD14 mRNA RQs and VE-cadherin mRNA RQs (p<0.0001).

6. ENDOTHELIAL PROGENITOR CELLS AND TYPE 1 DIABETES

6.1 Introduction

People who have type 1 diabetes experience deficient wound healing secondary to a lower capacity to form collateral vessels in areas of tissue ischaemia [174]. Until recently the reasons for this were not known, but with the identification of bone marrow-derived endothelial progenitor cells (EPCs), it is now thought that the quantitative and qualitative changes seen in this cell group in people both with type 1 and type 2 diabetes are critical to the process of impaired neovascularisation [3, 80, 141, 175]. EPCs have been characterised by a number of methods. The most widely used are measurement of the phenotypic expression of early (CD34, VEGFR2 and VE-cadherin) and late (VE-cadherin and vWF) EPC markers, by the ability of EPCs to form mature endothelial cells in culture, and demonstration of EPC incorporation into new vessels in animal models of hind limb ischaemia [176]. The aim of this study was to investigate the effect of type 1 diabetes on EPCs, using real-time PCR as a novel method of assessment, in addition to the established methods of flow cytometry and cell culture.

6.2 Methods

EDTA anticoagulated blood samples (5mls) were collected from 45 people with type 1 diabetes attending the diabetes outpatient clinics at the Royal Infirmary of Edinburgh and from 34 non-diabetic subjects (control group A). Approval was obtained from the local medical research ethics committee (Lothian), and written informed consent was obtained from each participant. Glycosylated haemoglobin (as HbA1c) was assayed

using high-performance liquid chromatography adjusted to the Diabetes Control and Complications Trial; the local non-diabetic range was 5.0-6.5%. Total leukocyte RNA (1μg) was extracted from 1ml of blood using an extraction kit (Qiagen, Valencia, CA, USA) according to the protocol in **section 2.1**, and transcribed into complementary DNA using random hexamers (Promega, Madison, WI, USA), as described in **section 2.2**. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed on an ABI PRISM 7900 Sequence Detector (Applied Biosystems, Warrington, England) using gene expression kits, containing primers and TaqMan probes for amplification of CD34, VEGFR-2, VE-cadherin and vWF mRNA species (Applied Biosystems, Foster City, CA, USA) (**section 2.3**). Ribosomal 18s was used as the endogenous control. The relative quantity of each mRNA species was calculated as previously described [177].

In a parallel study, performed at a different laboratory, 100μl of the EDTA sample from each of 21 of the patients with type 1 diabetes and from 15 different non-diabetic control subjects (control group B) was directly stained and analysed for the phenotypic expression of CD34, VEGFR2 and VE-cadherin using anti-CD34-FITC (Becton Dickinson, Oxford, UK), anti-VEGFR2-PE (R&D systems), and anti-VE-cadherin-PE labelled monoclonal antibodies (see **section 2.5**). Appropriate negative controls (isotype and/or no antibody) were used to establish positive stain boundaries, and 50.000 events were counted and measured by flow cytometry using a FACS-Calibur flow cytometer (Becton-Dickinson, Oxford, UK). The percentage of cells positive for a particular marker was determined from the events counted.

In addition, 2ml of blood from 11 of the people with type 1 diabetes and from the 15 non-diabetic subjects (above) was subjected to centrifugation over a Ficoll gradient to separate out the mononuclear cell fraction (see **section 2.4**). The pelleted cells were resuspended in Endocult (composed from Endocult basal medium plus a 1/5 dilution of Endocult supplements) (Stem Cell Technologies, UK) and cultured on fibronectin using the same technique described by Hill et al [12]. At day 5 of the assay the EPC colony forming units (EPC-CFU) per well were counted and the number of EPC-CFUs per cells plated was calculated.

6.3 Results

No significant differences were found in the median RQ values of CD34, VEGFR2 and vWF mRNA species between patients with type 1 diabetes and control group A (**figure 6.1a-c, table 6.1**). However, the patient group had a significantly lower median RQ of VE-cadherin mRNA compared with the control group (**figure 6.1d**). Furthermore, no differences were found in the RQ values in patients with or without microvascular disease or in patients with poorly controlled diabetes (**table 6.1**). A significant positive correlation was found between vWF mRNA RQs and duration of diabetes (**figure 6.2**). However, no correlations were found between the RQs of the mRNA species of interest and HbA_{1c} levels (data not shown).

The patient group had a significantly lower median percentage of cells positive for CD34 compared to control group B (**figure 6.3**). However, there were no differences

found between the two groups for the other markers or for the percentage of cells expressing both CD34 and VEGFR2 (**table 6.2**).

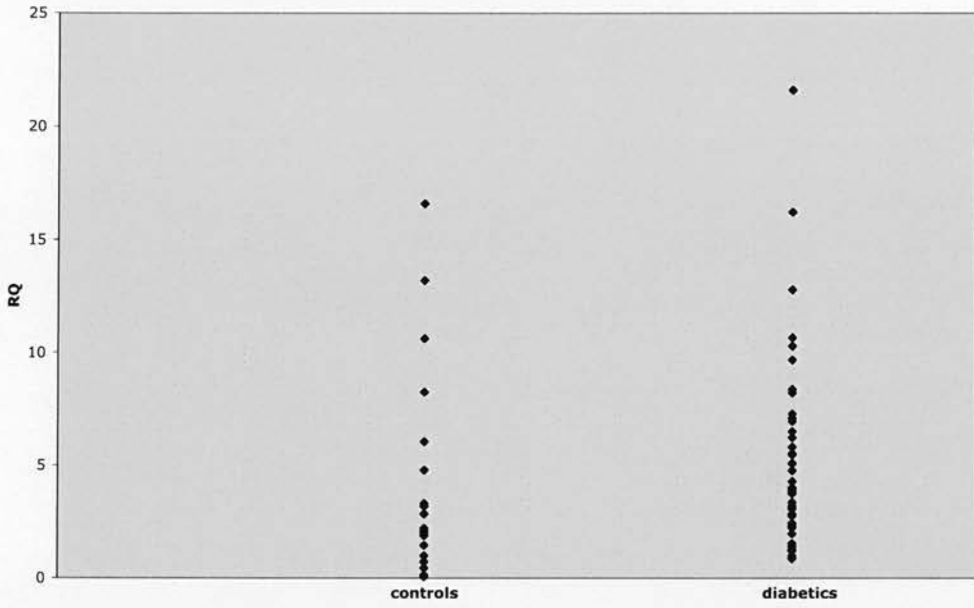
The median number of EPCs detected in control group B was significantly higher than in the patient group (**figure 6.4**).

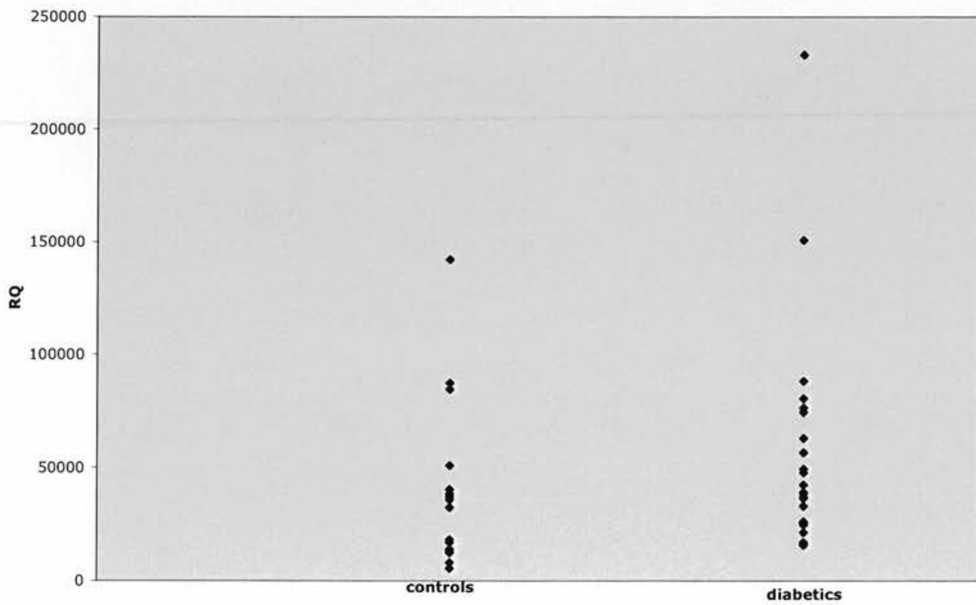
	Non-diabetic controls (A)	**Interquartile ranges**	**People with type 1 diabetes**	**Interquartile ranges**	**p value**
Number	37	-	45	-	-
Mean Age	40.9	-	37.4	-	0.24
CD34 mRNA RQ	3.660	1.897-8.022	4.015	2.452-6.518	0.5360
MVD+ MVD-	-	-	3.830 4.156	1.973-5.491 3.00-7.00	0.35
MVD-, HbA_{1C}>8 MVD-, HbA_{1C}<8	-	-	4.115 5.090	2.640-5.997 3.204-9.699	0.33
VEGFR2 mRNA RQ	1.302	0.541-2.660	1.487	1.018-2.603	0.3281
MVD+ MVD-	-	-	1.403 1.608	0.905-2.805 1.083-2.212	0.8773
MVD-, HbA_{1C}>8 MVD-, HbA_{1C}<8	-		0.8283 2.29	0.739-1.201 1.445-4.437	0.2571
VE-cadherin mRNA RQ	2.919	1.577-7.519	2.244	1.373-3.256	0.0435
MVD+ MVD-	-	-	2.930 1.997	1.854-3.447 1.369-2.773	0.1972
MVD-, HbA_{1C}>8 MVD-, HbA_{1C}<8	-	-	1.892 2.244	1.499-2.421 1.099-2.803	0.81
VWF mRNA RQ	2.170	1.249-3.595	2.597	1.474-4.229	0.18
MVD+ MVD-	-	-	2.230 3.149	2.033-3.067 1.303-4.805	0.48
MVD-, HbA_{1C}>8 MVD-, HbA_{1C}<8	-	-	2.091 3.596	1.303-4.362 2.602-4.946	0.42

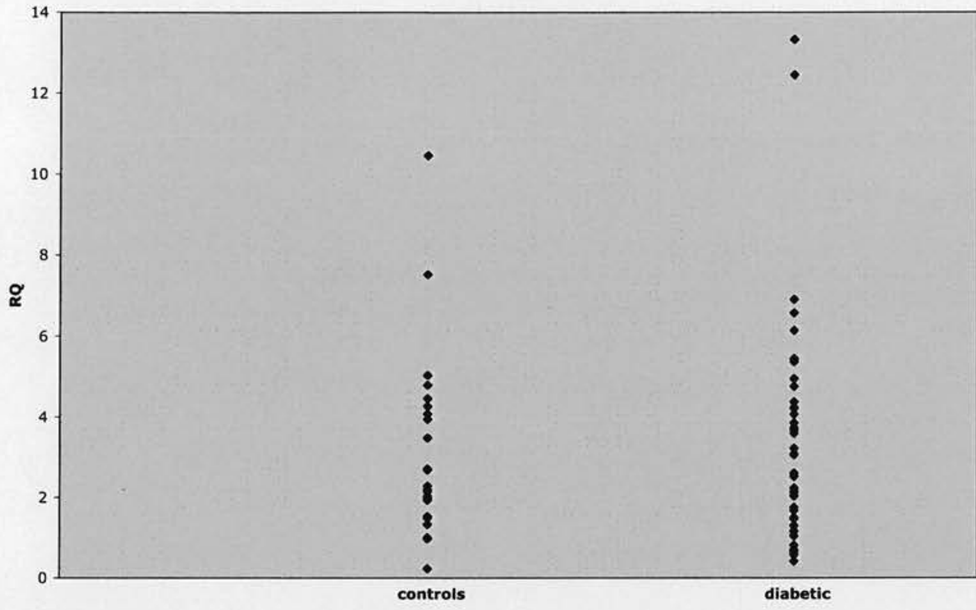
Table 6.1. Comparison between the diabetic group and non-diabetic group A of the markers of EPCs detected by real-time PCR. Results are expressed as medians with interquartile ranges. The unpaired t-test was used to compare mean ages of patient and control group. **MVD**: microvascular disease. **HbA1c**: Haemoglobin A1c.

	Non-diabetic controls (B)	Interquartile ranges	People with type 1 diabetes	Interquartile ranges	p value
Number	15	-	11 (cell culture) 21 (flow cytometry)		-
Mean Age	39.7	-	39.9 (cell culture) 34.7 (flow cytometry)		0.97 0.21
Median number of EPCs (per 10^4 cells)	0.23	0.16-0.335	0.032	0-0.059	0.004
Median %CD34+ cells	0.06	0.05-0.09	0.02	0.02-0.05	0.003
Median %VEGFR2+ cells	1.89	0.885-3.06	4	1.21-32.33	0.14
Median %VE-cadherin+ cells	10.55	6.865-13.11	12.6	8.38-16.98	0.26
Median % CD34+VEGFR2+ cells	24.39	5.73-37.345	15.08	0.222-43.5	0.35

Table 6.2. Comparison between the diabetic group and non-diabetic group B of the number of EPCs detected by flow cytometry and cell culture. Results are expressed as medians with interquartile ranges. The unpaired t-test was used to compare mean ages of patients and control group.


CD34 mRNA RQ controls v diabetics
RQ
25
20
15
10
5
0
controls
diabetics
a.


VEGFR2 mRNA RQ controls v diabetics
RQ
250000
200000
150000
100000
50000
0
controls
diabetics
b.


vWF mRNA RQ controls v diabetics
RQ
14
12
10
8
6
4
2
0
controls
diabetic
c.

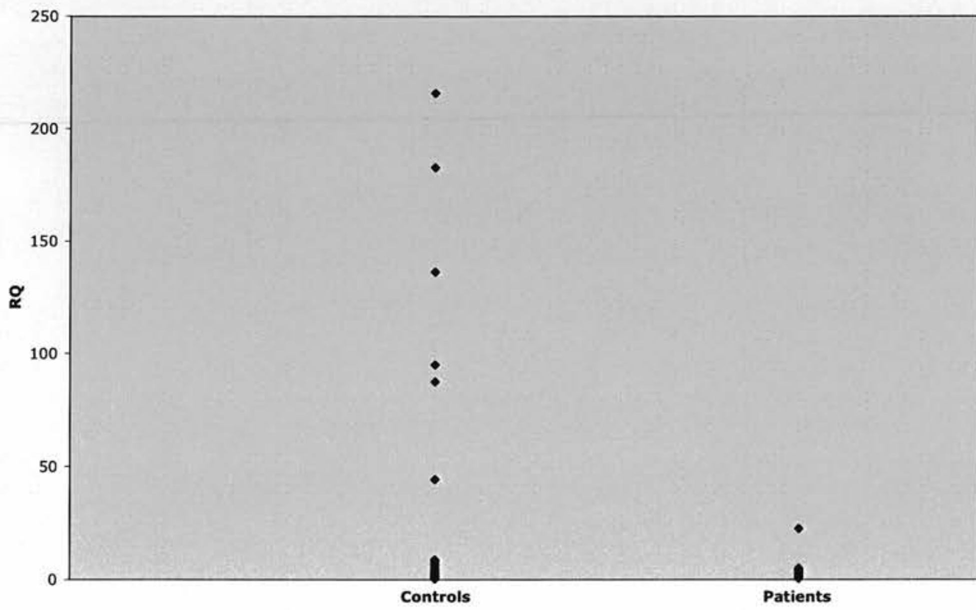

VE-cadherin mRNA RQ controls v diabetics
RQ
250
200
150
100
50
0
Controls
Patients
d.

Figure 6.1. The relative quantities of CD34 mRNA **(a)**, VEGFR-2 mRNA **(b)**, vWF mRNA **(c)** and VE-cadherin mRNA **(d)** in the group with diabetes compared to control group A. The median RQ of VE-cadherin mRNA was significantly reduced in the group with diabetes compared to non-diabetic group A (2.24 versus 2.9, $p<0.05$, **figure d**)

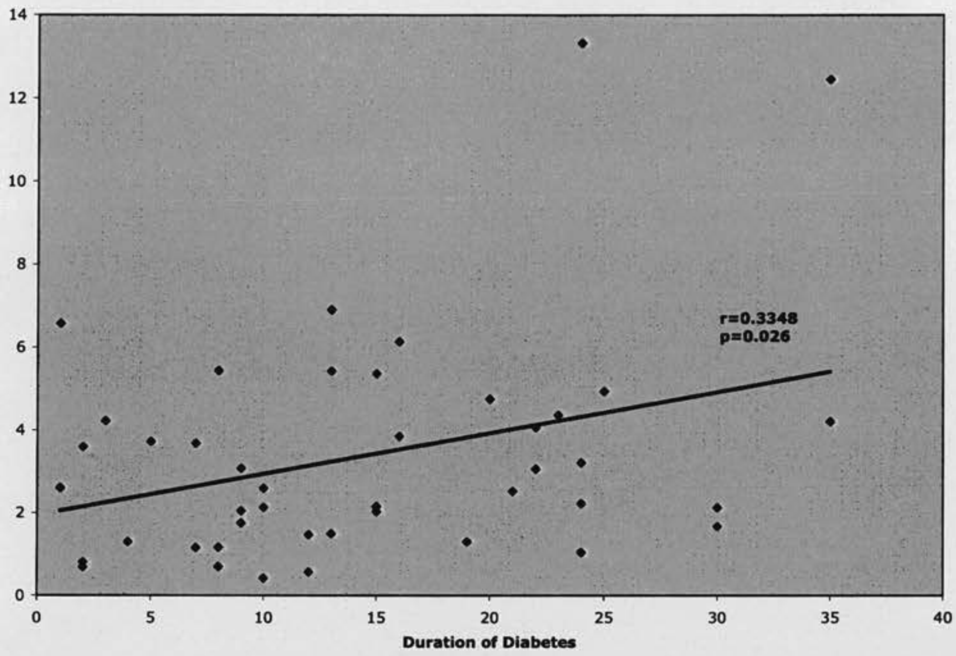


Figure 6.2. A positive correlation was observed between relative vWF mRNA level and duration of type 1 diabetes. This correlation was not related to age (see text).

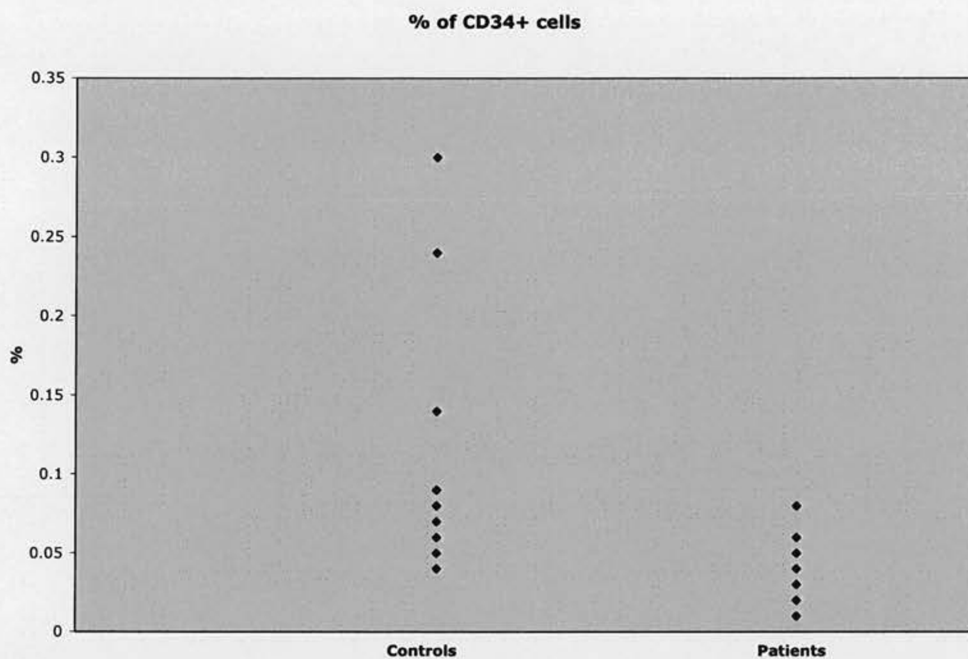


Figure 6.3. The percentage of cells expressing CD34 was significantly lower in the diabetic group compared to non-diabetic group B (0.02% versus 0.06%, $p<0.0005$).

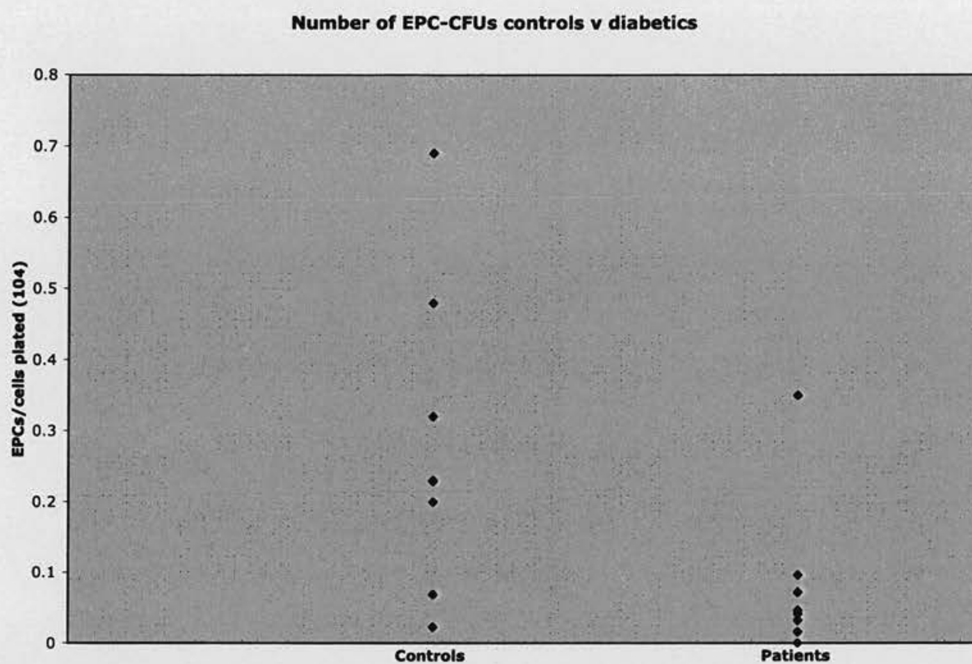


Figure 6.4. The number of EPCs able to form endothelial colonies was significantly less in the diabetic group compared to non-diabetic group B (0.032×10^4 versus 0.23×10^4 cells plated, $p<0.005$).

6.4 Discussion

The present study has shown that the group of people with type 1 diabetes had lower amounts of VE-cadherin mRNA, a reduced percentage of CD34+ cells, and lower numbers of EPC-CFUs in comparison with non-diabetic groups. VE-cadherin is an adhesion molecule involved in angiogenesis and is specific to vascular endothelium [178]. Moreover, it is thought to be a marker of mature EPCs [165]. Reduction in the RQ of VE-cadherin mRNA may reflect a lower number of EPCs in the diabetic group.

It has been demonstrated previously that the number of CD34+ cells extracted from people with type 1 diabetes did not differ significantly from the number of CD34+ cells extracted from non-diabetic control subjects [179]. Our findings contrast with this observation. The discrepancy may, in part, be a consequence of a lack of effective matching between diabetic and non-diabetic groups in the present study. In addition, Schatteman et al used a different technique for quantification of CD34+ cells and quoted absolute numbers instead of percentages [179]. However, with data showing that CD34+ cells accelerate vascularisation and healing in diabetic mouse skin wounds, the lower percentage of CD34+ cells observed in the present group of diabetic patients may have functional relevance to the number of EPCs capable of participating in neovascularisation [180]. This observation is strengthened by the present data which has demonstrated fewer EPC-CFUs in culture compared to non-diabetic controls, which is consistent with previous results [80, 179].

Although a significant difference in vWF mRNA RQ was not demonstrable between the diabetic and non-diabetic groups, a direct correlation between vWF mRNA RQ and duration of diabetes was observed in the patient group (**figure 6.2**). This was not apparent when vWF mRNA RQ and age in the non-diabetic subjects were compared ($r=0.30$, $p=0.07$), implying that age itself was not the factor affecting vWF mRNA levels. This is perhaps unsurprising as vWF is known to be a surrogate marker of endothelial damage, and can predict the development of microvascular disease in patients with diabetes [181, 182].

The major limitations of the present study were the lack of reproducibility of results using markers of EPCs other than VE-cadherin and CD34 (**tables 6.1&6.2**), and the lack of correlation between the different methods of analysis of the same marker. For example, although a significant reduction occurred in the percentage of CD34+ cells in the diabetic group, this was not reflected in the PCR data for CD34. One possible explanation for this is that two different non-diabetic groups were used. It highlights, however, the difficulty in measuring EPCs, which comprise a very small subset of the circulating cell pool.

In summary, using a combination of three different techniques, the present study suggests that circulating EPC numbers are lower than normal in people with type 1 diabetes. This may have important functional consequences in relation to their ability to form collateral vessels as part of the vascular repair mechanism.

7. ENDOTHELIAL PROGENITOR CELLS AND CHRONIC RENAL FAILURE

7.1 Introduction

Patients with renal disease are known to have high morbidity and mortality rates from cardiovascular disorders secondary to atherosclerosis, such as myocardial infarction and stroke [183]. There are a number of potential reasons for this, but the process of impaired vascular repair secondary to reduced numbers and/or impaired function of EPCs as a cause has become a focus for further research. It has recently been demonstrated that the number of EPCs in peripheral blood is significantly reduced in patients with advanced renal failure when compared to age- and sex- matched controls [82]. This has also been found to be the case in patients on maintenance haemodialysis [81, 184]. Moreover, it has been observed that uraemic serum can exert an inhibitory effect on the differentiation capacity of EPCs in vitro, and on their capacity to migrate and form tube-like (vascular) structures [82]. One contributory factor to EPC deficiency in patients with renal disease could be the lack of erythropoietin (Epo) [185]. EPO is an 18-kDa protein consisting of a 166-amino-acid polypeptide chain linked by two disulphide bonds. It undergoes post-translational glycosylation, which is essential for its biological activity. It is encoded on a gene on the long arm of chromosome 7 and, in adult life; this is expressed by the peritubular fibroblast-like interstitial cells of the kidney. Epo circulates to the bone marrow and binds to Epo receptors (EpoRs) on erythroid precursors [186]. Mature endothelial cells have also been shown to express EpoRs [187]. Furthermore, Epo has been shown to induce a proangiogenic response in cultivated mature endothelial cells, as evidenced by stimulation of endothelial cell proliferation, migration, endothelin-1 release, and increase in cytosolic-free calcium

concentration [188-191]. With this background in mind, this study has been designed to assess the effect of Epo on EPCs, by measuring changes in the RQ values of EPC mRNA in patients with chronic renal failure who are commenced on recombinant human Epo for the treatment of renal anaemia [192].

7.2 Methods

Five patients with stable CRF, who were felt to be candidates for EPO, were identified. There were three males and two females, with a mean age of 65 years and a mean serum creatinine of 327μmol/l. None of the patients had suffered form a recent acute coronary syndrome or systemic inflammatory condition. 5ml of EDTA anticoagulated blood was collected from each patient before they were commenced on EPO (Neorecormon®, with dose ranges from 4000 to 8000units/week), and then at serial time points up to 8 weeks after commencement of therapy.

Total leukocyte RNA was extracted from 2.5ml of each sample using a standard extraction kit (Qiagen) (**section 2.1**). The amount of RNA was determined by optical densitometry. The RNA was then reverse transcribed using reverse transcriptase (Promega), and the complementary DNA used in a real-time PCR system (**section 2.2**). Primer-probe sets for CD34, VEGFR-2 and von-Willebrand factor (vWF) were purchased through Applied Biosystems "assays-on-demand" (Warrington, UK), and the 96 well plates run on an ABI 7900HT thermal cycler. The endogenous control used was

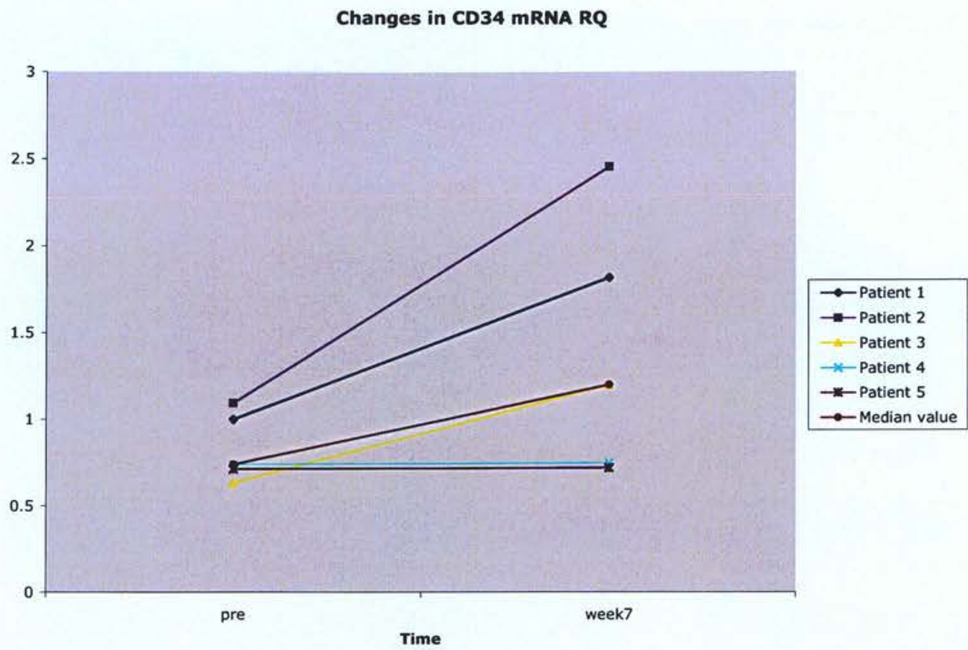
ribosomal 18s. Data generated was used to calculate the RQ of each mRNA species at each time point (**sections 2.3 & 3.3**).

The remainder of each sample was spun down to separate off the plasma fraction, which was then used in a quantitative sandwich enzyme immunoassay system (R&D Systems) to measure the amount of VEGF at each time point (**section 2.6**).

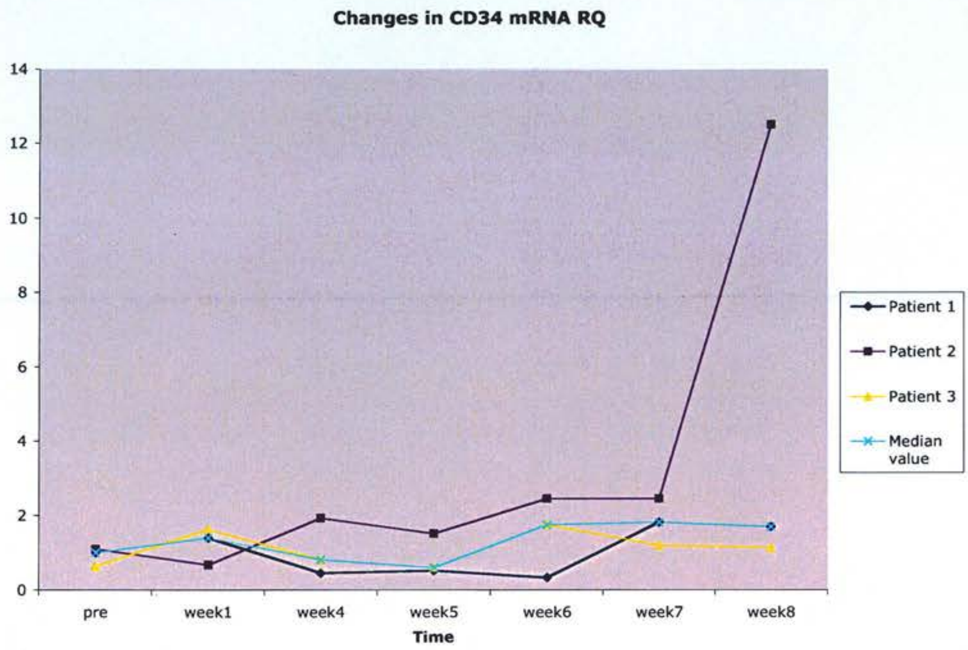
7.3 Results

CD34						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	1.00	1.09	0.63	0.73	0.71	0.73
Week1	1.40	0.67	1.63			1.40
Week2	1.11	1.08	-			1.09
Week3	0.68	-	1.05			0.86
Week4	0.45	1.93	0.81			0.81
Week5	0.51	1.51	0.59			0.59
Week6	0.33	2.44	1.75			1.75
Week7	1.81	2.45	1.19	0.74	0.71	1.19
Week8	1.69	12.49	1.13			1.69

Table 7.1. Compared to baseline there is an increase in the median CD34 mRNA level of 62% (p=0.06) by week 7 after commencement of Epo. This is depicted graphically in **Figures 7.1a&b**. The peak value was observed at week 8 after starting Epo.



a.



b.

Figure 7.1

CD133						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	1.00	-	0.38	-	-	0.69
Week1	0.49	0.34	1.11			0.49
Week2	0.33	-	-			0.33
Week3	-	-	0.59			0.59
Week4	-	0.46	0.67			0.57
Week5	0.66	0.18	-			0.42
Week6	-	0.90	0.19			0.54
Week7	0.65	0.40	-	-	-	0.53
Week8	0.73	-	0.22			0.48

Table 7.2. Compared to baseline there appears to be very little change in the level of CD133 mRNA. Too few data points were present for statistical analysis. This is represented graphically in **Figure 7.2**.

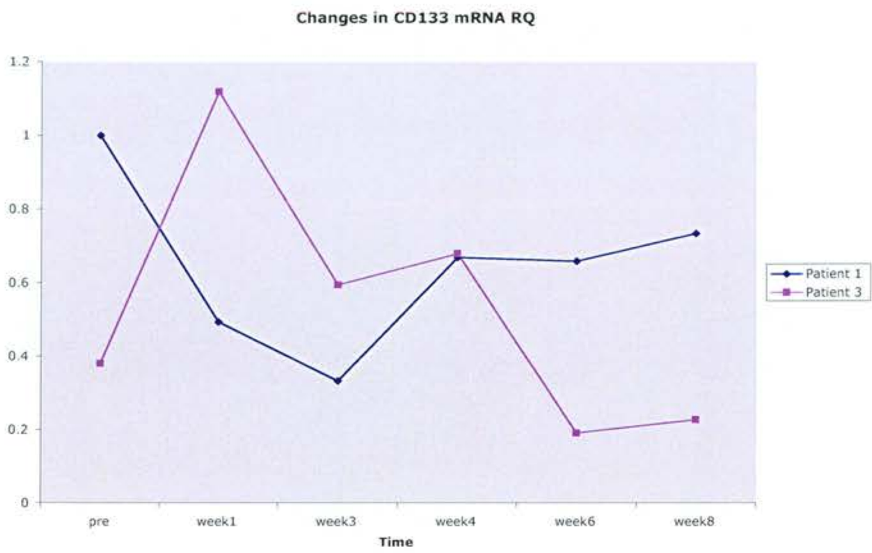
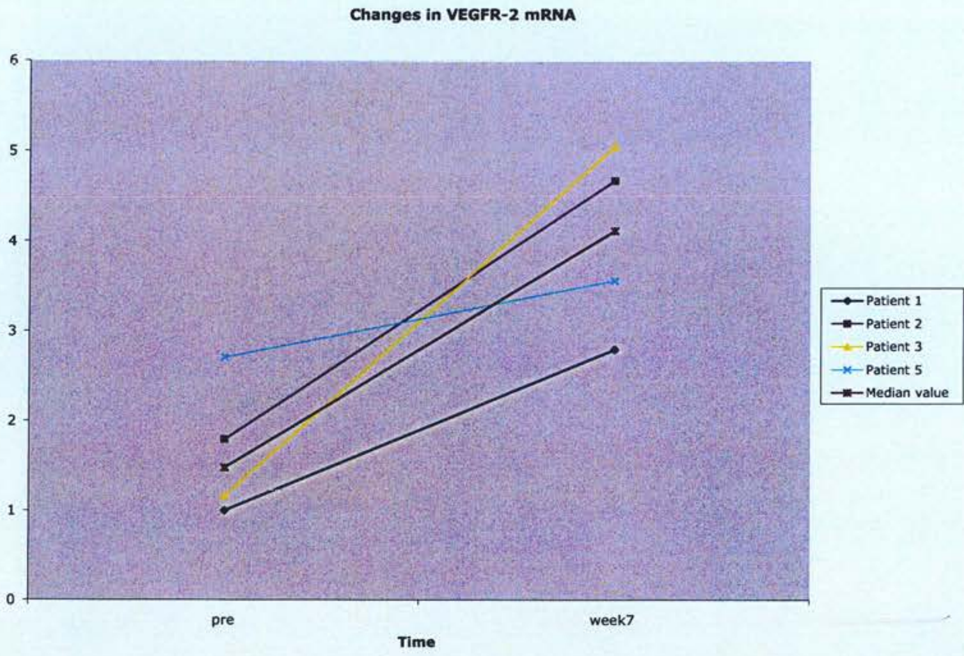


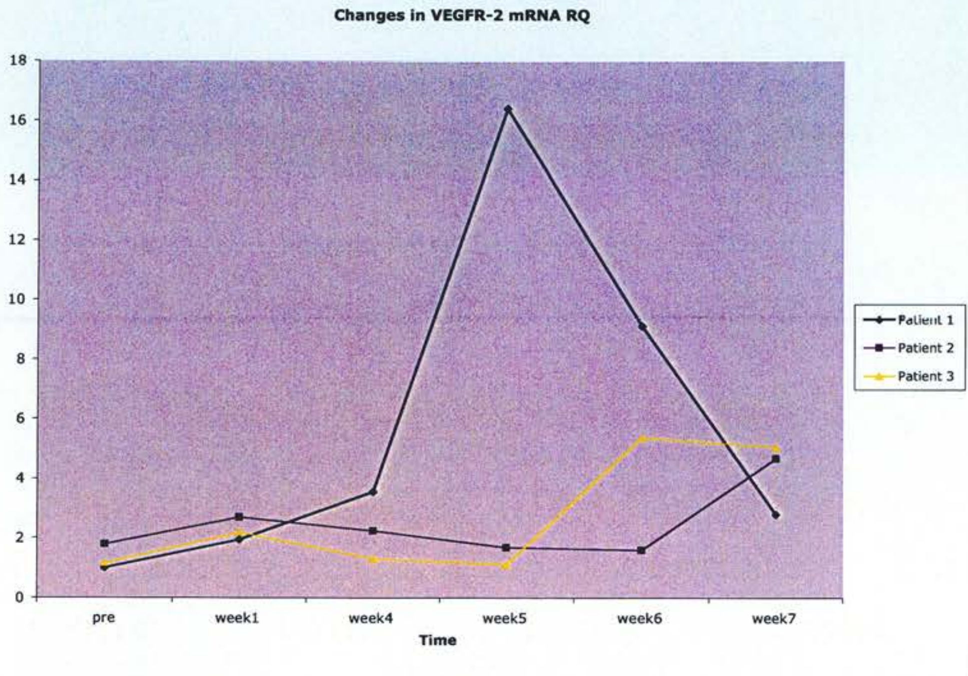
Figure 7.2

VEGFR-2						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	1.00	1.79	1.16	-	2.70	1.47
Week1	1.94	2.69	2.19			2.19
Week2	2.10	1.39	-			1.75
Week3	5.89	-	2.51			4.20
Week4	3.55	2.24	1.30			2.24
Week5	16.39	1.68	1.11			1.68
Week6	9.11	1.59	5.36			5.36
Week7	2.79	4.67	5.05	-	3.55	4.11
Week8	1.27	-	1.42			1.35

Table 7.3. Compared to baseline there is an increase in the median level of VEGFR-2 mRNA of 179% by week 7 after commencement of Epo ($p<0.05$). The peak increase appears to be by week 6 after starting Epo. This is depicted graphically in **Figures 7.3a&b**.



a.

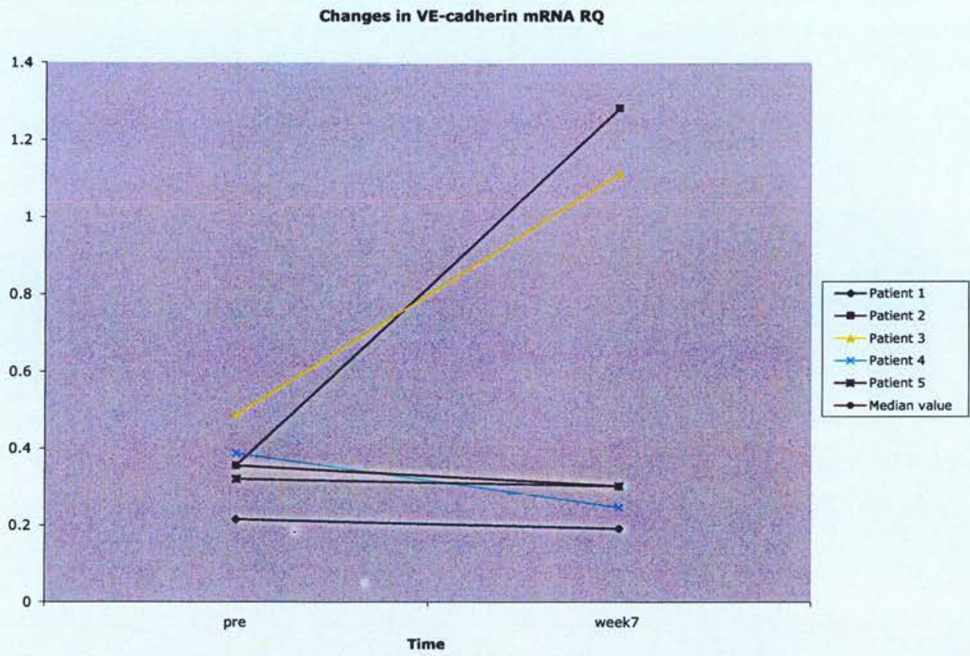


b.

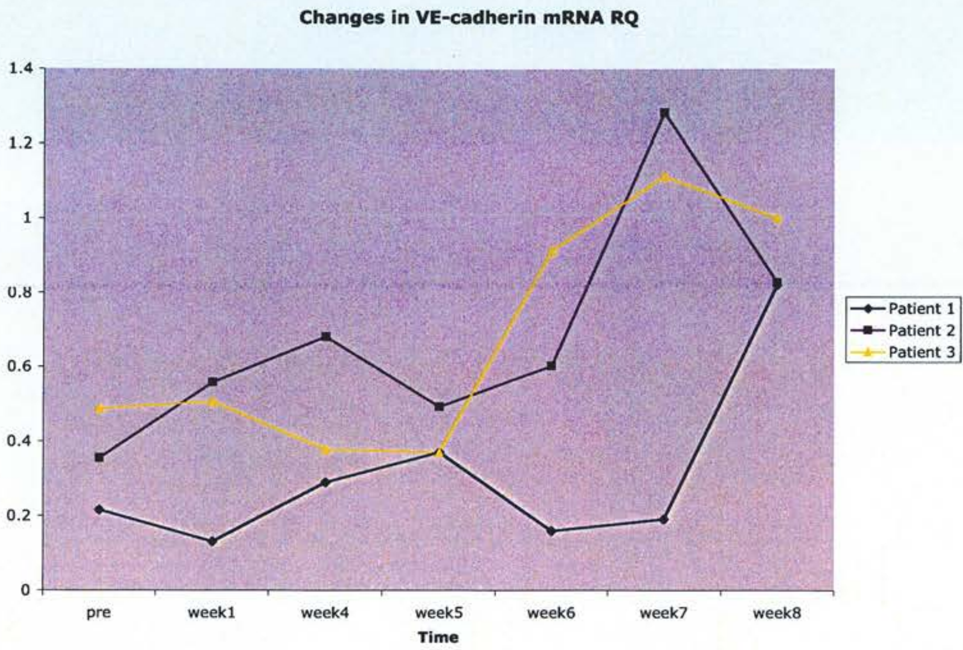
Figure 7.3

VE-cadherin						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	0.21	0.35	0.48	0.38	0.32	0.35
Week1	0.13	0.55	0.50			0.50
Week2	0.32	0.48	-			0.40
Week3	-	-	0.95			0.95
Week4	0.28	0.68	0.37			0.37
Week5	0.37	0.49	0.37			0.37
Week6	0.15	0.60	0.91			0.60
Week7	0.19	1.28	1.11	0.24	0.30	0.30
Week8	0.82	0.82	1.00			0.82

Table 7.4. Compared to baseline there is no change in the median value of VE-cadherin mRNA by week 7 after commencement of Epo (**figure 7.4a**). However, as depicted in **figure 7.4b**, there appears to be an upward trend in the level of VE-cadherin expression in patients 1, 2 and 3 by week 8 after starting Epo.



a.

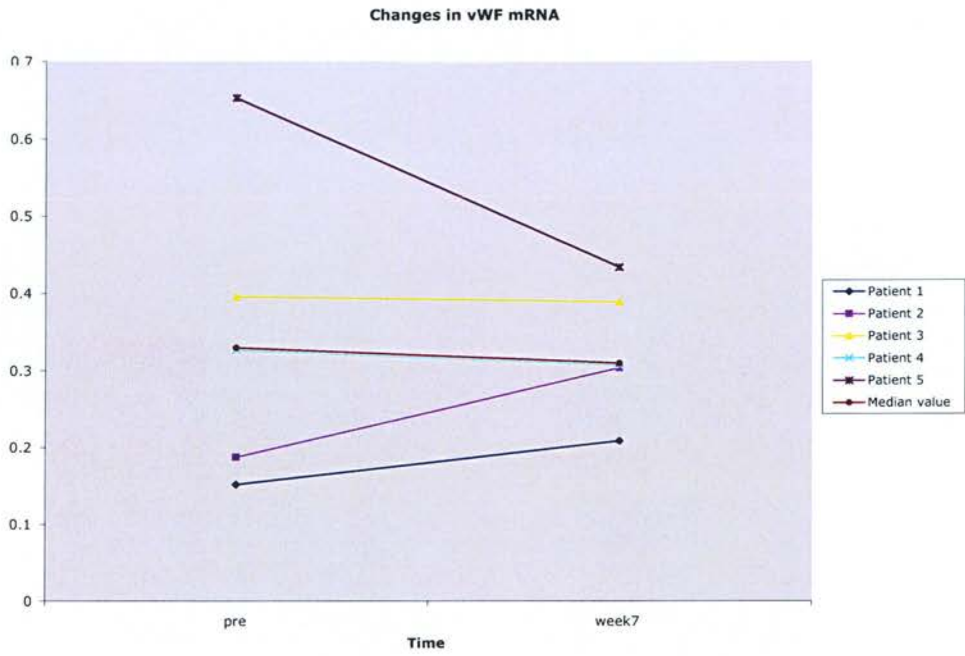


b.

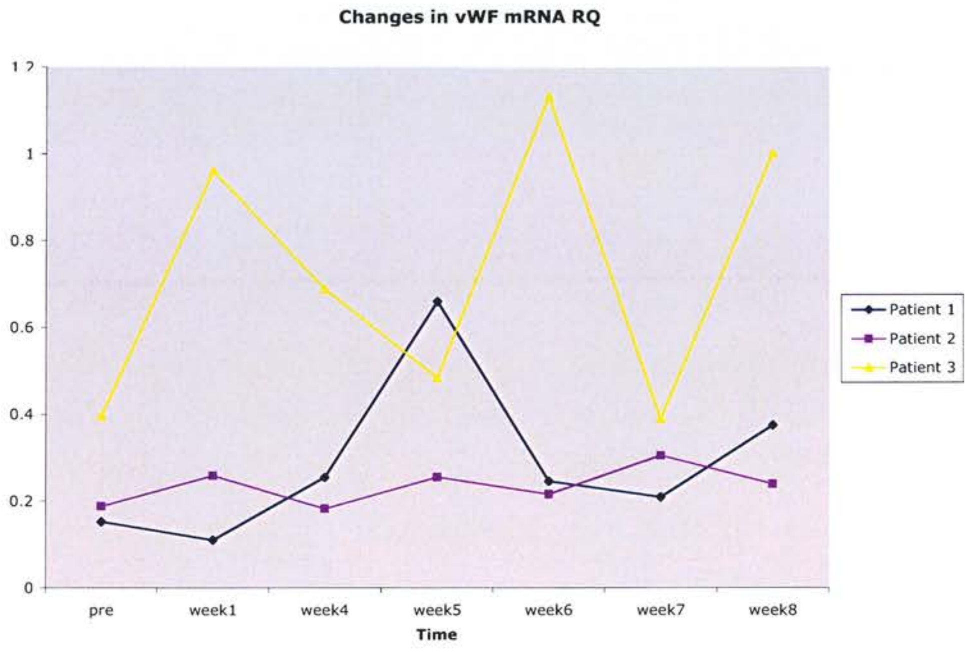
Figure 7.4

vWF						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	0.15	0.18	0.39	0.32	0.65	0.32
Week1	0.10	0.25	0.96			0.25
Week2	0.24	0.18	-			0.21
Week3	0.29	-	0.46			0.38
Week4	0.25	0.18	0.68			0.25
Week5	0.65	0.25	0.48			0.48
Week6	0.24	0.21	1.13			0.24
Week7	0.20	0.30	0.38	0.30	0.43	0.30
Week8	0.37	0.23	1			0.37

Table 7.5. Compared to baseline there is no change in the median value of vWF mRNA by week 7 after commencement of Epo. This is depicted graphically in **Figures 7.5a&b**.



a.

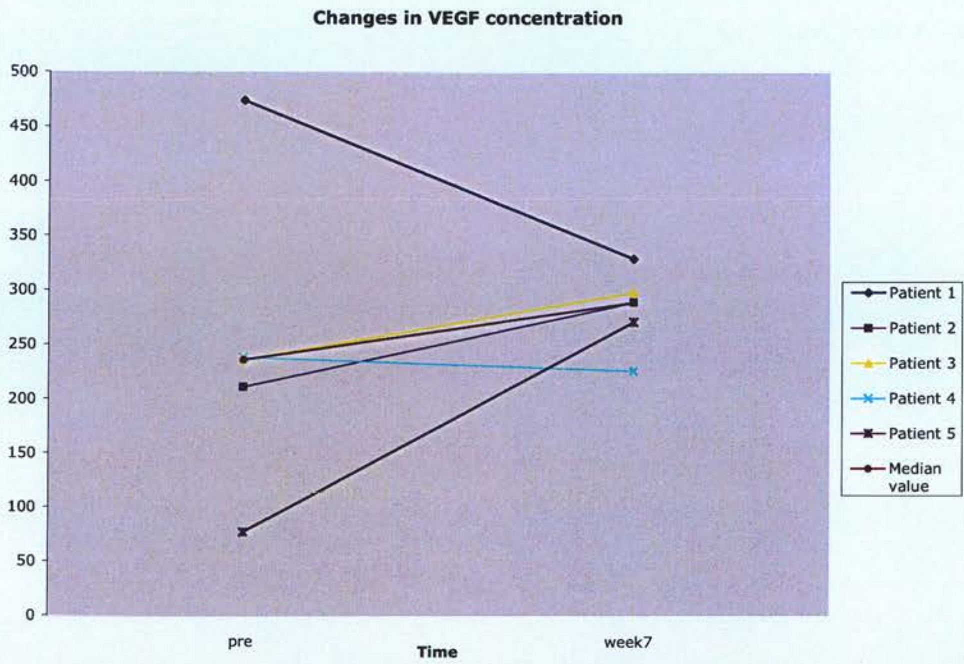


b.

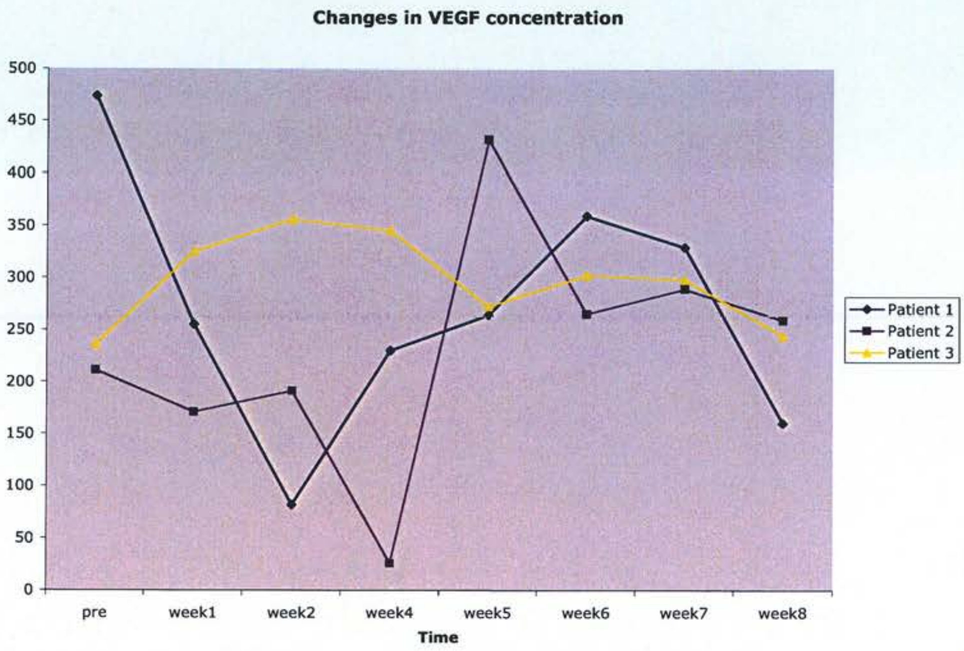
Figure 7.5

VEGF						
	Patient1	**Patient2**	**Patient3**	**Patient4**	**Patient5**	**Median Value**
Pre Epo	474	211	236	238	77	236
Week1	255	171	325			255
Week2	82	191	356			191
Week3	189	312	0			250
Week4	230	26	345			230
Week5	264	432	272			272
Week6	359	265	302			302
Week7	329	289	298	226	271	289
Week8	160	259	244			244

Table 7.6. Compared to baseline there was no significant change in the median value of VEGF by week 7 after the commencement of Epo. This is depicted graphically in **Figures 7.6a&b**.



a.



b.

Figure 7.6

7.4 Discussion

The effect of human recombinant Epo on the transcription of CD34, CD133, VEGFR-2 and VE-cadherin (all markers of EPCs) and vWF (as a marker of mature endothelial cells) has been investigated in a group of patients with chronic renal failure, using a PCR based technique for quantitation of mRNA for these proteins. In addition, the effect of Epo on the amount of plasma VEGF protein in the same patients has been investigated, using an ELISA based technique.

The number of patients recruited to this study was very small. In addition, we did not examine EPCs by flow cytometry or in cell culture in this study. As a result it was difficult to draw any firm conclusions from the results that could be based on solid statistical analysis. However, there appeared to be an increase in the median level of CD34 mRNA, although this change did not quite reach statistical significance (**figure 7.1**). In addition, there was a statistically significant increase in the median level of VEGFR-2 by week 7 after the commencement of Epo (**figure 7.3a**). Furthermore, there was an increase in the median level of VE-cadherin by week 8 of EPO treatment, but this did not reach statistical significance (**figure 7.4**). These changes in suggest that EPC levels may be increased following commencement of Epo. The lack of a significant change in the median amount of vWF mRNA would suggest that mature endothelial cells are not affected in the same way (**figure 7.5**). This would certainly fit with the current understanding regarding Epo and EPC stimulation, where Epo has been shown to be a powerful stimulatory agent for EPC proliferation and functional activity [90, 193,

194]. A marked and persistent stimulation of EPC recruitment in vitro and in vivo was demonstrable even at sub therapeutic doses of Epo when used to treat renal anaemia. In our study therapeutic doses of Neorecormon® were used. Our data did not show any change in the amount of plasma VEGF following commencement of Epo (**figure 7.6**). It has been known now for some time that VEGF is an important cytokine in the stimulation of EPCs form the bone marrow [31]. Our results suggest that EPCs may be mobilised by a VEGF independent route. This would fit with other groups who have demonstrated that Epo can directly stimulate EPCs via the Akt tyrosine kinase signalling pathway [90, 193, 195]. We could not demonstrate a change in the amount of CD133 mRNA (**figure 7.2**). This was due in part to the low subject numbers studied, but also to the fact that there was failure of amplification at may of the time points in each series. The reason for this is uncertain, but it may just reflect the very low level of PCR product from a cell that is found in low numbers in the periphery, resulting in a fall below the limit of detection in the system used. Another observation that is difficult to explain is the variation observed in the RQ of a particular marker in a patient over the period of the study. For example the variation in the amount of vWF mRNA in patient 3 (**figure 7.5b**), or the transient increase in the level of VEGFR-2 mRNA at week 5 following commencement of Epo in patient 1 (**figure 7.3b**). This may be due to natural variation in the level of these mRNA species, but may also be due to a transient vascular insult in the patient. Von-Willebrand factor, for example, has been shown to be a surrogate marker of vascular injury, and VEGFR-2 levels have been shown to increase following an episode of renal ischaemia-reperfusion [181, 182, 196, 197].

Stimulation of EPCs by Epo could represent a new therapeutic strategy in cardiovascular medicine in order to prevent the sequelae of atherosclerotic vascular disease. This approach may be particularly useful in patients with renal failure, a population at high risk of cardiovascular complications. In this respect, administration of Epo might be indicated at an earlier stage of renal failure than currently recommended.

8. CONCLUSION

Identification and enumeration of EPCs present in the adult remains difficult and non-standardised. The reasons for this are numerous, and may partly explain the discrepancy in results and lack of correlation between different assays supposedly measuring the same marker in this thesis. Primarily, it is unclear whether EPCs are truly bone marrow derived from primitive cells (a putative haemangioblast) or from differentiated cells (like monocytes) that acquire an endothelial cell-like phenotype [22, 23, 198]. Similarly, it remains to be clearly established to what degree vessel wall derived cells can contribute to the circulating pool of endothelial progenitors. For example, it has recently been shown that the vessel wall may also contain highly proliferative EPCs capable of forming colonies with re-plating potential (HPP-ECs) [199, 200]. Furthermore, the pathway of HSC maturation is not fully defined and it is possible that expression of CD34 and CD133 may be continuously up and down-regulated. It has been suggested that stem cells are continuously altering their phenotype and these alternations are reversible [201]. Thus, many described cell phenotypes could represent a single cell in different functional states. Finally, it has been suggested that rather than a hierarchical transition from stem cells to progenitor cells, fluctuation continues to exist in which stem cells adjust their phenotype depending on the cell kinetic state activated by their genetic program or micro environmental stimuli [202]. The data presented in this thesis also suggests that no single marker is truly specific for EPCs. Rather, a number of markers are likely to be needed. This is particularly important when utilising these marker combinations to best identify CEC and EPCs prior to clinical application of such cells.

No single assay appears to reliably measure EPCs, suggesting that either there are differences in the sensitivity of detection of a particular assay and/or that different cell populations are being detected. A limitation in using singleplex real-time PCR in the studies is that the assumption has to be made that each amplicon is being amplified from the same cell or cell population. This may not be the case, however. One potential way to confirm this would be to perform in-situ RT-PCR to detect gene expression directly within a cell population. With the knowledge that the putative population of EPCs is likely to be less than 1% of the leucocyte pool, a limitation of the flow cytometry assay used in the studies is that 50,000 events may not have been a sufficient number to capture this population. Other studies have used nearer 1 million events in such "rare event analysis". Finally, with recent studies showing that that progenitor cells adherent to fibronectin after 2 days in culture may contain the definitive EPC population, a limitation of the "Hill" culture assay used in the studies is that it will detect monocyte-derived EPCs only [203].

In general, it is very difficult to interpret and compare results between studies because each group uses their particular EPC definition based on their experimental and/or clinical outcomes. CD34 and CD133 are both haematopoietic markers, but these populations are only partially overlapping. Similarly, VEGFR2 and VE-cadherin antigens are present in subpopulations of each fraction and only a low percentage of these cells co-express together. Therefore, there is only a small chance that all these different EPC definitions refer to the same cell population. The combination of the expression of these three markers could reflect a hierarchy of EPC differentiation or

cells in different kinetic states, which would explain why different groups achieved different results. It may also explain why using the in vitro functional EPC assay (EPC-CFU), which could be thought of as a more global measure of the EPC numbers, appeared to provide more interpretable results in this thesis than the real-time PCR or flow cytometry data, which may have measured different cells at different stages of maturity. Despite this, however, all three techniques used appeared to demonstrate a change in EPCs, whether it was due to exogenous vascular insult, or secondary to endogenous vasculopathy or administration of a recombinant protein. A more accurate definition for EPCs will be required first before further assay comparisons can be performed.

9. ABSTRACTS AND PRESENTATIONS RELATING TO WORK IN THIS THESIS

1. Millar, C. G., Stirling D., Woodward, A., Ludlam, C. A. *Endothelial progenitor cells are mobilised following elective abdominal aortic aneurysm repair.* Abstract book of the Scottish Stem Cell Network Meeting, 2005: p. 50.

   (Abstract presentation at the Scottish Stem cells Network Meeting, Edinburgh 2005)

2. Millar, C. G., Stirling, D., Woodward, A., Rae, M., Ludlam, C. A. *CD34 and VEGFR-2 mRNA expression is reduced but VE-cadherin and CD14 mRNA expression is increased in patients after elective aortic aneurysm repair.* Br J Haematol, 2005. **121**(suppl. 1): p. 9.

   (Abstract presentation at the Annual Scientific Meeting of the British Society for Haematology, Manchester 2005)

3. Millar, C. G., Stirling, D., Macdonald, R., Mills, N. L., Newby, D. E., Ludlam, C. A. *CD34 and VE-cadherin mRNA expression is reduced in patients undergoing coronary angiography.* Br J Haematol, 2005. **121**(suppl. 1): p. 9.

   (Abstract presentation at the Annual Scientific Meeting of the British Society for Haematology, Manchester 2005)

4. Millar, C. G., Stirling, D., Mills, N. L., Newby, D. E., Ludlam, C. A. *Endothelial progenitor cells are mobilised in a group of patients following coronary angiography.* Abstract book of the International Society of Thrombosis and Hemostasis Meeting in Sydney., 2005.

   (abstract presentation at the ISTH meeting, Sydney 2005)

5. Millar, C. G., Stirling, D., Woodward, A., Ludlam, C. A. *A reduction is seen in peripheral blood mRNA expression of CD34 and VEGFR-2, but VE-cadherin and CD14 mRNA expression is increased in a group of patients following elective abdominal aortic aneurysm repair.* Abstract book of the International Society of Thrombosis and Haemostasis Meeting in Sydney., 2005.
(abstract presentation at the ISTH meeting, Sydney 2005)

6. Millar, C. G., Tura, O., Zammitt, N., Stirling D., Barclay, G. R., Frier, B. M., Turner, M. L., Ludlam, C. A. *Endothelial progenitor cells are reduced in patients with type 1 diabetes.* Abstract book of the British Society of Haemostasis and Thrombosis Meeting in Chester., 2005.
(Oral presentation at the British Society of Haemostasis and Thrombosis Meeting, 28-30th September 2005, Chester)

7. Mills, N. L., Millar, C. G., Tura, O., Stirling, D., Barclay, R. G., Newby, D. E., Turner, M., Laing, N., Ludlam, C. A. *Percutaneous coronary intervention increases endothelial progenitor cell mobilisation in patients with ischaemic heart disease.* J Am Coll Cardiol, 2006. **47**(4, suppl. A).
(Abstract presentation at the 55th annual congress of the American College of Cardiologists, Georgia 2006)

8. Millar, C. G., Mann, S., Stirling D., Turner, N., Ludlam, C. A. *Recombinant human erythropoietin stimulates endothelial progenitor cell mobilisation in patients with chronic renal failure: implications for prevention of atherosclerotic complications.* Br J Haematol, 2006. **133**(suppl.1): p. 78.
(Abstract presentation at the Annual Scientific Meeting of the British Society for Haematology, Edinburgh 2006)

9. Millar, C. G., Stirling, D., Mills, N. L., Newby, D. E., Ludlam, C. A. *The effect of coronary angiography and percutaneous intervention on total white cell counts and differential counts in patients with chronic stable angina: part of a systemic inflammatory response?* Br J Haematol, 2006. **133**(suppl.1): p. 78.
(Abstract presentation at the Annual Scientific Meeting of the British Society for Haematology, Edinburgh 2006)

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