The Influence of Age, Cardiorespiratory Fitness, Exercise and Sedentary Behaviours on Circulating Angiogenic Cells and Cell Surface Receptor Expression

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Doctor of Philosophy Thesis

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A thesis submitted in partial fulfilment of the requirements of Edinburgh Napier University, for the award of Doctor of Philosophy

April 2016

Abstract

Cardiovascular disease (CVD) is the biggest killer of people in western civilisation. Age is a significant risk factor for the development for CVD, and treatments and therapies to address this increased risk are crucial to quality of life and longevity. Exercise is one such intervention which has been shown to reduce CVD risk. Age is also associated with endothelial dysfunction, reduced angiogenic capabilities, and reduced ability to repair the vessel wall. Circulating angiogenic cells (CACs) are a subset of circulating cells which assist in the repair and growth of the vasculature and in the maintenance of endothelial function. Reductions in these cells are observed in those with vascular disease compared to age-matched healthy controls. Exercise may reduce CVD risk by improvements in number and/or function of these CACs.

Data was collected from human volunteers of various ages, cardiorespiratory fitness (CRF) levels and latent viral infection history status to investigate the effects of chronological age, CRF, viral serology and other lifestyle factors, such as sedentary behaviours and exercise on CACs. The levels of CACs in these volunteers were measured using four-colour flow cytometry using various monoclonal antibodies specific to cell surface markers that are used to identify specific subsets of these CACs. In addition, the response to acute exercise of a specific subset of these CACs, termed 'angiogenic T-cells' (T_{ANG}) were investigated, in a group of well-trained males aged 20-40 years, using a strenuous submaximal exercise bout.

Advancing age was associated with a decline in various subsets of CACs, including bone marrow-derived $CD34^+$ progenitors, putative endothelial progenitor cells (EPCs) and also T_{ANG} cells. Individuals with a higher CRF were more likely to have higher circulating numbers of T_{ANG} cells, particularly in the $CD4^+$ subset. CRF did not appear to modulate $CD34^+$ progenitors or EPC subsets. Increasing sitting time was associated with reduction in T_{ANG} cells, but after correcting for the effects of fitness, sitting time no longer negatively affected the circulating number of these cells. Acute exercise was a powerful stimulus for increasing the number of T_{ANG} cells (140% increase), potentially through an SDF-1:CXCR4-dependent mechanism, but more studies are required to investigate this. Latent CMV infection was associated with higher number of T_{ANG} cells ($CD8^+$), but only in 18-40 year old individuals, and not in an older age group (41-65 year old). The significance of this has yet to be understood.

In conclusion, advancing age may contribute to increased CVD risk partly due to the observed reductions in angiogenic cells circulating in the peripheral compartment. Maintaining a high CRF may attenuate this CVD reduction by modulating T_{ANG} cell number, but potentially not $CD34^{+}$ progenitor or EPC subsets. Acute exercise may offer a short window for vascular adaptation through the mobilisation of T_{ANG} cells into the circulation.

Dedication

I dedicate this Ph.D thesis to my Dad (Geoff Ross, 1954-2016). Dad, your thirst for knowledge inspired me as a boy to continually investigate the world around me. I will miss you every day, and I strive to make you proud. Thank you, for everything.

Acknowledgements

I would like to thank all participants who volunteered for the studies presented in this thesis. Thanks must go to Peter Tormey for his assistance with data collection for *Chapter 7*. I would also like to show my appreciation for all the postgraduate research students who helped me throughout the journey of this Ph.D.

To the technicians, Russell Wilson, Marina Mocogni, and Steven Curnyn. Thanks for your technical assistance, wise words and putting up with me over the last 4 years.

To Peter Tormey and Lesley Ingram. It has been an absolute pleasure to go through this journey with you both. You were a constant source of advice and active discussion, which helped make the last 4 years much easier than it could have been. Thank you both.

To my supervisory team. To Dr. Richard Simpson and Dr. Eva Malone. You were both a constant source of technical and academic expertise, which has helped me grow into the scientist I am today.

To my Director of Studies, Dr. Geraint Florida-James. I am lucky enough to have had the opportunity to study for a Ph.D under your supervision. It has been my absolute pleasure. A constant source of advice, wisdom and light-hearted discussion. I look forward to working with you for some years to come.

To my friends and family. You have supported me in everything I have done. I could not have done it without you all.

Finally to Sarah. Words cannot do justice to how much you mean to me. Your unwavering support during these Ph.D years has always been appreciated. I am forever in your debt. This has been as much your accomplishment as it is mine.

Publications

The following published papers and communications have been produced from the work contained within this thesis. Published papers and communications are provided in *Appendix 5*.

Published Papers

Ross, M.D; Malone, E., Florida-James, G. (2016). Vascular ageing and exercise: Focus on cellular reparative processes. *Oxidative Medicine and Cellular Longevity*. DOI: 10.1155/2016/3583956

Published Communications

Ross, M.D; Malone, E; Simpson, R; Florida-James, G. (2015). The influence of age and cardiorespiratory fitness on circulating angiogenic T cells and CXCR4 expression. American College of Sports Medicine 62nd Annual Meeting, 6th World Congress in Exercise Medicine, 26th-30th May, San Diego, California, USA

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List of Abbreviations and Symbols

ACS- Acute Coronary Syndrome

ACSM- American College of Sports Medicine

AGE- Advanced Glycation End Products

ANOVA- Analysis of Variance

BM- Bone Marrow

BMI- Body Mass Index

BP- Blood Pressure

c-src- Tyrosine-Protein Kinase CSK

CAC- Circulating Angiogenic Cells

cGMP- Cyclic GMP

CHD- Coronary Heart Disease

CI- Confidence Intervals

CMV - Cytomegalovirus

CO_2- Carbon Dioxide

CRF- Cardiorespiratory Fitness

CRP- C-Reactive Protein

CV- Cardiovascular

CVD – Cardiovascular Disease

CVS- Cardiovascular System

CXCR4 – CXC-Chemokine Receptor 4

CXCR7- CXC-Chemokine Receptor 7

DBP- Diastolic Blood Pressure

DNA- Deoxyribonucleic Acid

EC-CFU- Endothelial Cell Colony Forming Units

EDTA- Ethylene Diamine Tetraacetic Acid

ELISA- Enzyme Linked Immunosorbent Assay

EMP- Endothelial Microparticles

eNOS- Endothelial Nitric Oxide Synthase

EPC - Endothelial Progenitor Cell

FACS- Fluorescence-Activated Cell Sorting

FMD- Flow-Mediated Dilatation

FRS – Framingham Risk Score

G-CSF - Granulocyte Colony Stimulating Factor

GC- Guanylate Cyclase

GTP- Guanosine Triphosphate

H_2O_2- Hydrogen Peroxide

HbA1c- Glycated Haemoglobin

HCMV- Human Cytomegalovirus

HDL-C- High Density Lipoprotein Cholesterol

HPA- Hypothalamic-Pituitary-Adrenal Axis

HR- Heart Rate

HR_{max}- Maximal Heart Rate

HUVEC- Human Umbilical Vein Endothelial Cell

IAT- Individual Anaerobic Threshold

IFN-y- Interferon-y

IL-6- Interleukin-6

IL-8- Interleukin 8

IRP – Immune Risk Profile

JAK-2- Janus Kinase 2

L-NAME- N^G-Nitro-L-Arginine Methyl Ester

LDL-C- Low Density Lipoprotein Cholesterol

LVEF- Left Ventricular Ejection Fraction

mAb- Monoclonal Antibody

MAP- Mean Arterial Pressure

MAPK- Mitogen-Activated Protein Kinase

MCP-1- Monocyte Chemoattractant Protein-1

MFI- Mean Fluorescence Intensity

mKitL- Membrane-Bound Kit Ligand

MMP-9- Matrix Metalloproteinase-9

MNC- Mononuclear Cell

mRNA- Messnger Ribonucleic Acid

NaCl- Sodium Chloride

NCD- Non-Communicable Disease

NFkB- Nuclear Factor kB

NO- Nitric Oxide

O_2- Oxygen

OxLDL- Oxidised Low Density Lipoprotein

PAD- Peripheral Arterial Disease

PBMNC- Peripheral Blood Mononuclear Cells

PBS-BSA- Phosphate-Buffered Saline Bovine Serum Albumin

PECAM-1- Platelet Endothelial Cell Adhesion Molecule

PGI2- Prostacyclin

PHA- Phytohemagglutinin

PI3K- Phosphatidyl Inositol-3 Kinase

pJAK-2- Phosphorylated Janus Kinase 2

PWV- Pulse Wave Velocity

$\dot{Q}$- Cardiac Output

RA- Rheumatoid Arthritis

Raf- Ras-Associated Factor

Ras- Rat Sarcoma

RNS- Reactive Nitrogen Species

ROS- Reactive Oxygen Species

SBP- Systolic Blood Pressure

Sca1- Stem Cell Antigen-1

PAI-1- Plasminogen Activator Inhibitor 1

SDF-1- Stromal-Derived Factor 1 (CXCL12)

SIRT-1- Sirtuin-1

Sit-Q7d- Self Reporting 7 Day Sitting Questionnaire

sKitL- Soluble Kit Ligand

T1DM- Type 1 Diabetes Mellitus

T2DM- Type 2 Diabetes Mellitus

T_{ANG} - Angiogenic T Cell

TC- Total Cholesterol

TCR- T-Cell Receptor

TNF-α- Tumour Necrosis Factor-α

TREC- T-Cell Receptor Excision Circles

TT- Time Trial

VCAM-1- Vascular Cell Adhesion Molecule-1

VE-Cadherin- Vascular Endothelial Cadherin-1

VEGF - Vascular Endothelial Growth Factor

VEGFR2 – Vascular Endothelial Growth Factor Receptor 2

$\dot{V}O_2$- Oxygen Uptake

$\dot{V}O_{2max}$ - Maximal Oxygen Uptake/Maximal Aerobic Capacity

VSMC- Vascular Smooth Muscle Cell

vWf- von Willebrand Factor

WBC- White Blood Cell

WHO- World Health Organisation

W_{max}- Maximal Aerobic Power Output

WT- Wild-Type

Chapter 1: Introduction

Rates of cardiovascular disease (CVD) are higher in Scotland and in the north of England in comparison to the rest of the United Kingdom, with over 50,000 'premature'[1] deaths in the whole of the United Kingdom from CVD in 2009 (Scarborough *et al.*, 2010). These high rates of CVD have been attributed to lifestyle factors, such as smoking, diet and increasing physical inactivity among the general population (Kontis *et al.*, 2014).

CVD can come in a variety of guises, including coronary heart disease (CHD) which is identified as a narrowing of the coronary arteries by a build-up of a fatty plaque, cerebrovascular disease which is defined as all disease affecting blood vessels that supply the brain (Scarborough *et al.*, 2010), and peripheral arterial disease (PAD), identified as a narrowing or blockages of the arteries of the lower extremity, often leading to claudication[2].

Exercise has been shown to improve quality of life (Tang *et al.*, 2009a), improve left ventricular function (Turan *et al.*, 2006), reduce blood pressure (Liu *et al.*, 2012), improve endothelial function[3] (Ades *et al.*, 2011) as well as reduce mortality rates in those with CVD (Wisløff *et al.*, 2006; Aijaz *et al.*, 2008; Sakamoto *et al.*, 2009). In fact, exercise capacity has been deemed an independent predictor of mortality in PAD patients (Leeper *et al.*, 2013), as well as in healthy men (Lee *et al.*, 2011) even when considering smoking status, history of congenital heart disease (Leeper *et al.*, 2013) and body mass index (BMI) (Lee *et al.*, 2011). The effect is so profound, that general practitioners, surgeons and cardiac rehabilitation specialists promote the use of exercise training and testing throughout a period of rehabilitation from surgery or cardiac-related illnesses (Mezzani *et al.*, 2009; Guazzi *et al.*, 2012; Martin *et al.*, 2012; Mezzani *et al.*, 2012).

There are many reported mechanisms for the beneficial effects of regular exercise on cardiovascular health, from reduced chronic low grade inflammation, improved organ and tissue perfusion, improved cardiac mechanics (improved left ventricular ejection fraction, increased stroke volume), reduced circulating pro-inflammatory cytokines to improved blood vessel growth. The focus of this thesis will be on blood vessel growth and repair.

Blood vessel growth and repair involves two distinct mechanisms, angiogenesis and post-natal vasculogenesis. Angiogenesis is the process of the growth of blood vessels from

[1] 'Premature' meaning death before the age of 75
[2] Pain experienced in the legs, often after a period of walking in PAD patients
[3] The ability of the blood vessels to dilate after an ischaemic challenge

pre-existing vessels. This is achieved through endothelial cell proliferation, which is often stimulated by cells that have a paracrine[4] effect on the endothelium. Alternatively, post-natal vasculogenesis describes the growth of blood vessels involving non-resident endothelial cells e.g. the incorporation of bone marrow (BM)-derived stem/progenitor cells which differentiate into endothelial cells. Blood vessel growth is reduced with increasing age (Rivard *et al.*, 1999; Sadoun and Reed, 2003; Reed and Edelberg, 2004), therefore increasing the risk of CVD. In addition, endothelial dysfunction is a hallmark of ageing (Black *et al.*, 2008; Black *et al.*, 2009) and progression of CVD (Gokce *et al.*, 2003; Green *et al.*, 2011; Hafner *et al.*, 2014), and it has been shown that $CD34^+$ progenitors can be beneficial in maintaining endothelial health, and are themselves independently associated with endothelial function (Sibal *et al.*, 2009; Bruyndonckx *et al.*, 2014). Both endothelial progenitor cells (EPCs) (Asahara *et al.*, 1997; Hur *et al.*, 2004; Urbich *et al.*, 2005) and angiogenic T lymphocytes (T_{ANG}) (Hur *et al.*, 2007) play an important part in blood vessel growth and repair. EPCs can act both in angiogenesis and post-natal vasculogenesis (depending on phenotype) (Asahara *et al.*, 1997; Hur *et al.*, 2004), and T_{ANG} acts by stimulating angiogenesis through secretion of growth factors and cytokines which act on endothelial cells (Hur *et al.*, 2007; Kushner *et al.*, 2010b). Circulating numbers of these cells have been identified to be reduced in those with vascular disease (Fadini *et al.*, 2005; Schmidt-Lucke *et al.*, 2005; Fadini *et al.*, 2006; Sibal *et al.*, 2009; Jung *et al.*, 2010; Shantsila *et al.*, 2012) or vascular risk factors (Vasa *et al.*, 2001; Weil *et al.*, 2011). These cells have also been reported to be decreased in number with age (Thijssen *et al.*, 2006; Hur *et al.*, 2007), as well as impaired functionality[5] with increasing age (Hoetzer *et al.*, 2007; Kushner *et al.*, 2010a; Xia *et al.*, 2012a; Xia *et al.*, 2012b). Therefore, it is apparent that these cells are of utmost importance in maintaining vascular homeostasis in health and disease, and methods of increasing their number and/or function are required. Some methods include diet (Chan *et al.*, 2011; Fernández *et al.*, 2012; Heiss *et al.*, 2012) as well as infusion of fully functional progenitor cells (Losordo *et al.*, 2012), not excluding the administration of exogenous mobilising factors (Powell *et al.*, 2005; Bruno *et al.*, 2006), however these studies have all focused on $CD34^+$ progenitor cells. There has been some evidence to show beneficial impact of protein on T-cell trafficking (Witard *et al.*, 2014), as well as research showing polyunsaturated fatty

[4] One cell having an effect on a cell in close vicinity through the secretion of hormones, cytokines or growth factors

[5] Cell migration to SDF-1 and/or VEGF, re-endothelialization of induced injury of carotid artery in mice (EPCs only), reduced cytokine release

acids acting to reduce production of interleukin-2 by T-cells (Wallace *et al.*, 2001). This is in addition to several studies indicating a modulatory role of diet T-cell number and function (Papathanassoglou *et al.*, 2006; Ma *et al.*, 2007; Maganto-García *et al.*, 2011), but with no research as of yet specifically on $CD31^+$ T-cells, this may offer potential for future studies. Additionally, the effect of infusion of these cells or administration of mobilising factors on these cells has also yet to be investigated. It is important to note that the efficacy and safety of these procedures mentioned have yet to be standardised and fully elucidated.

Other methods proposed to increase circulating numbers of EPCs and T_{ANG} cells, as well as improve cellular function, have included regular exercise. In fact, acute and regular exercise training has shown increases in circulating number and function of EPCs (Adams *et al.*, 2004; Laufs *et al.*, 2005a; Van Craenenbroeck *et al.*, 2008; Van Craenenbroeck *et al.*, 2011; Fernandes *et al.*, 2012; Choi *et al.*, 2014). There is no current evidence to show any effect of exercise (acute, chronic or cardiorespiratory fitness) on T_{ANG} cells.

Recently, research has shown that sedentary behaviour can be deleterious on cardiometabolic health (Hamilton *et al.*, 2007; Katzmarzyk *et al.*, 2009; Gibbs *et al.*, 2014; Chau *et al.*, 2015), with negative impact on insulin sensitivity (Reynolds *et al.*, 2014) and endothelial function (Nosova *et al.*, 2014), in addition to being associated with increases in arterial stiffness (van Duijnhoven *et al.*, 2010) and increases in circulating biomarkers of endothelial damage (Boyle *et al.*, 2013). Currently, the impact of sedentary behaviours on circulating angiogenic cells (CAC) have yet to be addressed, and it is unknown whether regular exercise can attenuate any negative effects sitting time can have on these cells.

The studies comprised within this thesis are aimed at examining the effects of age, cardiorespiratory fitness (CRF), exercise and sedentary behaviours on CACs and cell surface receptor expression. The thesis is presented in eight subsequent chapters. These chapters are detailed as follows:

Chapter 2: This chapter reviews the current literature on the importance of the endothelium in cardiovascular health and the role of exercise and age on the endothelium and CACs.

Chapter 3: This chapter details the materials and methods used to collect the data presented in the subsequent studies

Chapter 4: This chapter presents the study investigating the influence of age and CRF on circulating $CD34^+$ progenitor cells and C-X-C chemokine receptor 4 (CXCR4) cell surface expression

Chapter 5: This chapter presents the study investigating the influence of age and CRF on circulating $CD31^+$ T-cells and CXCR4 cell surface receptor expression

Chapter 6: This chapter presents the study investigating the effect sedentary behaviour (sitting time and screen time) has on $CD34^+$ progenitors, and $CD31^+$ T-cells and how CRF may attenuate these effects.

Chapter 7: This chapter presents the study investigating the effects of acute exercise on the $CD31^+$ and $CD31^-$ T-cells in the circulation, and the role of the SDF-1:CXCR4 axis in the changes seen.

Chapter 8: This chapter presents the study investigating the role of cytomegalovirus (CMV) on $CD31^+$ T-cells.

Chapter 9: This chapter discusses the findings presented in this thesis. Conclusions are presented, as are limitations within the thesis, and future studies are proposed.

Keywords: Endothelium, Progenitor, T-Cell, Angiogenesis, Vasculogenesis, Exercise, Age.

Chapter 2: Literature Review

The following literature review will cover the main research in the areas of exercise in cardiovascular health and disease, as well as the protective role that endothelial progenitor cells (EPC) and angiogenic T-cells (T_{ANG}) play in preventing vascular disease. The effects of exercise, physical activity or inactivity and sedentary behaviour on the circulating number and function of these cells will also be addressed.

2.1 Cardiovascular Disease: Current State of Play

Heart and circulatory disease is the United Kingdom's biggest killer, with more than 1 in 4 deaths in men before the age of 75, and 1 in 5 deaths in women before the age of 75 accountable to CVD (Scarborough *et al.*, 2010). It is estimated that nearly 30% of all deaths around the world are caused by CVD (Lozano *et al.*, 2012). The death rates attributable to CVD differ depending on geographical region. Well-developed countries have much lower death rates attributable to CVD in comparison to less developed countries, with France, Spain, Denmark, Switzerland, USA and the UK all showing reduced age-corrected mortality rates due to CVD (149.6, 162.0, 173.5, 181.2, 235.5 and 205.2 respectively [deaths per 100,000 population]) in comparison to Republic of Moldova, Kazakhstan, Kyrgyzstan, Uzbekistan, Belarus, and Turkmenistan (790.3, 809.8, 841.8, 858.0, and 1017.4 respectively [deaths per 100,000 population]) (Go *et al.*, 2014; Nichols *et al.*, 2014). In Scotland, 16.2% of adults, aged 16+ had any CVD diagnosable condition (Bromley *et al.*, 2012). This is not simply a medical issue, but also an economic one, with CVD estimated to cost the UK economy in the region of £30 billion a year.

Despite the high prevalence, the death rate from CVD has been falling since the 1970s (Lozano *et al.*, 2012), mostly as a result of medical and surgical advancements, as well as reductions in major risk factors, for example smoking. As a population, we are becoming older, we are living longer and thus are exposed to more risk factors in our lifetime, and thereby age itself is a significant risk factor for developing CVD. In the UK, life expectancy for males increased from 68.7 years in 1970 to 77.8 years in 2010, and likewise female life expectancy increased from 75 to 81.9 years in the same time period (Wang *et al.*, 2012). Lozano *et al.* (2012) mathematically modelled the percentage change in death caused by non-communicable disease (NCD; e.g. CVD, diabetes and cancer) as a result of an ageing population, and estimated that there was a ~39% increase in deaths attributable to NCD as a direct consequence of more people living longer, and 22.9% as

a result of an increasing global population. These findings tell us that, although our life expectancy is increasing, we are not ageing 'successfully'.

In addition to age, other major risk factors for developing CVD are: smoking, gender, high blood pressure (hypertension), dyslipidemia and diabetes (Cupples and D'Agostino, 1987). It is only recently the focus has evolved to the role that physical activity and inactivity may play in the development, progression or the prevention of CVD. In 1953, a seminal study investigating the mortality rates in bus conductors and bus drivers showed that bus drivers had a significantly higher mortality rate from CVD than bus conductors (Morris *et al.*, 1953). The conclusion was that due to a higher level of physical activity, the bus conductors were 'protected' from developing CVD, whereas the inactive mode of work of the bus drivers led them to be susceptible. The study by Morris *et al.* (1953) has since been followed up by a plethora of studies suggesting that physical activity and physical fitness offers protection against mortality and CVD (Kurl *et al.*, 2003; Joyner and Green, 2009; Vigen *et al.*, 2012; Barry *et al.*, 2013; Berry *et al.*, 2013; Chomistek *et al.*, 2013; Holtermann *et al.*, 2015). Unsurprisingly, sedentary time (time spent seated or lying down per day being inactive) appears to have deleterious effects on health and longevity (Laufs *et al.*, 2005b; Hamilton *et al.*, 2007; Katzmarzyk *et al.*, 2009; van der Ploeg *et al.*, 2012; Wilmot *et al.*, 2012; Staiano *et al.*, 2014; Chau *et al.*, 2015), yet its importance has only recently come into the spotlight. With the current depth of evidence on physical fitness, activity and inactivity, these 3 lifestyle factors should be taken into account in disease risk stratification.

Physical activity reduces chronic low-grade inflammation (Mathur and Pedersen, 2008) which is a player in atherosclerotic lesion development (Libby *et al.*, 2002), improves capillary number and thus improves tissue perfusion (Hoier and Hellsten, 2014), improves cardiac function (Ehsani *et al.*, 1991; Turan *et al.*, 2006), reduces body fat (Sillanpää *et al.*, 2008), reduces fasting blood glucose (Gillen *et al.*, 2012) and reduces blood pressure (Liu *et al.*, 2012). Physical activity and physical fitness have many other benefits other than those which directly affect the cardiovascular system (CVS), increased insulin sensitivity (Babraj *et al.*, 2009), improved mood state, reduced risk of depression (Mammen and Faulkner, 2013), dementia (DeFina *et al.*, 2013), and can reduce incidence of some forms of cancer (Lee, 2003). Many have campaigned for physical activity to be prescribed by doctors, implying that exercise is a 'polypill'- one medication that can impact on a large number of physiological variables related to health (Fiuza-Luces *et al.*, 2013; Matheson *et al.*, 2013; Kraus *et al.*, 2015; Sanchis-Gomar *et al.*, 2015). A recent

meta-analysis (16 articles, >340,000 participants) by Huseyin and John (2013) found that exercise was comparable to drugs when the end-result was mortality risk. The number of exercise trials was only 54 in comparison to the 248 drug trials, yet the results showed that with respect to mortality from CHD, exercise (odds mortality 0.89, 0.74-1.04 95% credible intervals) was as effective as statins (0.82, 0.75-0.90), beta-blockers (0.85, 0.78-0.92), antiplatelets (0.83, 0.74-0.93), and ACE inhibitors (0.83, 0.72-0.96). Furthermore, exercise was the favoured intervention for stroke, as when compared with anticoagulants and antiplatelets, odds ratios were significantly reduced in exercise (0.09 [0.01 – 0.70 95% credible intervals] vs. anticoagulants, 0.10, [0.01 – 0.62] vs. antiplatelets). Exercise was not always the best treatment however, for example in heart failure, diuretics had a greater impact on reducing mortality than exercise (0.19 [0.03 – 0.66 95% credible intervals] vs. 0.79 [0.59-1.00]). More studies are required in order to fully understand the role and importance of exercise in medical treatment and prevention.

Despite the clear health benefits of physical activity, we are becoming an increasingly sedentary population. It is estimated that over the past 50 years physical activity levels have reduced by 20% (UK-Active, 2014), thus the American College of Sports Medicine's (ACSM) minimum guidelines for physical activity, which are to accumulate 30 minutes of moderate intensity aerobic exercise 5 days per week (ACSM, 2013) are not currently being met by the majority of the UK population (NISRA, 2007; NHS, 2009; Bromley *et al.*, 2013; WGSD, 2014). A recent meta-analysis suggests it may not be that we need to meet these guidelines for everyone, as the biggest effect on all-cause mortality may be going from lowest level of leisure time physical activity to the next (Myers *et al.*, 2004; Kelly *et al.*, 2014), with no need to reach very high levels of physical activity, as for most, this would be difficult to manage.

Periods of inactivity reduces insulin sensitivity (Rimbert *et al.*, 2004; Reynolds *et al.*, 2014), vascular function (Nosova *et al.*, 2014), can accelerate atherosclerotic plaque formation in mice (Laufs *et al.*, 2005b) and is associated with high levels of plasma inflammatory cytokines C-reactive protein (CRP) and interleukin-6 (IL-6) (Fischer *et al.*, 2007). Recently, the clinical effects of physical inactivity have been called the 'diseasome', with physical inactivity being labelled a promoter of disease (Arias-Loste *et al.*, 2014). A simple measure, such as daily sitting time can be used as a surrogate measure of inactivity and can discriminate those who are at high risk of all-cause mortality, with those reporting high levels of sitting time at greater risk (van der Ploeg *et al.*, 2012; Wilmot *et al.*, 2012), and thus this can easily be part of a general practitioner's monitoring

list to classify patients as low or high risk and treat accordingly. However, there exists a dichotomy, where a large number of those who sit for long periods at work, may take part in high intense training, for example recreational runners, cyclists and triathletes. The inter-play between fitness and sitting time must therefore be considered, and the effect of one attenuating the effects of the other must be investigated.

The attributable fraction for mortality by CVD and all-cause mortality in the UK by physical inactivity is reported to be 10.5% and 16.9% respectively (Lee *et al.*, 2012b), yet it was estimated that if physical inactivity was eliminated, life expectancy would only increase by 1.07 years. One way of interpreting this is to suggest that by reducing physical inactivity and sedentary behaviour may not extend our life to a great extent, but it may help us grow older 'successfully' without the burden of disease, and improve quality of life. The current evidence suggests that physical inactivity levels may not be associated with an increased CVD risk, due to the influence of the covariates, such as moderate and vigorous physical activity levels (Pulsford *et al.*, 2015), therefore the potential impact of covariates must be considered for any studies investigating physical activity/inactivity.

Working groups have been set up with the specific goal of improving physical activity levels, and reducing sedentary time. For example, *The Lancet Physical Activity Observatory* has the following goals:

1. To reduce the global prevalence of physical inactivity among adults from 31% to 28%
2. Increase the proportion of adolescents engaging in at least 1 hour per day of vigorous and moderate-intensity physical activity from 21% to 24%
3. Reduce the proportions of coronary heart disease, type 2 diabetes, breast cancer, colon cancer, and premature deaths worldwide that are attributable to physical inactivity by 10%
4. Increase the proportion of peer-reviewed scientific publications on physical activity that come from low-income and middle-income countries over the total number of publications by 10%.

Taken from Kohl 3rd et al., 2012

The World Health Organization (WHO) have charged their 25 member states to reduce physical inactivity levels by 10% by 2025 (WHO, 2010). The fact that these organisations

are publishing these guidelines and goals highlights the pandemic of physical inactivity, so much so that there is more media attention being paid to it (Figure 2.1).

Figure 2.1. Media headlines concerning physical activity and inactivity levels. Sources: www.bbc.co.uk/news/health, www.theguardian.co.uk/society/health

2.2 The Endothelium

The endothelium is the inner layer of all blood vessels, and in the case of capillaries, the only layer. The endothelial cells function to control diffusion and transport nutrients, gases and other signalling proteins from the blood and into the tissues (and vice versa), and control the adhesion, rolling and migration of leukocytes to sites of infection and tissue damage. This cell is also crucial in the regulation of blood flow distribution throughout the body. The endothelium, under certain physiological conditions, can release vasoactive substances, for example nitric oxide (NO) and prostacyclin (PGI_2). Vasodilatation (the increase in diameter of the vascular lumen to allow greater blood flow distal to the vessel) predominantly occurs via endothelial cell production and secretion of NO (Furchgott and Zawadzki, 1980) which subsequently acts on vascular smooth muscle

cells (VSMCs) to stimulate relaxation, and the end result being a widening of the vessel lumen (figure 2.2).

The endothelial cells contain an isoform of NO synthase termed endothelial NO synthase (eNOS) (Novella *et al.*, 2013), which, when activated, cleaves the nitrogen group from the amino acid L-arginine and combines it with an oxygen molecule to form NO. Activation of eNOS can occur via shear stress (the force of blood against the vessel wall) (Huang *et al.*, 2015a) as well as other inflammatory mediators, such as bradykinin (Bae *et al.*, 2003) which leads to the observed vasodilatation of inflammation. Shear stress provides a mechanical signal, which is 'sensed' by the glycocalyx (a carbohydrate rich extracellular layer anchored to the endothelial cell membrane). The glycocalyx offers a buffer between erythrocytes and endothelial cells, thereby offering cellular protection from mechanical disruption. Glycocalyx disruption by shear stress activates the enzyme phosphatidyl inositol-3 kinase (PI3K) leading to activation of protein kinase B. This enzyme phosphorylates eNOS resulting in NO production from L-arginine and oxygen. NO diffuses to neighbouring VSMCs where it binds to the haem group of guanylyl cyclase which then catalyses the production of cyclic guanosine monophosphate (cGMP) from guanosine triphosphate (GTP). The cGMP produced activates a protein kinase G which phosphorylates phospholamban to reduce the inhibition of calcium-ATPase (Ca^{2+}-ATPase) pumps, leading to an expulsion of Ca^{2+} from the cytoplasm, and thus reduced Ca^{2+} bioavailability within the cytoplasm to bind troponin to reveal binding sites on actin for myosin to bind in order for cross-bridge contraction to occur, thus causing relaxation. The second mechanism by which NO causes VSMC relaxation and thus vasodilatation is by activating potassium channels in the VSMC membrane, which hyperpolarises the muscle cell, leading to relaxation (Stankevičius *et al.*, 2011). The importance of the endothelium in vasodilatation was exhibited in animal studies, where dilatation of arteries was dependent on the presence of an intact endothelial cell layer (Smiesko *et al.*, 1985; Pohl *et al.*, 1986).

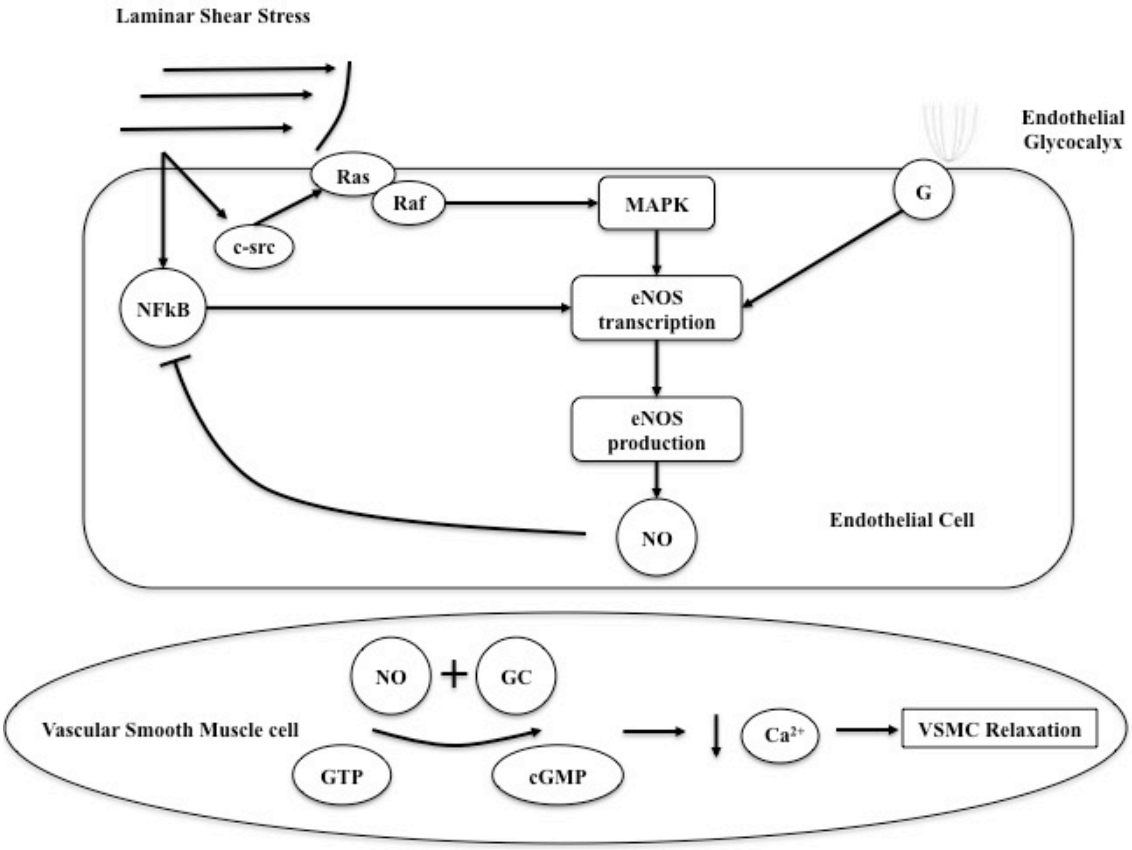

Figure 2.2. Endothelium-dependent vasodilation. *Figure shows the production of nitric oxide via mechanotransduction of laminar shear stress, diffusion across to the vascular smooth muscle cells and cause relaxation with resulting vessel lumen widening. Adapted from Harrison et al. (2006), Mochizuki et al. (2003) and Lincoln et al. (2001). c-src- Tyrosine-protein kinase CSK, Ras- Rat Sarcoma, Raf- Ras-Associated Factor, MAPK- Mitogen-Activated Protein Kinase, eNOS-endothelial nitric oxide, NO-nitric oxide, NFkB- Nuclear Factor kB, G- G protein, GC- Guanylate Cyclase, GTP- Guanosine Triphosphate, cGMP- Cyclic Guanosine Monophosphate , Ca^{2+} cytosolic calcium.*

The ability of endothelial cells to produce and release NO for VSMC relaxation is commonly known as endothelial function. This can be measured *in vivo* by flow-mediated dilatation (FMD). FMD utilises the ischaemic hyperaemic response to assess the ability of the blood vessels to dilate to an ischaemic challenge. Importantly, FMD seems to offer prognostic information regarding the vascular health of individuals who are asymptomatic and those with CVD. Indeed several studies have shown FMD to independently predict future CV events (Gokce *et al.*, 2003; Meyer *et al.*, 2005; Yeboah *et al.*, 2007; Shechter *et al.*, 2009; Yeboah *et al.*, 2009; Green *et al.*, 2011; Hafner *et al.*, 2014) confirming the role of a dysfunctional endothelium in CVD onset or progression.

The physiological basis for a role of a dysfunctional endothelium in CVD is the assumption that it reflects NO bioavailability. NO not only functions in the regulation of

vascular tone, but it is also anti-atherogenic, and inhibits platelet and leukocyte adhesion to the endothelial cell wall (Moncada *et al.*, 1991; Cooke and Tsao, 1994). The endothelial cell production of NO important for dilation of resistance vessels, but it is also the role the endothelium plays in repairing and growing of new blood vessels by endothelial proliferation that is important to maintain homeostasis. For example, after tissue injury, insufficient blood vessel formation can lead to tissue death as in CVD, and delayed wound healing (Watt and Fox, 2005; Martin-Rendon *et al.*, 2009; Critser and Yoder, 2010). Therefore, CVD and other vascular-related diseases (for example diabetes mellitus) often display a lopsided cardiovascular (CV) maintenance system, whereby endothelial repair fails to keep up with endothelial damage.

2.2.1 The Role of the Endothelium in Progression of Cardiovascular Disease and Ageing

Endothelial dysfunction often precedes CVD, and as mentioned previously can predict CV events (Green *et al.*, 2011) and mortality (Shechter *et al.*, 2009). Anderson *et al.* (2011) found that in 1574 men, aged 49.4 ± 9.9 years, FMD did not predict subsequent CV events. The follow up period in this study was limited (only 9 years), and CV events only occurred in 71 of the men (4.5%), so results from the study need to be interpreted with caution. In addition, FMD is limited by the measure variation, which can be up to 20%. The majority of evidence supports the hypothesis that the endothelium plays a significant role in onset and progression of vascular diseases (Gokce *et al.*, 2003; Shechter *et al.*, 2009; Li and Förstermann, 2013; Miller *et al.*, 2013; Hafner *et al.*, 2014; Liao *et al.*, 2014; Manganaro *et al.*, 2014).

The vascular endothelium in disease states has been reported to be in replicative senescence, which may contribute to endothelial dysfunction, as senescent endothelial cells contain reduced levels of eNOS activity and reduced NO release upon shear stress stimulation (Matsushita *et al.*, 2001). Oxidative stress caused by excessive levels of reactive oxygen species (ROS; for example free oxygen radicals, oxygen ions and peroxides) or reactive nitrogen species (RNS; for example NO, superoxides), which are known to be increased in CVD, may also contribute to endothelial dysfunction (Taddei *et al.*, 2001). Oxidative stress has been associated with atherosclerosis (Stocker and Keaney, 2004), as oxidised low density lipoprotein (oxLDL) stimulates macrophages to migrate from blood into the sub-endothelial space, to internalise the oxLDL which then cause the

macrophages to transition to foam cells - a key process in the initial development of an atherosclerotic lesion. Park *et al.* (2007) were able to demonstrate that in aged mice there is also a concomitant increase in ROS production by NADPH-oxidase which may contribute to endothelial dysfunction. Reducing production of ROS molecules (by treatment with N^G nitro-L-arginine methyl ester [L-NAME] or diphenyliodonium) leads to improved endothelial function (Hamilton *et al.*, 2001; Csiszar *et al.*, 2002) highlighting the role of ROS in endothelial dysfunction.

Advancing age is often characterised with a dysfunctional endothelial phenotype (Taddei *et al.*, 2001; Muller-Delp, 2006; Soucy *et al.*, 2006; Black *et al.*, 2008; Black *et al.*, 2009), apoptosis of aged endothelial cells (Wang *et al.*, 2013) and altered intracellular signalling (Soucy *et al.*, 2006). Oxidative stress appears to play a role in age-related endothelial dysfunction, as excessive ROS leads to cellular dysfunction and apoptosis (Dikalov, 2011) as a consequence of excessive hydrogen peroxide (H_2O_2) production leading to DNA damage and senescence. Aged vascular tissue exhibit greater levels of superoxide anions (O_2^-) than their younger counterparts (Hamilton *et al.*, 2001; Chrissobolis and Faraci, 2008; Mayhan *et al.*, 2008), a potential source of cellular H_2O_2 production. Studies have paradoxically shown an increase in eNOS content within aortic rings from old rats (22 months old) in comparison to young (2 months old) (Luttrell *et al.*, 2013) despite reduced endothelial function, a potential overcompensatory mechanism to increase the drive for NO production.

2.2.2 The Protective Role of Exercise on the Endothelium

Exercise offers anti-atherogenic effects, preventing or reversal of plaque formation in the vasculature (Szostak and Laurant, 2011; Huang *et al.*, 2015b; Madssen *et al.*, 2015). This can be achieved through modulation of endothelial adhesion receptor expression via shear-stress stimulation (Ando *et al.*, 1994; Sheikh *et al.*, 2003). Exercise results in an increase in cardiac output ($\dot{Q}$), and thus greater blood flow through the vasculature. This increase in blood flow consequently creates a greater shear stress stimulus, which is the shearing effect of circulating cells across the endothelial cell layer. Greater levels of laminar shear stress reduce adhesion receptor expression on the endothelium (Ando *et al.*, 1994; Sheikh *et al.*, 2003) whereas lower levels and oscillatory/disturbed patterns of shear stress increases adhesion receptor expression (Chappell *et al.*, 1998; Sheikh *et al.*, 2003), commonly seen at bifurcation points in the arterial tree (points where vessels branch off)

(Chiu and Chien, 2011). These adhesion receptors (commonly platelet-endothelial adhesion molecule-1, PECAM-1; vascular cell adhesion molecule-1, VCAM-1) mediate the adhesion, rolling and extravasation of leukocytes across the vascular wall. Although the migration of leukocytes is very important for immunosurveillance, it can also be detrimental, as leukocyte infiltration of VSMCs is one of the initial steps for atherosclerotic plaque formation.

There is a an abundance of data indicating that exercise training results in a greater FMD, indicating improved endothelial function (Black *et al.*, 2008; Farsidfar *et al.*, 2008; Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Birk *et al.*, 2012; Luk *et al.*, 2012; Murias *et al.*, 2013; Ashor *et al.*, 2015). Importantly also, sedentary behaviour results in the opposite, a reduction in FMD scores (Demiot *et al.*, 2007; Thosar *et al.*, 2012; Januszek *et al.*, 2014; Thosar *et al.*, 2015a). This is because there is a lack of increased stimulus of shear stress across the endothelium, which is required to an extent, for improvements in FMD to be seen as a result of exercise training (Birk *et al.*, 2012).

2.2.3 Angiogenesis

The development of the vasculature occurs in the early stage of foetal development, and is termed vasculogenesis (Risau *et al.*, 1988). The source of this vascular development includes haematopoietic progenitor cells from the embryonic yolk sac and placenta (Caprioli *et al.*, 2001) and angioblasts, a subset of peripherally located blood island cells (Patel-Hett and D'Amore, 2011). Embryonic blood vessel development proceeds as angioblasts differentiate to form endothelial cells, form a lumen and deposit a basal lamina. An important family of signalling molecules involved in embryonic vascular development is the vascular endothelial growth factor (VEGF) family, including VEGF-A, VEGF-B and VEGF-C. Embryos lacking a single copy of VEGF (VEGF-$A^{+/-}$) (Carmeliet *et al.*, 1996) or a receptor for VEGF-A (VEGFR1 or VEGFR2) die early in development stages due to an inability to regulate vasculogenesis (Fong *et al.*, 1995) or to form sufficient vascular networks respectively (Shalaby *et al.*, 1995). VEGF-deficient skeletal muscle also causes a regression of capillary number indicating a key role of VEGF to maintain skeletal muscle blood flow (Tang *et al.*, 2004). VEGF is such an important regulator in angiogenesis, that anti-VEGF therapy has been used in cancer patients to inhibit the growth of tumours (Ebos *et al.*, 2009)

Angiogenesis, the growth and formation of new blood vessels from pre-existing blood vessels, occurs in the adult. This post-natal vascular growth involves the splitting or sprouting of pre-existing vessels via a process of basement membrane degradation and endothelial cell tip formation. The process is highly dependent upon VEGF as with embryonic vascular growth. Angiogenesis may be stimulated by hypoxia as a consequence of hypoxia-inducible factor-1–α (HIF-1α)-induced expression of VEGF (Kelly *et al.*, 2003). It may also be stimulated by shear stress across the endothelium, as increased shear stress increases capillary endothelial cell growth *in vitro* (Ando *et al.*, 1987).

Angiogenesis is crucial for adult vascular homeostasis, and this ability is reduced with advancing age, shown both in mice (Rivard *et al.*, 1999; Sadoun and Reed, 2003; Reed and Edelberg, 2004; Wang *et al.*, 2011) and in humans (Gunin *et al.*, 2014). The age-related decline in angiogenic capabilities may be one mechanism behind age being a significant risk factor for development and progression of CVD. After all, angiogenesis would be of benefit to restoring blood flow to ischemic tissues. On the other hand, angiogenesis is a crucial process in the development of tumours. Accelerated angiogenesis provides a vascular network which provides more nutrients for the developing tumour to continue to grow (Folkman, 1995).

Muscle tissue capillarity is reduced in cardiovascular-related disease states, as well as in metabolic syndrome (Frisbee *et al.*, 2014) and this loss of vasculature is associated with a reduced exercise capacity (Potus *et al.*, 2014). Both the loss of vasculature with disease and the link to reduced exercise performance indicates that maintenance or improvement in vascularisation of muscle tissue is key for prevention of vascular-related diseases and also for those who are taking part in an exercise training regime to promote aerobic fitness. Therefore the role of exercise in promoting angiogenesis may be of importance to both sporting and diseased populations.

Exercise has a remarkable ability to stimulate blood vessel morphological changes. This encapsulates the ability of exercise to reduce vessel stiffness (Fujie *et al.*, 2014), improve vascular function (Goto *et al.*, 2003; Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Tinken *et al.*, 2010) as well as increase vessel number (Laufs *et al.*, 2004; Chinsomboon *et al.*, 2009; Geng *et al.*, 2010; Bellafiore *et al.*, 2013), and this would result in more efficient delivery of oxygen (O_2) and nutrients to the exercising muscle. Exercise-stimulated angiogenesis allows for more blood flow to skeletal muscle for future exercise endeavours.

An increase in maximal oxygen consumption ($\dot{V}O_{2max}$) as a consequence of regular exercise training may be due in part to the increase in blood vessel number. It has been shown that the increases in vessel number are due to increases in gene expression, tissue content and circulating levels of pro-angiogenic growth factors, for example VEGF (Breen *et al.*, 1996; Gustafsson *et al.*, 1999; Ross *et al.*, 2014), and HIF-1α (Gustafsson *et al.*, 1999).

These capillary changes are fibre-type specific (Gute *et al.*, 1996; Waters *et al.*, 2004), therefore the increases in pro-angiogenic growth factors must originate from the contractile tissue. VEGF has been shown to originate within skeletal muscle, located near the contractile elements (Hoier *et al.*, 2013; Uchida *et al.*, 2015), and during exercise, VEGF is transported from within the muscle fibre to the extracellular space via intracellular vesicles. The subsequent release of VEGF into interstitial space can then have an effect on surrounding capillaries and their endothelial cells, stimulating endothelial tip cell formation (Gerhardt *et al.*, 2003) as well as acting as a chemoattractant to attract endothelial proliferation and vessel growth in the direction of the metabolically active tissue.

2.3 Post-Natal Vasculogenesis - The Discovery of Endothelial Progenitors

Until recently it was thought that adult vessel formation was by the process of angiogenesis, which is the growth and formation of new blood vessels from pre-existing vessels, which is highly flow-dependent (Ando *et al.*, 1987; Hudlicka, 1991).

In 1997, Asahara *et al.* (1997) discovered that by isolating $CD34^+$ cells from human peripheral blood by magnetic sorting and plating these cells on fibronectin-coated plates, that they formed spindle and tube-like structures *in vitro*. After 7 days in culture, these cells began to express endothelial-lineage markers such as VEGF, PECAM (CD31), and E-selectin and would stain positively for eNOS. These cells were also able to secrete NO under stimulation by VEGF and acetylcholine, which is a key characteristic of mature endothelial cells. To confirm their roles in vascular growth, the researchers used a mouse hindlimb ischemic model and administered human $CD34^+$ or $CD34^-$ cells. Athymic nude mice were used in this study to prevent graft-versus-host complications. The mice which were administered with $CD34^+$ cells were found to have these cells incorporated into

vessels in the ischemic hindlimb. Authors failed to assess recovery of blood flow in the mice. Various studies have confirmed the incorporation of $CD34^+$ cells into the vascular wall and differentiate into mature endothelial cells (Galasso *et al.*, 2013; Shi *et al.*, 2013).

The topic of the origin of these cells has been much debated. Studies have shown that these cells are derived from BM progenitors (Asahara *et al.*, 1999a; Reyes *et al.*, 2002). Although the BM may be the origin of these cells, a small proportion circulate in peripheral blood, with reports of EPCs accounting for between 0.0001 and 0.01% of all circulating mononuclear cells (MNCs) (Case *et al.*, 2007). Asahara *et al.* (1999a) investigated whether these progenitors were derived from BM, and used a murine BM transplant model, in which they lethally irradiated mice, transplanted them with BM overexpressing ß-galactosidase (lacZ) regulated by the Tie-2 promotor (murine VEGF receptor), or from mice with a null mutation for flk-1 (containing a promoter-less lacZ gene). With the use of tumour, wound healing, ischemic hindlimb and myocardial infarction models, they observed flk-1/lacZ and Tie-2/lacZ transcripts within the tumour tissue, in addition to EPCs located within neovascularised vessels in the remaining models. These cells were also observed to be localised in the lungs, spleen, liver, intestine, skin, ovary and uterus vasculature, albeit in small number, indicating that these BM-derived cells do not simply play a role in repairing vasculature, but also in organ maintenance.

Reyes *et al.* (2002) obtained BM from healthy human volunteers, and excluded $CD45^+$ cells, as these cells are leukocyte precursors, and cultured the remainder of the BM cells on fibronectin plates, with VEGF as an endothelial-specific medium to encourage endothelial cell marker expression. After 9-14 days, these cells showed increased expression of VCAM-1, E-selectin, von Willebrand factor (vWf), PECAM (CD31), vascular endothelial-cadherin (VE-cadherin). These cells were then implanted into mice with Lewis lung carcinoma spheroids and the researchers discovered increased vascular mass in those that received the adult progenitor cell-derived endothelial cells than those who did not (x 1.45-fold). The contribution of BM-derived progenitors to tumour vasculature has been debated, with some authors finding no contribution of these cells to vascular growth in murine tumour models or matrigel plug experiments (Purhonen *et al.*, 2008). This may be due to the lack of mobilisation of BM-derived progenitors observed in this study, and therefore the abundance of circulating BM-derived progenitors and the ability of these cells to significantly contribute to the vascular growth observed in the tumour would be limited.

Some researchers have contested the view that true endothelial precursors (cells that can differentiate into mature endothelial cells) are derived from the BM, although Asahara *et al.* (1999a) and Reyes *et al.* (2002) clearly show that BM-derived cells contribute to vascular growth in mice. It may be that these BM-derived cells are not true endothelial precursors, rather a cell line that can instigate and support endothelial cell growth by paracrine means through secretion of pro-angiogenic growth factors, such as VEGF, interleukin-8 (IL-8) and granulocyte colony-stimulating factor (G-CSF) (Hur *et al.*, 2004). Some have suggested that EPCs are found to be resident within the vascular wall, and stimulated into differentiation upon demand. In mice, the adventitia of aortic root vessels contained clusters of cells positive for stem cell antigen-1 (Sca1) which had the potential to differentiate into a VSMC (Hu *et al.*, 2004), however there was no evidence in this study to suggest they could differentiate into an endothelial cell. In adult human arteries, the adventitia contained $CD34^+$ cells, which were found to promote vessel formation *in vivo* (Zengin *et al.*, 2006). These cells could also form endothelial-like cells in the presence of VEGF *in vitro*, in addition to forming capillary-like structures (Pasquinelli *et al.*, 2007). In contrast, adventitial $Sca1^+$ cells from adult mice thoracic aorta failed to express endothelial cell antigens (Passman *et al.*, 2008), suggesting these adventitial $Sca1^+$ cells have the ability to differentiate into VSMCs alone. It is likely that vascular repair and growth proceeds in a complex fashion, with contribution of circulating EPCs that can differentiate into endothelial cells, circulating haematopoietic cells, which can support endothelial repair and growth through paracrine means, resident endothelial cell proliferation, and tissue-resident progenitor cells (both endothelial and VSMC resident progenitors) which differentiate when stimulated to do so. The exact contribution of each to a single episode of vascular repair or insult is not known, and may differ depending on location on the vascular tree (Schwartz and Benditt, 1976).

Whether circulating EPCs participate in endothelial differentiation, or promote vasculogenesis by secreting pro-angiogenic growth factors, they may be of use as a cellular biomarker for endothelial function (Oliveras *et al.*, 2008) or for an individual's vascular growth and repair ability.

2.3.1 The Mobilisation and Recruitment of Bone Marrow-Derived Endothelial Progenitor Cells

For EPCs to exert their vasculo-reparative potential they must first be mobilised from the BM. Stem or progenitor cells often express C-X-C Chemokine Receptor 4 (CXCR4) (Hattori *et al.*, 2001; Wojakowski *et al.*, 2004; Kaminski *et al.*, 2007; Ziaei *et al.*, 2014) which is a seven-transmembrane-spanning G protein-coupled receptor and is the receptor for the ligand SDF-1. Stem/progenitor cell number within the BM is tightly regulated by the relationship between SDF-1 and CXCR4, more commonly known as the SDF-1:CXCR4 axis. Stromal cells within the BM express and release SDF-1, which acts to prevent stem/progenitor cell release into the circulation. SDF-1 can be cleaved by CD26 which can be located on stem/progenitor cells (Christopherson *et al.*, 2002), in addition to proteinases such as elastase and cathepsin G causing the inactivation of SDF-1, which themselves are released by neutrophils (Levesque *et al.*, 2003). Therefore neutrophils may be a key cellular regulator of $CXCR4^+$ stem/progenitor cell mobilisation.

G-CSF (Takahashi *et al.*, 1999; Natori *et al.*, 2002; Powell *et al.*, 2005; Pitchford *et al.*, 2009; Fu *et al.*, 2015), and VEGF (Asahara *et al.*, 1999b; Pitchford *et al.*, 2009; Tashiro *et al.*, 2014) are both known to stimulate the mobilisation of progenitor cells from the BM. VEGF appears to only mobilise BM-progenitor cells with a receptor for it, e.g. VEGFR2, as indicated by a loss of mobilisation of BM progenitors by VEGF that did not express this marker, for example hematopoietic stem cells (Pitchford *et al.*, 2009). G-CSF is often used in the clinical settling to mobilise BM progenitor cells into the peripheral circulation for transplantation and use in medicine. Alongside VEGF, G-CSF may play a role in the maintenance of circulating progenitor cells as well as the dynamic changes to the desired requirements for these cells for tissue repair.

Once mobilised, progenitor cells need to travel to where they are required for tissue repair. This is a process called ‘homing’, a process governed by a chemotactic gradient. EPCs express VEGFR2 in addition expressing CXCR4, therefore can home to sites of tissue damage and ischemia by travelling along a gradient of VEGF (Tang *et al.*, 2009b; Williamson *et al.*, 2013) and SDF-1 (Yamaguchi *et al.*, 2003). In fact the migratory capacity of EPCs is often assessed by their ability to migrate across a semi-permeable membrane to differing concentrations of either VEGF or SDF-1 (Peichev *et al.*, 2000), and blocking with antibodies or antagonists to VEGFR2 (Tang *et al.*, 2009b) or CXCR4 impair or completely block migration of these cells (Sun *et al.*, 2013).

2.3.2 Characterisation of Endothelial Progenitor Cells

There is widespread debate on the exact antigenic characterisation of circulating EPCs as well as their exact role in vascular repair. They are typically enumerated by fluorescence activated cell sorting (FACS) and flow cytometry, identifying cell surface antigens that appear on cultured cells that can form tubular-like structures *in vitro*.

Cell surface antigens of EPCs include a stem cell antigen (for example CD34, CD133), and a marker of endothelial cell lineage (e.g. VEGF, vWf, CD31). As mentioned, the cells that express these markers in the combination of a stem cell marker and endothelial marker have the ability to form tube-like structures, and express eNOS, a key marker of putative endothelial precursor cells (Asahara *et al.*, 1997). CD34 is often used as a stem cell marker for the identification of EPCs, however, CD34 can be expressed by mature endothelial cells (Fina *et al.*, 1990) but is generally accepted to be a reliable marker for progenitors for a number of different tissue-types (Sidney *et al.*, 2014). It therefore follows that more markers may be required to discriminate between circulating EPCs and circulating endothelial cells which may have detached from the vessel wall. Markers such as CD133 (also known as AC133) and the negative selection of CD45 have both been mooted as potential markers to use, not just to discriminate between circulating mature endothelial cells, but also between EPCs and hematopoietic stem cells which also express CD34 (Case *et al.*, 2007).

There appears to be two distinct subsets of EPCs, each one playing divergent roles in endothelial repair. These have been termed ‘early’ and ‘late’ EPCs, or outgrowth cells. They have been termed so because of their appearance in culture. Early EPCs appear early in culture (but die after 4 weeks), they secreted pro-angiogenic cytokines and growth factors, such as VEGF, IL-8, whereas late EPC appear late in culture (up to 12 weeks), produce more NO than the early EPC subset, and formed capillary tube-like structures to a greater extent than early EPCs (Hur *et al.*, 2004). These subsets also differed in their gene expression profiles, with late EPCs expressing greater levels of VEGFR2 and VE-cadherin messenger RNA (mRNA), suggestive of being more of an endothelial cell phenotype. Taken together, it can be suggested that late EPCs have potentially greater ability to differentiate into endothelial cells, whereas early EPCs have greater potential to act in a paracrine manner, by producing and secreting pro-angiogenic factors to stimulate endothelial cell proliferation.

Case and colleagues (2007) identified $CD34^+CD133^+VEGFR2^+$ cells, muted to be EPCs, however these cells failed to form vessels to a significant extent. The authors referred to these cells as a subset of hematopoietic cells and not EPCs, and these cells were also found to be expressing CD45 to a high level. This study however failed to investigate gene expression profiles, or compare to a $CD34^+CD133^-VEGFR2^+$ fraction, but they did compare endothelial cell colony forming unit (EC-CFU) measures between $CD34^+CD45^+$ and $CD34^+CD45^-$. The $CD45^-$ fraction was able to form EC-CFU, however the $CD45^+$ cell fraction did not. Hence, positive or negative selection of CD45 may offer a means to measure distinct EPC populations. This inability of $CD45^+$ or $CD133^+$ progenitors to form endothelial-like cells has been seen elsewhere (Timmermans *et al.*, 2007). Recent studies still use $CD34^+CD133^+$ or $CD34^+CD45^+$ as antigenic characterisation of EPCs, yet the literature shows that these cells are probably not true endothelial precursors. These cells may still offer some prognostic information as they may contribute to endothelial homeostasis through paracrine mechanisms. Cell surface antigens of both 'early' and 'late' putative EPCs are summarised in table 2.1.

Table 2.1. Phenotyping of endothelial progenitors.

	CD34	**CD133**	**CD45**	**VEGFR2**	**CD31**	**vWf**	**CXCR4**
'Early' EPC	+	+	+	+	+	+	+/-
'Late' EPC	+	-	-/dim	+	+	+	+/-

2.3.3 Endothelial Progenitors in Cardiovascular Disease and Diabetes

Since endothelial progenitors play a role in vascular repair (whether that be paracrine or by adhering to the endothelium and differentiating into mature endothelial cells) it seems pertinent to investigate if they play a role in preventing CVD or vascular dysfunction. Many studies have found that in those with CVD or CV risk factors, circulating EPCs are either low in the circulation or are functionally impaired in comparison to age-matched healthy controls (Hill *et al.*, 2003; Fadini *et al.*, 2005; Schmidt-Lucke *et al.*, 2005; Walter *et al.*, 2005; Fadini *et al.*, 2006; Xiao *et al.*, 2007; Sibal *et al.*, 2009; Jung *et al.*, 2010; Liu and Xie, 2012; Rouhl *et al.*, 2012; Shantsila *et al.*, 2012; Teraa *et al.*, 2013; Vemparala *et*

al., 2013; Barsotti *et al.*, 2014; Bruyndonckx *et al.*, 2014; Liao *et al.*, 2014). Circulating EPC counts are also associated with endothelial function (Sibal *et al.*, 2009; Bruyndonckx *et al.*, 2014) potentially implicating the role of EPCs in maintaining endothelial homeostasis. Hill *et al.* (2003) published data that appeared to suggest that EC-CFU number, not traditional cardiovascular risk factors (Framingham Risk Scores) were associated with endothelial function. Some studies show no such differences between CVD and 'healthy' controls (Padfield *et al.*, 2013), with one study showing that $CD34^+$ circulating progenitor cells offered more prognostic information relating to risk of mortality than $CD34^+VEGFR2^+$ EPCs (Patel *et al.*, 2015). Interestingly, when researchers investigated $CD34^+CXCR4^+$ numbers, those in the lowest tertile group had a hazard ratio of 2.77 for risk of death in comparison to those in the highest tertile. The addition of CXCR4 expression analysis to EPC studies investigating CVD risk and risk of mortality may add more physiological understanding behind the causes of vascular disease as CXCR4 could be potentially used as an indirect indicator of ability to migrate. Indeed blockade of CXCR4 expression in EPCs has been shown to significantly reduce *in vitro* EPC migration, adhesion and re-endothelialisation (Xia *et al.*, 2012a).

The number of late EPCs have been shown to be predictive of mortality rate, with higher numbers associated with a lower mortality rate in heart failure patients (Alba *et al.*, 2013b). Pelliccia *et al.* (2013) observed that in people with stable angina who had undergone percutaneous coronary intervention, those with higher numbers of $CD34^+CD45^-VEGFR2^+$ cells had greater risk of a primary event than those with lower circulating number of these cells. Those in the highest tertile for these circulating cells were at significantly greater risk of a follow up event within 5 years, with <50% of those individuals being event free at the 5-year end point of the study. It is currently unclear why these authors found these results, but may be linked to the potential participation of EPCs in the progression of atherosclerosis (Massot *et al.*, 2013), however evidence is limited. The role of EPCs in the progression or prevention of atherosclerosis has been investigated with much interest in the past decade. Some reports show that EPCs, either by association (Fadini *et al.*, 2006) or by infusion are linked with either reduced atherosclerotic plaque number or burden, or with a decrease in plaque progression (Yao *et al.*, 2012; Tousoulis *et al.*, 2013). Interestingly, upon stimulation by tumour necrosis factor-α (TNF-α), late EPCs isolated from humans secreted significant levels of plasminogen activator inhibitor 1 (PAI-1) and monocyte chemoattractant protein-1 (MCP-1) (Zhang *et al.*, 2009). PAI-1 is linked with atherosclerosis (Peng *et al.*, 2008) due

to its role in preventing the dissolution of fibrin and helps with clotting. In fact PAI-1 is expressed within atherosclerotic plaques, and expression is increased with increasingly progressed plaques (Padró *et al.*, 1997). MCP-1 is involved in the adhesion of monocytes to the vascular wall (Gerszten *et al.*, 1999), a process involved in atherosclerotic plaque development. Gosling *et al.* (1999) deleted the MCP-1 gene from alipoprotein B^+ transgenic mice and found that there was a significant reduction in monocyte recruitment to atherosclerotic lesions in comparison to mice with the MCP-1 gene intact. Therefore both PAI-1 and MCP-1 could be considered a pro-inflammatory mediator of atherosclerosis. EPCs secreting these factors is a surprising function, and paves the way for the potential for EPCs to be involved in atherosclerosis development and progression. These observations could suggest that there may be evidence for a pro-inflammatory environment shifting EPCs from an anti-inflammatory to a pro-inflammatory phenotype.

In the studies showing reduction in circulating number of EPCs, the reduction seen may be due to an exhaustion of the BM pool of these cells because of a chronic requirement for vascular repair. In those with CVD, there is evidence of extensive endothelial damage and dysfunction. Endothelial microparticles (EMPs) are blebs of cells that are released by endothelial cells undergoing apoptosis (Jimenez *et al.*, 2003), and several studies have found that EMPs circulating in the blood are elevated in those with CVD (Bernal-Mizrachi *et al.*, 2004; Sinning *et al.*, 2011) and diabetes (Sabatier *et al.*, 2002), suggestive of both endothelial damage and a need for endothelial repair. Taken with the documented evidence of endothelial dysfunction with CVD (Gokce *et al.*, 2003; Shechter *et al.*, 2009; Li and Förstermann, 2013; Miller *et al.*, 2013; Hafner *et al.*, 2014; Liao *et al.*, 2014; Manganaro *et al.*, 2014) it appears that EPCs are at increased demand, and thus it may be that the signal for increased repair may deplete BM of progenitors. For example, an infusion of VEGF in mice has been shown to reduce number of both hematopoietic and mesenchymal stem and progenitor cells within the BM after five days (Tashiro *et al.*, 2014). It could be that the chronic need for EPCs in the CVS could eventually diminish BM progenitors as a result of inadequate production of progenitors. Critical limb ischemia patients have been seen to not only have reduced number of circulating EPCs, but also reduced number of BM $CD34^+$ cells compared to healthy controls (Teraa *et al.*, 2013). Over time this would mean reduced number appearing in the circulation that was seen in the latter study. The authors attributed this to the increased systemic inflammation that is seen in CVD.

The reduction in circulating progenitors may also be due to an impaired mobilisation process. Teraa *et al.* (2013) found a reduced matrix metalloproteinase-9 (MMP-9) activity in those with critical limb ischemia versus healthy controls, a finding that was accompanied by the reduction in circulating EPCs and in BM $CD34^+$ progenitor cell numbers. MMP-9 is reported to play a role in the mobilisation of progenitors from the BM (Huang *et al.*, 2009; Jujo *et al.*, 2010; Ling *et al.*, 2012), thought to be via cleaving BM SDF-1 (Jin *et al.*, 2008), leaving $CXCR4^+$ progenitors free to enter the circulation.

With acute coronary syndromes (ACS) we see an increase in $CD34^+/CD133^+$ progenitor and EPC mobilisation in the acute phase after a myocardial infarction or other ischaemic event, e.g. ischaemic stroke (Shintani *et al.*, 2001; Wojakowski *et al.*, 2004; Assmus *et al.*, 2012; Sobrino *et al.*, 2012; Martí-Fàbregas *et al.*, 2013; Paczkowska *et al.*, 2013; Sepp *et al.*, 2014; Regueiro *et al.*, 2015). The changes in circulating EPC are accompanied by release of VEGF and SDF-1 (Shintani *et al.*, 2001; Wojakowski *et al.*, 2004; Sobrino *et al.*, 2012; Paczkowska *et al.*, 2013; Chen *et al.*, 2015). The extent of mobilisation of these progenitor cells is positively associated with the rise in SDF-1 (Chen *et al.*, 2015). EPCs and other tissue-specific progenitor cells are mobilised as a consequence of ischaemic mobilising factors being released by the ischaemic tissue in order to stimulate or participate in repair or the growth of collateral vessels to improve recovery of blood flow to the ischaemic area. Co-factors, such as diabetes, may also impair this process, a process which will be discussed later in this section.

Type 1 (T1DM) and type 2 diabetic mellitus (T2DM) patients also appear to present with a lower circulating number and function of EPC progenitors than healthy controls (Fadini *et al.*, 2005; Hamed *et al.*, 2009; Sibal *et al.*, 2009; Yue *et al.*, 2011; Jiraritthamrong *et al.*, 2012; Lombardo *et al.*, 2012; Meng *et al.*, 2013; Spinetti *et al.*, 2013), an observation that is augmented with additional vascular conditions (Fadini *et al.*, 2005). Glycated haemoglobin (HbA1c) (Yue *et al.*, 2011), high levels of circulating glucose (Hamed *et al.*, 2009), advanced glycation end products (AGEs) (Li *et al.*, 2012) and oxidative stress have all been observed to affect progenitor cell number and function, through impairing mobilisation from the BM, increasing apoptosis of these cells (Jung *et al.*, 2010; Spinetti *et al.*, 2013), or by interrupting normal cell signalling within the cell to reduce NO production (Hamed *et al.*, 2009). Spinetti *et al.* (2013) also observed differences in capillarity of the BM within T2DM patients who underwent hip surgery. In a multiple regression model the reduction in capillarity of the BM was associated with duration of the diabetes, and fasting glucose levels. The microvascular reductions could cause

disrupted nutrient supply for stem/progenitor cell production within the BM. There was also an accumulation of fat tissue within the BM with T2DM. The reduced vascularity of the BM may not only suggest a potential impaired mobilisation but also an impaired maintenance of the progenitor pool, as was observed.

Paracrine mechanisms within EPCs also appear to be hindered in diabetic conditions. Hyperglycaemic conditions *in vitro* reduces secretion of VEGF and NO (Zhang *et al.*, 2013), and although this was performed *in vitro* for a period of 24 hours, the elevated fasting glucose that were observed with T2DM may chronically affect EPC paracrine functions thus reducing the body's ability to maintain endothelial health. Impaired mobilisation has been seen in diabetics (Gallagher *et al.*, 2007; Barthelmes *et al.*, 2013; Fadini *et al.*, 2013; Westerweel *et al.*, 2013; Albiero *et al.*, 2014), one study showing MMP-9-dependent mobilisation being impaired (Ling *et al.*, 2012).

Impairments in function, mobilisation and survival of EPCs in those with T1DM or T2DM may be potential process in common vascular complications that often develop with diabetes (Lockhart *et al.*, 2011; Llauradó *et al.*, 2012; Roberts and Porter, 2013; Sawada *et al.*, 2014). For an in-depth review of progenitor cells and vascular regeneration in diabetes, readers are directed to the work by Fadini *et al.* (2014).

Due to their apparent role in endothelial homeostasis and vascular repair, and potential role in helping to prevent vascular dysfunction with CVD and age, it is unsurprising that researchers and clinicians are interested in their therapeutic use in medicine. Recently, the use of stem and progenitor cells in medicine has increased. Although the clinical use is still in its infancy, there has been a lot of research in the use of these cells to help treat or prevent disease. With respect to EPCs, researchers have isolated these cells from several populations, grown them in a cell culture lab and then re-injected or transfused them into the patient and assessed efficacy for treatment as well as their safety in this setting. Many researchers have found success in neovascularisation, recovery of blood flow or reduction of infarct sizes in mouse models (Kalka *et al.*, 2000; Chen *et al.*, 2012; Schuh *et al.*, 2012; Chang *et al.*, 2013; Chen *et al.*, 2013; Iskander *et al.*, 2013). Studies in humans are sparse, but so far promising preliminary findings have been shown, for example reduced risk of morality or hospitalisation with those administered with BM-progenitor cells compared to those who did not receive these cells (Schächinger *et al.*, 2006; Assmus *et al.*, 2010). Despite these promising preliminary results, studies investigating long-term effects (>5 years) are warranted before these treatments are

declared ‘safe’. For further information on stem cell therapies for vascular medicine, readers are directed to the reviews by Asahara *et al.* (2011) and Lasala and Minguell (2011). Despite the promise of the therapeutic use of BM-derived progenitor cells in translational vascular medicine, it is likely the cost needed to produce a sufficient number of cells for human vascular or cardiac repair will be too great. Therefore it may be more efficient to promote EPC function and number through non-pharmacological means.

Table 2.2. Endothelial progenitors and vascular-related disease states.

Reference	Subject Characteristics	EPC Assay	Findings
Barsotti *et al.,* 2014	14 non ischaemic heart disease, no CAD (75yrs) 22 presence of CAD (76yrs)	Circulating EPCs and myocardial tissue resident EPCs Flow cytometry $CD34^{+}CD45^{dim}VEGFR2^{+}$	Lower circulating EPC in CAD group Greater EPCs in myocardial tissue in CAD
Bruyndonckx *et al.,* 2014	57 obese children (15yrs) 30 normal weight children (15yrs)	Circulating EPCs Flow Cytometry $CD34^{+}CD45^{dim}VEGFR2^{+}$	Circulating EPCs lower in obese children EPCs significantly positively associated with peripheral arterial tonometry.
Fadini *et al.,* 2006	66 cIMT < Median (42yrs) 71 cIMT >Median (48yrs)	Circulating EPCs Flow Cytometry $CD34^{+}VEGFR2^{+}$	Reduced circulating EPCs in those with high cIMT (32% lower)
Hill *et al.,* 2003	45 men, no symptomatic CAD 50yrs	EC-CFU assay	Significant negative association between EPCs and Framingham Risk Score (FRS) Increased circulating EPCs associated with increased endothelial function
Jung *et al.,* 2010	19 patients with vascular disease without DM (63yrs) 20 patients with vascular disease and with DM (62yrs)	Circulating EPCs Flow Cytometry $CD34^{+}VEGFR2^{+}$ $CD34^{+}CD133^{+}VEGFR2^{+}$	No differences in circulating EPCs No relationship between EPCs and peripheral arterial tonometry

CAD- Coronary Artery Disease, cIMT- Carotid Intima Media Thickness, DM- Diabetes Mellitus, EC-CFU- Endothelial Cell Colony Forming Units, EPC- Endothelial Progenitor Cells, FRS- Framingham Risk Score.

Table 2.2. Endothelial progenitors and vascular-related disease states (continued).

Reference	Subject Characteristics	EPC Assay	Findings
Liao *et al.,* 2014	19 CHD (55yrs) 17 CAD & PAD (53yrs) 21 DM (52 yrs) 20 Healthy controls (51yrs)	Circulating EPCs Flow Cytometry $CD34^{+}CD45^{dim}VEGFR^{+}$	Lower circulating EPCs in DM + CHD + CAD & PAD compared to healthy controls EPCs inversely associated with BMI, SBP, Age, HbA1c, LDL-C
Padfield *et al.,* 2013	201 patients undergoing coronary angiography for ACS or angina	Circulating EPCs Flow Cytometry $CD34^{+}VEGFR2^{+}$ $CD34^{+}CD133^{+}VEGFR2^{+}$	Circulating EPCs not related to CAD extent or clinical outcomes
Rouhl *et al.,* 2012	32 hypertensive patients with CSVD (65yrs) 29 hypertensive patients without CSVD (63 yrs)	Circulating EPCs Flow Cytometry $CD31^{+}CD34^{+}CD45^{dim}VEGFR2^{+}$ EC CFU EPC Telomerase assay	Decrease in circulating EPCs in those with CSVD compared to those without CSVD No difference in EPC colony forming units No difference in telomerase activity
Shantsila *et al.,* 2012	50 heart failure patients (LVEF<40%) (66yrs) 40 disease control (CAD but normal LVEF) (64yrs) 40 healthy controls (61yrs)	Circulating EPCs Flow Cytometry $CD34^{+}VEGFR2^{+}$	Heart failure patients displayed decrease in EPCs compared to healthy controls Decrease in circulating EPCs in CAD compared to healthy controls No difference between heart failure patients and CAD but normal LVEF patients.

BMI- Body Mass Index, CAD- Coronary Artery Disease, CHD- Coronary Heart Disease, CSVD- Cerebral Small Vessel Disease, DM- Diabetic Mellitus, EC-CFU- Endothelial Cell Colony Forming Units, EPC- Endothelial Progenitor Cells, HbA1c- Glycated Haemoglobin LDL-C- Low Density Lipoprotein Cholesterol, LVEF- Left Ventricular Ejection Fraction, PAD- Peripheral Arterial Disease, SBP- Systolic Blood Pressure,.

Table 2.2. Endothelial progenitors and vascular-related disease states (continued).

Reference	Subject Characteristics	EPC Assay	Findings
Teraa *et al.,* 2013	101 critical limb ischaemia patients (65yrs) 37 healthy controls (no PAD) (62yrs)	Circulating and bone marrow EPCs Flow Cytometry $CD34^+VEGFR2^+$ CAC paracrine activity measured	Decrease in circulating EPCs in critical limb ischaemia patients compared with controls MNC CXCR4 expression is decreased in bone marrow in critical limb ischaemia patients CAC paracrine function decreased in critical limb ischaemia patients
Vemparala *et al.,* 2013	57 CAD (43yrs) 57 controls (40yrs)	Circulating EPCs Flow Cytometry $CD34^+VEGFR2^+$ EPC telomere length and telomerase activity	Decreased circulating EPCs in CAD Decrease in telomere length and activity in CAD
Walter *et al.,* 2005	Healthy human volunteers and stable CAD patients (n=unknown)	Circulating EPCs Flow Cytometry **?** + CXCR4 expression JAK-2 phosphorylation measured by immunoblotting	CXCR4 cell surface expression on EPCs in CAD similar to controls Phosphorylation of JAK-2 decreased basally, and stimulated (with SDF-1α) in CAD compared to controls
Xiao *et al.,* 2007	571 adults (40-79yrs)	Circulating EPCs EPC culture (n=571) EC-CFU (n=542)	EPC Culture: Positive association with EPC culture number of cells and FRS Decrease in EPC associated with increase in cIMT SDF-1α strongest determinant of EPC culture numbers and EC-CFU

CAC- Circulating Angiogenic Cells, CAD- Coronary Artery Disease, cIMT- Carotid Intima Media Thickness, EC-CFU- Endothelial Cell Colony Forming Units, EPC- Endothelial Progenitor Cells, FRS- Framingham Risk Score, JAK-2- Janus Kinase-2, MNC- Mononuclear Cells, PAD- Peripheral Arterial Disease,

Table 2.3. Diabetes Mellitus and endothelial progenitors.

Reference	Subject Characteristics	EPC Assay	Findings
Albiero *et al.,* 2014	29 DAN (52yrs), 10yrs duration of diabetes 112 no DAN (59yrs), 12yrs duration of diabetes Experimental diabetic mouse model	Circulating PCs Flow Cytometry $CD34^+$ Experimental mouse model: Basal and ischaemia-induced mobilisation of EPCs ($CD34^+Flk1^+$)	DAN associated with decrease in $CD34^+$ cells Impaired mobilisation of EPCs in diabetic mouse.
Barthelmes *et al.,* 2014	Mouse model of diabetes 16 wks hyperglycaemia vs. non-diabetic control mice	Bone marrow and circulating EPCs Flow Cytometry $Lin1^-VEGFR2^+$	Mobilisation of $Lin^-VEGFR2^+$ cells attenuated in diabetic mice
Fadini *et al.,* 2005	24 diabetics with PVD (69yrs) 16 diabetics without PVD (70yrs) 11 PVD without diabetes (68yrs) 17 without PVD (50yrs)	Circulating EPCs Flow Cytometry $CD34^+VEGFR2^+$	PVD associated with 47% decrease in circulating EPCs
Fadini *et al.,* 2013	24 diabetics (10 T1DM, 14 T2DM) (49yrs) 14 non-diabetics (40yrs)	Circulating EPCs Flow Cytometry $CD34^+/CD34^+CD133^+/CD34^+VEGFR2^+/$ $CD133^+VEGFR2^+/CD34^+CD133^+VEGFR2^+$ Mobilisation to administration of G-CSF	Lower mobilisation of $CD34^+CD34^+CD133^+/CD34^+VEGFR2^+/$ $CD133^+VEGFR2^+$ in diabetics compared to healthy controls
Gallagher *et al.,* 2007	Induction of diabetes in 6-12wk old mice	Circulating EPCs Flow Cytometry $Tie2^+VEGFR2^+$	Decrease in circulating EPCs in diabetic mice compared to non-diabetic mice.

DAN- Diabetic Autonomic Neuropathy, EPC- Endothelial Progenitor Cells, PC- Progenitor Cells, PVD- Peripheral Vascular Disease, T1DM- Type 1 Diabetes Mellitus, T2DM- Type 2 Diabetes Mellitus,.

Table 2.3. Diabetes Mellitus and endothelial progenitors (continued).

Reference	Subject Characteristics	EPC Assay	Findings
Hamed *et al.,* 2009	23 T2DM patients (60yrs) 15 healthy controls (56yrs)	Circulating EPCs Flow Cytometry $CD34^+VEGFR2^+$ EC-CFU EPC SOD activity, O_2^- generation, NO production	Circulating EPCs decreased in T2DM Decrease in EC-CFU in T2DM Decrease in EPC NO production in T2DM Increase in SOD activity in EPCs from T2DM patients Increase in O_2^- generation in EPCs from T2DM patients
Jiraritthamrong *et al.,* 2012	Peripheral blood from healthy adult volunteers	Circulating EPCs cultured in low, medium and high levels of glucose *In vitro* vessel formation capacity Ang-1 gene expression measured in EPCs	Decreased *in vitro* vessel forming capacity in high glucose conditions compared with control Ang-1 mRNA decreased in EPCs cultured in high glucose conditions
Ling *et al.,* 2012	31 T2DM (69yrs) 31 non-diabetic controls (68yrs)	Circulating EPCs Flow Cytometry $CD34^+CD133^+CD45^{dim}VEGFR2^+$ Measured 1, 3, 5, 7, 14 and 28 days post AMI	Decreased mobilisation of EPCs in response to AMI in T2DM Despite greater release of SDF-1α
Lombardo *et al.,* 2012	54 T2DM (53yrs) 24 healthy controls (51yrs)	Circulating EPCs Flow Cytometry Early: $CD34^+CD133^+VEGFR2^+$ Late: $CD31^+VEGFR2^+CD144^+$	Decreased circulating 'late' EPCs in T2DM compared to healthy controls No difference in 'early' EPCs

AMI- Acute Myocardial Infarction, Ang-1- Angiopoietin-1, EC-CFU- Endothelial Cell Colony Forming Units, EPCs- Endothelial Progenitor Cells, NO- Nitric Oxide, SDF-1α- Stromal-Derived Factor 1α, SOD- Superoxide Dismutase, T2DM- Type 2 Diabetes Mellitus.

Table 2.3. Diabetes Mellitus and endothelial progenitors (continued).

Reference	Subject Characteristics	EPC Assay	Findings
Sibal *et al.,* 2009	74 T1DM (26yrs) 80 healthy controls (25yrs)	Circulating EPCs Flow Cytometry $CD34^+VEGFR2^+/CD133^+VEGFR2^+$/ $CD34^+CD144^+/CD133^+CD144^+$	Decrease in $CD34^+CD144^+/CD133^+VEGFR2^+$/ $CD133^+CD144^+$ EPCs in T1DM compared to controls $CD34^+CD144^+$ positively correlated with FMD in T1DM
Westerweel *et al.,* 2013	Experimentally induced diabetes in mice	Circulating and bone marrow EPCs Flow Cytometry $Sca1^+Flk1^+$ Resting and after mobilisation induced by G-CSF/SCF	Bone marrow EPCs similar between diabetic and non-diabetic mice Circulating EPCs decreased in diabetic mice Mobilisation of Sca1+Flk1+ attenuated in diabetic mice
Yue *et al.,* 2011	234 T2DM (57yrs) 121 controls (57yrs)	Circulating EPCs Flow Cytometry $CD34^+VEGFR2^+$ $CD133^+VEGFR2^+$	Decrease in both subsets of EPCs in T2DM Those with HbA1c <6.5% had higher levels of EPCs than those with poor glucose control in T2DM Inverse relationship with arterial stiffness and EPCs

HbA1c- Glycated Haemoglobin, EPCs- Endothelial Progenitor Cells, G-CSF- Granulocyte Colony-Stimulating Factor, SCF- Stem Cell Factor, T1DM- Type 1 Diabetes Mellitus, T2DM- Type 2 Diabetes Mellitus.

2.3.4 Endothelial Progenitors and Ageing

CVD risk increases with age (Kannel and Gordan, 1978). One proposed mechanism for this age-related increased risk is the reduced ability for vascular growth and repair in response to vascular trauma, as shown by reduced endothelial repair after balloon injury in rat model (Torella *et al.*, 2004). There is also a clear age-related impairment in angiogenesis (Rivard *et al.*, 1999; Sadoun and Reed, 2003; Reed and Edelberg, 2004; Wang *et al.*, 2011; Gunin *et al.*, 2014), in addition to increased apoptosis of capillary endothelial cells surrounding skeletal muscle (Wang *et al.*, 2013). Endothelial function is also reduced with advancing age (Spier *et al.*, 2004; Heiss *et al.*, 2005; Soucy *et al.*, 2006; Prisby *et al.*, 2007; Black *et al.*, 2008; Black *et al.*, 2009). This ageing effect on the endothelium could be due, in part, to impairments in mobilisation or function of EPCs. There is some evidence to support this, with observations that age results in reductions in circulating endothelial progenitor cells (Thijssen *et al.*, 2006; Thum *et al.*, 2007) and reductions in progenitor cell function (Heiss *et al.*, 2005; Hoetzer *et al.*, 2007; Thum *et al.*, 2007; Xia *et al.*, 2012a; Xia *et al.*, 2012b; Williamson *et al.*, 2013; Yang *et al.*, 2013).

Thijssen *et al.* (2006) observed significantly reduced $CD34^{+}VEGFR2^{+}$ EPCs in old (67-76 years) versus young men (19-28 years), however the underlying reason for this reduction was not addressed. Heiss *et al.* (2005) observed no change in progenitor cell number, but measures of function (survival, migration to VEGF, and proliferation) were both impaired in old humans (61 years) compared to younger individuals (25 years). Migration and proliferative abilities of the EPCs were independent predictors of endothelial function in both age groups. The reduced function of EPCs in old subjects has been observed elsewhere, both Xia *et al.* (2012a) and Xia *et al.* (2012b) used *in vivo* mouse models to investigate the effect of aged human EPCs on regeneration after carotid artery injury. They found the extent to which the mouse was able to repair the vessel was age-dependent, with the mice receiving the 'young' EPCs displaying greater repair as evidenced by greater re-endothelialisation. Interestingly, other functional measures such as migration as well as adhesion of these cells under stimulation was reduced in EPCs from aged (~68yrs) compared to young (~26yrs) human subjects. The authors attributed this deleterious ageing effect to intracellular signalling of the cells, more specifically the phosphorylation of CXCR4's downstream target, janus kinase-2 (JAK-2). Under stimulation with SDF-1, which binds to CXCR4, there appeared to be significantly less phosphorylated JAK-2 as a result, which was independent of CXCR4 expression on the cell surface of the EPCs as there was no difference in CXCR4 expression between the

two age groups. Therefore it may not be cell number and/or CXCR4 expression, but intracellular signalling which is affected by age. In the aged population in these two studies, there was also a reduction in circulating number of EPCs which may be one effect of ageing, and the aspects of EPC functional decline with age being related to intracellular mechanisms.

Kushner *et al.* (2010a) stimulated isolated EPCs from young and old humans with the stimulant phytohemagglutinin (PHA) for 72 hours. The authors found a reduced release of the pro-angiogenic growth factor G-CSF in the old subjects, with no change in another pro-angiogenic factor, IL-8. Reduction in secretion of such factors may contribute to the reduced angiogenic potential in aged people. In another study, the same group measured telomere length in these cells. Telomeres are replicative DNA sequences (TTAGGG) at the end of chromosomes that protect the DNA genetic sequence from damage. As a result of repeated replications, telomeres can shorten, and telomere length has long been used as a marker for cellular ageing. Continuous telomere degradation leads to loss of its function, which has been associated with senescence (cell being unable to replicate further) or cellular apoptosis. In old people, EPCs appear to have reduced telomere lengths (Kushner *et al.*, 2009) even in apparently healthy men, without diabetes or history of CVD. The reduced telomere length was not different between young and middle aged men however, indicating that function may not be affected until we are in our 60s, as was apparent with reductions in EPC migration in old people but not with middle aged subjects (Hoetzer *et al.*, 2007).

Mandraffino *et al.* (2012) conducted a very interesting study, where they measured circulating $CD34^+$ progenitors in male and female octogenarians. They also measured ROS levels, BMI, and high-density lipoprotein cholesterol (HDL-C) and then followed them up for a period of 7 years. $CD34^+$ progenitor cell numbers were significantly fewer at time of enrolment in those who had died at the end of the 7-year follow up, in comparison to those who remained alive. The number of these progenitor cells were significantly inversely correlated with levels of ROS, which may partly explain the reduced number of EPCs in those who died by the end of the 7-years, as those who had died by the end of the study had significantly higher baseline levels of ROS compared to survivors at the time of enrolment. Despite not investigating endothelial-specific progenitor cells, this study highlights the importance of maintaining high levels of progenitors later in life to sustain tissue repair.

As the study by Mandraffino *et al.* (2012) alluded to, the circulating environment may be affecting the progenitor cells and causing the potential dysfunction seen with age. For example, $CD34^+$ progenitors from aged mice (19-26 months) expressed reduced Notch signalling factors (Delta, Notch protein) when incubated with their own serum, however when incubated with serum from young mice (2-3 months), $Delta^+$ and $Notch^+$ satellite $CD34^+$ cells were significantly enhanced, with a concomitant improvement in muscle tissue regeneration (Conboy *et al.*, 2005).

Ageing may cause the depletion and functional loss of progenitor cells through many means. Oxidative stress (Mandraffino *et al.*, 2012; Rimmelé *et al.*, 2014), DNA damage (Rimmelé *et al.*, 2014; Walter *et al.*, 2015) and potentially BM depletion of these progenitor cells (de Haan and Van Zant, 1999; Rauscher *et al.*, 2003; Dedeepiya *et al.*, 2012). One study found no changes in BM content of $CD34^+$ progenitors with age (Povsic *et al.*, 2010), however this has been contested (Abraham, 2013). Indeed, in support of Abraham (2013) several studies have observed declines in BM content of $CD34^+$ progenitor cells with age (de Haan and Van Zant, 1999; Rauscher *et al.*, 2003; Dedeepiya *et al.*, 2012). The study by Povsic *et al.* (2010) only recruited participants with median age of 62 and recruited 107 participants compared to 332 for the study by Dedeepiya *et al.* (2012), as well as lacking functional data. The majority of the evidence suggests that there is in fact a depletion of BM progenitors with age which may explain, in part, the reduction seen in the circulation with age.

The evidence from the research over the last 20 years indicates that EPC dysfunction and reduction in circulating number with age may play an important role in the age-related increase in CVD risk. It is hence important that we maintain EPC number and function throughout the lifespan to prevent NCD, which appears to be potentially modified with pharmaceuticals as well as modifying lifestyle factors. The effects of CVD, diabetes and age on EPCs are depicted in figure 2.3.

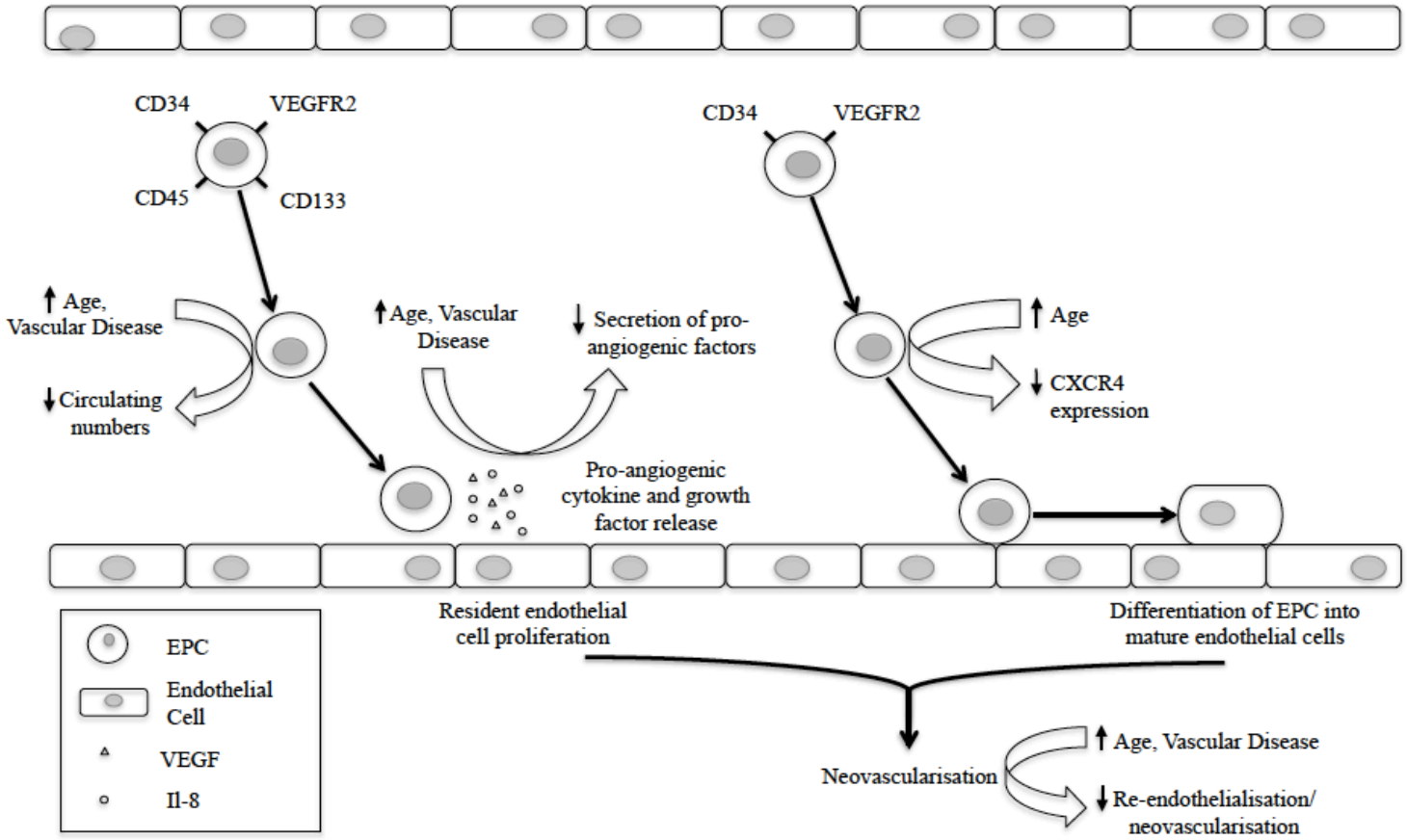

Figure 2.3. Effect of age, CVD and diabetes on circulating EPCs. *EPCs circulate in small number in the blood and can promote neovascularisation through paracrine secretion of growth factors and stimulate resident endothelial cell proliferation, as well as differentiating into endothelial cells. The deleterious effects of age and vascular disease on EPCs are shown and have been found to reduce CXCR4 EPC cell surface expression, inhibit secretion of endothelial growth factors and reduce circulating numbers in the blood, leading to impaired neovascularisation.*

2.3.5 Exercise and Physical Activity- Modulating Endothelial Progenitor Cells

Regular exercise has many benefits, and has been prescribed to help individuals rehabilitating from cardiovascular events. General practitioners and cardiac rehabilitation personnel have proposed that exercise reduces the risk of recurring cardiac events, as well as improving other health parameters such as lowering blood pressure (Mughal *et al.*, 2001), and increasing left ventricular ejection fraction (LVEF) (Oberman *et al.*, 1995; Turan *et al.*, 2006). Since EPCs are reduced or dysfunctional in individuals with vascular-related diseases, it is proposed that exercise, by either acutely or chronically improving either number or function of EPCs, can reduce cardiovascular risk and improve vascular function. It is also proposed that EPCs play an important role in vascular adaptations to exercise in athletes by contributing to capillarisation, resulting in increased oxygen supply to the working muscle and improved exercise capacity (Murias *et al.*, 2011). There have been several reviews on the influence of exercise, both acute and chronic, on EPC number and/or function (Lenk *et al.*, 2011; Silva *et al.*, 2012; Volaklis *et al.*, 2013). These have often focused on specific populations (for example healthy or CVD patients), and lack detailed mechanisms of the influence of exercise on EPC number and/or function.

Acute exercise is known to mobilise EPCs and enhance functional ability of these cells in the post-exercise recovery period of up to 72 hours, depending on the intensity and duration of the exercise bout (Adams *et al.*, 2004; Rehman *et al.*, 2004; Laufs *et al.*, 2005a; Van Craenenbroeck *et al.*, 2008; Möbius-Winkler *et al.*, 2009; Van Craenenbroeck *et al.*, 2010b; Sandri *et al.*, 2011; Ross *et al.*, 2014; Chang *et al.*, 2015). Several studies have failed to show any changes or even a decrease in progenitor cell count post-exercise (Thijssen *et al.*, 2006; Adams *et al.*, 2008; Rummens *et al.*, 2012), but there were many differences amongst these studies, including the subject population recruited, blood sampling time-points, the processing and analysis of EPCs, in addition to the duration, intensity and modality of the exercise bout.

Sandri *et al.* (2011), Scalone *et al.* (2013) and Van Craenenbroeck *et al.* (2011) all compared the response of circulating number of EPCs to a maximal exercise test between individuals with CVD and apparently healthy controls. The results showed that although EPCs increased in response to the exercise bout in both groups, the response was attenuated in individuals with CVD. The control population in the study by Sandri *et al.* (2011) was not age-matched to the population with CVD, and was significantly younger than the CVD group, whereas the study by Van Craenenbroeck *et al.* (2011) successfully

age-matched their participants. The latter study also included a young population, thus investigating the effect of age on the ability to mobilise EPCs to a given exercise bout. They showed that the older control group had an attenuated response to exercise, however the response in the old group was still significantly greater than the CVD. Previous work by Van Craenenbroeck *et al.* (2010a) found no increase in circulating EPCs in either CVD patients or healthy sedentary control subjects 10 minutes following a graded exercise bout, although the EPCs had increased migratory capacity to both VEGF and SDF-1 *in vitro*. Interestingly, this study recorded an improved function of these cells, possibly before any rise in circulating EPCs, which could be as a result of increased CXCR4 surface expression induced by increased shear stress (Xia *et al.*, 2012b) caused by increases in cardiac output. This improved functional ability of EPCs has been seen in other studies, with improved EPC migration to a chemoattractant such as VEGF or SDF-1 *in vitro* (Laufs *et al.*, 2005a; Yang *et al.*, 2007; Van Craenenbroeck *et al.*, 2010b).

The effect of EPC responses to exercise appears to be duration-dependent. Laufs *et al.* (2005a) found an increase in circulating $CD34^{+}VEGFR2^{+}$ cells after 30 minutes running exercise at both 100% and 80% of the individual's anaerobic threshold (IAT), however no such increase was found after 10 minutes running at 80% of IAT. When extended to a marathon, Adams *et al.* (2008) found no change in the same cellular phenotypic EPCs ($CD34^{+}VEGFR2^{+}$). This study only measured EPCs pre- and immediately post-exercise. Any EPC changes during or several hours after the exercise bout would have been missed. EPC mobilisation during an exercise bout and 24 hours post-exercise has been investigated by Möbius-Winkler *et al.* (2009), and a >5-fold increase in $CD34^{+}VEGFR2^{+}$ cells towards the end of a 4-hour cycling bout at IAT was observed. This increase did last up to 24-hours post-exercise when the EPCs returned to baseline levels. A 3.5-fold increase in $CD133^{+}VEGFR2^{+}$ EPCs towards the end of the exercise bout was also recorded which lasted up to 24-hours post-exercise until numbers returned to baseline levels.

Increases in circulating EPC numbers are accompanied by increases in circulating VEGF (Adams *et al.*, 2004; Möbius-Winkler *et al.*, 2009; Sandri *et al.*, 2011; Ross *et al.*, 2014), SDF-1 (Chang *et al.*, 2015), G-CSF, MMP-9 (Ross *et al.*, 2014) or increased NO production (Yang *et al.*, 2007). Some studies have not observed increases in circulating SDF-1 or G-CSF (Yang *et al.*, 2007; Van Craenenbroeck *et al.*, 2011). SDF-1 may play a greater role in homing of EPCs to ischaemic tissue during exercise, as SDF-1 can be expressed on ischaemic tissue cell surface membranes (van Solingen *et al.*, 2011). The

data suggests that both VEGF and NO may play a greater role in exercise-induced EPC mobilisation than SDF-1 or G-CSF despite the ability of SDF-1 and G-CSF to mobilise EPCs from the BM after exogenous administration (Powell *et al.*, 2005; Prokoph *et al.*, 2012). Further studies in VEGF and NO knockout mice will help elucidate the mechanisms behind VEGF and NO-induced EPC mobilisation in response to exercise. Indeed, acute ingestion of dietary nitrate mobilises $CD34^{+}VEGFR2^{+}$ and $CD133^{+}VEGFR2^{+}$ cells in healthy volunteers, thought to be via SDF-1, as SDF-1 was also increased by acute nitrate ingestion (Heiss *et al.*, 2012).

MMP-9 may also contribute to exercise-induced progenitor cell changes. Circulating levels of MMP-9 have been found to be increased post-exercise in studies showing increases in circulating EPCs (Ross *et al.*, 2014) and haematopoietic progenitor cells (Wang *et al.*, 2014b). Reduced level of MMP-9 activity is associated with impaired EPC mobilisation in diabetics (Ling *et al.*, 2012). MMP-9 acts through transforming stromal cell membrane-bound Kit ligand (mKitL) to soluble Kit ligand (sKitL) within the BM (Heissig *et al.*, 2002). Exogenous sKitL stimulates the mobilisation of progenitor cells in both $MMP\text{-}9^{+/+}$ and $MMP\text{-}9^{-/-}$ mice. MMP-9 up-regulation within the BM was stimulated by increases in plasma SDF-1 and VEGF, and therefore exercise may stimulate progenitor cell mobilisation through SDF-1 or VEGF-induced activation of MMP-9 and subsequent conversion of mKitL to sKitL. MMP-9 may also act by cleaving the SDF-1:CXCR4 bonds that may themselves prevent progenitor cell escape from BM (Jin *et al.*, 2008).

In summary, it is clear that there are beneficial effects of acute exercise on EPCs, whether that is by mobilising EPCs from the BM or by increasing their functional abilities potentially through mechanotransduction of shear stress and consequent up-regulation of CXCR4. However, these effects may be short-lived (Möbius-Winkler *et al.*, 2009) and thus regular exercise training may confer a more long-lasting effect.

As with acute exercise studies, most (Laufs *et al.*, 2004; Steiner *et al.*, 2005; Hoetzer *et al.*, 2007; Sarto *et al.*, 2007; Cesari *et al.*, 2009; Manfredini *et al.*, 2009; Van Craenenbroeck *et al.*, 2010a; Schlager *et al.*, 2011; Sonnenschein *et al.*, 2011; Fernandes *et al.*, 2012; Xia *et al.*, 2012a; Choi *et al.*, 2014) but not all studies (Thijssen *et al.*, 2006; Luk *et al.*, 2012), report an increase in number of circulating EPCs or functional capacities of EPCs after a period of exercise.

Xia *et al.* (2012a) used a murine model of carotid artery injury to investigate the influence of exercise training in old men (~68 years old) on EPC function. Pre- and post-exercise

training (30 minutes per day, 3 days per week, 12 weeks aerobic exercise) human EPCs were isolated and cultured. These EPCs were then injected into the left carotid artery of athymic nude mice after carotid injury. Endothelial regeneration was evaluated by measuring area of re-endothelialisation in the denuded area 3 days after injecting the EPCs. The re-endothelialisation of the denuded carotid artery was dramatically improved by injection of the EPCs from elderly men post-exercise training in comparison to EPCs from the elderly men at baseline. This improvement was accompanied by increases in CXCR4 intracellular protein expression and the ratio of p-JAK-2:JAK-2 (measure of intracellular signalling from CXCR4 to JAK-2) was improved. These findings of improved function have been seen elsewhere (Sonnenschein *et al.*, 2011), as well as improved EPC migration towards VEGF (Hoetzer *et al.*, 2007; Van Craenenbroeck *et al.*, 2010a; Schlager *et al.*, 2011) or SDF-1 (Van Craenenbroeck *et al.*, 2010a), adhesion to human umbilical cord vein endothelial cells (HUVEC) (Xia *et al.*, 2012a), and secretion of various cytokines, and soluble factors, e.g. NO (Sonnenschein *et al.*, 2011)

The reported improvements in endothelial function with exercise training appear to be associated with increases in resting levels of circulating EPCs. Steiner *et al.* (2005) found that the increases in EPCs were positively associated with the improvements in FMD in asymptomatic CVD patients. The higher level of circulating EPCs over time may aid in the repair of the vascular endothelium and some EPCs may adhere to the vascular lumen wall and differentiate into mature endothelial cells. These newly differentiated cells may aid in the vascular function by secreting NO to greater levels than the dysfunctional cells they may have replaced. Although EPCs may not be the only potential cause of improvements in endothelial function, the improved FMD seen with exercise training may be in part due to improvements in EPC number and function.

With respect to age, it appears that endurance training may offset the decline in EPC number and function associated with ageing (Yang *et al.*, 2013). The conclusion was based on cross-sectional data comparing young (21-33 years old) vs. old (59-72 years old), and sedentary versus endurance-trained individuals. The authors then conducted a 3-month exercise training regime (3 x a week, 30 minutes per session) in young and the old sedentary subjects. Both the young and the old sedentary participants exhibited an increase in EPC number as a result of the training regime, despite the younger group displaying an attenuated increase compared with the old group. The older participants further increased their EPC migratory capabilities *in vitro*. These increased levels of EPCs and function were inversely correlated with PWV, indicating reduced arterial stiffness.

Thijssen *et al.* (2006) surprisingly reported a decrease in EPCs in aged men after eight weeks of endurance training. The authors suggested that a reduced bioavailability of NO in the old men was to account for this decrease. There has been evidence of reduced NO availability in advancing age in humans (Di Massimo *et al.*, 2006). However, Sonnenschein *et al.* (2011) was able to report an increase in NO production by EPCs as a result of a regular moderate-intensity exercise training programme in subjects with the metabolic syndrome, with an average age of 58. This suggests that exercise as a stimulus can significantly up-regulate NO production and release in vascular cells (Taddei *et al.*, 2000). Conversely, Brinkley *et al.* (2009) reported no significant changes in circulating nitrate/nitrite (measure of NO in the circulation) in older individuals after a period of 4 weeks of exercise training. There is also evidence that suggests NO prevents endothelial senescence through the activation of telomerase (Vasa *et al.*, 2000), and an age-related decline in NO bioavailability may contribute to endothelial senescence and apoptosis. It also may be that the inability to upregulate eNOS and subsequent NO production after exercise training in older adults leads to increased EPC senescence and apoptosis resulting in lower circulating functional EPCs. The other possible cause of reduced EPC circulating numbers in this study after the training period is the method by which they chose to analyse the EPCs. The authors quantified EPCs by flow cytometry within 24 hours of incubation with antibodies. This long time delay could be the cause for this contrasting finding, as the fluorescent tag of the antibodies used may have degraded over this time period.

The longitudinal change of $\dot{V}O_{2max}$ appears to modulate EPC changes as a result of an exercise programme. Changes in EPC are associated with changes in $\dot{V}O_{2max}$ (Cesari *et al.*, 2013). Conversely, a reduction in $\dot{V}O_{2max}$ was associated with a reduction in EPC count. The participants without an increase or even a decrease in EPC count post-exercise rehabilitation programme were those with a significantly higher maximal aerobic power output (W_{max}) than those who had displayed an increase in EPCs (133W versus 119W). It could be that those with already higher levels of functional fitness and functionality will not respond with a positive change of EPCs to an exercise programme as those with a lower level of fitness. This is not due to differing responses of plasma VEGF post-exercise, as this angiogenic factor and EPC-mobilising factor has been reported to be increased post-exercise independent of training status (Kraus *et al.*, 2004)

Intriguingly, increases in EPC function as a result of an exercise training programme may be predicted by the individuals responses to an acute bout of exercise. The extent of

improvement in EPC migration as a result of 6 months exercise training was highly associated with the individual's migratory capacity improvements post-graded exercise test (r=0.780) (Van Craenenbroeck *et al.*, 2010a). Therefore those most responsive to an acute bout of exercise will gain the most from an exercise training program. On the other hand, those who are not responsive to an acute bout of exercise may not benefit greatly from an exercise training programme. This has become a focal discussion point within exercise physiology circles, with the 'trainability' of individuals causing some to state that some people will not respond to training and therefore should not be pushed towards an exercise training program. Data to back this theory has come within the last 5 years, with studies showing large variations in physiological changes to a personalised training program (Timmons *et al.*, 2010; Keller *et al.*, 2011; Bacon *et al.*, 2013).

Contrary to the majority of the literature investigating activity and fitness on EPCs, Alba *et al.* (2013a) and Alba *et al.* (2013b) found that EPCs ($CD34^+VEGFR2^+$) were inversely associated with peak $\dot{V}O_2$. However, EPCs were analysed by flow cytometry after peripheral blood MNC isolation using Ficoll-density gradient. There is debate over whether this procedure will give the greatest yield of EPCs, and results in less background noise during the flow cytometric enumeration. On the other hand, by density gradient centrifugation there is a risk of losing a large number of cells, and considering that these EPCs are reported to make up only 0.0001 and 0.01% of MNCs (Case *et al.*, 2007), it may be more beneficial to enumerate EPCs using whole blood. Therefore, results such as those seen from Alba *et al.* (2013a) and Alba *et al.* (2013b) must be interpreted with caution. It may also be the case that those with greater functional capacity (as assessed by peak $\dot{V}O_2$) may not have the 'need' for the increased progenitor cell number, therefore there is no increased requirement for their mobilisation into the peripheral circulation. These two studies did not include measures of VEGF or SDF-1, which themselves would have given a clearer picture of why they observed this surprising inverse relationship between progenitor cell numbers and peak $\dot{V}O_2$. Conversely, Povsic *et al.* (2013) found low number of circulating progenitor cells ($CD34^+$) in 60-81 year old men with poor 6 minute walking test scores, however no relation to other physical function tests such as grip strength or balance was demonstrated.

The mechanisms by which there is an improvement in EPC function due to regular exercise training are several-fold. Firstly, exercise training can reduce basal levels of inflammation (Goldhammer *et al.*, 2005; Stewart *et al.*, 2007; Timmerman *et al.*, 2008) and oxidative stress (McArdle and Jackson, 2000; Roberts *et al.*, 2002; Bloomer and

Fisher-Wellman, 2008; Durrant *et al.*, 2009), both of which are associated with reduced function of these cells (Witkowski *et al.*, 2010). In addition, the regular increases of shear stress may act as a biomechanical signal to the EPCs, with the mechanical disruption of the cell membrane by the increased blood flow flowing past stimulating intracellular signalling cascades involved in upregulating functional proteins. Shear stress has been consistently shown to affect EPC biology through intracellular signalling mechanisms (Yang *et al.*, 2012), promoting endothelial differentiation (Ye *et al.*, 2008; Obi *et al.*, 2009; Cheng *et al.*, 2012; Cui *et al.*, 2012; Obi *et al.*, 2012; Cheng *et al.*, 2013), increased NO production (Tao *et al.*, 2006) and migration (Xia *et al.*, 2012b) which itself may be a direct consequence of increased CXCR4 expression. It also appears that NO plays a significant role in the changes in EPCs in the circulation with exercise. In eNOS knockout mice (eNOS-$^{-/-}$) there was an attenuated response of EPCs to exercise training in comparison to wild-type mice, and in wild-type mice fed with an eNOS inhibitor (N^G-nitro-L-arginine methyl ester; L-NAME) there was no significant change in circulating EPCs (Laufs *et al.*, 2004), highlighting the importance of NO in exercise-induced BM mobilisation of EPCs.

2.3.6 Physical Inactivity and Vascular Health- Potential Deleterious Effect on Endothelial Progenitor Cells

Physical inactivity, a risk factor for CVD development, and for mortality, may also affect progenitor cell number and/or function. It is known that inactivity deleteriously affects vascular health (Laufs *et al.*, 2005b; Thijssen *et al.*, 2010; Boyle *et al.*, 2013; Thosar *et al.*, 2015a). Enforced detraining after several weeks of training was shown to significantly reduce circulating EPCs, as well as decrease vascular function as assessed by reactive hyperaemia (Witkowski *et al.*, 2010). These 2 outcome measures as a direct result of detraining were also positively associated, linking a possible reduction in EPC availability to a subsequent deleterious effect on the vasculature. Unsurprisingly there was a high level of oxidative stress, as measured by ROS after the detraining period, whereas the ROS level was reduced after a period of pre-conditioning exercise training. Schlager *et al.* (2011) found improvements in EPC migration and circulating numbers after three and six months of regular exercise training in PAD patients, however after six months of detraining the migratory ability of these cells returned to baseline levels. EPC circulating numbers (as expressed as % of MNCs) remained higher than baseline despite a trend for

reduction from 6 months of training. Figure 2.4 summarises the effects of acute and regular exercise training on EPCs.

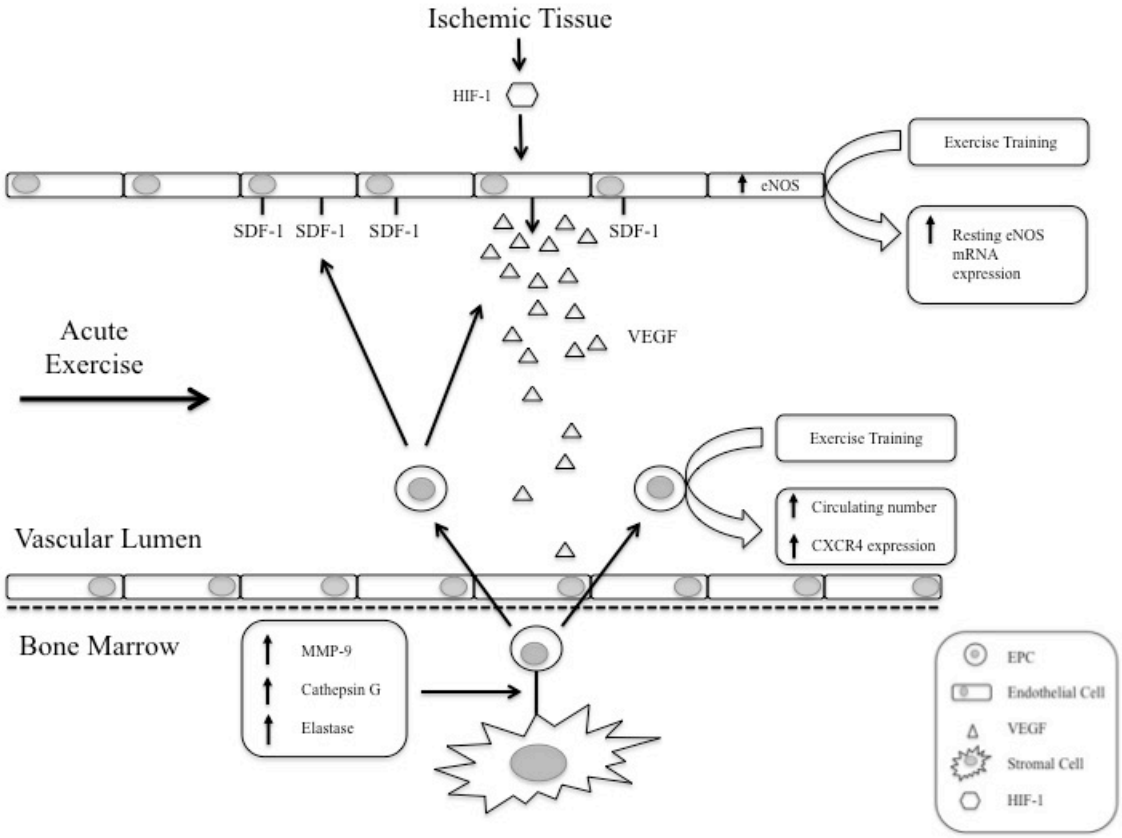

Figure 2.4. The effect of acute and regular exercise training on number and function of EPCs. *Acute exercise causes the proteolysis of stromal bond with EPCs allowing EPCs to transmigrate into the circulation. Once in the circulation, EPCs home to areas of high chemoattractant concentration (e.g. VEGF and SDF-1). Acute exercise-induced tissue ischaemia causes HIF-1-mediated increase in endothelial VEGF release. Exercise training causes increased EPC circulating numbers, and potentially increased CXCR4 cell surface expression. In addition, exercise training stimulates increases in eNOS mRNA expression in endothelial cells. Taken together exercise training results in improved neovascularisation via improvements in EPC number and function, and increases endothelial function as a result of its direct effects on endothelial cells.*

It is clear that exercise training in a wide population of people (CVD patients, aged) can increase EPC circulating levels as well as improve their function which has been aligned to enhanced vascular repair, endothelial function and reduced arterial stiffness thus improving overall cardiovascular health and reducing the risk of potential future vascular-related events.

2.4 Angiogenic T Cells- A New Prospect for Neovascularisation

A variety of leukocytes are known to have angiogenic and vasculo-supportive properties. Polymorphonuclear neutrophils can release IL-8 (Cassatella, 1995) and VEGF (Gaudry *et al.*, 1997), both of which are pro-angiogenic. Culture supernatant from the activated cells also stimulate capillary growth (Schruefer *et al.*, 2005). This supernatant contained significant quantities of IL-8 and VEGF within one hour of stimulation, highlighting their proangiogenic capacity. Both nude mice (deficient in all components of the T-cell population) and CD8 knockout mice (CD8 is an antigen for cytotoxic T-cells) exhibit impaired angiogenic responses to damage or blood vessel occlusion (Stabile *et al.*, 2003; Stabile *et al.*, 2006). To further demonstrate the angiogenic properties of T-cells, $CD3^+$ cells express VEGF mRNA in both CD4 and CD8 sub-fractions which is expressed to a greater extent by exposure to hypoxia (Freeman *et al.*, 1995). Further support for the role of T-cells in post-natal vasculogenesis and endothelial repair came recently from Wu *et al.* (2015), who provided evidence of $CD3^+$ T-cell involvement in vessels after experimental myocardial infarction in mice. Of all the cells that were located in the microvasculature that were not of vessel origin, 21% of these were $CD3^+$ T-cells. However, recovery of blood flow and cardiac functional measures were not performed on the mice.

Hur and colleagues (2007) isolated peripheral blood MNCs and attempting to isolate EPCs. In the central cluster they identified a population of $CD3^+$ T-cells co-expressing CD31. CD31 is an adhesion molecule which regulates leukocyte adhesion and trafficking on the endothelium (Muller, 1995). These $CD3^+CD31^+$ T-cells were required for the optimal *in vitro* differentiation and proliferation of EPCs which were co-cultured (Hur *et al.*, 2007). These 'angiogenic T-cells' (T_{ANG}) secreted VEGF, IL-8 and G-CSF to a greater extent than the $CD31^-$ sub-fraction of T-cells, and the T_{ANG} cells also had a greater capacity for adhesion to HUVECS, and they also migrated towards SDF-1 significantly more than the $CD3^+CD31^-$ cells. It was also found that the T_{ANG} cells expressed CXCR4, which would explain their ability to migrate towards SDF-1. Blocking of CXCR4 on T_{ANG} with a neutralising antibody significantly reduced migration, suggesting that the transendothelial migration of these cells may depend on the SDF-1:CXCR4 axis. The authors injected these cells into mice with hindlimb ischaemia and assessed capillarity and blood flow recovery for 21 days after ligation of the femoral artery. Blood flow recovery and capillarity was greater in the mice which were injected with T_{ANG} cells in comparison to those mice injected with $CD3^+CD31^-$ cells. This improved ability of the

double positive cells to promote vascular growth and capillaries may be linked through greater adhesion onto vessel wall due to the expression of CD31, as well as potentially through CXCR4 signalling as the researchers found that 97% of $CD3^+CD31^+$ cells expressed CXCR4, whereas only 37% of $CD3^+CD31^-$ cells expressed CXCR4, potentially opening up a relationship between expression of CD31 and CXCR4 and expression of CD31 may also regulate CXCR4.

Kushner *et al.* (2010b; 2010c) corroborated these findings through T_{ANG} cells migrating to both SDF-1 and VEGF. In addition to the previous findings showing that these cells secrete VEGF, IL-8 and G-CSF, Kushner *et al.* (2010b) stimulated the T_{ANG} cells with PHA and found that in addition to IL-8 and G-CSF, the cells also released MMP-9, a key enzyme involved in the degradation of the basement membrane in the process of angiogenesis. The authors also stated that these cells may be more susceptible to apoptosis by the relatively greater intracellular levels of caspase-3 and cytochrome c. Cytochrome c is involved in the caspase-9-dependent activation of caspase-3 (Li *et al.*, 1997), which itself is involved in cellular apoptosis (Faleiro *et al.*, 1997). Their susceptibility to apoptosis may be due to their status of being recent thymic emigrants (Ashman and Aylett, 1991; Kilpatrick *et al.*, 2008), as these cells display more T-cell receptor excision circles (TREC), an indicator of these cells recently passaging through the thymus (Huehn *et al.*, 2004). Recent thymic emigrants have yet to proliferate to a great extent, and highly proliferative and highly differentiated T-cells are less susceptible to apoptosis, (Spaulding *et al.*, 1999), so this finding is not a surprise.

Other studies have observed CD31 in T-cells being linked to modulating immunological functions, e.g. T-cell and B-lymphocyte interactions (Clement *et al.*, 2015). CD31 signalling in T-cells appeared to attenuate joint inflammation associated with rheumatoid arthritis (RA) in mice as indicated by CD31 agonism in T-cells causing a reduction in T:B-cell interaction, which is a classic process within arthritis and inflammation (Clement *et al.*, 2015). The reduced T:B-cell interaction was accompanied by significantly reduced inflammation in ankle joints of mice, as well as reduced inflammatory cytokines TNF-α and interferon-γ (IFN-γ). T_{ANG} cells may play a role in preventing RA as the circulating number of these cells are low in patients with RA (Rodríguez-Carrio *et al.*, 2014).

Bird *et al.* (1993) demonstrated that CD31 may not assist in T-lymphocyte migration. This group performed transmigration experiments with T-cells across a human umbilical vein endothelial cell (HUVEC) monolayer, and found predominantly $CD31^-$ T-cells in

the transmigrated population. This could be due to a transient loss of CD31 expression as cells migrated across the HUVEC layer. The transmigrated cells were also depleted of CD45RA. The expression of CD31 and CD45RA on the T-cells basally were highly correlated, suggesting that $CD3^+CD31^+$ T-cells may be predominantly of naïve phenotype. Therefore, highly differentiated T-cells, which lack CD45RA and express high levels of CD45RO may have a greater potential to migrate. Yet Stockinger *et al.* (1992) demonstrated that after activation, $CD3^+$ cells may lose CD45RA and upregulate CD45RO cell surface expression with no changes in CD31 cell surface expression seven days after activation with PHA. In contrast, Fornasa *et al.* (2010) demonstrated that memory T-cells may not downregulate cell surface CD31 expression, but rather enzymatically shed it from the membrane. The evidence from Bird *et al.* (1993) suggests that CD31 may not be required for T-cell migration, yet more recent evidence supports the role of CD31 in T-cell transendothelial migration by blocking CD31 suppressing $CD4^+$ T-cell migration across TNF-α-treated human dermal microvascular cells (Manes *et al.*, 2010).

CD31 monoclonal antibody (mAb) binding on human monocytes has been demonstrated to elicit functional effects, such as respiratory burst (Stockinger *et al.*, 1990). CD31 mAb binding had no effect on calcium release or proliferation in $CD3^+$ T-lymphocytes (Stockinger *et al.*, 1992), with the authors stating that CD31 appeared to have no physiological function on T-cells. Recently, Hur *et al.* (2007) stimulated $CD3^+CD31^-$ and $CD3^+CD31^+$ T-cells isolated from human volunteers and observed a significantly greater release of pro-angiogenic cytokines and growth factors (VEGF, IL-8) in the $CD31^+$ fraction compared to the $CD31^-$ subset. In addition the $CD31^+$ subset demonstrated greater adhesion to HUVECs and greater transendothelial migration than $CD31^-$ T-cells. The greater transendothelial migration was attenuated by adding CXCR4 antibody, suggesting that the greater migration abilities of $CD31^+$ T-cells compared to $CD31^-$ T-cells may be due to the SDF1:CXCR4 axis. The blunting effect of the CXCR4 antibody still did not blunt the transendothelial migration enough to similar levels seen with the $CD31^-$ population, suggesting that the presence of CD31 on T-cells may still aid migration independent of CXCR4.

Several studies have implicated the CD31 molecule in immunomodulatory roles, with genetic deletion of CD31 leading to increased T-cell-mediated inflammation involved in collagen-induced arthritis (Tada *et al.*, 2003) and increased allograft rejection and increased amplification of $CD31^-$ T-cells indicating reduced regulatory T-cell-mediated

immunosuppression (Ma *et al.*, 2010). Kishore *et al.* (2012) suggested that CD31 may be a key molecule involved in controlling T-cell effector functions through attenuation of T-cell receptor (TCR) activation and reduced Zap-70 phosphorylation which reportedly results in T-cell expansion and effector functions (Ma *et al.*, 2010). The suppression of T-cell clonal expansion could be mediated by CD31-activated signalling as a result of interaction of $CD31^+$ naïve T-cells with antigen-presenting dendritic cells, however the downstream cascade is not fully understood. Kishore *et al.* (2012) demonstrated that wild type (WT) mice displayed reduced T-cell responses (migration and phosphorylation of Akt) to chemokine CXCL10 compared to $CD31^{-/-}$ mice. It is interesting to note that the effects observed were only seen in activated T-cells, not naïve T-cells. The transformation of naïve T-cells to activated T-cells also appears to alter the distribution of CD31 on the T-cell surface membrane, with an even distribution in naïve T cells, and clustering of CD31 in activated T-cells (Kishore *et al.*, 2012). The significance of this has yet to be identified.

In summary, it appears that CD31 expression on T lymphocytes, in addition to being linked with vasculogenic properties, may also regulate T-cell activation and prevent T-cell hyper-reactivity, which may play a role in prevention of auto-immune diseases, thus confirming that these cells may have a variety of roles within both the immune and cardiovascular systems.

2.4.1 Angiogenic T Cells in Vascular Disease

Due to their potential role in vasculogenesis, as shown by modulating HUVEC tube formation and their requirement for optimal EPC growth (Hur *et al.*, 2007) several researchers have investigated whether T_{ANG}, or the lack of T_{ANG}, play a role in CVD prevention or development. Thus far, T_{ANG} levels (reported as a % of total $CD3^+$ cells) have been observed to be inversely related with Framingham Risk Score (FRS; a scoring system that aims to give risk profile for CVD onset within 10 years) as well as age (Hur *et al.*, 2007). The small sample number means that more investigation is required (n=58). Weil *et al.* (2011) failed to observe a relationship between T_{ANG} and forearm blood flow responses to acetylcholine, or to observe an association with the FRS, possibly as the study may have been underpowered to investigate this (n= 24). The authors suggested that T_{ANG} play only a minor role in endothelial function maintenance, but more studies are required with a greater number of participants to fully elucidate the role of T_{ANG} in

vascular function. The authors did find a relationship of migration to SDF-1 or VEGF and forearm blood flow response to acetylcholine, and also found an inverse association with FRS. It may be that function of these cells, not necessarily circulating number are more predictive or vascular risk/function. Therefore CXCR4 expression would also need to be also measured to assess if this is affected by age and/or CVD. So far no studies to date have measured CXCR4 expression in relation to mean fluorescence intensity (MFI-surrogate marker for level of expression on the cell surface which can be analysed by flow cytometry), or intracellular mRNA/protein levels.

Rouhl *et al.* (2012) defined T_{ANG} as $CD3^{+}CD31^{+}CXCR4^{+}$ cells, however they failed to provide a measure of relative expression of CXCR4 on the T_{ANG} cells. Despite this, T_{ANG} co-expressing CXCR4 were lower in hypertensives exhibiting small vessel cerebrovascular disease in comparison to those without small vessel cerebrovascular disease (age-matched and no differences in medications). No measures of oxidative stress, cellular function or vascular function were made therefore mechanisms or reasons behind this possible link between T_{ANG} number and vascular health have yet to be elucidated.

CD31 expression on T-cells may play a regulatory role in atherosclerosis, preventing plaque neovascularisation, atherosclerotic lesion progression, as well as preventing T cell infiltration of plaques (Groyer *et al.*, 2007). The role in atheroprotection of $CD31^{+}$ T-cells most probably is not due to its vasculogenic effects, but could be attributed to their non-activated status. T-cell activation is associated with a loss of CD31 expression (Zehnder *et al.*, 1992), and thus CD31 expression on T-cells may represent a population on non-activated cells. CD31 on $CD4^{+}$ T-cells also play a regulatory role on $CD8^{+}$ cytotoxic T-cells and prevented their inflammatory effects on VSMCs in abdominal aortic aneurysms (Caligiuri *et al.*, 2006). The exact role of the adhesion molecule CD31 on T-cells in the protection of the vascular wall from inflammatory processes has yet to be fully examined.

In patients with RA T_{ANG} cells expressing CXCR4 were reduced versus healthy controls, but more interestingly, in those with RA, those who had also suffered a cardiovascular event since their diagnosis of RA had a significantly greater decrease in T_{ANG} (versus healthy controls) than those who were diagnosed with RA and were event free (Rodríguez-Carrio *et al.*, 2014). The reduction in T_{ANG} could potentially account for increased vascular risk in those with RA through either reduction in vasculoprotection or an associated increase in systemic inflammation.

Although $CD3^+CD31^+$ T_{ANG} cells have been investigated for their vascular properties, other subsets of T-cells may be able to stimulate repair in vascular events. $CD3^+CXCR4^+$ could be potentially used as a biomarker for general cardiovascular risk without the requirement for the CD31 antibody marker. Teraa *et al.* (2013) found that T lymphocytes expressing CXCR4 were higher in critical limb ischemia patients compared to healthy controls, and interestingly CXCR4 expression in BM was lower in critical limb ischemia patients. The lower level of $CXCR4^+$ cells in BM in these patients could mean an increased release of the $CXCR4^+$ cells as there may be a greater signal to mobilise these cells from the BM for repair and to recovery blood flow to ischemic tissues. Akin to EPC responses to myocardial infarction, there may be an increased signal for circulating $CXCR4^+$ cells to be mobilised into the circulation, whether they be $CXCR4^+$ progenitors or $CXCR4^+$ lymphocytes, to stimulate rapid repair and attempt to recover blood flow to the ischaemic limb or tissue, highlighting the importance to include general cell subsets for analysis (e.g. $CD3^+CXCR4^+$, $CD34^+$, $CD34^+CXCR4^+$).

By reducing the proportion of $CD3^+CD31^+$ cells and thus resulting in an increase in $CD31^-$ T-cells may promote inflammation which could have negative effects on the endothelium. $CD31^-$ T-cells, when stimulated, produce and release more IFN-γ than $CD31^+$ T-cells (Caligiuri *et al.*, 2006). Björkbacka *et al.* (2015) found an inverse relationship between IFN-γ-expressing $CD4^+$ T-cells and coronary events. The role of $CD31^-$ T-cells in cardiovascular health has yet to be investigated in full.

2.4.2 Angiogenic T Cells and Ageing

Age is a risk factor for the development of CVD and thus it is important to assess the role T_{ANG} may play in preventing CVD, and if there is an age-related effect on these cells which would have a knock-on effect on cardiovascular risk. The literature supports the hypothesis that there is a reduction in T_{ANG} cells, as these cells were significantly lower in older (24% of $CD3^+$ T-cells) compared with middle-aged (38%) and young (40%) men, and this was accompanied by a marked reduction in migration to both SDF-1 and VEGF in the middle-aged and old groups in comparison to the young group (Kushner *et al.*, 2010c). The data suggests that functional impairment may be the first step prior to a reduction in the proportion of $CD3^+CD31^+$ T-cells with age. Telomere length, as with EPCs, progressively declined with age in this study, and this telomeric reduction may be

associated with migration in these cells, potentially linking telomere length and cellular function.

Thymic involution occurs with advanced age resulting in a decrease in thymic output of naïve T-cells (Fagnoni *et al.*, 2000; Simpson, 2011), however, the changes in T_{ANG} cells were in the absence of $CD3^+$ T-cell number, suggesting other age-related mechanisms are at play. Telomerase activity is linked with CXCR4 expression, at both the protein and mRNA level, as well as SDF-1-stimulated migration in fibroblasts (Qu *et al.*, 2008). The age-related decline in telomere length and telomerase activity may be partially responsible for age-related dysfunction of T_{ANG}, and decline in number, however no studies have investigated the link between telomere length or telomerase activity and intracellular T_{ANG} signalling with specific focus on the CXCR4:SDF-1 axis.

2.4.3 Angiogenic T Cells and Acute Exercise

Acute exercise has a dramatic effect on circulating T-lymphocyte numbers. Acute exercise, depending on intensity and duration (Campbell *et al.*, 2009) can elicit large increases in circulating T-cells, from 64% to 181% in healthy human volunteers (Simpson *et al.*, 2007; Campbell *et al.*, 2009). This is reportedly due to increases in cardiac output and subsequent shear stress on the vascular endothelium (detaching adherent lymphocytes) and by increases in catecholamines, such as epinephrine and norepinephrine (Simpson *et al.*, 2007). Interestingly, exercise acutely preferentially mobilises T-cells with a highly differentiated phenotype compared to naive T-cells (Simpson *et al.*, 2010; Turner *et al.*, 2010; LaVoy *et al.*, 2014), potentially due to the greater expression of ß-adrenergic receptors found on the differentiated T-cells. However, to date, there is no evidence to demonstrate the effect of acute exercise on CD31-expressing T-cells, but there is evidence to suggest that T-cells expressing adhesion receptors, such as CD56 and integrins) demonstrate a greater ingress and subsequent egress from the peripheral circulation compared to cells that do not express these receptors (Goebel *et al.*, 2000; Shephard, 2003; Simpson *et al.*, 2006). The exact mechanism for the preferential redistribution of cells expressing adhesion receptors is not yet known.

Evidence strongly suggests that acute exercise may provide a short window of vascular adaptation due to acute increases in CACs, such as EPCs (Adams *et al.*, 2004; Rehman *et al.*, 2004; Laufs *et al.*, 2005a; Van Craenenbroeck *et al.*, 2008; Möbius-Winkler *et al.*,

2009; Sandri *et al.*, 2011; Scalone *et al.*, 2013; Ross *et al.*, 2014; Chang *et al.*, 2015), pro-angiogenic monocytes (Rehman *et al.*, 2004; Bonsignore *et al.*, 2010; Avolio *et al.*, 2015), as well as documented evidence of improved CAC function post-exercise (Chang *et al.*, 2015). These cells are reported to mobilise into the circulation due to exercise-induced increase in SDF-1α (Chang *et al.*, 2015), a ligand for CXCR4, a receptor often located on these progenitor and CAC subtypes (Adams *et al.*, 2013; Hur *et al.*, 2013; Abdallah *et al.*, 2014). T-cells also express this chemokine receptor (Abbal *et al.*, 1999; Hur *et al.*, 2007; Besedovsky *et al.*, 2014), therefore, the CXCR4:SDF-1α axis may play a role in the T-cell redistribution observed with acute exercise.

If $CD31^+$ T-cells display a similar redistribution in response to acute exercise as other T-cell subpopulations do (Simpson *et al.*, 2008; Ingram *et al.*, 2015), then this may offer a short period of time whereby these cells can act on the endothelium to promote endothelial health.

2.5 The Role of C-X-C Chemokine Receptor 4 in Vascular Repair and Regeneration

Cell migration is a key process for many cells which need to enter and exit the peripheral blood compartment in order to perform their effector functions. For circulating angiogenic cells (CAC), like EPCs and T_{ANG} cells, the ability to migrate to ischaemic tissue may be a key function which will underline their effect on prevention of vascular disease. A key player in the cell's ability to migrate is CXCR4, a seven trans-membranous protein, which is a receptor for the ligand SDF-1 (Yamaguchi *et al.*, 2003).

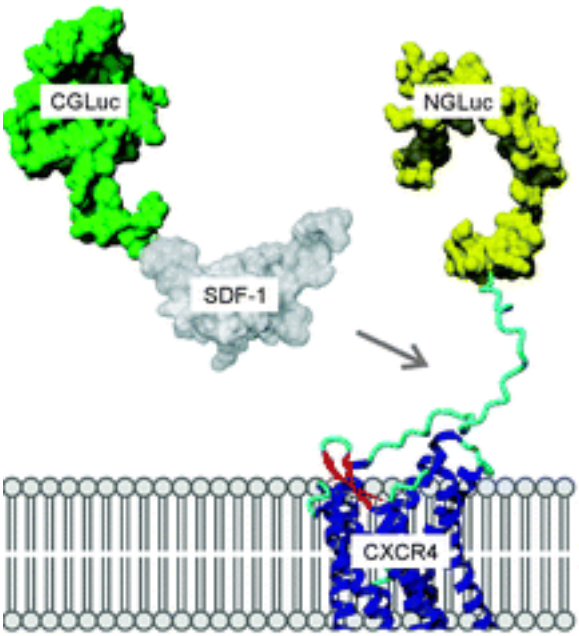

Figure 2.5. SDF-1 binding with extracellular N-terminus of CXCR4. From Kuil *et al.* (2012).

The importance of CXCR4 is shown when deletion of CXCR4 in mice results in perinatal lethality, and in those mice with CXCR4 deficiency, the BM number of hematopoetic stem cells was reduced (Sugiyama *et al.*, 2006), indicating a role for CXCR4 in haematopoiesis. CXCR4 may also play an important role in homing of T-cells to the thymus (Calderón and Boehm, 2011). Interestingly, CXCR4 also acts as an 'entry' protein for human immunodeficiency virus (HIV) into $CD4^+$ T-cells (Hesselgesser *et al.*, 1998).

CXCR4 and SDF-1 (the ligand for CXCR4) is critical for development of vasculature during development as shown by reduced cardiac vascularisation in SDF-1 and CXCR4 mutant mice (Ivins *et al.*, 2015) as well as vascular abnormalities in embryonic development in SDF-1 and CXCR4-deficient mice (Ara *et al.*, 2005). The process of SDF-1-induced trafficking of $CXCR4^+$ MNCs to sites of vascularisation is thought to play a significant role in embryonic vascular development (Ivins *et al.*, 2015). In the adults, circulatory increases in SDF-1, as caused by secretion by hypoxic tissue (Ceradini *et al.*, 2004; De Falco *et al.*, 2004), signals $CXCR4^+$ cells to move into the circulation from the BM and other lymphoid tissues (Hattori *et al.*, 2001; Heissig *et al.*, 2002) and home to areas of vascular damage or sites of ischaemia (Yao *et al.*, 2003). Here, $CXCR4^+$ cells can migrate to ischaemic tissue along a gradient of SDF-1 to where they can exert effector functions, either directly differentiating into mature endothelial cells (Mao *et al.*, 2014),

or stimulate endothelial cell proliferation by paracrine means (Hur *et al.*, 2007; Hur *et al.*, 2013).

The ability of T-cells and progenitor cells to migrate to SDF-1 is highly dependent upon the cell surface expression of CXCR4 (Walter *et al.*, 2005; Bryant *et al.*, 2012; Xia *et al.*, 2012b; Adams *et al.*, 2013; Mao *et al.*, 2014) or the signalling between CXCR4 and its downstream target, JAK-2 (Walter *et al.*, 2005; Xia *et al.*, 2012a). The study by Hur *et al.* (2007) demonstrated that $CD31^+$ T-cell migration was significantly blunted by blocking CXCR4, yet this did not fully impair migration to levels similar to $CD31^-$ T-cells, which was blunted compared to the $CD31^+$ fraction. This indicates that CXCR4 is required for optimal cell migration, yet not essential.

There is evidence to show that ageing and vascular disease results in a reduced number of $CXCR4^+$ BM-derived cells (Xu *et al.*, 2011), and blocking of CXCR4 with antibodies reduce the ability of EPCs to migrate to both SDF-1 and VEGF, in addition to an impaired vascular regeneration with disrupted CXCR4 signalling (Walter *et al.*, 2005), yet these researchers observed no differences in cell surface expression of CXCR4 on EPCs between CAD patients and age-matched healthy controls indicating that cell surface expression may not be the determining factor behind their ability to stimulate revascularisation, but instead it may the signalling cascade, from CXCR4 to JAK-2 which may be disrupted in vascular disease or age.

2.6 Summary

Circulating angiogenic cells (CAC) are important to the maintenance and growth of the vasculature. Ageing is a significant risk factor for the onset and progression of CVD, and thus interventions which promote 'successful' ageing to reduce the risk of disease and mortality have been highly sought after. Exercise, whether it be acute or chronic exercise interventions, has been shown to improve circulating number and/or function of some CAC subsets, and also provides many more benefits such as improved blood pressure profile, reduced cholesterol and oxidative stress which may promote 'successful' ageing. However, further research is required into some of these cellular subsets to elucidate the effects of age, CRF and exercise on biomarkers of endothelial health.

2.7 Hypotheses and Aims of the Study

The overarching aim of this thesis is to investigate the effects of chronological age, CRF, acute exercise and sedentary behaviours on CACs and CXCR4 cell surface expression. The specific aims and hypotheses for each of the studies conducted were:

Chapter 4:

1. The aim of this study was to examine the effect of chronological age and CRF (as measured by estimated $\dot{V}O_{2max}$) on circulating $CD34^+$ progenitor cells and the cell surface expression of a key migratory and mobilisation factor, CXCR4 in apparently healthy men.

Hypothesis: advancing age is associated with reduced number of $CD34^+$ progenitors and CXCR4 cell surface expression, which can be attenuated by increasing CRF.

Chapter 5:

2. The primary aim of this study was to examine the effect of chronological age and CRF (as measured by estimated $\dot{V}O_{2max}$) on circulating $CD31^+$ T-cells and the cell surface expression of CXCR4 in apparently healthy men.
3. The secondary aim of this study was to investigate any effects of age and/or CRF on $CD4^+$ and $CD8^+$ T-cells expressing CD31 as well as any impact of cardiometabolic risk factors, such as BMI, waist circumference, fasting glucose and inflammatory cytokines.

Hypothesis: advancing age is associated with reduced number and proportion of $CD31^+$ T-cells with concomitant decline in CXCR4 cell surface expression. CRF attenuates the effect of age on these cells either through CD4 or CD8-dependent mechanism.

Chapter 6:

4. The primary aim of this study was to examine the effect of sedentary behaviours on both $CD34^+$ progenitor cells, $CD31^+$ T-cells and other cardiometabolic risk factors, such as BMI, waist circumference, fasting glucose and inflammatory cytokines.

5. The secondary aim of this study was to assess if CRF attenuates any deleterious effect sedentary behaviours have on any CAC subset or cardiometabolic risk factors.

Hypothesis: Increasing sitting time and screen time will be associated with reduced number of $CD34^+$ progenitor cells and $CD31^+$ T-cells, which will be attenuated by increasing CRF levels.

Chapter 7:

6. The primary aim of this study was to evaluate the changes in $CD31^+$ and $CD31^-$ T-cells to an acute bout of strenuous exercise
7. The secondary aim of this study was to assess the relationship between changes in circulating SDF-1 and the changes in $CXCR4^+$ T-cells expressing CD31.

Hypothesis: Strenuous exercise causes an ingress of $CD31^+$ T-cells, more so than $CD31^-$ T-cells and this ingress is associated with CXCR4 expression on the cell surface of these cells.

Chapter 8:

8. The primary aim of this study was to evaluate the impact of persistent viral infection (cytomegalovirus; CMV) on $CD31^+$ T-cells
9. The secondary aim of this study was to assess the $CD4^+$ and $CD8^+$ T_{ANG} cell differences between CMV seropositive versus seronegative individuals
10. The final aim of this study was to assess the impact CMV plays in the age-related decline in $CD31^+$ T-cells.

Hypothesis: CMV is linked with reduced number of $CD31^+$ T-cells as a result of $CD4^+$ and $CD8^+$ T-cell changes within CMV seropositive individuals. In addition, CMV will be linked with accelerated ageing of the $CD31^+$ T-cell pool.

Chapter 3: General Materials and Methods

3.1 Participants

Inclusion criteria for enrolment in this study were male, non-smoking, non-obese (BMI <35), no known cardiovascular risk factors, free from infectious disease for 6 weeks, and between the ages of 18 and 65 years. Smokers were excluded because of the deleterious effect of smoking on EPC number (Paschalaki *et al.*, 2013). Those with excessive alcohol consumption (>14 drinks per week) were excluded as this has been found to significantly affect baseline circulating EPC number (Xiao *et al.*, 2007). Participants who were taking medication affecting the immune system, routinely using ibuprofen and/or aspirin, anti-depressants or medications affecting blood pressure were excluded from the study. The study was approved by Edinburgh Napier University Faculty Research Integrity Committee and written informed consent *(Appendix 2)* was obtained from all participants.

3.2 Resting Measures

Participants visited the Human Performance Centre at 9 am after an overnight fast, and participants refrained from ingesting caffeine from the night before, as well as refraining from any alcohol intake 24 hours prior to participation. Participants were also required to avoid strenuous exercise for 3 days prior to the visit.

Firstly, participants were measured for height and body mass. From this BMI was calculated as:

$$BMI = \frac{Body\ Mass\ (kg)}{(Height\ [m]^{2})}$$

Waist and hip measures were also taken to calculate waist-to-hip ratio, which is also considered a cardiovascular risk factor (de Koning *et al.*, 2007). Waist circumference was measured at the midpoint between the lowest palpable rib and the iliac crest (World Health Organisation, 2008) and hip circumference was taken around the widest proportion of the buttocks with the participant standing upright (World Health Organisation, 2011). Blood pressure (BP) was measured using an automated blood pressure cuff (Nonin Puresat Avant 2120, Nonin Medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA) after 5 minutes rest in a supine position on the non-dominant arm. BP was measured twice and the average systolic and diastolic measures were recorded.

3.3 Venepuncture

Venepuncture was performed with the participant in a supine position after 5 minutes of rest. A 21-gauge needle and collection kit (BD Biociences, USA) was used for collection of peripheral blood. Blood samples were evacuated into 6mL tubes spray-coated with Ethylene Diamine Tetraacetic Acid (EDTA) anticoagulants using the BD Vacutainer Safety-Lok™ system (BD Biosciences, USA). To ensure that the blood was not contaminated with mature endothelial cells due to puncture of the vein, the first 3mL was discarded. Four x 6 mL EDTA tubes and 3 x 6 mL serum tubes (BD Biosciences, USA) were used for determination of circulating EPCs and T_{ANG} cells (EDTA blood) and for determination of fasting glucose, lipids, associated cytokines and growth factors (serum).

For the acute exercise study (*Chapter 7: The Effect of Acute Exercise on the Ingress and Egress of CD31$^+$ T-Cells*) venepuncture was performed pre-exercise (10km running time trial [TT]), immediately post- and 1 hour post-exercise to investigate the kinetics of T_{ANG} cells as a result of an exercise-stressor. Blood samples for this study were used to enumerate $CD3^+CD31^+$ T-cells (see *3.6 Flow Cytometric Enumeration of Angiogenic T-cells*).

3.4 Isolation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMNCs) were isolated using density gradient centrifugation. Briefly, 3 x 6 mL EDTA peripheral blood was mixed 1:1 with sodium chloride (NaCl; 0.9% Sodium Chloride; Baxler, UK). Following this, 6 mL blood + NaCl solution was layered onto 3 mL Lymphoprep™ (Axis-Shield plc, Scotland) solution in a 15 mL centrifuge tube (x 4 tubes). These tubes were centrifuged at 800 x *g* for 30 minutes at 22ºC. After centrifugation, the PBMNC band on the erythrocyte-serum interface was carefully aspirated into one 50mL centrifuge tube, and 40 mL NaCl (0.9%) solution was added prior to centrifugation at 250 x *g* for 10 minutes at 22ºC. The supernatant was discarded and the pellet resuspended in 40 mL phosphate buffered saline-bovine serum albumin (PBS-BSA; 0.01M phosphate buffer, 0.0027 M potassium chloride, 0.137 M sodium chloride, pH 6.5-7.5, 1% BSA) prior to re-centrifugation at 250 x *g* for 10 minutes at 22ºC. Once again the supernatant was discarded and the pellet resuspended in 1 mL

PBS-BSA. The sample was then stored at 4ºC in the dark until incubation with antibodies for T_{ANG} analysis (see *3.6 Flow Cytometric Enumeration of Angiogenic T Cells*).

3.5 Flow Cytometric Enumeration of Endothelial Progenitor Cells

EPCs were quantified on a flow cytometer (BD FACS Calibur; BD Biosciences, USA). Briefly, 200 µL of EDTA whole peripheral blood was incubated with 20 µL of FcR Blocking Reagent (Miltenyi Biotec, Germany) for 15 minutes in the dark at 4°C, followed by incubation with 10 µL CD34-FITC, 10 µL CD45-APC (BD Biosciences, USA), 15 µL VEGFR2-PE, and 10 µL CXCR4- PE-Cy5 (BD Biosciences, USA) for 45 minutes at 4°C in the dark. CXCR4 receptor expression was analysed as CXCR4 is an important homing receptor for EPCs, and enhanced CXCR4 expression may enhance the endothelial repair capacity of EPCs (Sainz and Sata, 2007; Chen *et al.*, 2010; Chen *et al.*, 2012). 2 mL Pharm Lyse™ (BD Biosciences, USA) was added, and left to incubate at 4°C in the dark for 20 minutes prior to flow cytometric detection. For each sample, 500,000 $CD45^+$ events ($CD34^+CD45^{dim}VEGFR2^+$ EPC analysis) were collected for analysis. Samples containing no antibody for VEGFR2 and CXCR4 were used as negative controls to set electronic gating to determine positive events in positive samples. Following data acquisition, flow cytometric data was analysed using FCS Express v3.0 (De Novo, Los Angeles, USA). EPCs and $CD34^+$ progenitors are expressed as percentage of total MNCs as advised by Van Craenenbroeck *et al.* (2013a) due to the error associated with calculating circulating $CD34^+$ progenitor cells based on total whole blood cell (WBC) count.

The colour-dot plots of flow cytometric enumeration of progenitor cells are shown in figure 3.1.

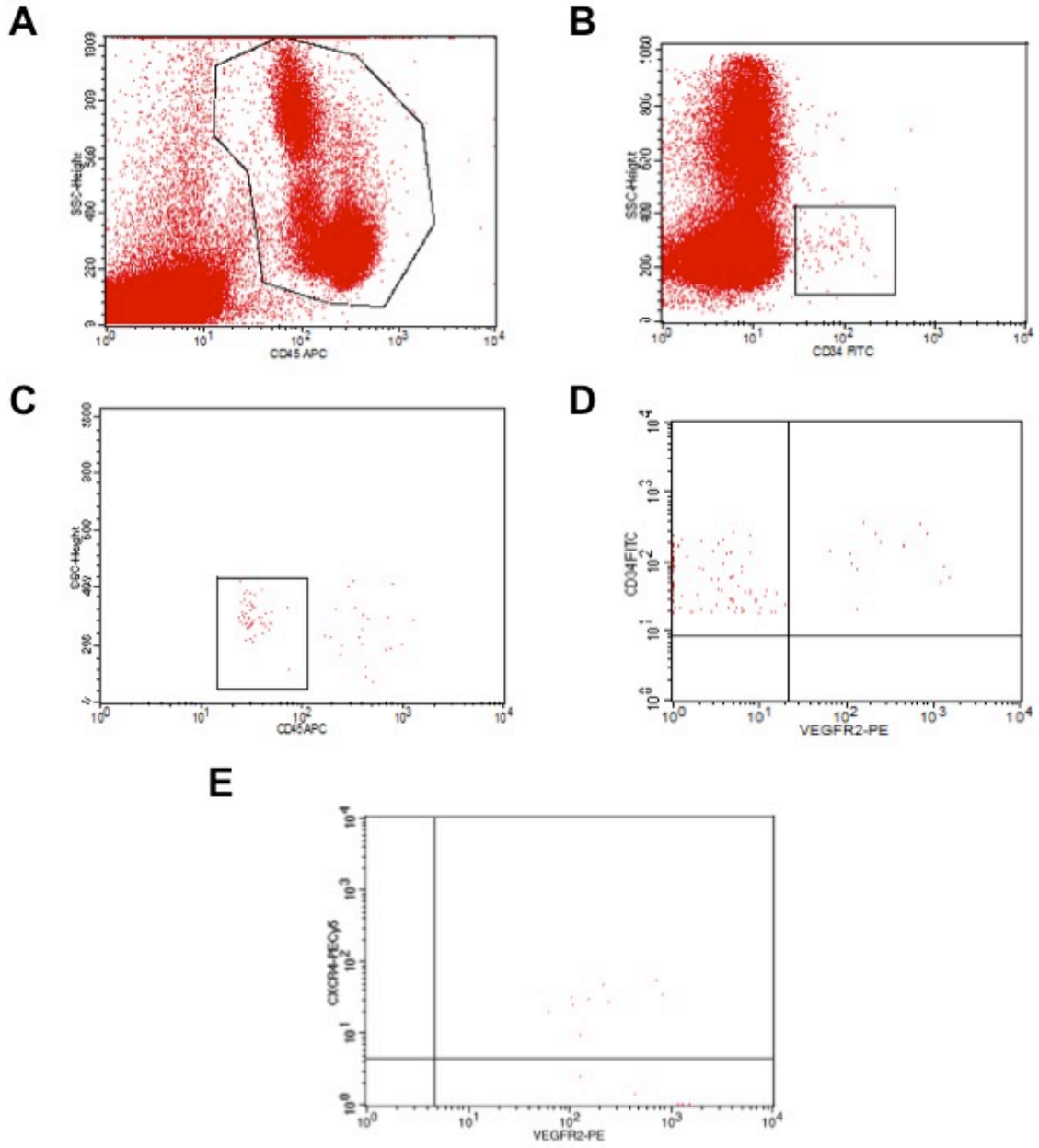

Figure 3.1. Flow cytometric enumeration of EPCs. *A- $CD45^+$ gating of mononuclear cells. B- Identification of $CD45^+CD34^+$ cells. C- Identification of $CD45^+CD34^+$ cells. D- $VEGFR2^+$ subset of $CD34^+CD45^{dim}$ cells (EPCs). E-CXCR4 expression on $CD45^{dim}CD34^+VEGFR2^+$ EPCs.*

3.6 Flow Cytometric Enumeration of Angiogenic T Cells

Angiogenic T cells (T_{ANG}) were quantified on a flow cytometer (BD FACS Calibur; BD Biosciences, USA). Briefly, 0.5 x 10^6 PBMNCs were incubated with 1 µL anti-CD3 APC, anti-CD31 FITC and anti-CXCR4 PE-Cy5 (BD Biosciences, USA) for 45 minutes at 4°C in the dark. Immediately prior to flow cytometric enumeration 500 µL PBS-BSA was added. Lymphocyte gate was identified using a forward-scatter and side-scatter plot. A minimum of 100,000 gated lymphocyte events were collected per sample. Isotypes for CD31 (FITC-Anti-Mouse Isotype; BD Biosciences, USA) and CXCR4 (PE-Cy5 Anti-Mouse Isotype; BD Biosciences, USA) were used (in matched concentrations) as controls to distinguish positive and negative events. In 50 participants aged 30-65, T_{ANG} were also analysed for the expression of CD4 (1 µL anti-CD4 PE [BD Biosciences, USA]) and CD8 (1 µL anti-CD8 PE [BD Biosciences, USA]). Following data acquisition, data was analysed using FCS Express v3.0 (De Novo, Los Angeles, USA). The percentage of all lymphocytes and lymphocyte subsets expressing CD3, CD31 and CXCR4 were analysed, and total $CD3^+$, $CD3^+CD31^+$, $CD31^+CD31^+CXCR4^+$ T-cell subsets were calculated by multiplying the percentage of lymphocytes expressing the surface markers of interest by total lymphocyte count as enumerated by Automated Hematology Analyser XS-1000i (Sysmex, Japan). The intra-assay coefficient of variation was calculated as 1.34% for T_{ANG} ($CD3^+CD31^+$) cell enumeration.

The colour-dot plots, contour plots, and histograms of flow cytometric enumeration of T_{ANG} are shown in figure 3.2.

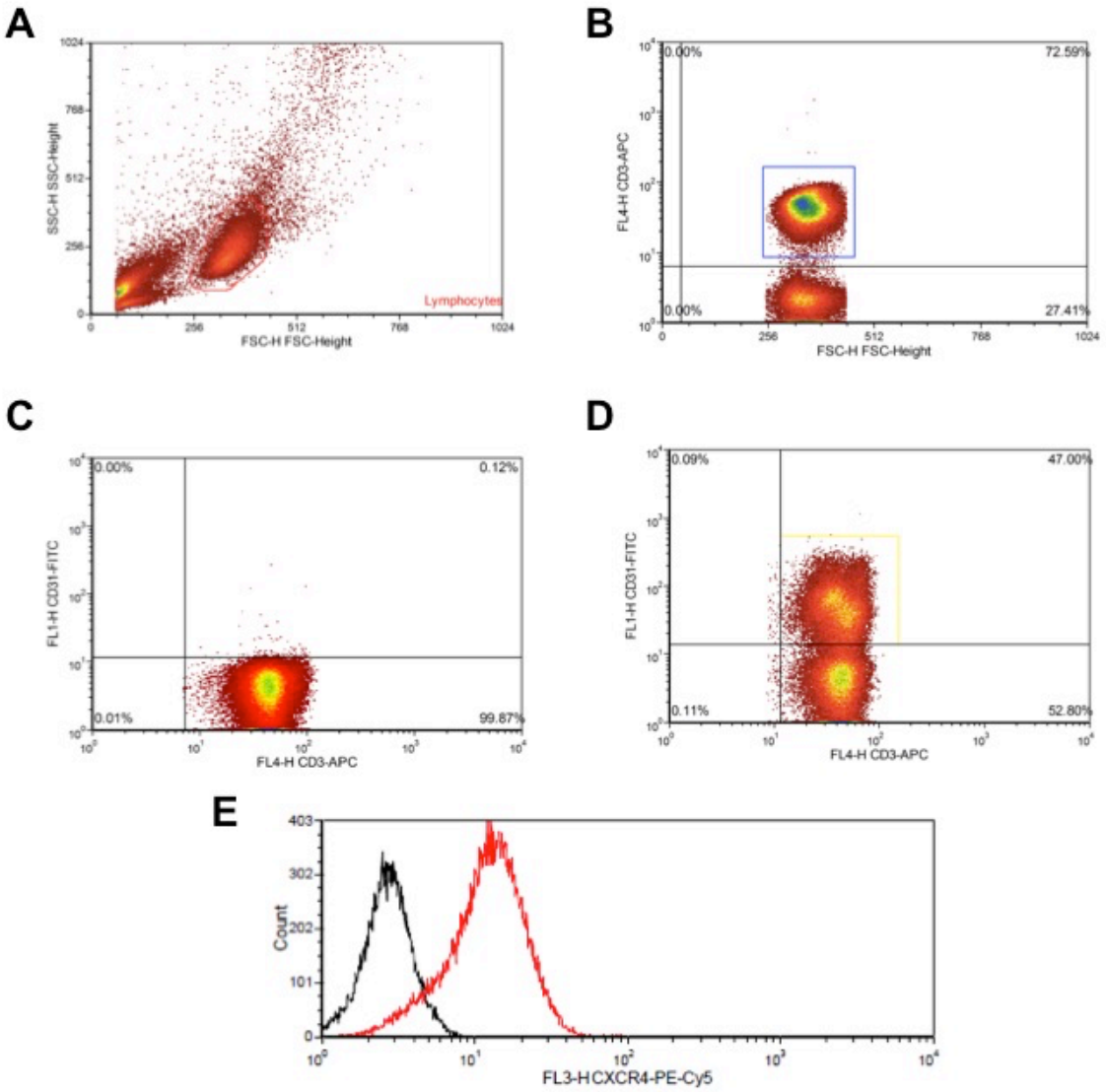

Figure 3.2. Flow cytometric enumeration of T_{ANG} cells and coexpression of CXCR4. *A- Side scatter vs. forward scatter for identification of lymphocyte gate. B- $CD3^+$ gating of lymphocytes. C- Isotype and D-positive sample for $CD31^+$ T cells. E- Isotype and positive sample for $CXCR4^+$ events in T_{ANG} cells ($CD3^+CD31^+$).*

3.7 Haematological Parameters

EDTA whole blood was used to attain a differential white blood cell count immediately after blood collection (Sysmex Automated Hematology Analyser XS-1000i, Sysmex, Japan). Samples were measured in duplicate and averaged**.** Quality control samples were used prior to each blood sample being quantified for differential white blood cell count (e-Check XS, Sysmex, Japan).

3.8 Analysis of Growth Factors, Cytokines and Cytomegalovirus Serostatus

Aliquots of serum were prepared and stored at -80°C, for determination by enzyme-linked immunosorbent assay (ELISA). For the following markers analysis was performed on -80°C stored serum: VEGF, SDF-1, IL-6 and IL-8 (R&D Systems, Inc, USA) and also used for CMV analysis (BioCheck Inc, USA). Standards of known concentrations were used to produce either concentration curve (VEGF, SDF-1, IL-6 and IL-8) or to determine fluorescence cut-off for determination of CMV serostatus.

Serum is known to contain significantly more VEGF than plasma VEGF, as a result of a significant contribution of platelet-derived VEGF (Maloney *et al.*, 1998). VEGF can be released in the serum as time for serum clotting increases, and thus time that serum was allowed to clot before processing was significantly controlled and maintained to limit additional error in the measurement of circulating VEGF.

Fasting glucose, triglycerides, total cholesterol, HDL-C and low-density lipoprotein cholesterol (LDL-C) was measured in human serum by semi-automated spectrophotometry (RX Monza Clinical Chemistry Analyzer, Randox, UK). Samples were analysed in duplicate and averaged. Standards of known concentrations were used prior to batch analysis of frozen samples to calibrate the semi-automated spectrophotometer.

Intra-assay coefficient of variation data for differential leukocyte count, ELISA data and spectrophotometry analysis of serum cytokines and growth factors are given in table 3.1.

Table 3.1. Coefficients of Variation for Blood Sample Analysis

Assay	CoV (%)
Differential Leukocyte Count (Sysmex XS-1000i, Japan)	
Lymphocytes	2.37
ELISA (various)	
VEGF	7.81
SDF-1	4.10
IL-6	18.19
IL-8	14.12
CMV IgG	8.56
Semi-Automated Spectrophotometry (RX Monza, Randox, UK)	
Glucose	8.00
Total Cholesterol	1.18
HDL-C	2.13

CoV- Coefficient of Variation, ELISA- Enzyme-Linked Immunosorbent Assay

3.9 Submaximal Exercise Test Protocol

After providing resting blood sample, all participants completed a submaximal cycling exercise test to estimate maximum oxygen uptake ($\dot{V}O_{2max}$). The protocol (YMCA submaximal cycling test) (Golding *et al.*, 1989) consisted of 3-4 x 3 minute incremental stages, beginning at 50W at 50rpm. All tests were performed on a Veletron™ Dynafit Pro (Racer Mate®, Seattle, USA) stationary exercise bike. Heart rate (HR) was measured continuously throughout the test (Polar, Finland) and the average HR during the last minute of each stage (HR_{2min}+HR_{3min}/2) was used to determine each workload adjustment in the subsequent stage(s). $\dot{V}O_{2max}$ was measured throughout the test through breath-by-breath online analysis (LABManager v5.3.0, Cardinal Health, Germany). The test was terminated when the desired steady state HR was attained. $\dot{V}O_{2max}$ was estimated using equations provided by Adams and Beam (1998).

3.10 Statistical Analysis

Data were analysed using SPSS statistical package version 20.0 for Mac (SPSS Inc., IBM, USA). Specific details of the statistical tests used to analyse the data within each study are given in the subsequent chapters. Statistical significance was set at $p<0.05$.

Chapter 4: The Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cell Number and CXCR4 Cell Surface Expression

4.1 Introduction

Cardiovascular disease (CVD) is estimated to cause nearly 30% of all deaths worldwide (Lozano *et al.*, 2012). Risk factors include smoking, hypertension, diabetes, and ageing (Cupples and D'Agostino, 1987). Both CVD (Gokce *et al.*, 2003; Shechter *et al.*, 2009; Hafner *et al.*, 2014; Liao *et al.*, 2014) and ageing (Taddei *et al.*, 2001; Muller-Delp, 2006; Soucy *et al.*, 2006; Black *et al.*, 2009) are associated with endothelial dysfunction. This is due to a variety of factors, such as increased vascular oxidative stress (Landmesser *et al.*, 2002), increased endothelial apoptosis (Wang *et al.*, 2013), and also as a result of reduced CAC which support normal endothelial growth and also play a role in the repair of a damaged endothelium.

Asahara *et al.* (1997) discovered that $CD34^+$ cells could form vessel-like structures *in vitro.* These cells have been termed putative endothelial progenitor cells (EPCs) and have been found to be independent predictors of endothelial function (Sibal *et al.*, 2009; Bruyndonckx *et al.*, 2014) and are reduced or dysfunctional in CVD patients compared to age-matched healthy controls (Fadini *et al.*, 2005; Walter *et al.*, 2005; Sibal *et al.*, 2009; Jung *et al.*, 2010; Lin *et al.*, 2013; Teraa *et al.*, 2013; Barsotti *et al.*, 2014; Chan *et al.*, 2014). Ageing is associated with reduced number (Thijssen *et al.*, 2006; Thum *et al.*, 2007) and/or function (Heiss *et al.*, 2005; Hoetzer *et al.*, 2007; Thum *et al.*, 2007; Xia *et al.*, 2012a; Xia *et al.*, 2012b; Yang *et al.*, 2012; Williamson *et al.*, 2013) of these cells, therefore it is important to improve EPC profile throughout life to provide protection against the onset and progression of CVD.

Exercise and physical activity reduces the risk of CVD (Morris *et al.*, 1953; Kurl *et al.*, 2003; Vigen *et al.*, 2012; Schnohr *et al.*, 2015) which is potentially attributable to the improved endothelial function seen with exercise training (Spier *et al.*, 2004; Black *et al.*, 2008; Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Birk *et al.*, 2012; Mitranun *et al.*, 2014), which may be partly due to the increased number and function of EPCs observed after acute exercise (Laufs *et al.*, 2005a; Shaffer *et al.*, 2006; Yang *et al.*, 2007; Van Craenenbroeck *et al.*, 2008; Jenkins *et al.*, 2009; Möbius-Winkler *et al.*, 2009; Van Craenenbroeck *et al.*, 2011; Ross *et al.*, 2014; Chang *et al.*, 2015) and chronic exercise training (Laufs *et al.*, 2004; Steiner *et al.*, 2005; Hoetzer *et al.*, 2007; Manfredini *et al.*, 2009; Van Craenenbroeck *et al.*, 2010a; Schlager *et al.*, 2011; Sonnenschein *et al.*, 2011; Fernandes *et al.*, 2012; Xia *et al.*, 2012a; Yang *et al.*, 2013). Some studies have shown no changes in $CD34^+$ progenitor cell or EPC basal levels after an exercise training

intervention (Thijssen *et al.*, 2006; Witkowski *et al.*, 2010; Luk *et al.*, 2012), yet some, having found no changes in number, found changes in *in vivo* (Xia *et al.*, 2012a) or *in vitro* (Manfredini *et al.*, 2009; Sonnenschein *et al.*, 2011) function of these cells. Therefore, debate has arisen whether exercise training stimulates the body to maintain higher levels of these progenitor cells in the circulation by mobilising them from the BM, or whether the effect is to improve the function of these cells via modulating intracellular gene expression and protein production, for example increasing CXCR4 intracellular protein levels (Xia *et al.*, 2012a).

The aim of this study was therefore to evaluate the effect of ageing in a healthy male population on circulating number and CXCR4 cell surface expression of $CD34^+$ progenitor cells and EPCs, and to assess the role CRF plays on any age-related effects observed.

4.2 Materials and Methods

4.2.1 Participants

Male participants (n=112), aged between 18 and 65 years volunteered to participate in the study, and consisted of students and staff based at Edinburgh Napier University, in addition to general public from the Edinburgh area. Prior to their involvement in the study, participants were asked to give written informed consent, after which they completed a Physiological Screening Questionnaire (see *Appendix 3*). Participants were excluded from the study if they were smokers, had an excessive alcohol intake (>14 drinks/week); had a BMI>35; were currently taking medication affecting the immune system, currently taking antidepressants; routinely using ibuprofen or aspirin. Additionally participants were excluded from the study if they: reported major affective disorders such as human immunodeficiency virus infection, hepatitis, arthritis, central or peripheral nervous disorders, or had a history of stroke or cardiac events; suffered from known cardiovascular or autoimmune disease; reported infection in the 6 weeks prior to study enrolment; or were bedridden in the 3 months prior to the study, or suffer from known cardiovascular or autoimmune disease (n=10 in total excluded). Participants who met the inclusion criteria (n=102) were asked to respond to these criteria on the Physiological Screening Questionnaire (see *Appendix 3*). Participants were advised not to partake in any strenuous exercise for 72 hours prior to their visit to the Human Performance Laboratory.

Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee. Participant characteristics are shown in table 4.1.

Table 4.1. Participant characteristics (n=102). Values shown are mean ± SD

	All (n=102)	18-30 years (n=36)	31-50 years (n=42)	51-65 years (n=24)	p-value
Age (years)	39 ± 14	24 ± 3	41 ± 6*	58 ± 5*$^{\#}$	0.000
Height (m)	1.79 ± 0.08	1.82 ± 0.07	1.80 ± 0.08	1.78 ± 0.08	0.141
Body Mass (kg)	83.64 ± 10.16	86.18 ± 10.39	84.02 ± 9.40	79.15 ± 10.12*	0.029
BMI ($kg \cdot m^2$)	25.83 ± 2.60	26.06 ± 2.37	26.05 ± 2.45	25.09 ± 3.12	0.283
SBP (mmHg)	130 ± 15	126 ± 10	129 ± 13	140 ± 19*	0.000
DBP (mmHg)	78 ± 9	73 ± 8	80 ± 8	84 ± 9*	0.000
MAP (mmHg)	96 ± 10	90 ± 7	96 ± 9*	103 ± 11*	0.000
Waist-to-Hip Ratio	0.95 ± 0.05	0.92 ± 0.04	0.96 ± 0.04	0.97 ± 0.04	0.000
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	43.60 ± 9.48	44.19 ± 7.99	47.03 ± 9.62	36.70 ± 7.82*$^{\#}$	0.000

*BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure. * $p<0.05$ vs. 18-30 years, $^{\#}p<0.05$ vs. 31-50 years.*

4.2.2 Anthropometric and Characteristics

On arrival to the Human Performance Laboratory, after informed consent was given, participants were measured for height and body mass, with values used to calculate BMI. Resting blood pressure was measured after 5-minute rest in a supine position using an automated sphygmomanometer (Avant® 2120, Nonin Medical Inc., USA).

4.2.3 Blood Sampling

Blood was taken from participants after a 5 minute supine rest by a certified phlebotomist. Blood samples were drawn into 6mL vacutainers (BD Biosciences, USA), which were either coated in EDTA to prevent coagulation or serum gel. EDTA blood was used for EPC enumeration (see *4.2.5 Endothelial Progenitor Cell Number and CXCR4 Expression Quantification*), and serum was used for analysis of serum for chemotactic factors (see *4.2.6 Serum Analysis for Chemotactic Factors*).

4.2.4 Submaximal Exercise Test to Estimate Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) of each participant was estimated using a submaximal exercise test (YMCA) as previously described in general materials and methods *Chapter 3.15*. Briefly, participants exercised on a stationary cycle ergometer (Veletron™ Dynafit Pro; Racer Mate®, Seattle, USA) at 50rpm for 3-4 incremental stages, each stage lasting 3 minutes. The initial power output of the test was 50W and increased in subsequent stages depending on HR response. $\dot{V}O_{2max}$ was measured throughout the test through breath-by-breath online analysis (LABManager v5.3.0, Cardinal Health, Germany). The test was terminated when the desired steady state HR was attained (80% of $HR_{max,}$ as calculated by equation from Tanaka *et al.,* 2001). $\dot{V}O_{2max}$ was estimated using equations provided by Adams and Beam (1998).

4.2.5 Endothelial Progenitor Cell Number and CXCR4 Expression Quantification

Peripheral whole blood was used for EPC analysis. Whole blood was labelled with monoclonal antibodies anti-CD34-FITC, anti-CD45-APC, anti-VEGFR2-PE and anti-CXCR4-PE-Cy5 (all BD Biosciences, USA) and left to incubate at 4°C for 45 minutes in the dark as described in general materials and methods *Chapter 3.5*. Progenitor cell subsets in human whole blood were analysed using a BD FACSCalibur (BD Biosciences, USA) as detailed in general materials and methods *Chapter 3.5*. Total progenitors were considered $CD34^+$ cells with a low side scatter (SSC^{low}) and putative EPCs considered as $CD34^+CD45^{dim}VEGFR2^+$ cells (Van Craenenbroeck *et al.*, 2013b) as described in general materials and methods *Chapter 3.8*.

4.2.6 Serum Analysis for Chemotactic Factors

Serum chemotactic factors VEGF and SDF-1α were analysed by ELISA as described in general materials and methods *Chapter 3.12*. Fasting glucose, triglycerides, total cholesterol, HDL-C and LDL-C was measured in human serum by semi-automated spectrophotometry (RX Monza Clinical Chemistry Analyzer, Randox, UK). Samples were analysed in duplicate and averaged.

4.2.7 Statistical Analysis

All data were assessed for normal distribution. Not normally distributed data were logarithmically or square root transformed. Linear regressions were initially performed to assess relationships between age and CRF on circulating $CD34^+$ progenitor, EPC number or CXCR4 expression. Age and CRF were added to a regression model by hierarchical multiple regression analysis to assess the effect of CRF after correcting for chronological age (Ainslie *et al.*, 2008; Giannaki *et al.*, 2013; Ho, 2013). A two-way analyses of variance (ANOVA) were performed to detect any interaction between age and CRF on the progenitor cell subtypes. Fisher's Least Significance Difference (LSD) post-hoc tests were performed to identify locations of any significant differences. Significance was set at p-value 0.05. Data was analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA).

4.3 Results

4.3.1 The effect of age and estimated $\dot{V}O_{2max}$ on circulating $CD34^+$ and putative endothelial progenitors

The effects of age and $\dot{V}O_{2max}$ on circulating $CD34^+$ progenitor cells and putative endothelial progenitor cells assessed initially by linear regression analysis and are shown in table 4.2. Age was inversely associated with various CAC subsets ($CD34^+$ [r=-0.251, p=0.006], $CD34^+CXCR4^+$ [r=-0.305, p=0.001], $CD34^+CD45^{dim}VEGFR2^+$ [EPC] [r=-0.209, p=0.020], $CXCR4^+$ EPC [r=-0.252, p=0.007], and EPC CXCR4 mean fluorescence intensity (MFI) [r=-0.228, p=0.013]). $\dot{V}O_{2max}$ was not associated with basal circulating level of any CAC subset, yet other physiological variables were associated with CACs, with MAP ($CD34^+CXCR4^+$, r=-0.256, p=0.005), total cholesterol ($CD34^+$, r=-0.229,

p=0.010; $CD34^+CXCR4^+$, r=-0.214, p=0.014) and IL-8 ($CD34^+$ r=-0.223, p=0.019; $CD34^+CXCR4^+$, r=-0.240, p=0.013) were inversely associated with various CACs. SDF-1α, a selected putative mobilising factor for progenitor cells was positively correlated with $CD34^+$ progenitor cell subsets (EPC, r=0.239, p=0.009; $CXCR4^+$ EPC, r=0.221, p=0.016). There was no effect of fasting glucose or serum IL-6 on CAC subsets.

Since $\dot{V}O_{2max}$ was also significantly associated with age (r=-0.328, p=0.000), hierarchical multiple regressions were performed to control for the effect of chronological age, and to assess the independent effect of CRF on these CAC subsets (tables 4.3 and 4.4). After correction for age, $\dot{V}O_{2max}$ was still not associated with any CAC subsets (p>0.05).

Table 4.2. Univariate linear regression analysis on progenitor cell subsets in healthy males (n=102).

	Pearson's coefficient (p-value)		F-statistic (p-value)	
	Age	$\dot{V}O_{2max}$	Age	$\dot{V}O_{2max}$
$CD34^+$	-0.251 (0.006)*	-0.064 (0.266)	6.542 (0.012)*	0.393 (0.532)
$CD34^+CXCR4^+$	-0.305 (0.001)*	-0.142 (0.077)	10.220 (0.002)*	2.060 (0.154)
$CD34^+CXCR4^+$ MFI	0.039 (0.347)	-0.077 (0.221)	0.596 (0.442)	0.596 (0.442)
$CD34^+CD45^{dim}VEGFR2^+$	-0.209 (0.020)*	-0.040 (0.349)	4.332 (0.040)*	0.152 (0.697)
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$	-0.252 (0.007)*	-0.026 (0.401)	6.326 (0.014)*	0.064 (0.802)
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$ MFI	-0.228 (0.013)*	0.007 (0.474)	5.096 (0.026)*	0.004 (0.949)

*MFI- Mean Fluorescence Intensity, * significant $p<0.05$*

Table 4.3. Hierarchical multiple regression analysis on $CD34^+$ progenitor cell subsets in healthy males (n=102).

Progenitor cell subsets	Factors for Analysis	R^2	R^2 *Change*	*Beta Value*	SEB	*Standardised ß*	F-statistic (p -value)	t-statistic (p-value)
$CD34^+$	**Model 1**	0.063					6.542 (0.012)*	
	Age			-1.009	1.004	-1.285		-2.558 (0.012)*
	Model 2	0.083	0.020				4.347 (0.016)*	
	Age			-1.011	1.004	-1.340		-2.876 (0.005)*
	$\dot{V}O_{2max}$			-1.008	1.005	-1.158		-1.442 (0.147)
$CD34^+CXCR4^+$	**Model 1**	0.093					10.220 (0.002)*	
	Age			-1.020	1.006	-1.357		-3.197 (0.002)*
	Model 2	0.116	0.055				8.559 (0.000)*	
	Age			-1.025	1.006	-1.449		-3.844 (0.000)*
	$\dot{V}O_{2max}$			-1.024	1.009	-1.275		-1.252 (0.0130)
$CD34^+CXCR4^+$ MFI	**Model 1**	0.002					0.155 (0.694)	
	Age			1.000	1.002	1.094		0.394 (0.694)
	Model 2	0.006	0.005				0.314 (0.732)	
	Age			1.000	1.002	1.047		0.191 (0.849)
	$\dot{V}O_{2max}$			1.002	1.002	1.180		-0.687 (0.493)

*SEB- Standard error of the Beta value. MFI- Mean Fluorescence Intensity, * significant p<0.05*

Table 4.4. Hierarchical multiple regression analysis on putative endothelial progenitor cells in healthy males (n=102).

Progenitor cell subsets	Factors for Analysis	R^2	R^2 *Change*	*Beta Value*	SEB	*Standardised β*	F-statistic (p -value)	t-statistic (p-value)
$CD34^+CD45^{dim}VEGFR2^+$	**Model 1**	0.044					4.332 (0.040)*	
	Age			-1.015	1.007	-1.232		-2.081 (0.040)*
	Model 2	0.054	0.010				2.667 (0.075)	-2.275 (0.025)*
	Age			-1.017	1.007	-1.267		-1.1001 (0.319)
	$\dot{V}O_{2max}$			-1.011	1.011	-1.110		
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$	**Model 1**	0.064					6.326 (0.014)*	
	Age			-1.018	1.007	-1.287		-2.515 (0.014)*
	Model 2	0.073	0.016				3.640 (0.030)*	
	Age			-1.020	1.007	-1.323		-2.686 (0.009)*
	$\dot{V}O_{2max}$			-1.011	1.011	-1.107		-0.979 (0.330)
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$ MFI	**Model 1**	0.052					5.096 (0.026)*	
	Age			-1.012	1.005	-1.690		-2.257 (0.026)*
	Model 2	0.055	0.003				2.689 (0.073)	
	Age			-1.012	1.005	-1.754		-2.318 (0.023)*
	$\dot{V}O_{2max}$			-1.005	1.007	-1.148		-0.565 (0.573)

*SEB- Standard error of the Beta value, MFI- Mean Fluorescence Intensity (MFI), * significance, p<0.05*

One-way analyses of variance (ANOVA) were performed to determine any differences in progenitor number and CXCR4 cell surface expression between participants aged 18-30yrs, 31-50yrs and 51-65yrs.

One-way ANOVA analysis between age groups identified several significant differences (figures 4.1 and 4.2). $CD34^+$ and $CD34^+CXCR4^+$ circulating progenitors were significantly higher in the 18-30yrs group compared to the 31-50yrs ($CD34^+$=0.143% ± 0.011 [0.131-0.192, 95% CI]) vs. 0.113% ± 0.009 [0.094-0.132], p=0.031; $CD34^+CXCR4^+$=0.089% ± 0.010 [0.069-0.110] vs. 0.048% ± 0.006 [0.036-0.061], p=0.000) and 51-65yrs group ($CD34^+$=0.143% ± 0.011 [0.131-0.192, 95% CI]) vs. 0.106% ± 0.010 [0.085-0.126], p=0.033; $CD34^+CXCR4^+$=0.089% ± 0.010 [0.069-0.110] vs. 0.040% ± 0.006 [0.028-0.051], p=0.001). Circulating EPCs ($CD34^+CD45^{dim}VEGFR2^+$) only differed between the 18-30yrs and 31-50yrs groups (0.015% ± 0.003 [0.008-0.022] vs. 0.006% ± 0.001 [0.004-0.008], p=0.000), however the number of EPCs expressing CXCR4 was raised in the 18-30yrs group compared to both the 31-50yrs (0.011% ± 0.002 [0.007-0.016] vs. 0.004% ± 0.001 [0.003-0.006], p=0.002) and 51-65yrs group (0.011% ± 0.002 [0.007-0.016] vs. 0.006% ± 0.001 [0.003-0.008], p=0.026).

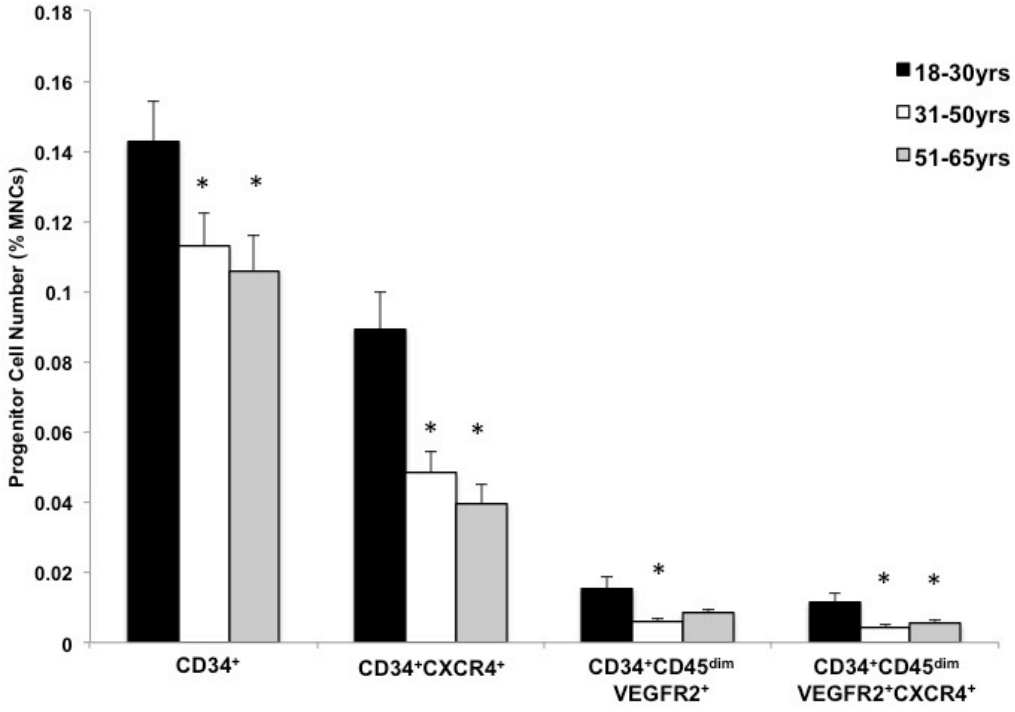

Figure 4.1. Circulating $CD34^+$ progenitor cells and EPCs in healthy males in age groups 18-30yrs, 31-50yrs and 51-65yrs. **p<0.05 different from 18-30yrs*.

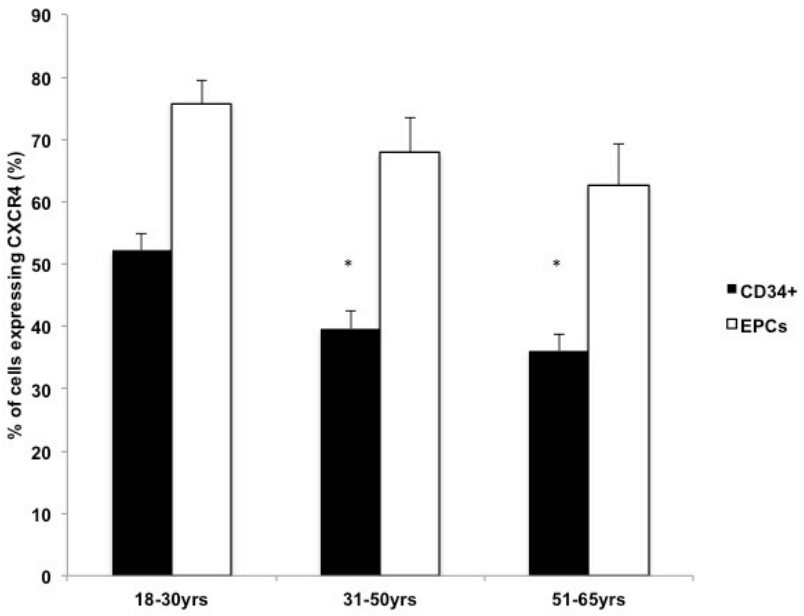

Figure 4.2. Percentage of $CD34^+$ and endothelial progenitors expressing CXCR4 in healthy males in age groups 18-30yrs, 31-50yrs and 51-65yrs. * *$p<0.05$ different from 18-30yrs.*

CXCR4 cell surface expression was assessed on $CD34^+$ and $CD34^+CD45^{dim}VEGFR2^+$ progenitor cells by flow cytometry and compared between males in age groups 18-30yrs, 31-50yrs and 51-65yrs by one-way ANOVA (figure 4.3). CXCR4 cell surface expression levels (as measured by flow cytometric MFI) was not different on $CD34^+$ progenitor cells between any age group. $CD34^+CD45^{dim}VEGFR2^+$ EPC CXCR4 cell surface expression was significantly reduced in 51-65yrs compared to 18-30yrs (19.87 ± 2.65 arbitrary units/AU [14.55-25.19] vs. 34.93 ± 4.61 AU [25.53-44.32], p=0.005) and 31-50yrs (19.87 ± 2.65 AU [14.55-25.19] vs. 32.99 ± 4.90 AU [23.05-42.93], p=0.017).

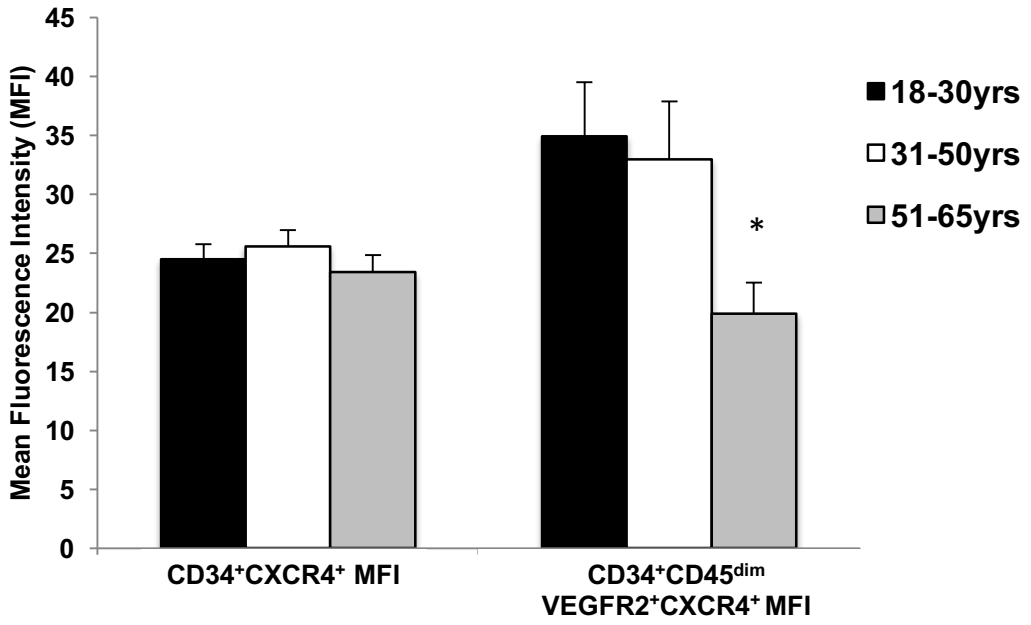

Figure 4.3. CXCR4 cell surface expression levels on circulating $CD34^+$ and $CD34^+CD45^{dim}VEGFR2^+$ progenitor cells in healthy males in age groups 18-30yrs, 31-50yrs and 51-65yrs. *$p<0.05$ *different from 18-30yrs and 31-50yrs*.

Participants were then grouped within their age-classifications (18-30yrs, 30-50yrs an 51-65yrs) into tertiles for estimated $\dot{V}O_{2max}$ (for CRF-grouped participant characteristics, see table 4.5). To further investigate the effect of CRF on these progenitor cells with age, a two-way factorial ANOVA was performed, with age groups (18-30yrs, 31-50yrs and 51-65yrs) and fitness groups (low CRF, moderate CRF, and high CRF- tertiles) as the fixed factors.

Table 4.5. Participant characteristics by cardiorespiratory fitness groupings (n=102).

Age Grouping	18-30yrs			31-50yrs			51-65yrs			p-value
$\dot{V}O_{2max}$ Category	Low CRF	Moderate CRF	High CRF	Low CRF	Moderate CRF	High CRF	Low CRF	Moderate CRF	High CRF	
N	12	12	12	14	14	14	8	8	8	NS
Age (years)	28 ± 3	24 ± 3	23 ± 3	43 ± 8	39 ± 5	42 ± 6	61 ± 4	55 ± 4	58 ± 5	NS
BMI ($h \cdot m^2$)	27.7 ± 2.4	24.7 ± 1.7*	25.9 ± 2.1*	26.2 ± 2.1	25.9 ± 2.8	26.1 ± 2.5	26.5 ± 2.9**	25.8 ± 3.5**	23.0 ± 2.0	<0.05
Waist (cm)	90.0 ± 5.1**	82.2 ± 3.8**	86.6 ± 4.5	95.3 ± 8.4	88.7 ± 6.8*	87.8 ± 8.6*	96.3 ± 9.5	92.5 ± 11.9	85.1 ± 3.3*	<0.05
Total Cholesterol ($mmol \cdot L^{-1}$)	3.34 ± 0.56**	3.22 ±0.62**	2.66 ± 0.75	3.49 ± 0.86	3.43 ± 1.2	3.78 ± 0.99	3.66 ± 1.15	3.38 ± 0.53	3.40 ± 0.59	<0.05
HDL-C ($mmol \cdot L^{-1}$)	1.21 ± 0.29	1.20 ± 0.16	1.31 ± 0.33	1.10 ± 0.31	1.18 ± 0.24	1.37 ± 0.29	1.28 ± 0.26	1.25 ± 0.11	1.40 ± 0.28	NS
LDL-C ($mmol \cdot L^{-1}$)	1.51 ± 0.50**	1.42 ± 0.61**	0.77 ± 0.61	1.66 ± 0.97	1.58 ± 1.1	1.37 ± 0.39	1.61 ± 0.98	1.38 ± 0.56	1.35 ± 0.69	<0.05
Fasting Glucose ($mmol \cdot L^{-1}$)	4.15 ± 0.97**	3.78 ± 0.65**	3.61 ± 0.54	4.48 ± 1.49	4.10 ± 0.56	4.49 ± 0.79	5.03 ± 0.73	4.42 ± 0.65	4.23 ± 0.65*	<0.05
MAP (mmHg)	93 ± 6	89 ± 7	88 ± 8	96 ± 10	95 ± 9	97 ± 8	105 ± 15	100 ± 17	104 ± 11	NS
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	35.8 ± 2.9**	43.8 ± 1.8* **	53 ± 5.5*	36.40 ± 5.27**	47.43 ± 3.2* **	57.27 ± 4.38*	28.23 ± 4.83**	37.27 ± 2.89** *	44.62 ± 3.92*	<0.05

*NS- No significant differences between CRF groups within age categories. * significantly different from low CRF, P<0.001, **significantly different from high CRF, p<0.05*

$CD34^+$ progenitor cells were largely unaffected by CRF, except in the 31-50yrs group, where there was an apparent decline in $CD34^+$ progenitor cells in the High CRF group compared to both the Low CRF (0.089 ± 0.014% [0.060-0.118] vs. 0.133 ± 0.017% [0.096-0.169], p=0.049) and Moderate CRF groups (0.089 ± 0.014% [0.060-0.118] vs. 0.119 ± 0.017% [0.083-0.155], p=0.049).

There was a significant age*CRF interaction found with the $CD34^+CXCR4^+$ progenitor cells (p=0.027). In the 31-50yrs group, the $CD34^+$ progenitor cells expressing $CXCR4^+$ were found to be higher in the Low CRF group and Moderate CRF in comparison to the High CRF groups (Low vs. High CRF: 0.061% ± 0.012 [0.036-0.086] vs. 0.023% ± 0.005 [0.012-0.034], p=0.001; Moderate vs. High CRF: 0.061% ± 0.011 [0.037-0.086] vs. 0.023% ± 0.005 [0.012-0.034], p=0.000) (figure 4.4B). Additionally, the percentage of $CD34^+$ progenitors expressing CXCR4 was also lower in the High CRF group compared to the Moderate CRF group (25.85 ± 3.72% [17.80-33.89], vs. 48.63 ± 4.63% [38.69-58.56], p=0.000) and compared to the Low CRF group (25.85 ± 3.72% [17.80-33.89], vs. 44.09 ± 4.67% [33.93-54.25], p=0.004) in the 31-50yrs group (figure 4.4C).

The intensity of expression of CXCR4 on these $CD34^+CXCR4^+$ progenitor cells, as quantified as MFI was not significantly different between age groups or between CRF groups, either as total groups, or within the different age subcategories (figure 4.5).

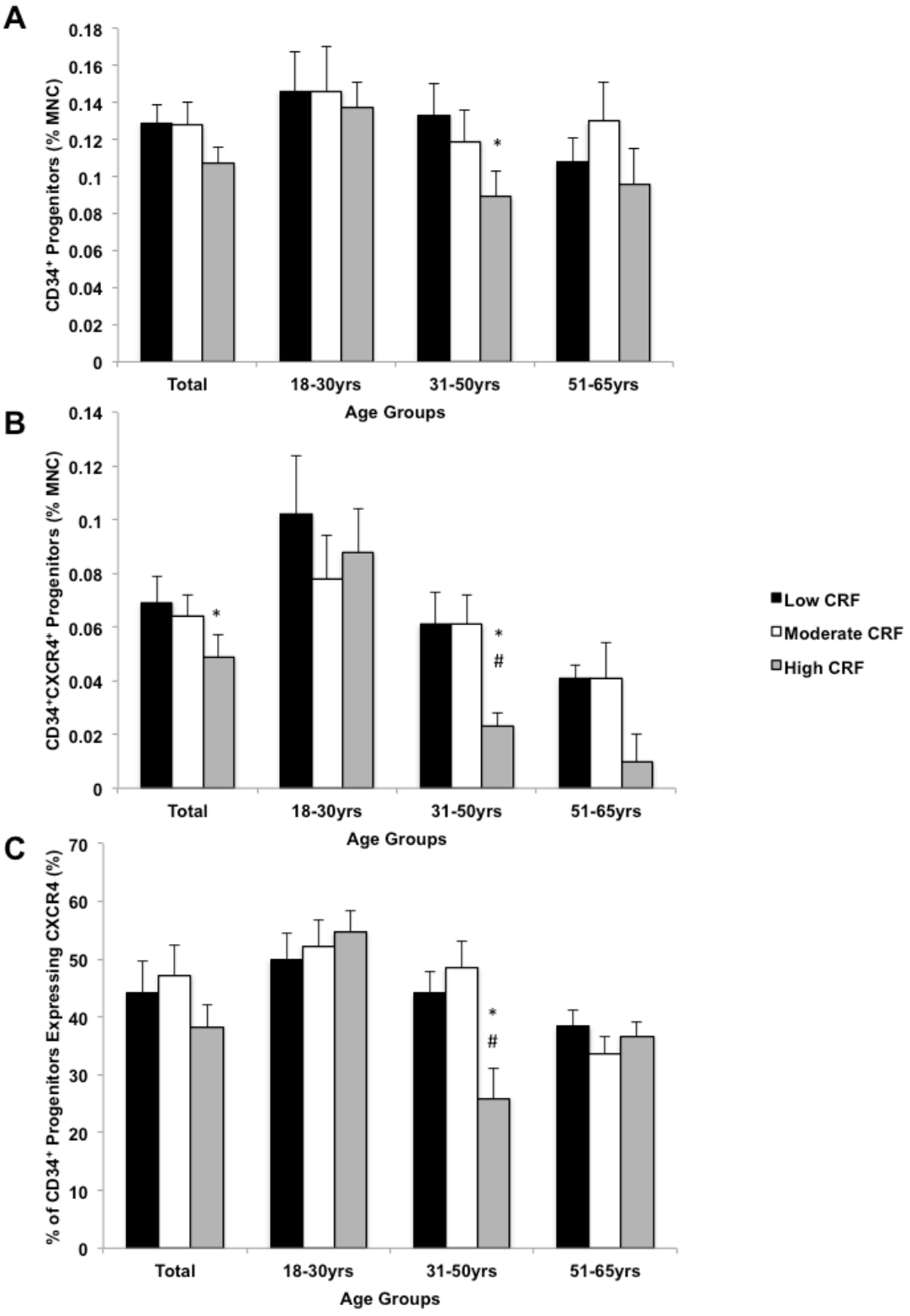

Figure 4.4. Effect of cardiorespiratory fitness on $CD34^+$ progenitors in men aged 18-30yrs, 31-50yrs and 51-65yrs (n=102 total). *A showing the circulating $CD34^+$ progenitor cell count, B showing the $CD34^+CXCR4^+$ circulating numbers, and C showing the proportion of total $CD34^+$ progenitors expressing CXCR4. * significantly different from Low CRF, $p<0.05$. # significantly different from Moderate CRF, $p<0.005$.*

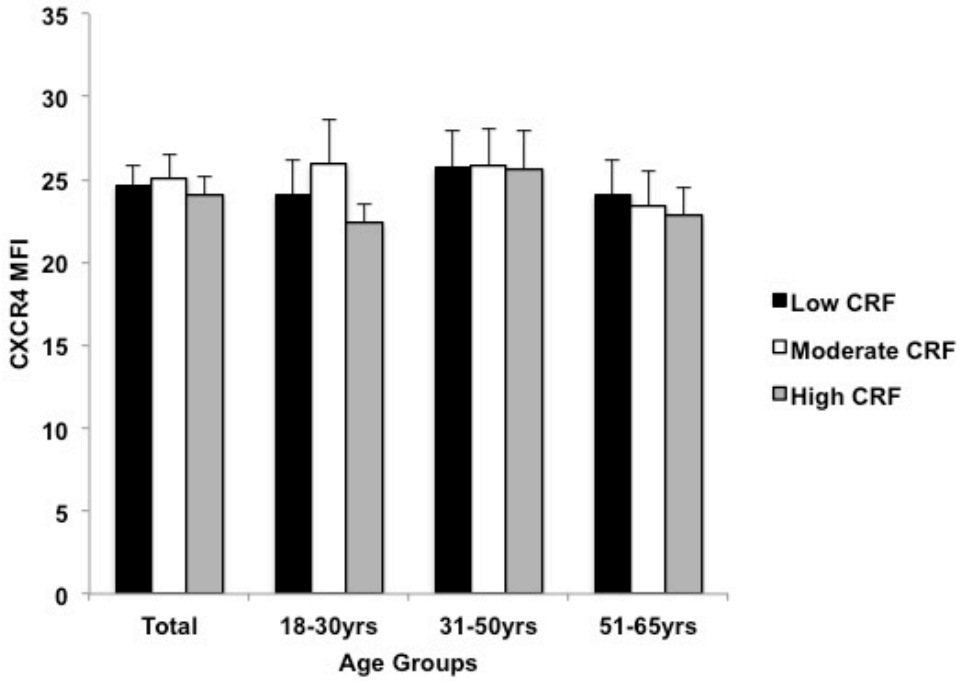

Figure 4.5. Level of CXCR4 cell surface expression on $CD34^+$ progenitor cells in healthy males grouped by age and cardiorespiratory fitness level. *MFI- Mean Fluorescence Intensity.*

There was no observed effect of CRF on circulating EPCs or CXCR4-expressing EPCs (figure 4.6). There appeared to be a progressive decline in all age groups from Low to High CRF in the percentage of EPCs expressing CXCR4, however this was not significant (figure 4.6C).

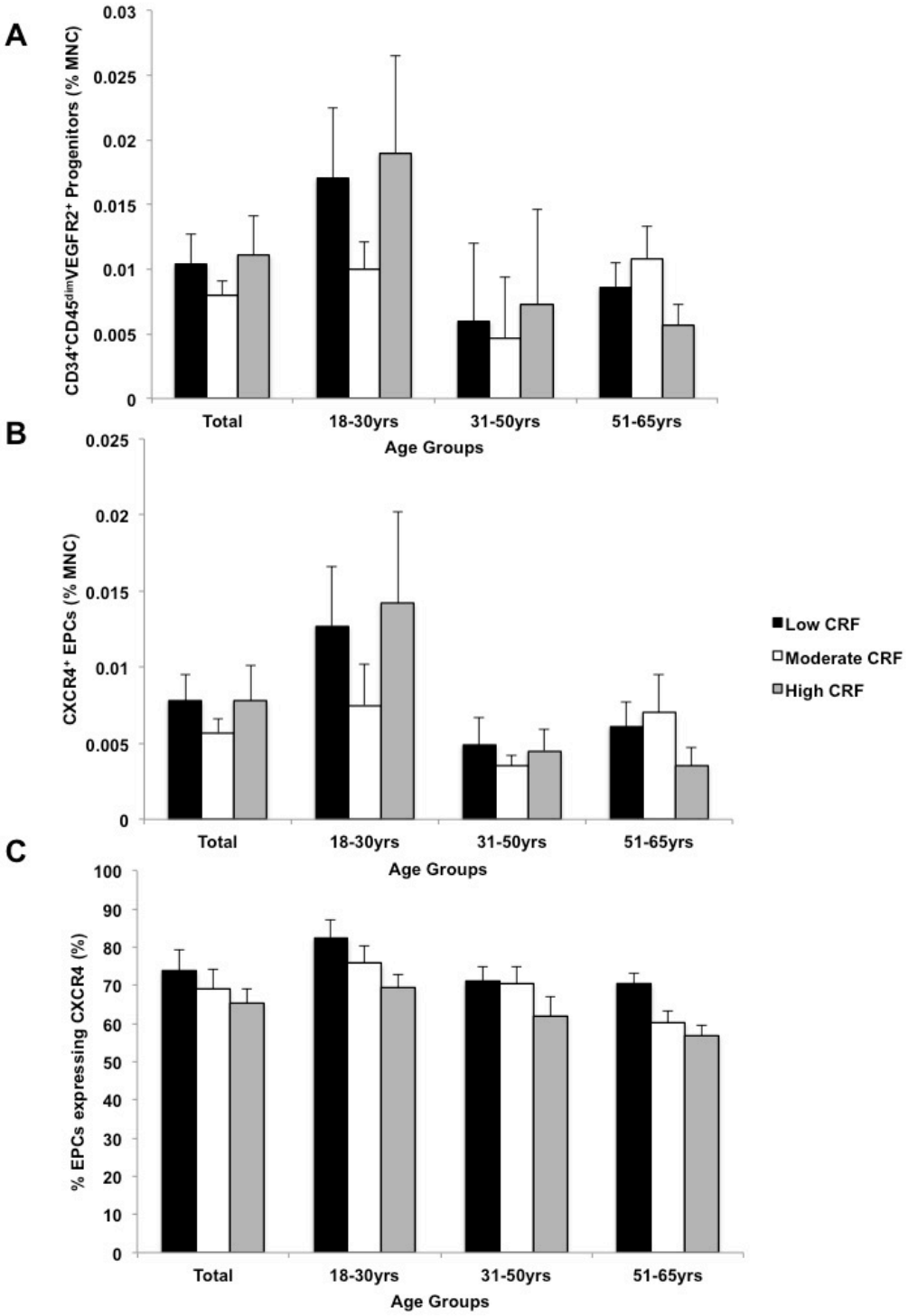

Figure 4.6. Effect of cardiorespiratory fitness on $CD34^{+}CD45^{dim}VEGFR^{+}$ putative endothelial progenitors in men aged 18-30yrs, 31-50yrs and 51-65yrs (n=102 total). *A- showing the circulating EPC count, B- showing the $CXCR4^{+}$ EPC circulating numbers, and C- showing the intensity of CXCR4 cell surface expression on $CD34^{+}CD45^{dim}VEGFR2^{+}$ progenitors. * significantly different from High CRF, $p<0.05$, ** significantly different from Moderate CRF, $p<0.05$.*

As with EPC number and CXCR4-expressing EPCs, there was no trend for changes in MFI with fitness levels amongst our participants in any age group (figure 4.7). No age*CRF interaction was observed (p=0.470).

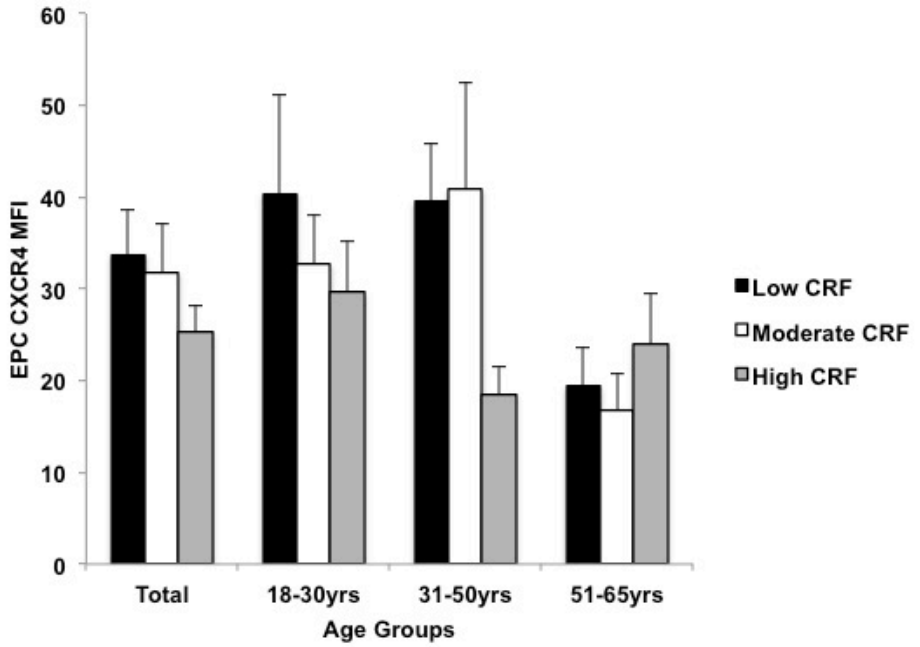

Figure 4.7. Level of CXCR4 cell surface expression on $CD34^{+}CD45^{dim}VEGFR2^{+}$ endothelial precursor cells in healthy males grouped by age and CRF level. *MFI- Mean Fluorescence Intensity.*

4.3.2 Effect of Age and CRF on Progenitor Cell Mobilising Factor SDF-1α

Since age and CRF appear to exert differential effects on circulating $CD34^{+}$ progenitors, the effect of these 2 factors on the circulating mobilising factor SDF-1α, was investigated. Two-way ANOVAs were performed with SDF-1α as the dependent variable, and age and CRF as the independent fixed factors.

Circulating SDF-1α was found to be reduced in circulation of those in the 51-65yrs group compared to the 18-30yrs group (2262.13 ± 67.41pg·mL^{-1} [2122.69-2401.57] vs. 2572.61 ± 91.57 pg·mL^{-1} [2386.71-2758.51], p=0.007) and the 31-50yrs group (2262.13 ± 67.41 pg·mL^{-1} [2122.69-2401.57] vs. 2373.04 ± 62.64 pg·mL^{-1} [2246.44-2499.63], p=0.004) (figure 4.8A).

Those with high levels of CRF displayed greater circulating SDF-1α than those with low CRF levels in the 31-50yrs group (2497.20 ± 105.33 pg·mL^{-1} [2269.64-2724.76] vs. 2192.05 ± 113.32 pg·mL^{-1} [1942.63-2441.48], p=0.017) (figure 4.8B). Circulating SDF-1α was significantly reduced in 18-30yrs group who displayed average levels of CRF group compared to the Low CRF group (2315.61 ± 165.32 pg·mL^{-1} [1951.73-2679.49] vs. 2852.46 pg·mL^{-1} [2538.94-3165.97] p=0.003). No other differences in circulating SDF-1α were observed.

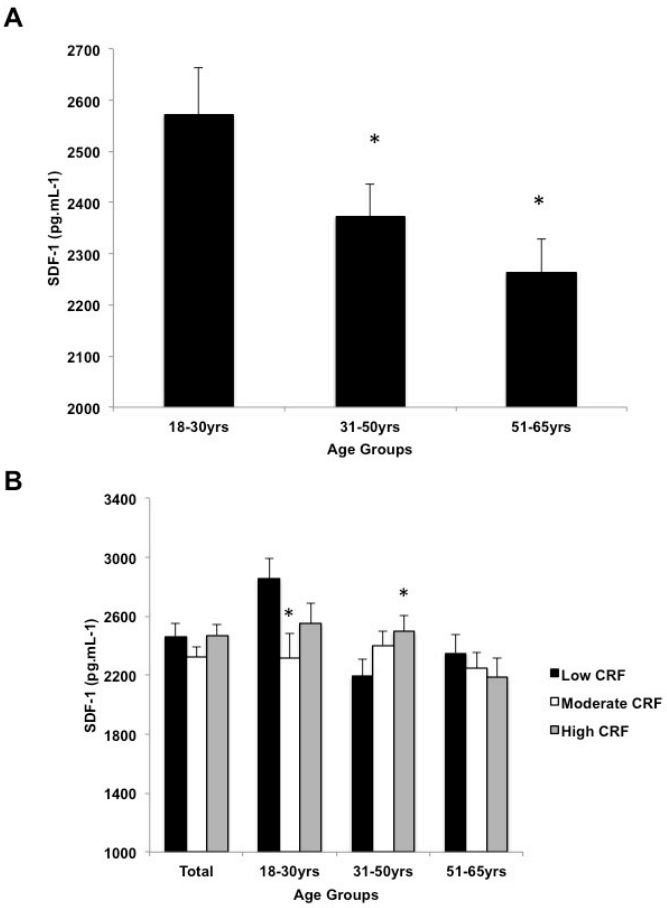

Figure 4.8. Effect of cardiorespiratory fitness and age on circulating SDF-1α in males aged 18-65yrs. *A- the effect of chronological age on circulating SDF-1α. * significantly different from 18-30yrs, p<0.05. B- Effect of cardiorespiratory fitness on circulating SDF-1α. * significantly different from Low CRF, # significantly different from Moderate CRF.*

4.3.3 Multiple Linear Regressions

To determine the most important factors on these circulating $CD34^+$ progenitors and $CD34^+CD45^{dim}VEGFR2^+$ putative endothelial progenitors, stepwise multiple level regressions were performed with age, BMI, waist circumference, blood pressure (mean arterial pressure [MAP]), cholesterol levels, fasting glucose, SDF-1α, VEGF and estimated $\dot{V}O_{2max}$ (table 4.6). Table 4.6 shows that $CD34^+$ progenitors and their subsets are affected by chronological age, SDF-1α, waist circumference and total cholesterol. Interestingly, waist circumference and total cholesterol were positively associated with $CD34^+CXCR4^+$ cell subset. $\dot{V}O_{2max}$ was interestingly negatively associated with $CD34^+CXCR4^+$ progenitor cell number, otherwise had no effect in the hierarchical multiple regression on EPCs and $CXCR4^+$ EPCs.

Table 4.6. Stepwise multiple linear regression model for $CD34^+$ progenitors (n=102).

Progenitor cell subsets	Predictors in Model	*R*	R^2	*Beta Value*	SEB	*Standardised ß*	F-statistic (p -value)	t-statistic (p-value)
$CD34^+$	**Age** **Waist**	0.372	0.138	-1.012 1.017	1.004 1.006	-1.391 1.325	7.613 (0.001)*	-3.338 (0.001)* 2.838 (0.006)*
$CD34^+CXCR4^+$	**Age** $\dot{V}O_{2max}$	0.358	0.131	-1.020 -1.024	1.006 1.019	-1.451 -1.276	8.536 (0.000)*	-3.840 (0.000)* -2.520 (0.013)*
$CD34^+CXCR4^+$ MFI	**Total Cholesterol**	0.356	0.127	1.127	1.033	2.270	14.342 (0.000)*	3.787 (0.000)*
$CD34^+CD45^{dim}VEGFR2^+$	**SDF-1α**	0.239	0.057	1.001	0.000	1.270	5.710 (0.019)*	2.390 (0.019)*
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$	**Age**	0.249	0.062	-1.018	1.007	-1.283	6.091 (0.015)*	-2.468 (0.015)*
$CD34^+CD45^{dim}VEGFR2^+CXCR4^+$ MFI	**Waist**	0.256	0.066	-1.021	1.009	-1.803	6.460 (0.013)*	-2.542 (0.013)*

SEB- Standard Error of the Beta Value, MFI- Mean Fluorescence Intensity

4.3.4 Differences in $CD34^+$ and EPC Quartiles

To identify differences in qualities in those with high circulating progenitors versus those with low circulating levels, one-way ANOVAs were performed between quartiles of $CD34^+$ frequency, $CD34^+CD45^{dim}VEGFR2^+$ frequency to detect differences in age, BMI, waist circumference, cholesterol profile, blood pressure, SDF-1α and estimated $\dot{V}O_{2max}$. Those participants displaying greater circulating $CD34^+$ progenitor cells tended to be younger and with a more favourable blood pressure profile than those with low levels of these circulating progenitor cells (table 4.7). As with the data already presented in this chapter on the effects of CRF on these $CD34^+$ progenitor cells, there was no discernable pattern in those with high or low circulating levels of EPCs (table 4.8).

Table 4.7. Differences in physical characteristics between quartiles of circulating $CD34^+$ progenitor cells.

$CD34^+$	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile	p-value
Age (years)	43 ± 13*	43 ± 14*	35 ± 24	34 ± 12	0.024
BMI ($kg \cdot m^2$)	25.8 ± 2.6	25.7 ± 3.1	26.4 ± 1.9	25.4 ± 2.7	0.576
Waist (cm)	89.5 ± 9.4	88.8 ± 7.4	90.7 ± 8.2	87.9 ± 7.7	0.659
MAP (mmHg)	100 ± 13*	97 ± 8	93 ± 8	93 ± 9	0.026
SDF-1α ($pg \cdot mL^{-1}$)	2423 ± 553	2410 ± 411	2356 ± 553	2480 ± 501	0.828
Fasting Glucose ($mmol \cdot L^{-1}$)	4.47 ± 0.75	4.18 ± 0.78	3.98 ± 1.11	4.27 ± 0.93	0.298
Total Cholesterol ($mmol \cdot L^{-1}$)	3.58 ± 1.04	3.39 ± 0.93	3.36 ± 0.94	3.16 ± 0.54	0.438
HDL-C ($mmol \cdot L^{-1}$)	1.27 ± 0.26	1.21 ± 0.24	1.29 ± 0.29	1.23 ± 0.32	0.746
LDL-C ($mmol \cdot L^{-1}$)	1.63 ± 0.97	1.52 ± 1.01	1.42 ± 0.82	1.25 ± 0.48	0.435
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	44.0 ± 10.0	44.8 ± 10.4	41.4 ± 9.3	44.1 ± 8.3	0.616

*BMI- Body Mass Index, MAP- Mean Arterial Pressure, HDL-C- High-Density Lipoprotein Cholesterol, LDL- Low-Density Lipoprotein Cholesterol. *p<0.05 vs. 3rd and 4th Quartile. Values are mean ± SD*

Table 4.8. Differences in physical characteristics between quartiles of circulating putative endothelial progenitor cells.

$CD34^{+}CD45^{DIM}VEGFR2^{+}$	1st Quartile	2nd Quartile	3rd Quartile	4th Quartile	p-value
Age (years)	42 ± 12	39 ± 13	41 ± 17	35 ± 13	0.303
BMI ($kg \cdot m^2$)	26.1 ± 3.0	26.1 ± 2.5	25.2 ± 1.9	25.9 ± 2.1	0.554
Waist (cm)	90.7 ± 9.4	90.1 ± 7.1	87.3 ± 8.0	87.9 ± 7.7	0.417
MAP (mmHg)	95 ± 11	97 ± 10	97 ± 8	93 ± 8	0.539
SDF-1α ($pg \cdot mL^{-1}$)	2442 ± 351	2456 ± 460	2320 ± 450	2451 ± 560	0.680
Fasting Glucose ($mmol \cdot L^{-1}$)	4.29 ± 1.27	4.37 ± 0.83	4.18 ± 0.69	4.04 ± 0.76	0.596
Total Cholesterol ($mmol \cdot L^{-1}$)	3.33 ± 0.83	3.43 ± 1.00	3.36 ± 0.96	3.36 ± 0.96	0.983
HDL-C ($mmol \cdot L^{-1}$)	1.20 ± 0.26	1.21 ± 0.27	1.25 ± 0.26	1.33 ± 0.30	0.325
LDL-C ($mmol \cdot L^{-1}$)	1.41 ± 0.89	1.55 ± 1.01	1.43 ± 0.72	1.42 ± 0.87	0.918
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	41.2 ± 10.3	43.9 ± 7.6	43.6 ± 8.8	45.6 ± 11.1	0.437

BMI- Body Mass Index, MAP- Mean Arterial Pressure, HDL-C- High-Density Lipoprotein Cholesterol, LDL- Low-Density Lipoprotein Cholesterol.

4.4 Discussion

The main findings of this study are that circulating $CD34^+$ progenitors are reduced in circulating numbers with advancing age, with numbers of $CXCR4^+$ progenitors also reduced. There appears to be minimal impact of CRF on these cells, with a reduction of $CD34^+$ progenitors and $CXCR4^+CD34^+$ progenitors in high CRF group in 31-50 year old participants. There were no such effects seen in the 18-30yrs and 51-65yrs groups. Fitness appears not to play a role in attenuating the age-related decline in these CACs, either age independent or dependent. Other factors appear to be more important, such as waist circumference, and cholesterol levels. Therefore, it cannot be discounted that CRF may play an indirect role on these CACs via modulating other health parameters such as cholesterol, waist circumference and other cardiometabolic risk factors. The largest predictor for circulating EPCs was circulating SDF-1α levels, a known circulating factor that assists in the mobilisation of progenitor cells from the BM and into the circulation. There also appears to be an age-related decline in circulating SDF-1α levels, potentially offering a mechanistic effect of advancing age on the observed decline in EPCs observed in this study. In addition, those with the highest number of $CD34^+$ progenitor cells appeared to be younger and displayed a more favourable blood pressure profile.

Advancing age is associated with endothelial dysfunction (Taddei *et al.*, 2001; Muller-Delp, 2006; Soucy *et al.*, 2006; Black *et al.*, 2008; Black *et al.*, 2009). There are many mechanisms postulated for the age-related decline in endothelial function, such as an increase in oxidative stress (Hamilton *et al.*, 2001; Taddei *et al.*, 2001) and increased rate of endothelial cell apoptosis (Wang *et al.*, 2013). Additionally, vascular regenerative potential may be reduced with advancing age, and cells that partake in the regeneration process, circulating or tissue-resident stem cells may also be impaired in the aged. Our data shows that advancing age is strongly related with reduction in several cell subsets of these circulating stem cells, $CD34^+$ and $CD34^+CD45^{dim}VEGFR2^+$ cells, with the latter termed endothelial progenitor cells (EPC). There was a 21% and 26% reduction in $CD34^+$ progenitor cells from 18-30yrs to 31-50yrs and 51-65yrs respectively, as well as a 46% and 55% in $CXCR4^+$ progenitor cells between the same age groups. EPCs were also reduced from 18-30yrs to 31-50yrs (60% reduction) and to 51-65yrs (47% reduction, $p=0.095$). As with $CD34^+$ progenitor cells, CXCR4-expressing EPCs followed the ageing trend, with a 64% and 46% reduction in cell number from 18-30yrs to 31-50yrs and 51-65yrs respectively. It was not only the $CXCR4^+$ cell number that was reduced, but the intensity of the cell surface expression on EPCs was also reduced in the 51-65yrs

compared to the 18-30yrs group, with a significant 43% reduction. Our data supports other studies that have found a reduction in circulating number of $CD34^+$ and EPCs with age (Thijssen *et al.*, 2006; Thum *et al.*, 2007), however, our data is the first to show that the $CD45^{dim}$ subset (reportedly 'true' endothelial precursors) of traditional $CD34^+VEGFR2^+$ EPCs is also affected negatively by age. In contrast, Heiss *et al.* (2005) found functional deficits in $CD34^+VEGFR2^+$ circulating cells rather than changes in number with age. These contrasting results highlight the importance for standardisation for EPC enumeration and quantification.

The reduction in circulating progenitors could be attributed to impaired mobilisation of these cells, reduced survival, reduced BM-resident vascular progenitors, or all three. Several studies have identified an age-related decline in progenitor cell number within the BM of aged versus young populations (Muschler *et al.*, 2001; Rauscher *et al.*, 2003; Stolzing *et al.*, 2008). These reductions in BM progenitor cell niche may be attributable to increased oxidative stress environment and senescence of these cells (Stolzing *et al.*, 2008). Consequently, reductions in the BM vascular progenitor cell niche may directly result in a reduction in numbers circulating in the peripheral blood compartment, further reducing the individual's ability for vascular repair.

In addition to a reduction of BM progenitor cell numbers, a reduced ability to mobilise these cells (Bosch-Marce *et al.*, 2007) may be a cause for the observed reduction seen in this study. Ageing was associated with a reduction in circulating SDF-1α in our study, a known mobilising factor for $CXCR4^+$ cells. Interestingly, SDF-1α was the only variable in the multiple regression model for $CD34^+CD45^{dim}VEGFR2^+$ cells ($r=0.239$, $p=0.019$) after correcting for age, fitness levels, circulating cholesterol, glucose and other anthropometric variables such as BMI and waist circumference. A 12% reduction in circulating SDF-1α was accompanied by a 47% reduction in $CD34^+CD45^{dim}VEGFR2^+$ cells from the 18-30yrs to the 51-65ys, although the reduction in EPCs from the young group to the 51-65yrs group was not significant ($p=0.095$). Despite these observations, Xing *et al.* (2006) found that in old mice, mobilisation of haematopoietic cells actually had a greater ability to enter the circulation than young mice after a intra-arterial dose of G-CSF was given. There are several studies showing an impaired mobilisation of BM progenitors in old mice versus young mice, whether that is to a burn wound stimulus (Zhang *et al.*, 2011) or exercise (Thijssen *et al.*, 2006). Zhang *et al.* (2011) investigated the mobilisation pattern of BM-derived vascular progenitors in response to a burn wound in both young and old mice- which is often used as a tissue repair model in mice. Wound-

induced SDF-1α expression was attenuated in the aged mice compared to the young mice, which had a knock-on effect of reduced progenitor cells entering the circulation, and subsequent reduced wound repair in comparison to the young mice. Although our study did not investigate stimulated release of progenitors, SDF-1α was reduced in our older individuals, which could be a significant predictor of basal progenitor cell levels.

This is the first study to our knowledge that demonstrates a reduction in $CXCR4^{+}CD34^{+}$ cells with age in humans. Aged atherosclerotic mice displayed reduced number of $CXCR4^{+}$ BM-derived cells (Xu *et al.*, 2011) which serves to strengthen the findings in our cohort. This may have implications for function of these cells as CXCR4 is a key receptor for these cells to migrate to ischaemic tissue expressing SDF-1α (Yamaguchi *et al.*, 2003). Indeed progenitor cell function appears to be reduced with age also (Heiss *et al.*, 2005; Hoetzer *et al.*, 2007; Thum *et al.*, 2007; Xia *et al.*, 2012b; Williamson *et al.*, 2013). These decrements in function may be somewhat linked to reduced CXCR4 cell surface expression, which would limit the cell's ability to migrate from the circulation to ischaemic tissue. This would have implications for the onset and progression of vascular disease. CVD patients display impaired CXCR4 signalling which was attributed to impaired vascular regeneration (Walter *et al.*, 2005) as blocking CXCR4 with neutralising antibodies reduced the ability of these EPCs to migrate to both SDF-1α and VEGF. When these human EPCs were incubated with CXCR4 antibody, EPC-mediated recovery of blood flow to ischaemic hind limb was significantly reduced. Corroborating with our data, these researchers found no difference in cell surface expression between CVD patients and age-matched healthy controls, indicating that CXCR4 cell surface expression may not be the most sensitive marker of CXCR4-mediated EPC dysfunction. Taken together, the reduction in circulating progenitor cell number and the reduced level of $CXCR4^{+}$ progenitor cells may contribute to the age-related endothelial dysfunction as a result of impaired endothelial regeneration and repair.

The data also indicates that before or after correction for age, there was no effect of CRF on these CAC subsets. This is in stark contrast to several studies which have demonstrated increases in $CD34^{+}$ cell number in aerobically fit versus unfit individuals, or as a result of a longitudinal exercise training programme (Laufs *et al.*, 2004; Steiner *et al.*, 2005; Hoetzer *et al.*, 2007; Sarto *et al.*, 2007; Cesari *et al.*, 2009; Manfredini *et al.*, 2009; Van Craenenbroeck *et al.*, 2010a; Schlager *et al.*, 2011; Sonnenschein *et al.*, 2011; Fernandes *et al.*, 2012; Xia *et al.*, 2012a; Choi *et al.*, 2014). Interestingly, Thijssen *et al.* (2006), Witkowski *et al.* (2010) and Luk *et al.* (2012) found no changes in circulating progenitor

cell number, and in some investigators who found no changes in number subsequently observed improvements in either *in vivo* (Xia *et al.*, 2012a) or *in vitro* (Manfredini *et al.*, 2009; Sonnenschein *et al.*, 2011) function.

It is likely that the differences in the data from the study presented and the studies showing increases in EPCs arises from the differences in the phenotype of EPC studied. Typically, EPCs have been defined as all circulating PBMNC co-expressing the stem cell antigen CD34 and an endothelial lineage antigen, VEGFR2 (Case *et al.*, 2007). This phenotype encompasses both cells that support endothelial growth through paracrine mechanisms and cells that have the ability to differentiate into mature endothelial cells. Van Craenenbroeck *et al.* (2013a) recommended using $CD34^{+}CD45^{dim}VEGFR2^{+}$ as the phenotypic identify of 'true' endothelial precursors since $CD45^{bright}$ fraction of these cells appear to be primarily haematopoietic in origin and function to secrete more pro-angiogenic cytokines and have less endothelial differentiating potential than $CD45^{dim}$ cells (Hur *et al.*, 2004; Timmermans *et al.*, 2007).

Our data suggests that regular exercise may not affect CXCR4 cell surface expression on these progenitor cells. Xia *et al.* (2012a) observed an increase in $CD34^{+}$ intracellular CXCR4 protein expression with exercise training in old subjects. This increased protein expression of this chemotactic receptor could lead to increased translocation of this receptor to the cell surface membrane, yet this was not assessed in this study. If this were to occur, this could apparently lead to the increased phosphorylation of JAK-2 after stimulation that was seen.

CXCR4 expression on these cells is heavily associated with the cell's ability to migrate to SDF-1α (Xia *et al.*, 2012a), which itself can be produced by ischaemic tissue and act as a potent stimulator for vasculogenesis. The age-related decline in endothelial regenerative ability may be linked to the reduced number of $CXCR4^{+}$ progenitors observed in our older cohort in the study, but by having a high CRF was potentially not strong enough to attenuate this age-related decline. CXCR4 is a cell surface receptor which can be upregulated when required, and thus a snapshot of the CXCR4 expression on these cells may not be a sensitive biomarker of cell function, and thus future studies should investigate the stimulated CXCR4 upregulation in these cells with age and exercise to further understand the effects of age and exercise on the function of these cells. Additionally, Walter *et al.* (2005) and Xia *et al.* (2012a) both are advocates for measuring the intracellular phosphorylation of JAK-2, a downstream target of CXCR4, to determine

mechanisms behind EPC dysfunction, as this measure was related to the extent to which these cells were able to either migrate to SDF-1α *in vitro*, or stimulate vascular repair *in vivo*. Therefore this may be a more sensitive measure to use in future studies to assess age- and exercise-related changes in vascular repair ability.

The data collected indicates that SDF-1α is a positive regulator of EPC number, as the multiple regression analysis revealed that SDF-1α was positively associated with EPC numbers. There is evidence that elevated levels of basal SDF-1α is linked with higher basal circulating levels of progenitor cells potentially a result of mobilisation from the BM (Aiuti *et al.*, 1997; Hattori *et al.*, 2001; Moore *et al.*, 2001) or enhanced survival within the circulatory environment (Sarto *et al.*, 2007; Zheng *et al.*, 2008; Yan *et al.*, 2012; Zhu *et al.*, 2012). Mesenchymal stem cell over-expression of SDF-1α, when transplanted into a mouse heart in a model of myocardial ischaemia, promoted greater incorporation of BM-derived progenitor cells and fewer apoptotic cells were found in the heart (Zhao *et al.*, 2009) providing evidence to its therapeutic potential to stimulate vasculogenesis. SDF-1α has been purported as potential therapeutic pharmacological agent to reduce morbidity in those with vascular disease due to its role in mobilisation of pro-angiogenic progenitors, ensuring survival of these cells and stimulating vascular growth. For an in-depth review of this topic, readers are directed to the work by Ho *et al.* (2012).

Paradoxically, in a cohort from the Framingham Heart Study, circulating higher circulating levels of SDF-1α was significantly associated with decreased $CD34^+$ cell frequency, yet higher levels of SDF-1α was a significant predictor for future CVD events and all-cause mortality in >3000 participants (Subramanian *et al.*, 2014). This is in stark contrast to several studies that have found positive association between SDF-1α and $CD34^+$ progenitor number (Moore *et al.*, 2001; Becchi *et al.*, 2008; Chang *et al.*, 2009). With regard to exercise, Laufs *et al.* (2004) found increases in circulating EPCs without any observable increases in circulating SDF-1α, suggesting that the exercise-induced improvements in basal EPCs was not due to circulating SDF-1α levels. We did see an ageing effect on SDF-1α levels, with serial decreases from 18-30yrs to 31-50yrs and again to 51-65yrs. In our stepwise multiple level regression analysis, after correction for age, SDF-1α was the only variable associated with $CD34^+CD45^{dim}VEGFR2^+$ EPCs, suggesting that they may be associated with modulating basal EPCs irrespective of age and exercise habits.

Recently, evidence is arising that SDF-1α may not act through modulating CXCR4 expression or EPC migration. Instead SDF-1α may act through C-X-C Chemokine Receptor 7 (CXCR7), and evidence suggests that the SDF-1α-CXCR7 axis may modulate cell survival and cell cycle initiation (Yan *et al.*, 2011; Torossian *et al.*, 2014), and EPC function (Dai *et al.*, 2011; Yan *et al.*, 2011). Therefore future studies could assess both CXCR4 and CXCR7 expression on these CACs to evaluate ageing and exercise effects.

4.5 Conclusion

Ageing is associated with reduced CAC numbers and $CXCR4^{+}$ BM-derived progenitors. CRF appeared to have little if any effect on these cells. Older individuals display reduced SDF-1α circulating levels, potentially causing the observed reduction in CACs in these individuals. Reduced SDF-1α and subsequent CAC circulating numbers may contribute to the well-described ageing-induced endothelial dysfunction and increased CVD risk.

Chapter 5: Influence of Age and Cardiorespiratory Fitness on Circulating Angiogenic T Cell Number and CXCR4 Cell Surface Expression

5.1 Introduction

Data from *Chapter 4* strongly suggests that age is negatively associated with a loss of EPCs, which may in turn have a deleterious effect on vascular health and angiogenesis. It is known that leukocytes can support angiogenesis (Gaudry *et al.*, 1997; Schruefer *et al.*, 2005). In 2007, Hur and colleagues (2007) isolated PBMNCs and cultured EPCs. In the central cluster of the culture they identified a sub-population of $CD3^+$ T-cells co-expressing CD31, which is itself an adhesion molecule (Muller, 1995). These cells are highly pro-angiogenic, being able to secrete high levels of VEGF, IL-8 and G-CSF to a significantly greater extent than $CD31^-$ fraction of these $CD3^+$ T-cells (Hur *et al.*, 2007; Kushner *et al.*, 2010b). In a hindlimb ischaemic mouse model, mice injected with these $CD3^+CD31^+$ cells showed greater blood flow recovery to the ischaemic limb and greater capillarity compared with mice injected with saline or $CD3^+CD31^-$ T-cells (Hur *et al.*, 2007). Unsurprisingly, these cells showed greater potential for adhesion to HUVECs than their $CD31^-$ counterparts, and they also migrated to a greater extent to SDF-1α than $CD3^+CD31^-$ T-cells which indicated that these cells must express CXCR4 (the receptor for SDF-1α) more than the $CD3^+CD31^-$ cells, which they did. As a result of their potent vasculogenic capabilities, these $CD3^+CD31^+$ cells were termed 'angiogenic T-cells', or T_{ANG}. The circulating number of these cells however has not been associated with endothelial function, but ability of these cells to migrate to SDF-1α, with improved migration to this factor positively correlating with improved forearm blood flow after an administered dose of acetylcholine (Weil *et al.*, 2011). This suggests that when enumerating this cell subset, investigators should also be investigating CXCR4 cell surface expression, and this may be involved in the function of these cells to migrate to ischaemic areas. Circulating T_{ANG} levels have been found to be negatively correlated with the Framingham Risk Score (FRS) (Hur *et al.*, 2007), reduced in patients with small vessel cerebrovascular disease (Rouhl *et al.*, 2012) as well as with advancing age (Hur *et al.*, 2007; Kushner *et al.*, 2010c), indicating that these cells may be implicated in the increased CVD risk with age, as a result of the imbalance between endothelial damage and repair.

Exercise may play a role in improving endothelial function and reducing CVD risk by association with elevated $CD31^+$ T-cell number and/or function. We have provided evidence that basal levels of $CD34^+$ and $CD34^+CD45^{dim}VEGFR2^+$ EPCs are unaffected by fitness level, an indicator of exercise training habits (see *Chapter 4*) and yet it is currently not known if exercise, either an acute exercise bout or chronic exercise training modulate the circulating levels of T_{ANG} or cell surface expression of CXCR4 of these

T_{ANG} cells. Therefore it was the aim of this study is to evaluate the effect of ageing in healthy males on circulating number and CXCR4 cell surface expression of $CD3^{+}CD31^{+}$ T-cells (T_{ANG}), and secondly to assess the role CRF plays in any age-related effects observed.

5.2 Materials and Methods

5.2.1 Participants

Participant information, inclusion and exclusion criteria are detailed in *Chapter 4* (*Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee. Participant characteristics are shown in table 4.1 (see *Chapter 4 - Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). For anthropometric characteristics, see *4.2.2 Anthropometric and Characteristics*.

5.2.2 Blood Sampling and PBMNC Isolation

Blood was taken from participants after a 5-minute supine rest by a certified phlebotomist. Blood samples were drawn into 6mL vacutainers (BD Biosciences, UK), which were either coated in EDTA to prevent coagulation or serum gel. EDTA blood was processed for PBMNC separation as previously described in general materials and methods *Chapter 3.7*. Isolated PBMNCs were then used for T_{ANG} number and CXCR4 enumeration (see *5.2.5 Angiogenic T Cell Number and CXCR4 Expression Quantification)*, and serum was used for analysis of serum for chemotactic factors (see *5.2.6 Serum Analysis for Chemotactic Factors and Inflammatory Markers*).

5.2.4 Submaximal Exercise Test to Estimate Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) of each participant was estimated using a submaximal exercise test (YMCA) as previously described in general materials and methods *Chapter 3.15*. Briefly, participants exercised on a stationary cycle ergometer (Veletron™ Dynafit Pro; Racer Mate®, Seattle, USA) at 50rpm for 3-4 incremental stages, each stage lasting 3 minutes. The initial power output of the test was 50W and increased in subsequent stages depending on HR response. $\dot{V}O_{2max}$ was measured throughout the test through breath-by-breath online analysis (LABManager v5.3.0, Cardinal Health, Germany). The test was terminated when the desired steady state HR was attained (80% of HR_{max}). $\dot{V}O_{2max}$ was estimated using equations provided by Adams and Beam (1998).

5.2.5 Angiogenic T Cell Number and CXCR4 Expression Quantification

Isolated PBMNCs ($0.5x10^6$) were labelled with monoclonal antibodies anti-CD3-APC, anti-CD31-FITC, anti-CD4/CD8-PE and anti-CXCR4-PE-Cy5 (all BD Biosciences, USA), and were left to incubate at 4°C for 45 minutes in the dark prior to flow cytometric analysis as detailed in general materials and methods *Chapter 3.9*. CXCR4 cell surface expression was analysed as a surrogate for migratory ability.

5.2.6 Serum Analysis for Chemotactic Factors and Inflammatory Markers

Serum chemotactic factor SDF-1α, in addition to inflammatory markers IL-6, and IL-8 were analysed by enzyme-linked immunosorbent assay (ELISA) as described in general materials and methods *Chapter 3.12*. Fasting glucose, triglycerides, total cholesterol, HDL-C and LDL-C was measured in human serum by semi-automated spectrophotometry (RX Monza Clinical Chemistry Analyzer, Randox, UK). Samples were analysed in duplicate and averaged.

5.2.7 Statistical Analysis

All data were assessed for normal distribution. Not normally distributed data were logarithmically transformed. Linear regressions were initially performed to assess relationships between circulating $CD3^+CD31^+$ cells (T_{ANG}) or CXCR4 expression and variables such as age, CRF ($\dot{V}O_{2max}$), BMI, MAP, circulating triglycerides, total cholesterol, HDL-C, LDL-C, fasting glucose, as well as SDF-1α, which may stimulate upregulation of CXCR4 cell-surface. Variables with a significant association with T_{ANG} cells were added to a regression model by hierarchical multiple regression. A one-way analysis of variance (ANOVA) was used to detect differences in T_{ANG} populations between age groups (18-30yrs, 31-50yrs, 51-65 years) and between age-adjusted $\dot{V}O_{2max}$ groupings. Two-way ANOVA were performed to detect any interaction between age and CRF on T_{ANG} cells and CXCR4 expression. Fisher's Least Significant Difference (LSD) post-hoc tests were performed to identify locations of any significant differences.

Data were analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA).

5.3 Results

5.3.1 The effect of age, CRF and other circulating factors on angiogenic T-cells

Advancing age was associated with a decrease in T_{ANG} cell number, expressed as either total $CD3^+CD31^+$ cell number (r=-0.410, p=0.000) or as a proportion of total $CD3^+$ T-cells (r=-0.534, p=0.000), however there was no association present for $CXCR4^+$ T_{ANG} number or intensity of expression (mean fluorescence intensity [MFI]) (see table 5.1). Estimated $\dot{V}O_{2max}$ was positively correlated with proportion of total $CD3^+$ T-cells expressing CD31 (r=0.284, p=0.002), and, like age, there was no association between $\dot{V}O_{2max}$ and CXCR4 expression on T_{ANG} cells.

Other measures of cardiometabolic health, such as total cholesterol ($CXCR4^+$ T_{ANG}, r=-0.228, p=0.011; % of T_{ANG} expressing CXCR4, r=-0.251, p=0.005), LDL-C ($CXCR4^+$ T_{ANG}, r=-0.234, p=0.009; T_{ANG} CXCR4 MFI, r=-0.203, p=0.020), and fasting glucose (T_{ANG} as % $CD3^+$, r=-0.261, p=0.004) were all negatively associated with various T_{ANG} cell subsets (data not shown). SDF-1α was only associated with rise in proportion of $CD3^+$ T-cells expressing CD31 (r=0.245, p=0.007), with no other relationship observed with CXCR4 expression on these cells.

Hierarchical multiple level regression analyses were performed to assess the relationship between CRF and T_{ANG} cells after correcting for age to see if CRF attenuates the age-related decline in these cells. After correction for age, CRF was not associated with any T_{ANG} subset or measure of CXCR4 cell surface expression. Results are shown in table 5.2.

Table 5.1. Univariate linear regression analysis on T_{ANG} cell number and CXCR4 cell surface expression subsets in healthy males (n=102).

	Pearson's coefficient (p-value)		F-statistic (p-value)	
	Age	$\dot{V}O_{2max}$	Age	$\dot{V}O_{2max}$
$CD3^+CD31^+$ T-cells (cells·μL^{-1})	-0.251 (0.006)*	0.144 (0.075)	6.542 (0.012)*	2.103 (0.150)
$CD3^+CD31^+$ T-cells (% of $CD3^+$)	-0.305 (0.001)*	0.258 (0.005)*	10.220 (0.002)*	7.050 (0.009)*
$CXCR4^+$ T_{ANG} (cells·μL^{-1})	0.039 (0.347)	0.002 (0.491)	0.155 (0.694)	0.001 (0.982)
%T_{ANG} expressing CXCR4	0.003 (0.486)	-0.034 (0.366)	0.001 (0.972)	0.117 (0.733)
MFI T_{ANG} CXCR4	0.041 (0.343)	0.009 (0.463)	0.163 (0.687)	0.009 (0.926)

*MFI- Mean Fluorescence Intensity. * significant $p<0.05$*

Table 5.2. Hierarchical multiple level regression analysis on $CD31^+$ T-cells in healthy men aged 18-65 (n=102).

Angiogenic T-Cell Subsets	Factors for Analysis	R^2	R^2 *Change*	*Beta Value*	SEB	*Standardised ß*	F-statistic (p -value)	t-statistic (p-value)
$CD3^+CD31^+$ T-cells (cells·μL^{-1})	**Model 1**	0.168					20.250 (0.000)*	
	Age			-6.654	1.479	-0.410		-4.500 (0.000)*
	Model 2	0.169	0.001					
	Age			-6.502	1.543	-0.401	10.102 (0.000)*	-4.213 (0.000)
	$\dot{V}O_{2max}$			0.813	2.257	0.034		0.360 (0.719)
$CD3^+CD31^+$ T-cells (% of $CD3^+$)	**Model 1**	0.289					40.215 (0.000)*	
	Age			-0.369	0.058	-0.537		-6.342 (0.000)*
	Model 2	0.303	0.014				21.321 (0.000)*	
	Age			-0.346	0.060	-0.505		-5.770 (0.000)*
	$\dot{V}O_{2max}$			0.125	0.088	0.124		1.419 (0.159)
$CXCR4^+$ T_{ANG} (cells·μL^{-1})	**Model 1**	0.023					2.361 (0.128)	
	Age			-1.009	1.005	-1.419		-1.537 (0.128)
	Model 2	0.025	0.002				1.255 (0.290)	
	Age			-1.009	1.007	-1.456		-1.584 (0.116)
	$\dot{V}O_{2max}$			-1.005	1.009	-1.102		-0.410 (0.683)
$\%T_{ANG}$ expressing CXCR4	**Model 1**	0.000					0.018 (0.894)	
	Age			-0.018	0.135	-0.013		-0.133 (0.894)
	Model 2	0.002	0.002				0.085 (0.918)	
	Age			-0.033	0.141	-0.024		-0.234 (0.815)
	$\dot{V}O_{2max}$			-0.081	0.206	-0.041		-0.391 (0.696)
MFI T_{ANG} CXCR4	**Model 1**	0.010					1.016 (0.316)	
	Age			0.017	0.017	0.100		1.008 (0.316)
	Model 2	0.012	0.002				0.576 (0.564)	
	Age			0.019	0.018	0.111		1.070 (0.287)
	$\dot{V}O_{2max}$			0.010	0.026	0.040		0.381 (0.704)

*SEB- Standard Error of the Beta Value; MFI- Mean Fluorescence Intensity. *p<0.05*

One-way analyses of variance (ANOVA) ($CD3^+CD31^+$/$CD3^+CD31^+CXCR4^+$) were performed to determine the effects of age on T_{ANG} cells and levels of and CXCR4 expression on these cells. With data available for $CD4^+$ and $CD8^+$ sub fractions only for the 31-50yrs and 51-65yrs groups, independent T-tests were performed to detect differences between age groups in these cells.

Figure 5.1 shows that T_{ANG} cells were reduced in circulation of those in the 31-50yrs and 51-65yrs group compared to the 18-30yrs group (631 ± 34 cells·μL^{-1} [563-699] vs. 751 ± 39 cells·μL^{-1} [672-831], p=0.014, and 543 ± 32 [476-610] vs. 751± 39 [672-831], p=0.000, respectively). There was no significant difference in $CD4^+$ or $CD8^+$ T_{ANG} cells between the 31-50yrs and the 51-65yrs groups. In addition, the percentage of total T-cells expressing CD31 were dramatically reduced in the 31-50yrs and 51-65yrs group compared to the 18-30yrs cohort (53.91 ± 1.39% [51.11-56.72) vs. 61.67 ± 1.26% [59.09-64.22], p=0.000; 49.15 ± 1.64% [45.77-52.54] vs. 61.67 ± 1.26% [59.09-64.22], p=0.000), and the 51-65yrs group also showing a reduction compared to the 31-50yrs group (49.15 ± 1.64% [45.77-52.54] vs. 53.91 ± 1.39% [51.11-56.72], p=0.027) (figure 5.2).

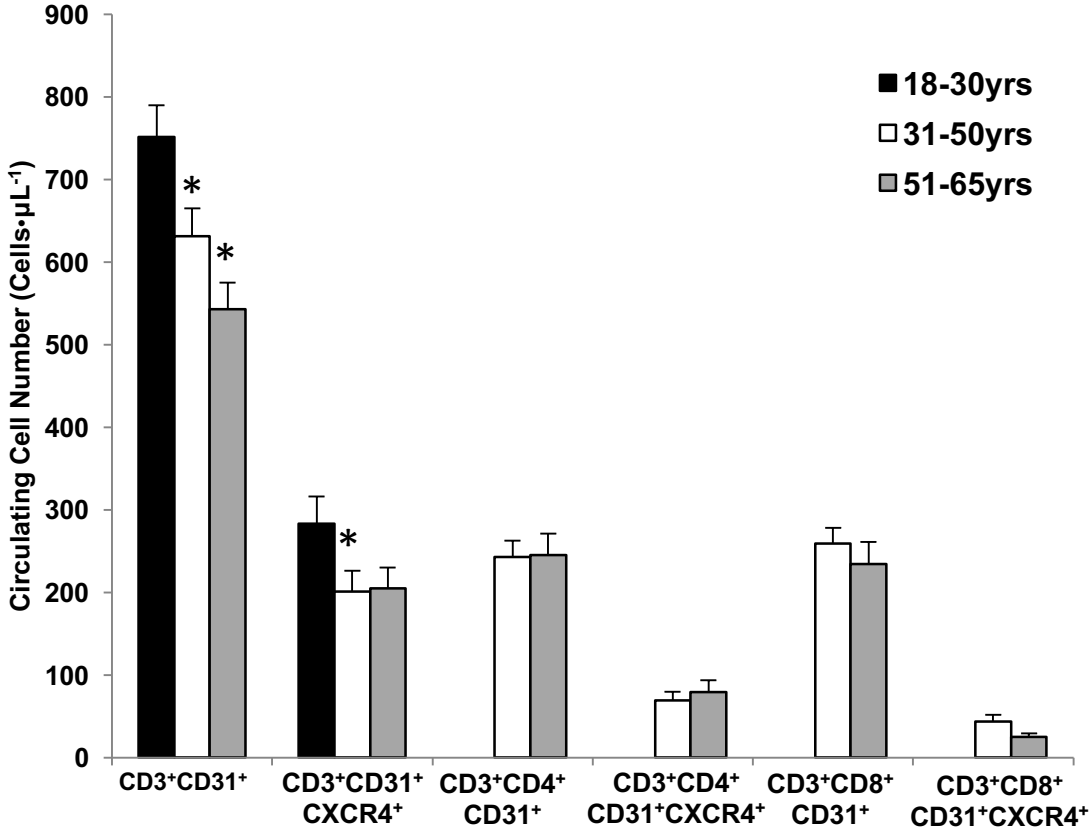

Figure 5.1. Circulating $CD3^+CD31^+$ T-cells in healthy males in age groups 18-30yrs, 31-50yrs and 51-65yrs. * *significantly different from 18-30yrs, p<0.05.*

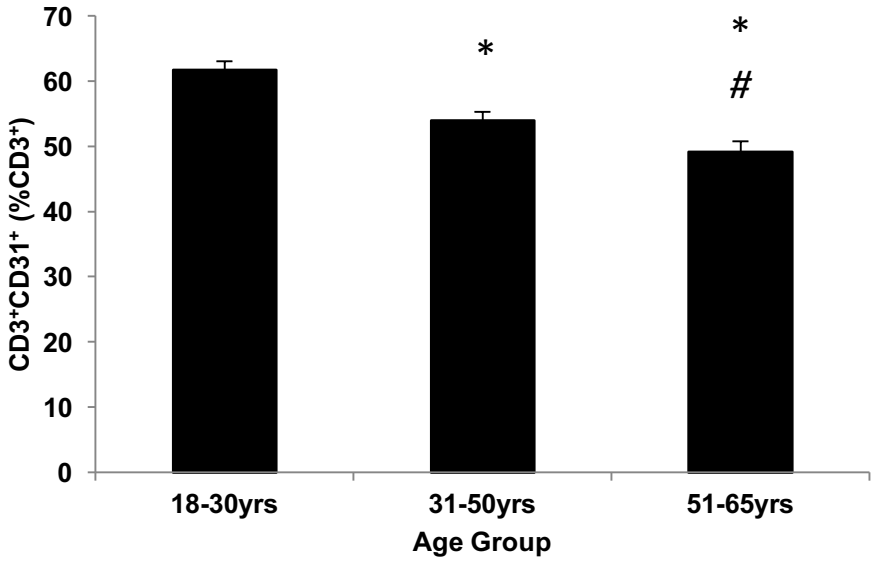

Figure 5.2. Percentage of $CD3^+$ T-cells expressing CD31 changes with age. **significantly different from 18-30yrs, $p<0.001$, # significantly different from 31-50yrs group, $p<0.05$.*

Interestingly, the percentage of $CD4^+$ and $CD8^+$ T-cells which express CD31 differ, with <35% of $CD4^+$ T-cells and >76% of $CD8^+$ T-cells expressing CD31 (figure 5.3A, p=0.002). Subsequent gating for CXCR4 cell surface expression revealed that the $CD4^+$ T_{ANG} cells had a greater proportion of $CXCR4^+$ cells than the $CD8^+$ T_{ANG} cells, with >30% $CD4^+$ T_{ANG} cells expressing CXCR4, with only ~ 15% of $CD8^+$ T_{ANG} cells doing so (31.56 ± 4.12% [23.27-39.85] vs. 14.80 ± 1.66% [11.45-49.51], p=0.006, figure 5.3B).

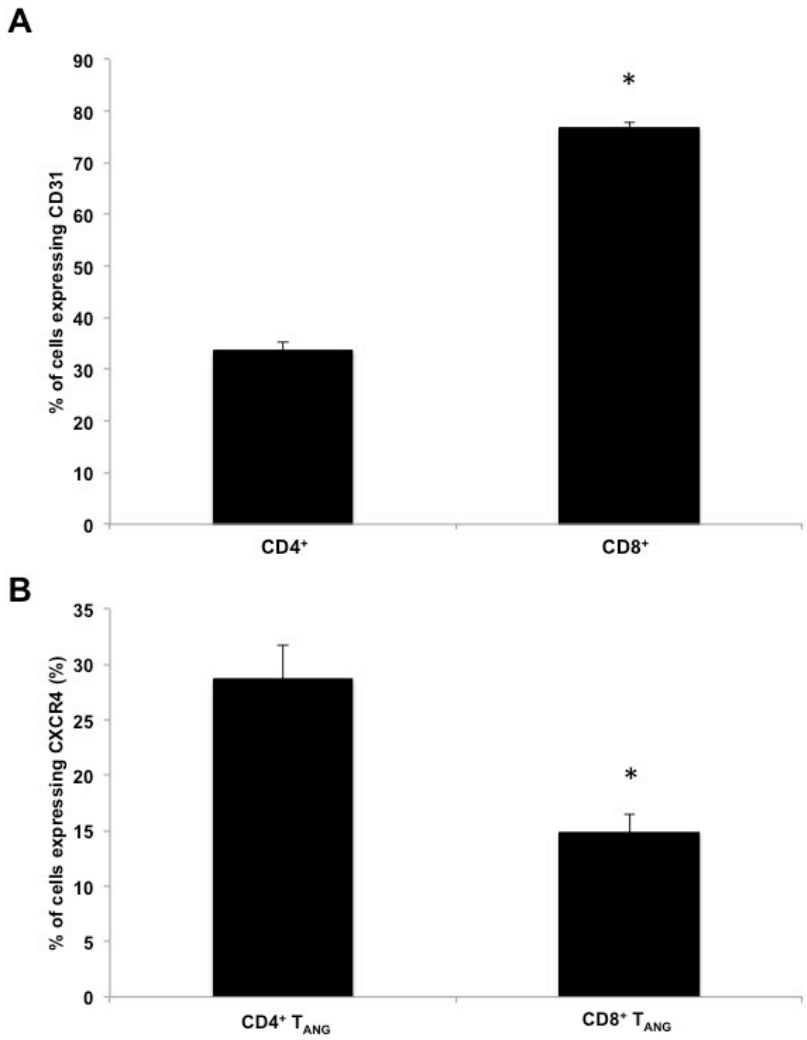

Figure 5.3. $CD4^+$ and $CD8^+$ T-cell expression of CD31 (A), and subsequent CXCR4 (B) cell surface expression (n=46). ***** *significantly different from* $CD4^+$, $p<0.05$.

Furthermore, the percentage of those $CD4^+$ and $CD8^+$ T-cells which co-express CD31 did not differ with age (figure 5.4A), yet the percentage of $CD8^+$ T_{ANG} cells expressing CXCR4 was reduced in our 51-65yrs cohort compared to the 31-50yr group (10.27 ± 1.35% [7.44-13.10] vs. 16.96 ± 2.50% [11.80-22.12], p=0.033) (figure 5.4B).

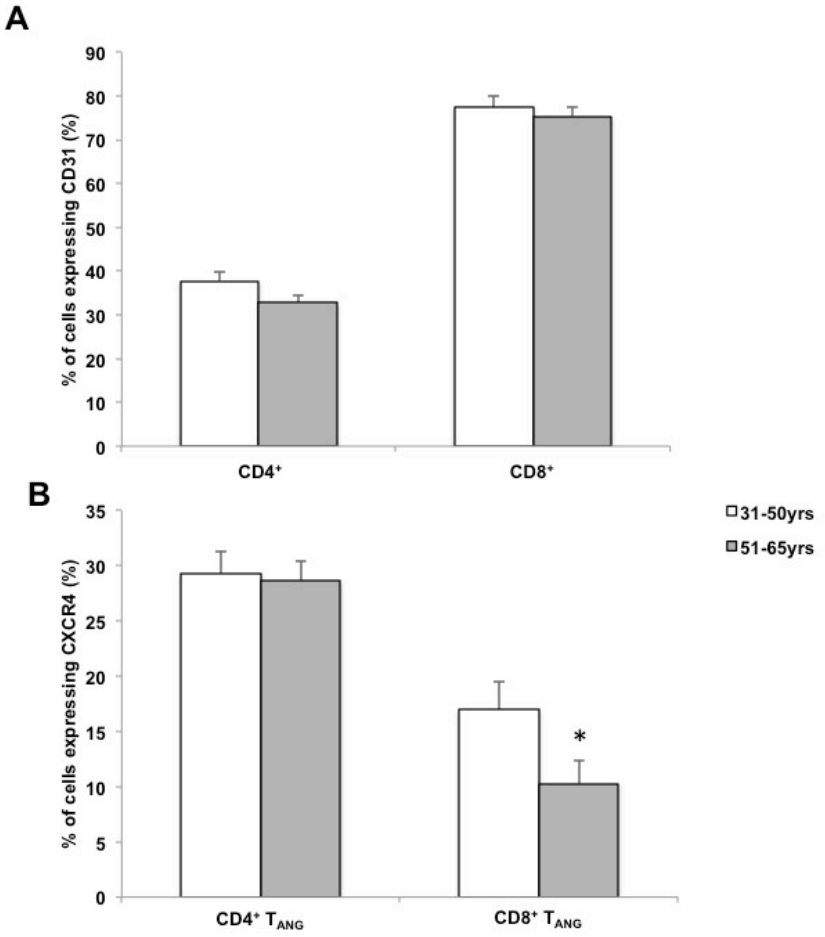

Figure 5.4. $CD4^+$ and $CD8^+$ expression of CD31 (A) and subsequent CXCR4 cell surface expression (B) between age groups. **significantly different from 31-50yrs, $p<0.05$.*

5.3.2 Cardiorespiratory Fitness and CD31$^+$ T-Cells

Participants were then grouped within their age-classifications (18-30yrs, 30-50yrs and 51-65yrs) into tertiles for estimated $\dot{V}O_{2max}$ (for CRF-grouped participant characteristics, see table 4.5**,** *Chapter 4: The Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR Cell Surface Expression*). To further investigate the effect of CRF on these T-cells with age, linear regressions were performed to assess the relationship of T_{ANG} cells and age within CRF sub categories.

Figure 5.5 shows the linear relationships between age and T_{ANG} cells within the CRF categories of low, moderate and high CRF. There is no difference in slope of the regression line, confirming the hierarchical multiple level regression analysis that if CRF plays a role in modulating T_{ANG} cell number, then it is not independent of age.

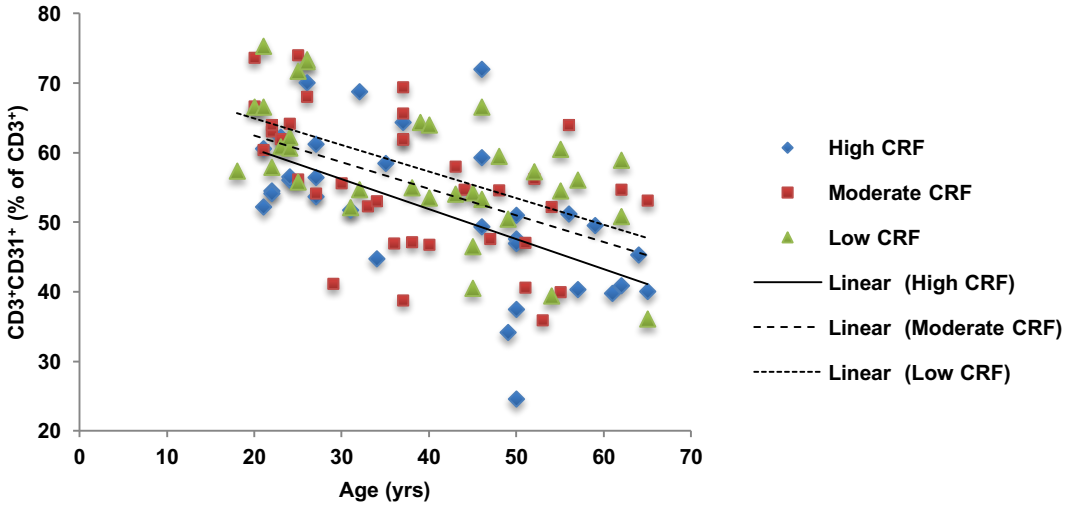

Figure 5.5. Linear regressions of T_{ANG} and age in low, moderate and high CRF categories.

A two-way factorial ANOVA was performed, with age groups (18-30yrs, 31-50yrs and 51-65yrs) and fitness groups (low CRF, moderate CRF, and high CRF- tertiles) as the fixed factors.

Analysis showed that $CD3^+CD31^+$ T-cells, when expressed as cells·μL^{-1} did not significantly differ between CRF groups in all age groups (figure 5.6). T_{ANG} cell numbers in the high CRF group was higher than in low CRF for 18-30yrs (816 ± 59 cells·μL^{-1} [687-946] vs. 522 ± 61 cells·μL^{-1} [522-788]), however this was not statistically significant (p=0.068), yet effect size calculations revealed that, although not significant, this was a moderate effect size (ES=0.78). No other notable differences were found.

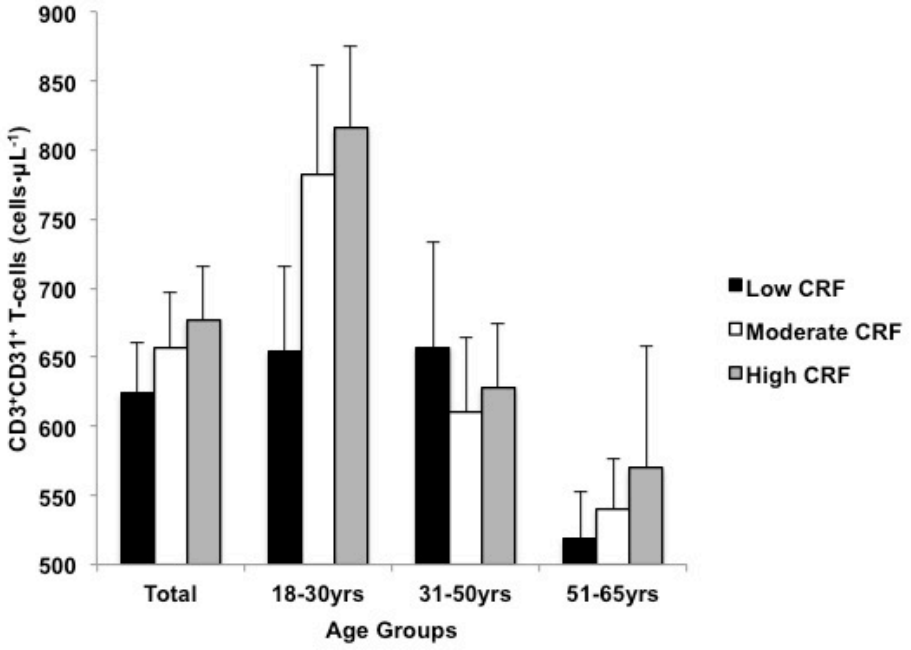

Figure 5.6. The effects of cardiorespiratory fitness on $CD31^+$ T-cells in healthy males aged 18-65yrs with age-group specific low, moderate and high $\dot{V}O_{2max}$ values.

Further sub-analysis of CD4 and CD8 proportion in both 31-50yrs and 51-65yrs revealed no differences in cell number between CRF groups (figure 5.7). Interestingly, the $CD4^+$ T_{ANG} cells appeared to be higher in the moderate CRF compared to low CRF participants in the 51-65yrs group, with a large effect size (301 ± 44 cells·μL^{-1} [189-413] vs. 187 ± 34 cells·μL^{-1} [104-271], ES=1.15). This suggests that $CD4^+$ T_{ANG} cells may be more susceptible to changes in CRF, rather than $CD8^+$ sub populations.

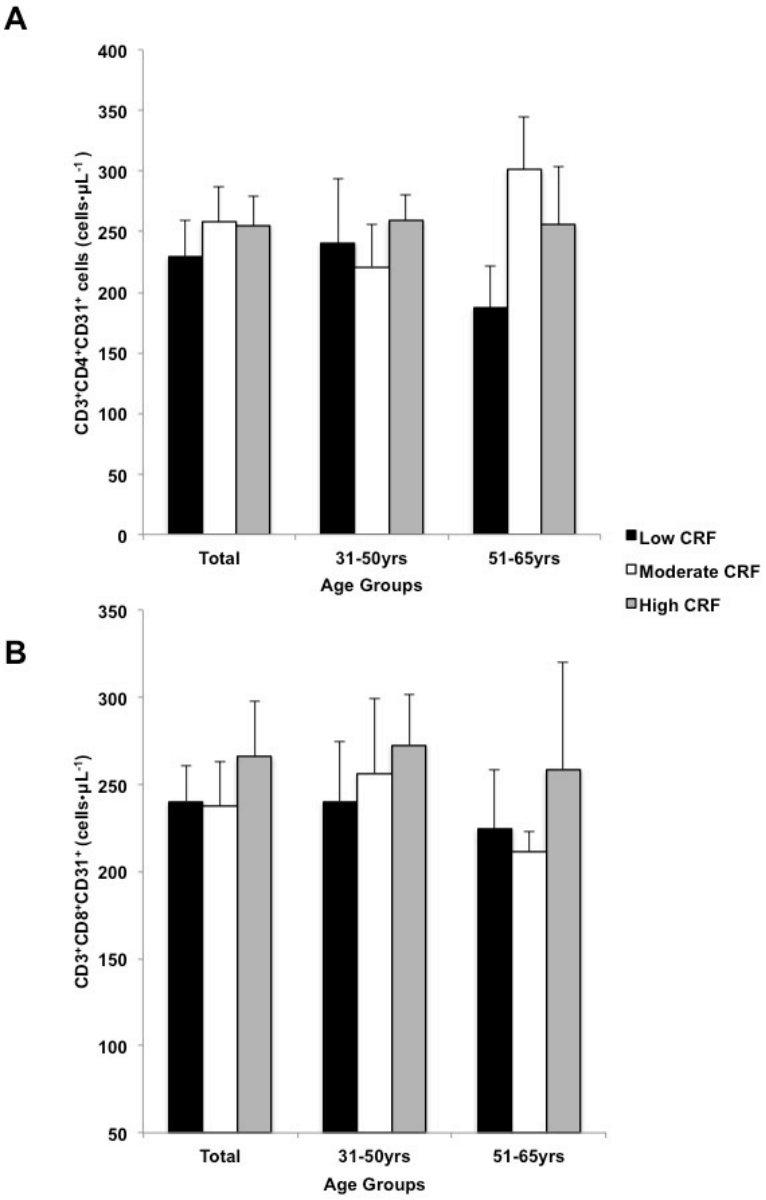

Figure 5.7. CD4/$CD8^+$ $CD31^+$ T-cells in healthy males aged 18-65yrs with age-group specific low, moderate and high $\dot{V}O_{2max}$ values.

Analysis of proportion of total T-cell pool revealed that high CRF was associated with greater percentage of T-cells expressing CD31 than low CRF in 18-30yrs (65.06 ± 2.01% [60.64-69.47] vs. 58.32 ± 1.49% [55.05-61.60], p=0.045), 51-65yrs (44.96 ± 1.96% [40.32-49.6] vs. 53.73 ± 2.34% [48.20-59.25], p=0.033) and total grouped participants (58.18 ± 1.45% [55.23-61.13] vs. 52.87 ± 1.66% [49.48-56.25], p=0.009) (figure 5.8).

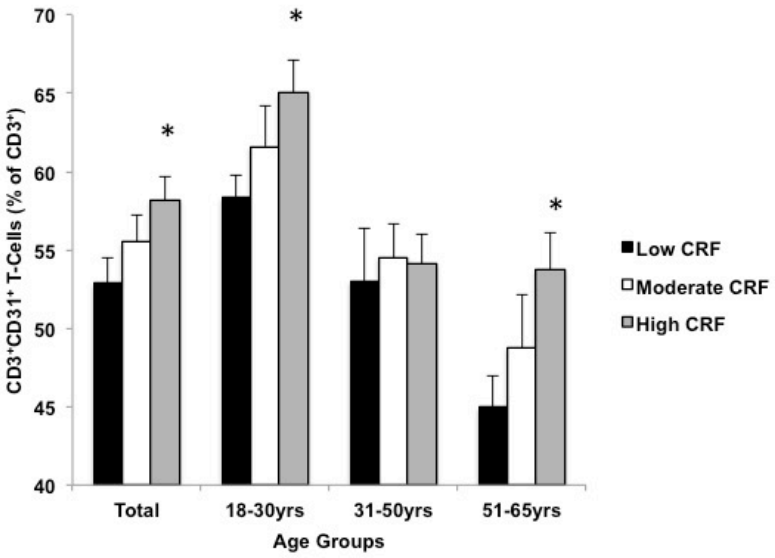

Figure 5.8. $CD31^+$ T-cell proportion of total T-cells in those with age-group specific low, moderate and high $\dot{V}O_{2max}$ values. * *p<0.05 significantly different from low CRF.*

The changes in T-cell make up with CRF could potentially be a result of changes within the $CD4^+$ T-cell pool, as shown by figure 5.9, with greater percentage of $CD4^+$ T-cells expressing CD31, with no changes observed in the $CD8^+$ T-cell pool. Participants in the moderate CRF and high CRF groups in the 51-65yrs group displayed greater percentage of $CD4^+$ T-cells expressing CD31 than participants in the low CRF group (38.32 ± 5.20% [24.96-51.67] vs. 23.51 ± 3.86% [14.06-32.97], p=0.009; 36.63 ± 2.52% [30.68-42.58], p=0.013 respectively). $CD4^+$ and $CD8^+$ T_{ANG} analysis was not performed in the 18-30yrs group in this study.

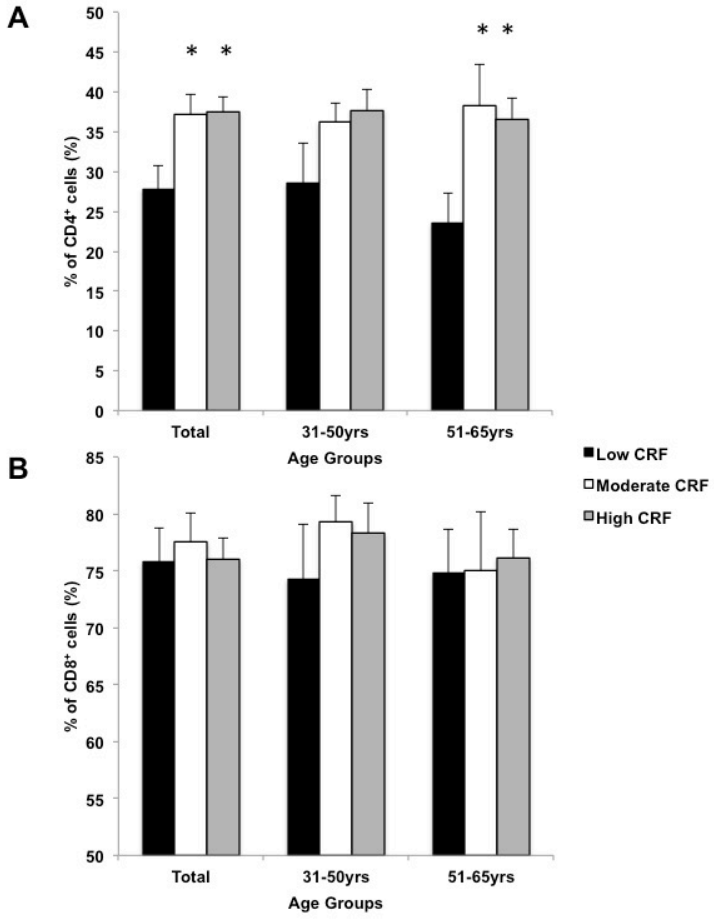

Figure 5.9. Changes in $CD4^+$ (A) and $CD8^+$ (B) T-cell CD31 expression with age and CRF levels. ** $p < 0.05$ significantly different from low CRF.*

There were no differences in CXCR4$^+$ T_{ANG} cell number between CRF groups in any age category. This was reflected by no changes in either CD4$^+$ or CD8$^+$ subsets of T_{ANG} cells (figure 5.10).

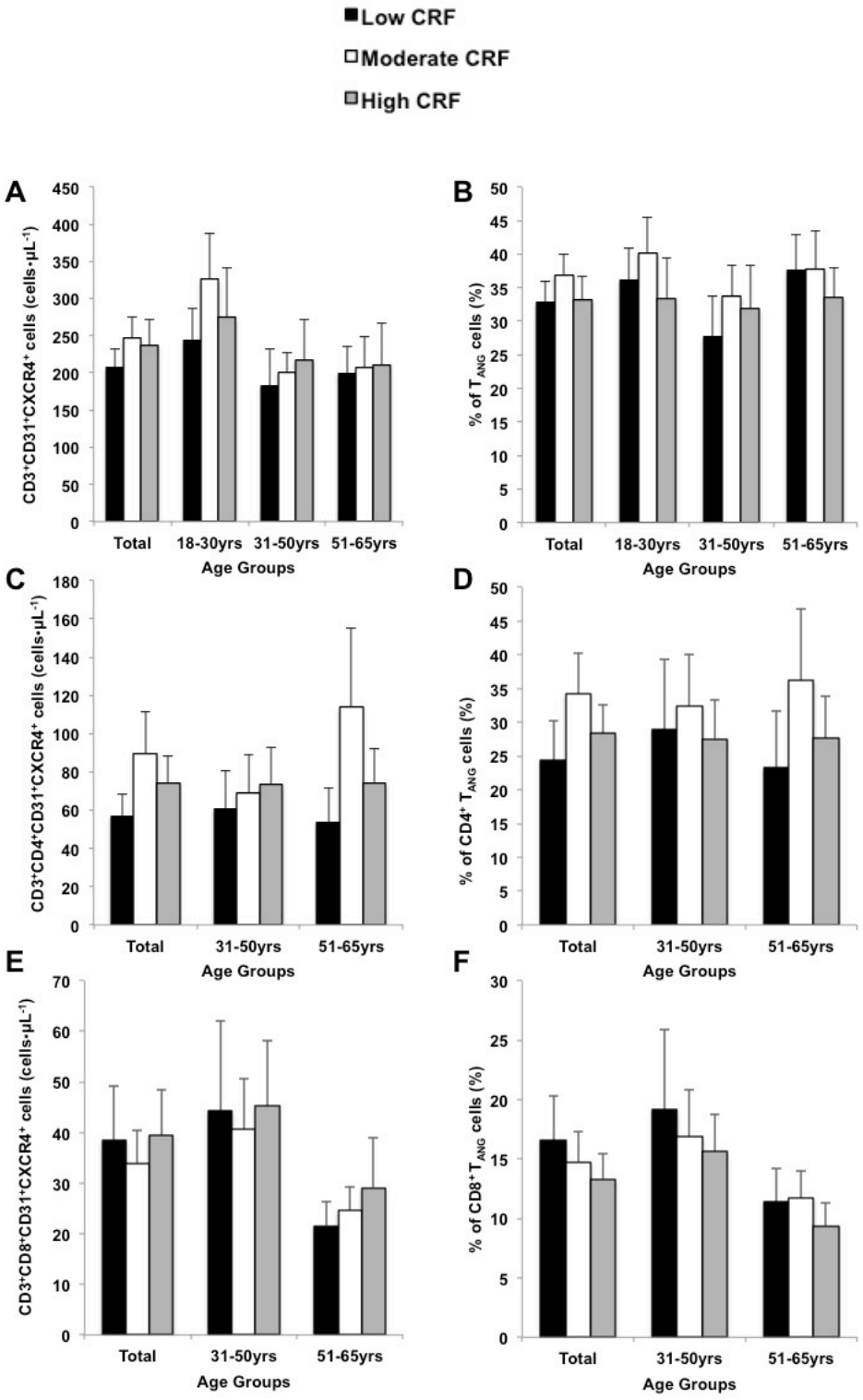

Figure 5.10. Age and cardiorespiratory fitness effects on CXCR4$^+$ T_{ANG} cells. *A+B- Total CD3$^+$CD31$^+$ T_{ANG} cells, C+D- CD4$^+$ T_{ANG}, E+F- CD8$^+$ T_{ANG}.*

Cell surface expression of CXCR4 was largely unchanged between CRF groups, with only $CD8^+$ T_{ANG} cells in the moderate CRF group showing reduced expression compared to the low CRF group (7.41 ± 0.29 [6.70-8.13] vs. 9.05 ± 0.89 [6.86-11.230, p=0.037) (figure 5.11). However, $CD4^+$ and total $CD3^+CD31^+$ cells showed no differences.

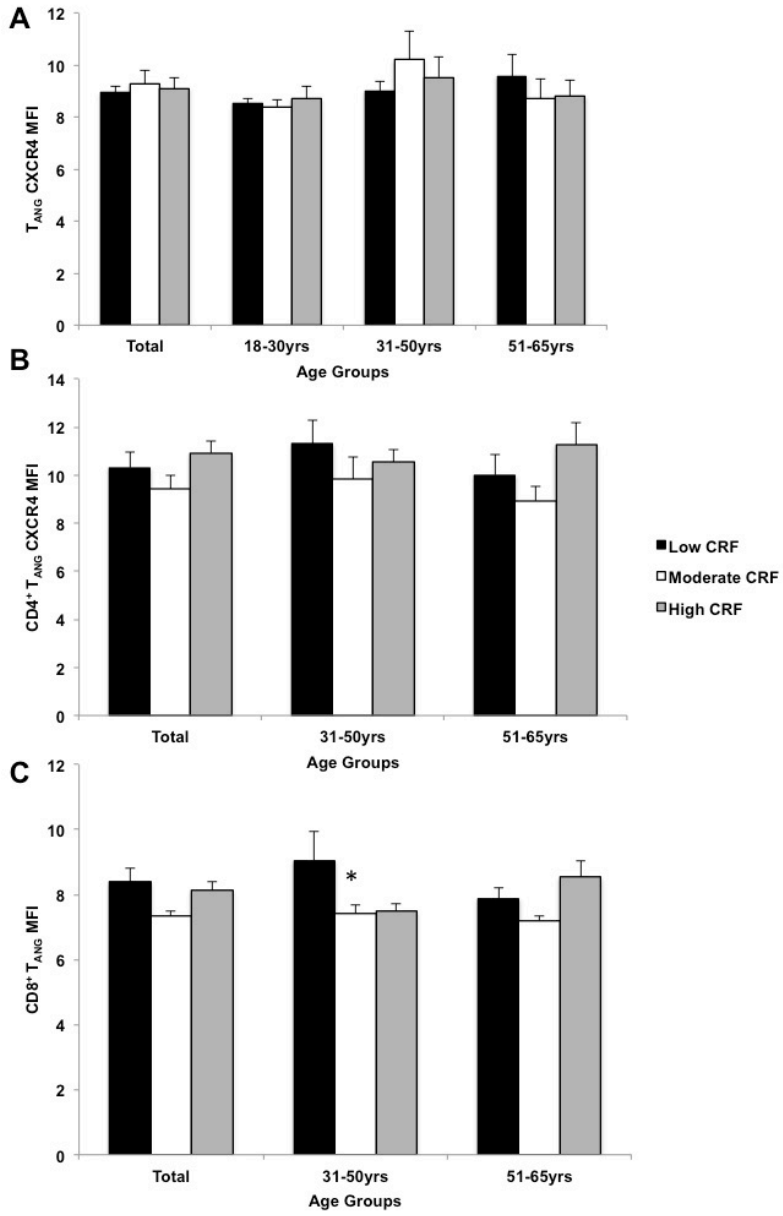

Figure 5.11. Cell surface expression levels of CXCR4 on $CD3^+CD31^+$ T-cells in different cardiorespiratory fitness groups. *A- $CD3^+CD31^+$ cell surface expression of CXCR4, B- $CD4^+$ T_{ANG} CXCR4 cell surface expression, C- $CD8^+$ T_{ANG} CXCR4 cell surface expression.*

5.3.3 Multiple Regression Analysis

To further assess the contributions to T_{ANG} cell number and CXCR4 expression, stepwise multiple level regression analyses were performed. The following variables that were collected in the study were entered: age, estimated $\dot{V}O_{2max}$, BMI, waist circumference, MAP, fasting glucose, total cholesterol, HDL-C, LDL-C, IL-6 and IL-8 and SDF-1α. Results are shown in table 5.3.

Stepwise multiple level regression showed that age is the greatest predictor for T_{ANG} circulating number, with LDL-C being related to CXCR4 expression on these cells. From the results presented in this chapter, unsurprisingly $\dot{V}O_{2max}$ was not included in any model. However, it was thought that SDF-1α may regulate $CXCR4^{+}$ T_{ANG} cell number, but this was not observed in this population of cells.

Table 5.3. Stepwise multiple linear regression model for $CD3^+CD31^+$ T_{ANG} cells (n=102).

T_{ANG} cell subsets	Predictors in Model	*R*	R^2	*Beta Value*	SEB	*Standardised ß*	F-statistic (p -value)	t-statistic (p-value)
$CD3^+CD31^+$ (cells·μL^{-1})	**Age**	-0.413	0.171	-6.706	1.486	-0.413	20.371 (0.000)*	-4.513 (0.000)*
$CD3^+CD31^+$ (% $CD3^+$)	**Age**	-0.541	0.293	-0.372	0.058	-0.541	40.586 (0.000)*	-6.371 (0.000)*
$CD3^+CD31^+CXCR4^+$ (cells·μL^1)	**LDL-C**	-0.220	0.048	-1.230	1.097	-1.660	5.032 (0.027)*	-2.243 (0.027)*
% T_{ANG} expressing CXCR4	**LDL-C**	-0.288	0.083	-6.410	2.144	-0.288	8.937 (0.004)*	-2.989 (0.004)*
T_{ANG} CXCR4 MFI	**N/A**	-	-	-	-	-	-	-

*SEB- Standard Error of the Beta Value; MFI- Mean Fluorescence Intensity, * p<0.05*

5.4 Discussion

Advanced age was associated with a decrease in $CD3^+CD31^+$ T-cell number, percentage of $CD3^+$ T-cells expressing CD31, and $CXCR4^+$ T_{ANG} cell number. In contrast to the $CD34^+$ progenitor cells (see *Chapter 4: The Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*), CRF appears to play a role in the modulation of these cells, with higher levels of $\dot{V}O_{2max}$ within the 18-30yrs and 51-65yrs group associated with higher levels of T_{ANG} cells (as a percentage of $CD3^+$ T-cells).

Our data shows that ageing results in a declined percentage of T-cells expressing the adhesion molecule CD31. There was an 8% and 13% reduction in proportion of T-cells expressing CD31 from the 18-30yrs group to the 31-50yrs and 51-65yrs group respectively. Additionally we observed a 16% and a 28% reduction in T_{ANG} cell number in the circulation between these groups. No changes in $CD4^+$ or $CD8^+$ T-cell expression of CD31 with age were observed, however $CD4^+$ and $CD8^+$ cells were only assessed in the 31-50 and 51-65yr cohort. Our results are in line with that of Kushner *et al.* (2010c) who found that young men (20-35 years old) had a significantly greater proportion of $CD31^+$ T-lymphocytes (40%) compared to middle-aged (36-55 years old) (38%) and old (56-75 years old) men (24%). The authors also measured T_{ANG} ability to migrate to both SDF-1α and VEGF and found a marked reduction in function of these cells in the middle-aged and old group. Interestingly, our data shows a reduced number of these cells expressing CXCR4 on their cell surface in 31-50yrs group compared to the younger, 18-30yrs group by 29%. The reduction in $CXCR4^+$ T_{ANG} cells could contribute to the reduced function of these cells with advancing age. If one works on the premise that function is directly correlated with CXCR4 MFI then despite the observation of $CXCR4^+$ T_{ANG} cells reducing with the age, the percentage of all T_{ANG} cells expressing CXCR4 was not affected by chronological age, indicating that these $CXCR4^+$ T_{ANG} cells themselves, although declining in number with age, the function of each cell may not, depending on the premise that function is directly correlated with CXCR4 MFI.

Reductions in T-cell pool are common with age, postulated to be a result of thymic involution which results in a decrease in thymic output of naïve T-cells (Fagnoni *et al.*, 2000; Simpson, 2011). It is highly likely that the reductions in circulating cell number of T_{ANG} cells are due somewhat to this thymic involution, however we also observed a decrease in percentage of total T-cells expressing CD31 with age suggesting other

mechanisms are also at work. From 31-50yrs to 51-65 years, there may have been a slight decrease in $CD4^+$ and $CD8^+$ $CD31^+$ T-cells, yet this was not significant. We did not measure CD4 and CD8 expression on T-cells in the 18-30yr cohort, and thus is a limitation of our study, as the biggest decline in T_{ANG} number and proportion was from 18-30yrs to 31-50yrs and 51-65yrs. Further research is required to elucidate whether it is the $CD4^+$ or the $CD8^+$ subset which is susceptible to 'immunoageing'. Interestingly, the $CD8^+$ T_{ANG} cells appear to lose CXCR4 expression from 31-50yrs to 51-65yrs, which could potentially contribute to the reduced migratory capacity of T_{ANG} cells with age as seen in the research by Kushner *et al.* (2010c).

After correcting for this age-related decline in T_{ANG} cells, there was no effect of CRF on these cells, indicating that there was no independent effect of fitness, and that regular aerobic exercise may not attenuate the negative effect of advancing age. However, there was an age-dependent effect on these cells with those with higher levels of CRF in certain age categories (18-30yrs, 51-65yrs) displaying greater number and proportion of T_{ANG} cells. These changes may be as a result of the effect on the $CD4^+$ population, with a higher number of these $CD4^+$ T-cells expressing CD31 in those with higher levels of CRF compared to those with low levels of CRF. There was no effect on the $CD8^+$ population or CXCR4 expression on these cells. Spielmann *et al.* (2011) demonstrated that CRF (also measured by estimating $\dot{V}O_{2max}$ through submaximal cycling test) was positively associated with levels of naïve $CD8^+$ T-cells and negatively associated with $CD4^+$ senescent T-cells, indicating that maintaining a high CRF may attenuate the progressive increase in senescent T-cells with age. In this study we did not measure markers of senescence (KLRG1 or CD57) however the percentage of $CD3^+CD4^+$ cells expressing CD31 declines with age ($r=-0.267$, $p=0.036$, data not shown). Evidence suggests that $CD4^+$ T-cells lose expression of CD31 after activation (Zehnder *et al.*, 1992) and differentiation (Demeure *et al.*, 1996) potentially implicating the loss of CD31 in the differentiation of $CD4^+$ T-cells from naïve to effector-type cells. In fact, $CD45RA^+$ effector memory cells also lack CD31 (Tanaskovic *et al.*, 2010) further suggesting the loss of these cells with 'immune ageing'.

CD31 may not only confer vascular protective capacity, but also may be beneficial for the immune system, as CD31 expression on these naïve T-cells may enhance the migration of these cells across the endothelium under an immune challenge for antigen priming (Zocchi *et al.*, 1996). As well as playing a role in migration of these cells to sites of infection or damage, the adhesion molecule has been reported to play a regulatory role

on $CD4^+$ T-cells, and on $CD8^+$ T-cells it appears to prevent their inflammatory effects on vascular smooth muscle cells (Caligiuri *et al.*, 2005). Taken together, the effects of exercise on the $CD31^+$ T-cells may be an immunomodulatory adaptation rather than a vasculogenic adaptation.

Pistillo *et al.* (2013) documented similar reductions in γδ T-cells with age. Most of these γδ T-cells do not express either CD4 or CD8 antigen. Around 15% (range 5-31%) of the total $CD3^+CD31^+$ T_{ANG} cells measured in this study did not express either CD4 or CD8 (measured in 31-65yr cohort only) indicating that some of the T_{ANG} cells may in fact belong to the γδ T-cell group. More research is required to elucidate if the age-related decline in these T_{ANG} cells are largely due to the documented decline in the γδ T-cell group (Pistillo *et al.*, 2013; Roux *et al.*, 2013).

It must be noted that although participants were measured for estimated $\dot{V}O_{2max}$, it cannot be concluded that regular exercise can modulate these cells unless further longitudinal exercise training studies are performed. Although it is highly likely that those with high CRF levels may also partake in more physical activity, there may be few participants in the cohort that, although expressing a high $\dot{V}O_{2max}$, may be relatively inactive.

5.5 Conclusion

Advancing age, as with $CD34^+$ progenitors, is associated with a reduction in T_{ANG} cell number, and proportion of total T-cells expressing CD31. This positive effect of CRF on these cells may provide another mechanism by which regular exercise is beneficial for the cardiovascular system and may help in the prevention of CVD.

Chapter 6: Sedentary Behaviours, Circulating Angiogenic Cells and Cardiometabolic Risk Factors

6.1 Introduction

CVD risk factors include smoking, hypertension, dyslipidaemia, and diabetes (Cupples and D'Agostino, 1987). Added to this is inadequate exercise and physical inactivity, which is linked to declines in cardiometabolic health. Several studies have reported that physical activity and CRF reduces the risk of CVD and mortality (Kurl *et al.*, 2003; Lee *et al.*, 2012a; Vigen *et al.*, 2012; Barry *et al.*, 2013; Berry *et al.*, 2013; Chomistek *et al.*, 2013; Holtermann *et al.*, 2015). Mechanistic effects of exercise include: reduced chronic low-grade inflammation (Mathur and Pedersen, 2008); improved cardiac function (Ehsani *et al.*, 1991; Turan *et al.*, 2006) and body composition (Sillanpää *et al.*, 2008); reduced fasting glucose (Gillen *et al.*, 2012); and improved tissue perfusion as a result of improved endothelial function (Rakobowchuk *et al.*, 2008; Black *et al.*, 2009; Tinken *et al.*, 2010; Birk *et al.*, 2012; Luk *et al.*, 2012; Mitranun *et al.*, 2014; Ashor *et al.*, 2015) and/or capillary number (Laufs *et al.*, 2004; Chinsomboon *et al.*, 2009; Geng *et al.*, 2010; Bellafiore *et al.*, 2013).

In contrast, sedentary time and physical inactivity behaviours are associated with ill-effects on cardiometabolic health and longevity (Laufs *et al.*, 2005b; Hamilton *et al.*, 2007; Katzmarzyk *et al.*, 2009; van der Ploeg *et al.*, 2012; Wilmot *et al.*, 2012; Stamatakis *et al.*, 2013; Gibbs *et al.*, 2014; Staiano *et al.*, 2014; Young *et al.*, 2014; Chau *et al.*, 2015). Even short periods of physical inactivity can reduce insulin sensitivity (Reynolds *et al.*, 2014), endothelial function (Nosova *et al.*, 2014), and increase systemic levels of inflammatory cytokines (Fischer *et al.*, 2007). Prolonged periods of inactivity and sedentary behaviour can also lead to a marked reduction in CRF (as measured as $\dot{V}O_{2max}$) (Saltin *et al.*, 1968; Ringholm *et al.*, 2011); and also associated with elevations in arterial wall thickness (van Duijnhoven *et al.*, 2010) and increases in the circulating level of biomarkers of endothelial damage (Boyle *et al.*, 2013). McGavock *et al.* (2009) reported that the loss of CRF and also reductions in cardiac output over a period of 3 weeks bed rest was significantly greater than the loss of CRF seen over 40 years of adulthood signifying the large impact physical inactivity has on the cardiovascular system.

However, CRF and physical inactivity are not synchronous with each other as there exists a dichotomy, whereby individuals who may be termed largely inactive (for example office workers) may still be termed as highly fit (with high levels of $\dot{V}O_{2max}$). Likewise, individuals may lead very active lives but may not be classified as highly fit. Therefore, the impact of CRF and physical inactivity on health must be investigated accordingly.

Circulating angiogenic cells (CACs) are a group of heterozygous circulating cells which participate in endothelial repair and regeneration (Asahara *et al.*, 1997; Hur *et al.*, 2007; Kushner *et al.*, 2010b; Xia *et al.*, 2012a). These cells include BM-derived progenitors ($CD34^+$ progenitors, $CD34^+CD45^{dim}VEGFR2^+$ endothelial precursor cells; EPCs) and $CD31^+$ T-cells (angiogenic T-cells; T_{ANG}). Both the BM-derived progenitors and the T_{ANG} cells (circulating number and function) are associated with endothelial function (Sibal *et al.*, 2009; Weil *et al.*, 2011; Bruyndonckx *et al.*, 2014) and also shown to be associated with CVD risk (Hill *et al.*, 2003; Fadini *et al.*, 2005; Schmidt-Lucke *et al.*, 2005; Fadini *et al.*, 2006; Xiao *et al.*, 2007; Sibal *et al.*, 2009; Jung *et al.*, 2010; Liu and Xie, 2012; Rouhl *et al.*, 2012; Shantsila *et al.*, 2012; Teraa *et al.*, 2013; Vemparala *et al.*, 2013; Berezin and Kremer, 2014; Castejon *et al.*, 2014; Chan *et al.*, 2014). We have shown that high levels of CRF result in greater number of T_{ANG} cells (*Chapter 5- Influence of Age and Cardiorespiratory Fitness on $CD31^+$ T-Cells and CXCR4 Cell Surface Expression*) with no change in the BM-derived progenitors (*Chapter 4- Influence of Age and Cardiorespiratory Fitness on Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). It is the aim of this study to therefore investigate whether self-reported physical inactivity affects CACs and other cardiometabolic risk factors in apparently healthy men.

6.2 Materials and Methods

6.2.1 Participants

Male participants (n=42), aged between 31 and 65 volunteered for the study, and consisted of a mixture of students and staff based at Edinburgh Napier University, in addition to general public from the Edinburgh area. Participant information, inclusion and exclusion criteria are detailed in Chapter 4 (*Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee.

Participant information, inclusion and exclusion criteria are detailed in Chapter 4 (*Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee.

6.2.2 Resting Measures

On arrival to the Human Performance Laboratory, after informed consent was given, participants were measured for height and body mass, with values used to calculate BMI. Waist and hip circumference were also measured as described in materials and methods *Chapter 3.2*. Resting blood pressure was measured after 5-minute rest in a supine position using an automated sphygmamometer (Nonin Puresat Avant 2120, Nonin Medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA). Participant characteristics are shown in table 6.1.

Table 6.1. Participant characteristics. Values shown are mean ± SD (Range).

	All (n=42)
Age (years)	48 ± 10 (31-65)
Height (m)	1.78 ± 0.07 (1.62-1.91)
Body Mass (kg)	82.02 ± 9.92 (69.10-108.90)
BMI ($kg \cdot m^2$)	25.780 ± 2.74 (21.20-33.09)
SBP (mmHg)	132 ± 13 (108-142)
DBP (mmHg)	81 ± 8 (63-99)
MAP (mmHg)	98 ± 9 (79-126)
Waist-to-Hip Ratio	0.97 ± 0.03 (0.90-1.04)
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	43.19 ± 11.41 (16.89-66.78)

BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure

6.2.3 Self-Reported Sitting Time

Self-reported sitting time was obtained from n=42 (age 31-65 years), with participants answering the question '*On average, how many hours do you usually spend sitting during a normal working day?*', with specific instruction to calculate based from when they woke up in the morning, to when they went to bed at night. These participants were also given a sitting time questionnaire, based on the 7 days prior to the laboratory visit (Sit-Q7d; *Appendix 4*), which quantifies the amount of sedentary time a person has spent in the 7 days leading up to the completion of the questionnaire. Recently the questionnaire has been shown to be accurate and a valid tool to measure physical inactivity (Wijndaele *et al.*, 2014). Domains of interest included 1) total sitting time, and 2) total screen time. Questionnaire data was coded via instructions given on the website: http://www.mrc-epid.cam.ac.uk/research/resources/.

6.2.4 Submaximal Exercise Test to Estimate Maximal Oxygen Uptake

Maximal oxygen uptake ($\dot{V}O_{2max}$) of each participant was estimated using a submaximal exercise test (YMCA) (Golding *et al.*, 1989) as previously described in general materials and methods *Chapter 3.11*. Briefly, participants exercised on a stationary cycle ergometer (Veletron™ Dynafit Pro; Racer Mate®, Seattle, USA) at 50rpm for 3-4 incremental stages, each stage lasting 3 minutes. The initial power output of the test was 50W and increased in subsequent stages depending on HR response. $\dot{V}O_{2max}$ was measured throughout the test through breath-by-breath online analysis (LABManager v5.3.0, Cardinal Health, Germany). The test was terminated when the desired steady state HR was attained (80% of HR_{max}). $\dot{V}O_{2max}$ was estimated using equations provided by Adams and Beam (1998).

6.2.5 Blood Sampling and PBMNC Isolation

Blood was taken from participants after a 5-minute supine rest by a certified phlebotomist. Blood samples were drawn into 6mL vacutainers (BD Biosciences, UK), which were either coated in EDTA to prevent coagulation or serum gel. EDTA blood (3 x tubes) was processed for PBMNC separation as previously described in general materials and methods *Chapter 3.4*, as well as for whole blood labelling for progenitor cell enumeration

(see *6.2.6 Circulating Progenitor Cell Number and CXCR4 Expression Quantification*). Isolated PBMNCs were then used for T_{ANG} number and CXCR4 enumeration (see *6.2.7 Angiogenic T Cell Number and CXCR4 Expression Quantification)*, and serum was used for analysis of serum for chemotactic factors and selected inflammatory markers (see *6.2.8 Quantification of Serum Inflammatory Markers*).

6.2.6 Circulating Progenitor Cell Number and CXCR4 Expression Quantification

Peripheral whole blood was used for progenitor cell analysis. Whole blood was labelled with monoclonal antibodies anti-CD34-FITC, anti-CD45-APC, anti-VEGFR2-PE and anti-CXCR4-PE-Cy5 (all BD Biosciences, USA), and left to incubate at 4°C for 45 minutes in the dark as described in general materials and methods *Chapter 3.5*. Progenitor cell subsets in human whole blood were analysed using a BD FACSCalibur (BD Biosciences, USA) as detailed in general materials and methods *Chapter 3.5*. CXCR4 cell surface expression was also analysed as a surrogate for migratory ability on these progenitor cell subsets.

6.2.7 Angiogenic T Cell Cell Number and CXCR4 Expression Quantification

Isolated PBMNCs ($0.5x10^6$) were labelled with monoclonal antibodies anti-CD3-APC, anti-CD31-FITC and anti-CXCR4-PE-Cy5 (all BD Biosciences, USA), and were left to incubate at 4°C for 45 minutes in the dark prior to flow cytometric analysis as detailed in general materials and methods *Chapter 3.6*. CXCR4 cell surface expression was analysed as a surrogate for migratory ability of T-cells.

6.2.8 Quantification of Serum Inflammatory Markers

Serum IL-6 was measured by enzyme-linked immunosorbent assay (ELISA) and serum glucose, total cholesterol (TC), HDL-C, LDL-C, triglycerides were quantified by clinical chemistry analyser as described in general materials and methods *Chapter 3.9*.

6.2.9 Statistical Analysis

Data was assessed for normal distribution using the Shapiro-Wilk test for normality. Not normally distributed data was logarithmically transformed (log_{10} or log_n).

Sit-Q7d questionnaire data was processed using the statistical package STATA version 14 (StataCorp LP, Texas, USA). Midpoint values were assigned for all categories (multiple choice answers typically given as ranges). Weekday data and weekend data were weighted 5- and 2-fold respectively, before dividing the summed data by 7 to give a single value for sitting time averaged for the previous 7 days (Wijndaele *et al.*, 2014). Data from the Sit-Q7d was also categorised and further analysed in terms of screen time.

Participants were grouped into clusters for total self-reported sitting time from both the Sit-Q7d or single question-based recall as well as total screen time (Sit-Q7d). Clusters were identified by hierarchical cluster analysis using Ward's method for identifying groupings, as well as using the squared Euclidean distance to measure intervals between groups (Mooi and Sarstedt, 2011). In all 3 cases, 4 groups were identified and thus participants were grouped into incremental groups of sedentary behaviour time, with group 1= lowest sedentary time, up to group 4= highest sedentary time (Sit-Q7d sitting time, single question-based sitting time, and Sit-Q7d screen time).

Univariate linear regressions were performed to assess the effect of sedentary behaviours ('*On average, how many hours do you usually spend sitting during a normal working day?';* Sit-Q7d: average sitting time/screen time for past 7 days) on various CACs ($CD34^+$, $CD34^+CD45^{dim}VEGFR2^+$, $CD3^+CD31^+$) and concurrent CXCR4 cell surface expression. In addition, linear regressions were also performed to assess the effect of sitting time on various biomarkers of cardiometabolic health (fasting glucose, total cholesterol, HDL-C, LDL-C, TC:HDL-C), $CXCR4^+$ cell-mobilising factor SDF-1α, and inflammatory biomarkers (IL-6, IL-8).

After regressions were performed and groupings were set, one-way analyses of variance (ANOVA) were performed to assess the effect of sitting time (Sit-Q7d and single-question-based, and screen time) on these CACs and cardiometabolic biomarkers. Fisher's Least Significant Difference (LSD) post-hoc tests were performed to identify locations of any significant differences. Where required, any differences in T_{ANG} cells were further explored using one-way ANOVA for $CD4^+$ and $CD8^+$ T_{ANG} cells.

Hierarchical multiple level regression analyses were performed to assess the relationship between sitting time and T_{ANG} cells after correction for CRF.

Sit-Q7d was processed using the statistical package STATA version 14 (StataCorp LP, Texas, USA). Data was analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA). Significance alpha was set at p=0.05.

6.3 Results

6.3.1 The Effect of Sedentary Behaviours on CACs and Cardiometabolic Biomarkers

Univariate linear regressions were performed for various sedentary behaviours on various CAC subsets ($CD34^+$, $CD34^+CD45^{dim}VEGFR2^+$, $CD3^+CD31^+$) and CXCR4 cell surface expression on these cells. Sitting time (reported from Sit-Q7d) was inversely associated with T_{ANG} cells (r= -0.291, p=0.031). There was a trend for a decrease in expression intensity of CXCR4 on $CD34^+$ progenitor cells (r= -0.242, p=0.061), and EPCs (r= -0.232, p=0.087) with increasing sitting time as well as a trend for an increase in percentage of T_{ANG} cells expressing CXCR4 with increasing sitting time (r=0.216, p=0.085) (table 6.2). Screen time was only associated with a decreased expression of CXCR4 on $CXCR4^+$ EPCs (r= -0.292, p=0.042).

Table 6.2. Univariate linear regression analyses for various sedentary behaviours on CAC subsets (n=42).

	Pearson's coefficient (p-value)		F-statistic (p-value)	
	Sitting Time (Sit-Q7d)	**Screen Time (Sit-Q7d)**	**Sitting Time (Sit-Q7d)**	**Screen Time (Sit-Q7d)**
$CD34^+$ (% MNCs)	0.118 (0.228)	-0.099 (0.266)	0.565 (0.457)	0.397 (0.532)
$CD34^+CXCR4^+$ (% MNCs)	0.134 (0.198)	0.118 (0.229)	0.734 (0.397)	0.563 (0.457)
% $CD34^+$ expressing CXCR4	0.012 (0.469)	0.132 (0.203)	0.006 (0.983)	0.705 (0.406)
MFI $CD34^+$ CXCR4	-0.242 (0.061)	-0.077 (0.314)	2.490 (0.122)	0.237 (0.629)
$CD34^+CD45^{dim}VEGFR2^+$ (% MNCs)	0.100 (0.278)	0.228 (0.088)	0.354 (0.556)	1.915 (0.175)
$CXCR4^+$ EPCs (% MNCs)	-0.011 (0.475)	0.149 (0.194)	0.004 (0.950)	0.768 (0.387)
% EPCs expressing CXCR4	-0.008 (0.479)	-0.156 (0.162)	0.003 (0.958)	0.994 (0.325)
MFI EPC CXCR4	-0.232 (0.087)	-0.292 (0.042)*	1.931 (0.174)	3.164 (0.084)
$CD3^+CD31^+$ T-cells (cells·μL^{-1})	-0.034 (0.415)	0.101 (0.262)	0.047 (0.830)	0.413 (0.524)
$CD3^+CD31^+$ T-cells (% of $CD3^+$)	-0.291 (0.031)*	-0.193 (0.110)	3.703 (0.061)	1.553 (0.220)
$CXCR4^+$ T_{ANG} (cells·μL^{-1})	0.128 (0.210).	0.140 (0.189)	0.664 (0.420)	0.796 (0.378)
%T_{ANG} expressing CXCR4	0.216 (0.085)	0.054 (0.368)	1.995 (0.170)	0.115 (0.736)
MFI T_{ANG} CXCR4	0.109 (0.246)	0.013 (0.469)	0.482 (0.492)	0.006 (0.937)

*MFI- Mean Fluorescence Intensity; EPC- Endothelial Progenitor Cell; MNC- Mononuclear Cells, *p<0.05*

To further investigate the effect of sitting time on T_{ANG} cells, univariate analyses were performed for sitting time and screen time measures on $CD4^+$ and $CD8^+$ T_{ANG} cells. Results are shown in table 6.3. The apparent decrease in T_{ANG} cells with increasing sitting time appears to be due to the decrease in $CD4^+$ cells expressing CD31 (r= -0.282, p=0.037), but not a decrease in $CD3^+CD4^+CD31^+$ cell number (r= -0.080, p=0.309), indicating that sedentary behaviour may be associated with the loss of CD31 expression on $CD4^+$ T-cells, rather than a decrease in $CD4^+$ cells. There were no relationships between sedentary behaviours and $CD8^+$ T_{ANG} cells. In addition, sitting time was positively associated with CD4:CD8 ratio (r= 0.333, p=0.017), a biomarker within the Immune Risk Profile (IRP).

Table 6.3. Univariate linear regression analyses for various sedentary behaviours on T_{ANG} subsets (n=42).

	Pearson's coefficient (p-value)		F-statistic (p-value)	
	Sitting Time (Sit-Q7d)	**Screen Time (Sit-Q7d)**	**Sitting Time (Sit-Q7d)**	**Screen Time (Sit-Q7d)**
$CD4^+$ T_{ANG} (cells·μL^{-1})	-0.080 (0.309)	0.117 (0.233)	0.254 (0.617)	0.544 (0.465)
% $CD4^+$ T-cells expressing CD31 (%)	-0.282 (0.037)*	-0.159 (0.161)	3.379 (0.074)	1.006 (0.322)
$CD3^+CD4^+CD31^+CXCR4^+$ (cells·μL^{-1})	0.104 (0.258)	0.179 (0.132)	0.430 (0.516)	1.288 (0.263)
% $CD4^+$ T_{ANG} expressing CXCR4	0.389 (0.007)*	0.207 (0.100)	6.788 (0.013)*	1.709 (0.199)
$CD8^+$ T_{ANG} (cells·μL^{-1})	0.002 (0.494)	0.014 (0.467)	0.000 (0.988)	0.007 (0.933)
% $CD8^+$ T-cells expressing CD31 (%)	-0.088 (0.292)	0.130 (0.209)	0.304 (0.584)	0.668 (0.419)
$CD3^+CD8^+CD31^+CXCR4^+$ (cells·μL^{-1})	0.076 (0.317)	0.083 (0.302)	0.229 (0.635)	0.272 (0.605)
% $CD8^+$ T_{ANG} expressing CXCR4	0.185 (0.123)	0.154 (0.168)	1.385 (0.246)	0.951 (0.335)
CD4:CD8 Ratio	0.333 (0.017)*	0.195 (0.111)	4.784 (0.033)*	1.539 (0.222)

Sitting time (r=0.243, p=0.060) and screen time (0.356, p=0.010) were positively associated with waist-to-hip ratio, and screen time was also correlated with TC:HDL-C ratio (r=0.359, p=0.010) as a result of the observed inverse relationship with HDL-C (r=-0.341, p=0.013) (table 6.4). No associations were seen for sedentary behaviours and other common cardiometabolic risk factors such as BMI, triglycerides, fasting glucose, inflammatory cytokine IL-6, BP and LDL-C.

Table 6.4. Univariate linear regression analyses for various sedentary behaviours on cardiometabolic biomarkers (n=42).

	Pearson's coefficient (p-value)		F-statistic (p-value)	
	Sitting Time (Sit-Q7d)	**Screen Time (Sit-Q7d)**	**Sitting Time (Sit-Q7d)**	**Screen Time (Sit-Q7d)**
BMI	0.047 (0.383)	-0.043 (0.395)	0.090 (0.766)	0.072 (0.789)
Waist Circumference	0.137 (0.194)	0.139 (0.190)	0.760 (0.388)	0.786 (0.381)
Waist-to-Hip Ratio	0.243 (0.060)	0.356 (0.010)*	2.518 (0.120)	5.795 (0.021)*
SBP	0.050 (0.376)	-0.079 (0.309)	0.101 (0.753)	0.253 (0.618)
DBP	0.119 (0.227)	-0.042 (0.395)	0.572 (0.454)	0.072 (0.790)
Fasting Glucose	0.054 (0.366)	0.012 (0.469)	0.119 (0.732)	0.006 (0.983)
IL-6	0.004 (0.491)	-0.101 (0.278)	0.000 (0.982)	0.352 (0.557)
Total Cholesterol	-0.063 (0.347)	0.125 (0.215)	0.158 (0.693)	0.635 (0.430)
HDL-C	0.009 (0.478)	-0.341 (0.013)*	0.003 (0.956)	5.278 (0.027)*
LDL-C	-0.089 (0.287)	0.201 (0.101)	0.323 (0.573)	1.685 (0.202)
TC:HDL	-0.033 (0.417)	0.359 (0.010)*	0.045 (0.837)	5.928 (0.019)*
Triglycerides	0.090 (0.285)	0.173 (0.136)	0.327 (0.571)	1.237 (0.273)

BMI- Body Mass Index, SBP-Systolic Blood Pressure; DBP- Diastolic Blood Pressure; HDL-C- High Density Lipoprotein Cholesterol, LDL-C- Low Density Lipoprotein Cholesterol

Participants were then grouped into sitting time (Sit-Q7d) and screen time groups based on hierarchical cluster analysis. Cluster analysis revealed 4 distinct groups per sedentary behaviour measure. Group 1-4 indicates increments of time spent in the sedentary behaviour domain. Characteristics of participants within these groups are shown in tables 6.5 (sitting time) and 6.6 (screen time).

Table 6.5. Participant characteristics based on sitting time cluster analyses (n=42).

	Group 1 (3-10hrs) (n=17)	**Group 2 (10-12.5hrs) (n=8)**	**Group 3 (12.5-16hrs) (n=11)**	**Group 4 (>16hrs) (n=6)**
Age (yrs)	46 ± 8	50 ± 11	47 ± 11	51 ±9
Height (m)	1.81 ± 0.06	1.76 ± 0.04	1.78 ± 0.04	1.76 ± 0.10
Body Mass (kg)	81.14 ± 7.32	79 ± 5.57	86.76 ± 13.72	79.8 ± 11.98
BMI ($kg \cdot m^2$)	24.92 ± 2.61	25.66 ± 1.88	27.29 ± 3.53	25.76 ± 1.59
SBP (mmHg)	132 ± 11	131 ± 4	132 ± 21	131 ± 15
DBP (mmHg)	81 ± 8	81 ± 9	80 ± 7	82 ± 11
MAP (mmHg)	98 ± 8	98 ± 7	97 ± 11	98 ± 12
$\dot{V}O_{2max}$ ($ml \cdot kg \cdot min^{-1}$)	47.39 ± 8.63	35.75 ± 13.80	44.89 ± 12.24	38.07 ± 8.71

SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure. Values shown are mean ± SD.

Table 6.6. Participant characteristics based on screen time cluster analyses (n=42).

	Group 1 (0.5-2hrs) (n=8)	Group 2 (2-3.5hrs) (n=12)	Group 3 (3.5-4hrs) (n=9)	Group 4 (4-6hrs) (n=11)
Age (yrs)	45 ± 10	45 ± 9	52 ± 10	47 ± 10
Height (m)	1.79 ± 0.06	1.79 ± 0.04	1.79 ± 0.07	1.77 ± 0.07
Body Mass (kg)	81.30 ± 6.67	85.35 ± 10.74	81.13 ± 12.47	78.36 ± 8.53
BMI ($kg \cdot m^2$)	25.55 ± 3.27	26.52 ± 3.01	25.41 ± 3.34	25.08 ± 1.82
SBP (mmHg)	132 ± 14	133 ± 12	137 ± 21	126 ± 9
DBP (mmHg)	82 ± 10	79 ± 8	85 ± 9	79 ± 6
MAP (mmHg)	99 ± 11	97 ± 8	102 ± 12	95 ± 6
$\dot{V}O_{2max}$ ($ml \cdot kg \cdot min^{-1}$)	50.40 ± 10.67*	47.86 ± 9.34*	37.10 ± 8.82	42.61 ± 9.83

*SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure. Values shown are mean ± SD. *p<0.05 vs. 3.5-4hrs group.*

One-way ANOVAs were performed for various CAC subsets in the various sedentary behaviour groups. Results are shown in tables 6.7 (sitting time) and 6.8 (screen time). There were no differences in $CD34^+$ progenitor cells amongst any sedentary behaviour domains. $CXCR4^+$ EPCs were found to be greater in sitting time 10-12.5hrs vs. 3-10hrs (0.0073 ± 0.0023% [0.0017-0.0129] 95% CI, vs. 0.0042 ± 0.0016% [0.0017-0.0066], p=0.032), 12.6-16hrs (0.0073 ± 0.0023% [0.0017-0.0129] 95% CI, vs. 0.0035 ± 0.0008% [0.0016-0.0053], p=0.017) and >16hrs (0.0073 ± 0.0023% [0.0017-0.0129] 95% CI, vs. 0.0058 ± 0.0034% [0.0031-0.0146], p=0.022). There were no other differences in EPCs between sitting time groups or screen time groups.

There was a trend for a decrease in proportion of $CD3^+$ T-cells expressing CD31 from those reporting >16hrs of sitting time per day compared to those reporting 3-10hrs (44.13 ± 5.41% [30.21-58.04] 95% CI, vs. 52.08 ± 2.59% [46.59-57.57], p=0.099, ES=0.66) (table 6.7). However, in contrast to the univariate regression analysis, there was no detectable difference in $CXCR4^+$ T_{ANG} cells between sitting time groups. There were no differences for T_{ANG} groups between screen time groups (table 6.8).

Table 6.7. One-way ANOVA results for daily average sitting time (Sit-Q7d) for CACs (n=42).

Variables	3-10hrs (n=17)	10-12.5hrs (n=8)	12.6-15hrs (n=11)	>16hrs (n=6)	p-value
$CD34^+$ (%MNC)	0.099 ± 0.014 (0.070-0.127)	0.107 ± 0.014 (0.075-0.139)	0.092 ± 0.012 (0.066-0.118)	0.105 ± 0.017 (0.061-0.149)	0.775
$CD34^+CXCR4^+$ (%MNC)	0.032 ± 0.006 (0.019-0.045)	0.042 ± 0.006 (0.029-0.056)	0.030 ± 0.005 (0.019-0.041)	0.033 ± 0.006 (0.017-0.049)	0.392
% $CD34^+$ expressing CXCR4	31.54 ± 3.69 (23.72-39.15)	40.67 ± 4.49 (30.07-51.27)	33.33 ± 4.70 (22.86-43.80)	31.31 ± 3.10 (23.35-39.27)	0.477
EPCs (%MNC)	0.0060 ± 0.0014 (0.0030-0.0091)	0.0091 ± 0.0028 (0.0023-0.0160)	0.0065 ± 0.0026 (0.0008-0.0122)	0.0074 ± 0.0037 (0.0021-0.0169)	0.167
$CXCR4^+$ EPCs (%MNC)	0.0042 ± 0.0016 (0.0017 -0.0066)*	0.0073 ± 0.0023 (0.0017-0.0129)	0.0035 ± 0.0008 (0.0016-0.0053)*	0.0058 ± 0.0034 (0.0031-0.0146)*	0.074
% EPCs expressing CXCR4	65.18 ± 9.33 (45.28-85.07)	56.87 ± 15.33 (19.35-94.39)	67.25 ± 8.25 (48.87-85.63)	69.92 ± 14.22 (33.38-106.47)	0.400
T_{ANG} (cells·µL^{-1})	540 ± 47 (441-638)	615 ± 48 (500-729)	624 ± 53 (507-741)	533 ± 80 (326-740)	0.534
T_{ANG} (% $CD3^+$)	52.08 ± 2.59 (46.59-57.57)	49.91 ± 2.58 (43.82-56.01)	52.58 ± 2.41 (47.21-57.95)	44.13 ± 5.41 (30.21-58.04)	0.344
$CXCR4^+$ T_{ANG} (cells·µL^{-1})	187 ± 41 (100-275)	192 ± 36 (106-277)	223 ± 52 (107-340)	237 ± 72 (53-237)	0.872
% T_{ANG} expressing CXCR4	32.61 ± 5.55 (20.86-44.37)	31.28 ± 5.44 (18.40-44.15)	35.09 ± 6.50 (20.60-49.58)	45.46 ± 11.12 (16.87-74.06)	0.622

*Values shown are mean ± SEM (95% CI). *P<0.05 vs. Group 2.*

Table 6.8. One-way ANOVA results for daily average screen time (Sit-Q7d) for CACs (n=42).

Variables	0.5-2hrs (n=8)	2-3.5hrs (n=12)	3.5-4hrs (n=9)	4-6hrs (n=11)	p-value
$CD34^+$ (%MNC)	0.127 ± 0.024 (0.070-0.184)	0.097 ± 0.010 (0.074-0.120)	0.079 ± 0.014 (0.046-0.112)	0.093 ± 0.012 (0.067-0.120)	0.289
$CD34^+CXCR4^+$ (%MNC)	0.040 ± 0.012 (0.012-0.068)	0.034 ± 0.005 (0.023-0.045)	0.027 ± 0.007 (0.010-0.043)	0.034 ± 0.005 (0.023-0.045)	0.758
% $CD34^+$ expressing CXCR4	29.65 ± 5.75 (16.05-43.26)	35.78 ± 4.55 (25.70-45.86)	31.89 ± 4.79 (20.55-43.22)	36.10 ± 4.14 (26.73-45.47)	0.750
EPCs (%MNC)	0.0075 ± 0.0025 (0.0014-0.0135)	0.0060 ± 0.0014 (0.0028-0.0092)	0.0039 ± 0.0012 (0.0010-0.0068)	0.0092 ± 0.0034 (0.0016-0.0168)	0.717
$CXCR4^+$ EPCs (%MNC)	0.0053 ± 0.0021 (0.0004-0.0102)	0.0048 ± 0.0013 (0.0020-0.0077)	0.0026 ±0.0012 (0.0003-0.0056)	0.0052 ± 0.0021 (0.005-0.0100)	0.708
% EPCs expressing CXCR4	69.34 ± 13.01 (13.57-100.10)	79.90 ± 7.55 (63.09-96.72)	46.67 ± 15.34 (10.39-82.94)	54.93 ± 9.66 (33.07-76.79)	0.429
T_{ANG} (cells·µL^{-1})	486 ± 63 (336-636)	616 ± 54 (497-736)	573 ± 55 (444-702)	609 ± 61 (471-746)	0.423
T_{ANG} (% $CD3^+$)	52.20 ± 3.41 (44.12-60.27)	53.87 ± 2.42 (48.54-59.20)	48.04 ± 4.55 (37.27-58.81)	50.86 ± 3.09 (43.87-57.86)	0.643
$CXCR4^+$ T_{ANG} (cells·µL^{-1})	165 ± 72 (6-337)	258 ± 45 (160-356)	165 ± 26 (103-228)	190 ± 59 (57-324)	0.455
% T_{ANG} expressing CXCR4	30.32 ± 9.71 (7.37-53.27)	40.27 ± 5.95 (27.18-53.36)	32.03 ±8.10 (12.88-51.18)	30.98 ± 7.08 (14.96-47.01)	0.727

Values shown are mean ± SEM (95% CI).

To further investigate differences in T_{ANG} cells, analysis of $CD4^+$ and $CD8^+$ T_{ANG} cells were performed between the various sedentary behaviour groups. There were no statistically significant differences between groups 1 (3-10hrs), 2 (10-12.5hrs), 3 (12.5-16hrs) and 4 (>16hrs) for sitting time and any $CD4^+$ or $CD8^+$ T_{ANG} group (figures 6.1 and 6.2). The difference in $CD4^+$ cells expressing CD31 between 3-10hrs and >16hrs approached significance, with lower proportion of $CD4^+$ T-cells expressing CD31 in participants reporting >16hrs of sitting per day compared to those reporting 3-10hrs of daily average sitting per day, with a moderate effect size (28.25 ± 4.60% [16.42-40.07] 95% CI, vs. 36.02 ± 2.59% [30.54-41.50], p=0.128, ES=0.71) (figure 6.1B). Additionally, $CD4^+$ T_{ANG} cells expressing CXCR4 appeared to be increased in those reporting the highest sitting time (>16hrs) compared to the lowest sitting time (3-10hrs), with a moderate effect size (41.62 ± 13.99% [5.66-77.58] 95% CI, vs. 27.41 ± 4.83% [17.17-37.66], p=0.175, ES=0.52) (figure 6.2B).

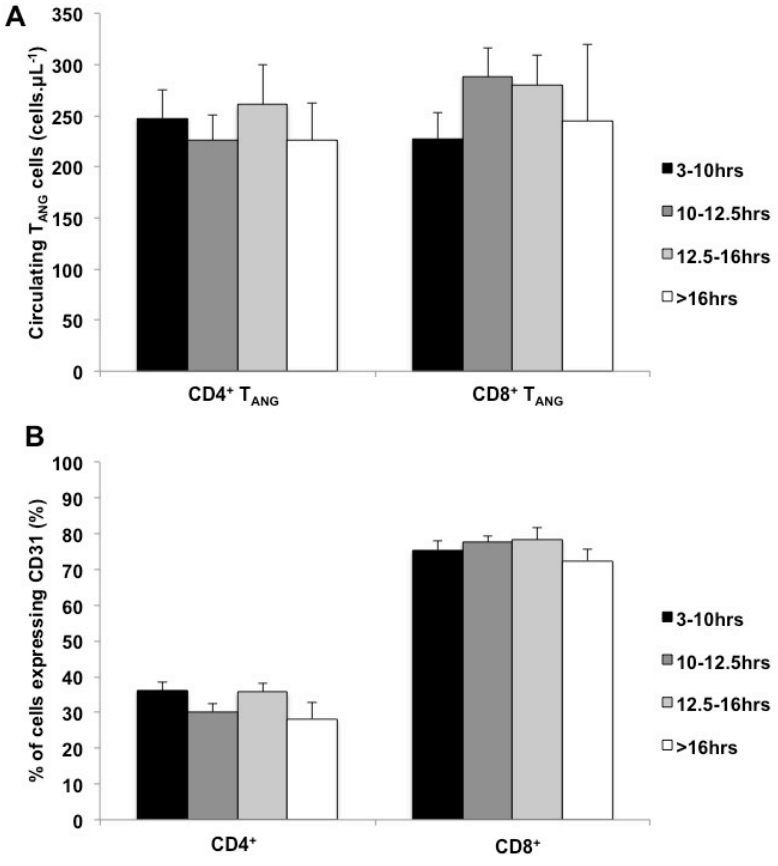

Figure 6.1. Circulating $CD4^+$ and $CD8^+$ T_{ANG} cells, expressed as cells·µL^{-1} (A) and percentage of $CD4^+$ and $CD8^+$ T-cells expressing CD31 (B) in different sitting time groups (groups 1-4). P=NS.

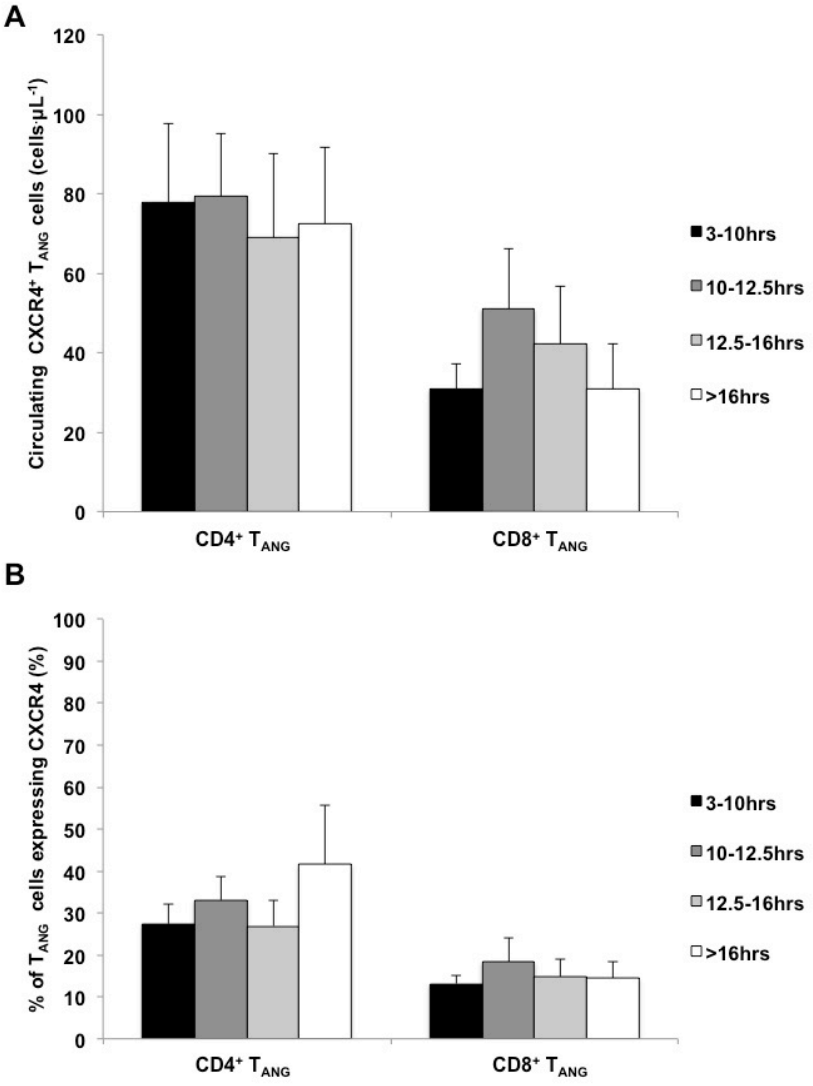

Figure 6.2. Circulating $CXCR4^+$ $CD4^+$ and $CD8^+$ T_{ANG} cells, expressed as cells·μL^{-1} (A) and percentage of $CD4^+$ and $CD8^+$ T_{ANG} cells expressing CXCR4 (B) in different sitting time groups (groups 1-4). P=NS.

As with the sitting time groups there were no significant differences in $CD4^+$ or $CD8^+$ T_{ANG} cell numbers (figure 6.3) or expression of CXCR4 (figure 6.4) with screen time. Interestingly, there was a trend for an increase in $CD4^+$ T_{ANG} absolute cell number in screen time group 4 compared to group 1 (282 ± 37 cell·μL^{-1} [198-367] 95% CI, vs. 212 ± 35 cells·μL^{-1} [130-294], p=0.191, ES=0.65), yet this difference was not significant despite a moderate effect size (figure 6.3A). This was not due to an increased relative expression of CD31 on $CD4^+$ T-cells (figure 6.3B), so therefore may be instead due to a greater absolute number of circulating $CD4^+$ T-cells.

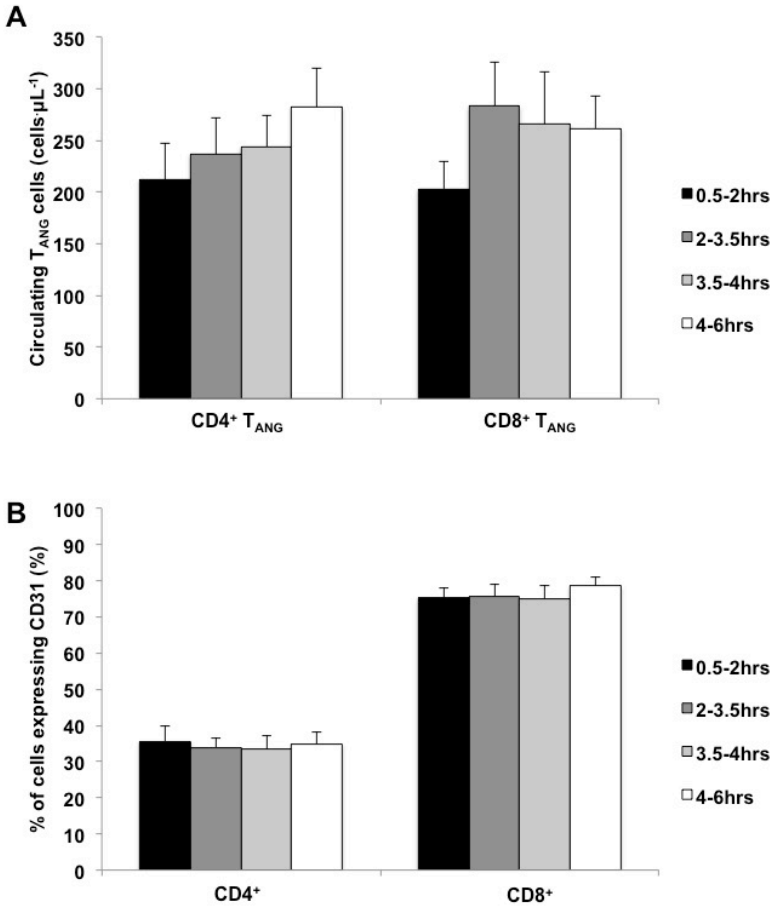

Figure 6.3. Circulating $CD4^+$ and $CD8^+$ T_{ANG} cells, expressed as cells·µL^{-1} (A) and percentage of $CD4^+$ and $CD8^+$ T-cells expressing CD31 (B) in different screen time groups (groups 1-4). P=NS.

There were no significant differences in CXCR4-expressing $CD4^+$ or $CD8^+$ T_{ANG} cells between screen time groups (figure 6.4).

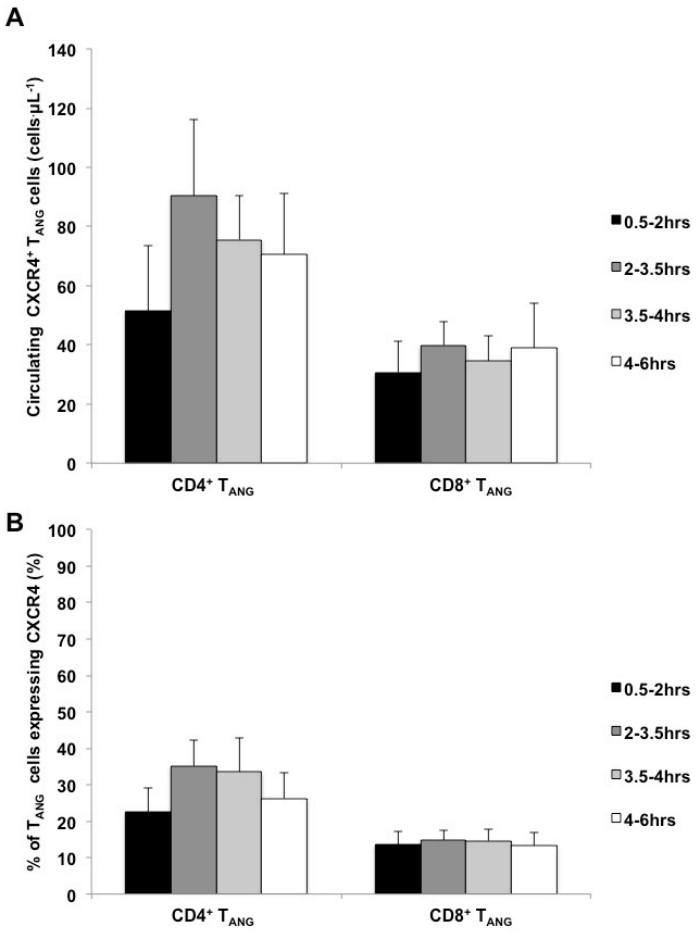

Figure 6.4. Circulating $CXCR4^+$ $CD4^+$ and $CD8^+$ T_{ANG} cells, expressed as cells·µL^{-1} (A) and percentage of $CD4^+$ and $CD8^+$ T_{ANG} cells expressing CXCR4 (B) in different screen time groups (groups 1-4). P=NS.

There were no observable differences between sedentary behaviour domain groups (sitting time and screen time) for the cardiometabolic risk factors measured: BMI, waist circumference, blood pressure, fasting glucose, IL-6, TC, LDL-C, TC:HDL-C and triglycerides (sitting time: table 6.9; screen time: Table 6.10). HDL-C was significantly lower in individuals reporting high levels of screen time compared to those reporting the lowest levels of screen time (4-6hrs: 1.27 ± 0.07mmol·L^{-1} [1.09-1.44] 95 % CI, vs. 0.5-2hrs: 1.51 ± 0.12mmol·L^{-1} [1.23-1.80], p=0.038), but no differences between sitting time groups were observed.

Table 6.9. One-way ANOVA results for daily average sitting time (Sit-Q7d) for cardiometabolic risk factors (n=42).

Variables	3-10hrs (n=17)	10-12.5hrs (n=8)	12.6-15hrs (n=11)	>16hrs (n=6)	p-value
BMI ($m \cdot h^2$)	25.32 ± 0.72 (23.75-26.88)	25.66 ± 0.66 (24.09-24.23)	26.89 ± 1.09 (24.38-29.39)	25.64 ± 0.78 (23.48-27.81)	0.169
Waist Circumference (cm)	89.81 ± 2.27 (84.90-94.71)	92.04 ± 2.72 (85.60-98.48)	92.53 ± 3.78 (83.81-101.26)	93.84 ± 2.93 (85.71-101.97)	0.353
SBP (mmHg)	132 ± 3 (126-138)	131 ± 1 (128-134)	133 ± 7 (116-150)	131 ± 8 (110-152)	0.993
DBP (mmHg)	81 ± 2 (76-86)	82 ± 3 (74-89)	82 ± 3 (76-88)	80 ± 5 (66-95)	0.975
Fasting Glucose ($mmol \cdot L^{-1}$)	4.68 ± 0.34 (3.94-5.42)	4.81 ± 0.37 (3.92-5.69)	4.55 ± 0.26 (3.94-5.16)	4.62 ± 0.37 (3.60-5.63)	0.808
IL-6 ($pg \cdot mL^{-1}$)	0.27 ± 0.03 (0.21-0.34)	0.30 ± 0.05 (0.19-0.41)	0.56 ± 0.20 (0.10-1.02)	0.34 ± 0.09 (0.09-0.59)	0.205
TC ($mmol \cdot L^{-1}$)	3.42 ± 0.15 (3.09-3.75)	3.74 ± 0.41 (2.78-4.70)	2.74 ± 0.28 (2.09-3.38)	3.21 ± 0.08 (2.98-3.44)	0.174
HDL-C ($mmol \cdot L^{-1}$)	1.44 ± 0.06 (1.30-1.58)	1.27 ± 0.11 (1.02-1.52)	1.23 ± 0.08 (1.04-1,42)	1.40 ± 0.11 (1.09-1.72)	0.088
LDL-C ($mmol \cdot L^{-1}$)	1.24 ± 0.17 (0.88-1.60)	1.64 ± 0.33 (0.85-2.43)	0.84 ± 0.29 (0.19-1.50)	1.09 ± 0.19 (0.58-1.61)	0.476
TC:HDL	2.43 ± 0.16 (2.09-2.77)	3.01 ± 0.29 (2.32-3.70)	2.30 ± 0.29 (1.64-2.96)	2.35 ± 0.18 (1.84-2.86)	0.370
Triglycerides ($mg \cdot dL^{-1}$)	143.51 ± 12.72 (116.02-170.99)	161.47 ± 17.65 (119.75-203.19)	129.21 ± 7.68 (111.51-146.91)	138.88 ± 12.24 (104.91-172.86)	0.318

BMI- Body Mass Index, SBP-Systolic Blood Pressure; DBP- Diastolic Blood Pressure; HDL-C- High Density Lipoprotein Cholesterol, LDL-C- Low Density Lipoprotein Cholesterol. Values shown are mean ± SEM.

Table 6.10. One-way ANOVA results for daily average screen time (Sit-Q7d) for cardiometabolic risk factors (n=42).

Variables	0.5-2hrs (n=8)	2-3.5hrs (n=12)	3.5-4hrs (n=9)	4-6hrs (n=11)	p-value
BMI ($m \cdot h^2$)	25.93 ± 1.27 (22.83-29.02)	26.15 ± 0.72 (24.51-27.78)	25.82 ± 1.28 (22.69-28.95)	24.92 ± 0.71 (23.24-26.59)	0.675
Waist Circumference (cm)	90.44 ± 4.04 (80.55-100.34)	90.78 ± 1.89 (86.50-95.07)	92.07 ± 4.45 (81.18-102.97)	89.26 ± 2.84 (82.56-95.97)	0.955
SBP (mmHg)	130 ± 5 (118-143)	133 ± 4 (124-142)	137 ± 9 (117-158)	126 ± 4 (118-135)	0.423
DBP (mmHg)	81 ± 4 (72-90)	81 ± 3 (75-86)	85 ± 4 (76-93)	78 ± 2 (74-82)	0.403
Fasting Glucose ($mmol \cdot L^{-1}$)	5.05 ± 0.65 (3.46-6.64)	4.59 ± 0.18 (4.19-4.99)	4.57 ± 0.28 (3.88-5.25)	4.36 ± 0.31 (3.64-5.09)	0.686
IL-6 ($pg \cdot mL^{-1}$)	0.33 ± 0.04 (0.24-0.42)	0.51 ± 0.19 (0.09-0.92)	0.31 ± 0.04 (0.20-0.41)	0.28 ± 0.06 (0.14-0.42)	0.521
TC ($mmol \cdot L^{-1}$)	3.37 ± 0.20 (2.88-3.88)	2.86 ± 0.26 (2.26-3.46)	3.66 ± 0.47 (2.52-4.81)	3.20 ± 0.22 (2.68-3.71)	0.301
HDL-C ($mmol \cdot L^{-1}$)	1.51 ± 0.12 (1.23-1.80)	1.31 ± 0.07 (1.16-1.47)	1.43 ± 0.10 (1.18-1.69)	1.27 ± 0.07* (1.09-1.44)	0.158
LDL-C ($mmol \cdot L^{-1}$)	1.04 ± 0.16 (0.65-1.43)	0.87 ± 0.25 (0.30-1.43)	1.48 ± 0.42 (0.45-2.51)	1.33 ± 0.24 (0.75-1.90)	0.337
TC:HDL	2.29 ± 0.19 (1.81-2.76)	2.22 ± 0.24 (1.68-2.75)	2.57 ± 0.26 (1.94-3.20)	2.58 ± 0.22 (2.05-3.11)	0.444
Triglycerides ($mg \cdot dL^{-1}$)	158.74 ± 24.46 (98.89-218.59)	132.65 ± 6.45 (118.05-147.24)	146.29 ± 7.66 (127.54-165.05)	117.56 ± 3.25 (109.86-125.25)	0.063

*BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, TC- Total Cholesterol, HDL-C- High-Density Lipoprotein Cholesterol, LDL-C- Low-Density Lipoprotein Cholesterol. Values shown are mean ± SEM. * $p<0.05$ vs. Group 1*

6.3.2 The Influence of Cardiorespiratory Fitness on the Effects of Sedentary Behaviour on CACs

The results indicate that increasing sedentary time affect $CD31^+$ T-cells (through a CD4-dependent mechanism). However due to the effect of CRF observed on these cells (see *Chapter 5- The Influence of Age and Cardiorespiratory Fitness on $CD31^+$ T-Cells and CXCR4 Cell Surface Expression*) we subsequently performed hierarchical multiple regression analysis to correct for CRF in the participants, and thus determine if CRF may attenuate the effects seen with sedentary behaviour. Results from the hierarchical multiple regression analysis for $CD31^+$ T-cells are shown in table 6.11.

Single univariate regression analysis, as shown previously in table 6.2 shows that increasing sitting time is associated with a decrease in $CD3^+CD31^+$ T-cells (% of total $CD3^+$ T-cells) (r=-0.291, p=0.031). After correcting for $\dot{V}O_{2max}$, sitting time was no longer associated with the proportion of these cells (t=-1.153, p=0.256 correcting for $\dot{V}O_{2max}$ from t=-1.924, p=0.061 uncorrected) (table 6.11). This was also the case for $CD4^+$ T-cells expressing CD31 (table 6.11). $CD4^+$ T-cells expressing CD31 were also inversely associated with sitting time (r=-0.282, p=0.037; table 6.3), but after correcting for $\dot{V}O_{2max}$, was no longer associated with sitting time (t=-1.144, p=0.260 correcting for $\dot{V}O_{2max}$ from t=-1.838, p=0.074 uncorrected) (table 6.11). The relationship between sitting time and CXCR4-expressing $CD4^+$ T_{ANG} cells (r=0.389, p=0.007) remained after correcting for CRF (t=3.032 p=0.004 correcting for $\dot{V}O_{2max}$ from t=2.605, p=0.013 uncorrected) (table 6.11).

Table 6.11. Hierarchical multiple regression analysis on $CD31^+$ T-cells (n=42).

Angiogenic T-Cell Subsets	Factors for Analysis	R^2	R^2 Change	*Beta Value*	SEB	*Standardised ß*	F-statistic (p -value)	t-statistic (p-value)
$CD3^+CD31^+$ T-cells (% of $CD3^+$)	**Model 1**	0.085					3.703 (0.061)*	
	Sitting Time			-0.011	0.006	-0.291		-1.924 (0.061)
	Model 2	0.179	0.095				4.264 (0.021)*	
	Sitting Time			-0.007	0.006	-0.178		-1.153 (0.256)
	$\dot{V}O_{2max}$			0.286	0.135	0.328		2.121 (0.040)*
% $CD4^+$ T-cells expressing CD31	**Model 1**	0.080					3.379 (0.074)	
	Sitting Time			-0.011	0.006	-0.282		-1.838 (0.074)
	Model 2	0.145	0.066				3.229 (0.051)	
	Sitting Time			-0.007	0.007	-0.184		-1.144 (0.260)
	$\dot{V}O_{2max}$			0.260	0.152	0.274		1.707 (0.096)
% $CD4^+$ T_{ANG} expressing CXCR4	**Model 1**	0.152					6.788 (0.013)	
	Sitting Time			0.032	0.012	0.389		2.605 (0.013)*
	Model 2	0.202	0.051				4.694 (0.015)	
	Sitting Time			0.039	0.013	0.480		3.032 (0.004)*
	$\dot{V}O_{2max}$			0.472	0.308	0.243		1.535 (0.133)

*SEB- Standard Error of the Beta Value; *p<0.05*

6.4 Discussion

Sitting time (as measured by self-report of average sitting time for 7 days prior to study participation; Sit-Q7d) was associated with a decrease in circulating $CD31^+$ T-cells. The observed association between sitting time and T_{ANG} cells was potentially due to a loss of CD31 expression on $CD4^+$ T-cells. However, this association, when correcting for CRF (estimated $\dot{V}O_{2max}$) disappeared for both total T_{ANG} cells and $CD4^+$ T_{ANG}. Furthermore, CXCR4-expression on $CD34^+$ progenitor cells and EPCs appeared to decline with increasing sitting time. Interestingly, screen time measures (as measured by self-report of average screen time [computer games, television, computer-related tasks] for 7 days prior to study participation; Sit-Q7d) were largely unrelated to CAC and cardiometabolic risk factor variables. Screen time was only associated with a decreased expression intensity of CXCR4 on CXCR4-expressing EPCs. Both sitting time and screen time were associated with several cardiometabolic risk factors (waist-to-hip ratio, TC:HDL ratio, HDL-C), but no associations were found between these two sedentary behaviours and other risk factors, such as fasting glucose, inflammatory cytokine IL-6 levels, BMI, BP, LDL-C and triglycerides.

One-way ANOVA analyses showed that those participants reporting the highest sitting time levels displayed greater $CXCR4^+$ $CD4^+$ T_{ANG} cells, however the effect size was only small-to-moderate (ES=0.52). This finding was not corroborated by the results in the univariate linear regression analyses. Similarly, $CD4^+$ T_{ANG} cells appeared to be greater in those reporting high levels of daily average screen time compared to those with the lowest levels of daily average screen time, but the effect size was only 0.65 and also not supported by the findings from the univariate linear regression analysis ($r=0.117$, $p=0.233$).

Those reporting high levels of sitting time are more likely to suffer from all-cause mortality (Katzmarzyk *et al.*, 2009; van der Ploeg *et al.*, 2012; Matthews *et al.*, 2015), in addition to other forms of sedentary behaviour associated with detrimental effects on cardiometabolic risk factors, such as fasting glucose, waist circumference (Gennuso *et al.*, 2013), triglycerides, and BMI (Chau *et al.*, 2014; Healy *et al.*, 2015). Our data shows that in apparently healthy males, sedentary behaviour is unfavourably associated with several cardiometabolic risk factors TC:HDL ratio, HDL-C, and waist-to-hip ratio. Healy *et al.* (2015) was able to demonstrate that by replacing 2 hours a day of sitting time with standing or stepping, improvements can be made in several cardiometabolic risk factors

such as lower BMI, waist circumference, fasting glucose, triglycerides, HDL-C:TC ratio, as well as higher HDL-C concentrations suggesting reduced CVD and diabetic risk with replacing sedentary time with activity time.

Sitting time is also associated with vascular dysfunction (Thosar *et al.*, 2012; Restaino *et al.*, 2015; Thosar *et al.*, 2015a) and vascular stiffness (van de Laar *et al.*, 2014; Younger *et al.*, 2015). Circulating $CD34^+$ progenitor and $CD31^+$ T-cells have been previously shown to be independently related to endothelial function (Weil *et al.*, 2011; Bruyndonckx *et al.*, 2014). As such we sought to investigate the effect of sitting time on these cells, as changes in these cells with sedentary behaviour may affect endothelial function and provide a mechanism for the observed reduced endothelial function observed in several epidemiological and interventional studies (Restaino *et al.*, 2015; Thosar *et al.*, 2015a; Thosar *et al.*, 2015b). Our data shows that those reporting higher levels of daily average sitting time were more likely to have a reduced percentage of $CD3^+$ T-cells expressing CD31. This was attributable to the reduced CD31 expression on $CD4^+$ T-cells. Paradoxically, there was a positive association between sitting time and CXCR4-expressing $CD4^+$ T-cells, indicating improved migratory function of these cells. This may be a physiological response to increase function of these $CD31^+$ T-cells to maintain homeostasis. As we observed a decrease in $CD31^+$ T-cells, this may be a response to partly reduce the homeostatic deleterious effect of sitting time on these cells. Cortisol, a stress hormone can upregulate CXCR4 cell surface expression in T-cells (Okutsu *et al.*, 2005), as well as by TNF-α and IL-6, but only in neuronal cells (Rostasy *et al.*, 2005) and glial cells (Ödemis *et al.*, 2002) respectively. More studies are required to investigate the mechanism by which sedentary lifestyle may lead to increases in CXCR4 cell surface expression, but could be due to rises in chronic low-grade inflammatory biomarkers as seen with sedentary behaviour (Yates *et al.*, 2012; Henson *et al.*, 2013), however we did not detect differences in IL-6, which can act as a pro-inflammatory cytokine (Gabay, 2006), between sitting time groups, and therefore may be IL-6-independent. However, IL-6 an act as both pro- and anti-inflammatory in nature (Scheller *et al.*, 2011), and thus is not an accurate reflection of an individual's pro-inflammatory state, and thus more accurate biomarkers are required to fully elucidate if physical activity or inactivity affect inflammation.

Due to the dichotomy of sedentary behaviour and CRF, where individuals can live a relatively sedentary lifestyle yet maintain high levels of $\dot{V}O_{2max}$ through brief periods of intense exercise, we sought to investigate whether the effects of sitting time remained

after correcting for CRF. Interestingly the negative impact of sitting time on total circulating T_{ANG} cells and $CD4^+$ T_{ANG} cells did not remain after correcting for CRF, indicating that the unfavourable health impact of a sedentary lifestyle can be attenuated by maintaining high levels of $\dot{V}O_{2max}$.

The impact of sedentary lifestyle on the immune system has been addressed before (Simpson and Guy, 2010), but has yet to be investigated. Thus far, the effects of acute and regular exercise has been shown to affect various aspects of the immune system, such as $CD4^+$ and $CD8^+$ T-cell levels in the blood (Simpson *et al.*, 2008; Turner *et al.*, 2010), immunoglobulin levels in saliva and blood, as well as *in vivo* and *in vitro* immune responsiveness (Chin A Paw *et al.*, 2000; Moro-García Marco *et al.*, 2013). We have shown that those with high levels of $\dot{V}O_{2max}$ have higher number of circulating T_{ANG} cells as a result of increased expression of CD31 on $CD4^+$ T-cells in an age-dependent manner (see *Chapter 5- Influence of Age and Cardiorespiratory Fitness on $CD31^+$ T-Cells and CXCR4 Cell Surface Expression*). The data shown in this study suggest that sedentary lifestyle may negatively impact on these cells. This could be one of many mechanisms by which sedentary lifestyle may lead to endothelial dysfunction. However, our data also imply that those living a sedentary lifestyle can eradicate some of the negative impacts of this lifestyle by attaining a high level of CRF potentially attainable through exercise training.

Limitations

This study has a few limitations. The quantification of sitting time and screen time were made purely by self-report, and lacks any accelerometer or step count measures of activity or inactivity. Ideally both measures would be made over a longitudinal study. The Sit-Q7d, although shown recently to display criterion-based validity (Wijndaele *et al.*, 2014), may be stronger for some criteria of sedentary time than others (e.g. screen time vs. total sedentary time), and thus further analysis of the results is required to reflect this. However reliable these self-report measures may be, these measures have limitations in participant recall of inactivity. Due to the low intensity nature of these sedentary behaviours, recall can prove difficult for participants. As a result, participants were asked to recall for only the past 7 days, potentially limiting the error associated with self-report.

Our study cohort was 43 in number, yet significantly underpowered to obtain sufficient statistical power to address the research question. We were unable to perform ANOVAs for sedentary time groups and CRF groups due to the low number of participants, and thus is a target for future study.

We estimated $\dot{V}O_{2max}$ from a submaximal exercise test (YMCA). Submaximal exercise tests have been widely used to estimate $\dot{V}O_{2max}$ (Akalan *et al.*, 2008; Faulkner *et al.*, 2009; Spielmann *et al.*, 2011). Many of the prediction equations rely on estimation of maximal heart rate (HR_{max}) of an individual to extrapolate data. Using HR_{max} prediction equations commonly underestimate HR_{max} (Gellish *et al.*, 2007) and thus be a limiting factor when using submaximal exercise tests to predict $\dot{V}O_{2max}$. However, both Beekley *et al.* (2004) and Garatachea *et al.* (2007) demonstrated that $\dot{V}O_{2max}$ as predicted using the YMCA submaximal cycling ergometer test, was not significantly different from $\dot{V}O_{2max}$ measured directly. However, there appears to be a gender difference, with women showing large discrepancies between predicted and measured $\dot{V}O_{2max}$ (Garatachea *et al.*, 2007). Despite limitations arising from predicting $\dot{V}O_{2max}$, submaximal exercise tests to estimate CRF in men can be used in a research setting.

Conclusion

Sitting time is negatively related with T_{ANG} cells through a CD4-dependent mechanism, and thus may be a potential mechanism of the observed vascular dysfunction observed with sedentary lifestyles. Regular exercise training to attain a high CRF may attenuate or negate the deleterious impact of sedentary behaviours on these CACs.

Chapter 7: The Effect of an Acute Endurance Exercise Bout on Circulating $CD31^+$ T-Cells

7.1 Introduction

Exercise provides many cardiovascular benefits, such reducing blood pressure (Liu *et al.*, 2012), improving fasting glucose levels (Gillen *et al.*, 2012), and reduces CVD risk and mortality rates (Kurl *et al.*, 2003; Lee *et al.*, 2012a; Vigen *et al.*, 2012; Barry *et al.*, 2013; Berry *et al.*, 2013; Chomistek *et al.*, 2013; Holtermann *et al.*, 2015). It is thought that regular acute exercise-induced increases in sub-population of CACs provide a mechanism for the vasculo-protection, angiogenesis and improved endothelial function seen with exercise. Acute exercise has been shown to increase some populations of these cells, specifically $CD34^+$ and $CD34^+VEGFR2^+$ putative EPCs (Adams *et al.*, 2004; Rehman *et al.*, 2004; Laufs *et al.*, 2005a; Möbius-Winkler *et al.*, 2009; Sandri *et al.*, 2011; Scalone *et al.*, 2013; Ross *et al.*, 2014; Chang *et al.*, 2015). This mobilisation of BM-derived cells can cause these cells to be elevated up to 72 hours in the recovery period, offering a sustained window for vascular-adaptation post-exercise.

$CD3^+CD31^+$ T-cells can act in a paracrine manner to secrete pro-angiogenic growth factors (e.g. VEGF, IL-8) to support endothelial cell growth and tube formation (Hur *et al.*, 2007). The ability of these cells to migrate is associated with endothelial function (Weil *et al.*, 2011), implicating them in vascular homeostasis. These angiogenic T-cells (T_{ANG}) have also been found to be reduced in individuals with cardiovascular risk factors (Hur *et al.*, 2007), as well as those with small vessel cerebrovascular disease (Rouhl *et al.*, 2012) in comparison to age-matched healthy controls. We have shown that advancing age is associated with reduced circulating numbers of these T_{ANG} cells, in addition to the CXCR4 cell surface expression of these cells, which could partially contribute to the age-related increased CVD risk (*Chapter 5: The Influence of Age and Cardiorespiratory Fitness on Angiogenic T-Cells and CXCR4 Cell Surface Expression*), yet individuals with a higher level of fitness have more T_{ANG} cells and a mechanism by which exercise can be beneficial for the vascular system.

T-cells are very responsive to acute bouts of exercise. Typically, there is a biphasic response in the circulation compartment of these $CD3^+$ cells to acute exercise (Simpson *et al.*, 2007; Turner *et al.*, 2010; Witard *et al.*, 2012). Initially, there is a lymphocytosis, where there is a large ingress of lymphocytes into the circulation immediately post-exercise followed by an egress of cells from the circulation (lymphocytopenia). The lymphocytosis observed is thought to be a result of shear stress-modulated detachment of lymphocytes from the vascular endothelial wall as well as by ß-adrenergic mechanisms

as a consequence of increased circulating levels of catecholamines during exercise (Simpson *et al.*, 2007). The increased cardiac output ($\dot{Q}$) seen with exercise provides the mechanical shear stress stimulus to detach lymphocytes from the endothelial wall. Acute exercise activates the hypothalamic-pituitary-adrenal axis (HPA), which results in the release of the catecholamines epinephrine and norepinephrine. These catecholamines stimulate the ß-adrenergic receptor-expressing T-cells to ingress into the blood. Cytotoxic $CD8^+$ T-cells, effector memory and senescent T-cells exhibit a greater ingress into the circulation during exercise (Simpson *et al.*, 2010; Turner *et al.*, 2010; LaVoy *et al.*, 2014), most likely due to greater expression of ß-adrenergic receptors found on these cells. Indeed the blocking of these receptors through the use of propranolol impairs the lymphocytosis observed with ß-agonist infusion (Schedlowski *et al.*, 1996). In immunosurveillance terms, this may be an evolutionary mechanism to allow for redistribution of these cytotoxic T-cells to be mobilised in response to a stressor to ward off infection (Dimitrov *et al.*, 2010). Preferential mobilisation of cells expressing $ß_2$ receptors, also occurs with T-cells co-expressing several adhesion molecules, such as integrins (Shephard, 2003; Simpson *et al.*, 2006) and CD56 (Goebel *et al.*, 2000; Simpson *et al.*, 2006), however the response of $CD31^+$ expressing T-cells to acute exercise has yet to be documented.

Cell surface receptor expression of CXCR4 may also be acutely modulated by exercise stimuli. Post-exercise plasma acutely stimulates the upregulation of CXCR4 on $CD3^+$ T-cell cell surfaces *in vitro*, and thought to be a result of the acute exercise increases in cortisol (Okutsu *et al.*, 2005). This effect appeared to primarily affect $CD4^+$ T-cells, with a smaller relative change in CXCR4 expression observed on the $CD8^+$ T-cell subset. Migration of these cells to SDF-1α also improved as a result of incubation with post-exercise plasma in comparison to pre-exercise plasma. However, no data as yet is currently available on the $CXCR4^+$ T-cell kinetics as a result of acute exercise bout, and the potential mechanism(s).

If these T_{ANG} ($CD3^+CD31^+$) are highly responsive to acute exercise, this may provide a potential mechanism for exercise-induced vascular adaptation, therefore it is the aim of this study to investigate the response of $CD3^+CD31^+$ T_{ANG} cells in addition to CXCR4 cell surface expression of these cells to an acute bout of exercise, as well as compare to their $CD3^+CD31^-$ counterparts.

7.2 Materials and Methods

7.2.1 Participants

Male participants (n=12), aged between 18 and 45 years volunteered to take part in the study, and consisted of a mixture of students and staff based at Edinburgh Napier University, in addition to general public from the Edinburgh area. Prior to their involvement in the study, participants were asked to give written informed consent, after which they completed a Physiological Screening Questionnaire (see *Appendix 3*). Participants were excluded from the study if they were smokers, had an excessive alcohol intake (>14 drinks/week), had a BMI>35, were currently taking medication affecting the immune system, antidepressants, routinely using ibuprofen, aspirin, reporting major affective disorders such as human immunodeficiency virus infection, hepatitis, arthritis, central or peripheral nervous disorders, previous stroke or cardiac events, reported infection in the 6 weeks prior to study enrolment, or were bedridden in the 3 months prior to the study, or suffer from known cardiovascular or autoimmune disease. Participants were advised not to partake in any strenuous exercise for 72 hours prior to their visits to the Human Performance Laboratory. Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee. Participant characteristics are shown in table 7.1.

Table 7.1. Participant characteristics (n=12). Values shown are mean ± SD (Range).

	All (n=12)
Age (years)	32 ± 7 (24-44)
Height (m)	1.82 ± 0.07 (1.65-1.97)
Body Mass (kg)	76.60 ± 8.41 (57.00-84.30)
BMI ($kg \cdot m^2$)	23.02 ± 1.72 (20.94-25.99)
SBP (mmHg)	124 ± 6 (117-135)
DBP (mmHg)	75 ± 6 (66-82)
$\dot{V}O_{2max}$ ($mL \cdot kg \cdot min^{-1}$)	58.33 ± 4.29 (50.00-67.30)
10km Time (min:ss)	43:01 ± 5:27 (35:10-54:34)

BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure.

Visit 1 (Assessment of $\dot{V}O_{2max}$)

7.2.2 Resting Measures

Participants visited the Human Performance Centre having refrained from ingesting caffeine from the night before as well as refraining from any alcohol intake 24 hours prior to participation. Participants were also required to avoid strenuous exercise for 3 days prior to the visit.

Participants were measured for height and body mass, from which BMI was calculated (see *3.2 Resting Measures*). Blood pressure (BP) was measured using an automated blood pressure cuff (Nonin Puresat Avant 2120, Nonin Medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA) after 5-minutes'

rest in a supine position on the non-dominant arm. BP was measured twice and the average systolic and diastolic measures were recorded.

7.2.5 Assessment of Maximal Oxygen Uptake ($\dot{V}O_{2max}$)

Maximal oxygen uptake ($\dot{V}O_{2max}$) of each participant was measured by graded treadmill exercise test to volitional exhaustion. The test was composed of 3-minute stages, beginning at a speed of 10km·h^{-1}, with subsequent stages increasing in pace by 3km·h^{-1}. After completing the 3rd stage at 16km·h^{-1}, the gradient of the treadmill was increased by 2.5% every minute until the participant reaches volitional exhaustion and cannot continue. Breath-by-breath online analysis was performed throughout the test (LABManager v5.3.0, Cardinal Health, Germany) to measure $\dot{V}O_2$ and respiratory exchange ratio (RER). $\dot{V}O_{2max}$ was identified and recorded as the final 8-breath averaged $\dot{V}O_2$. HR was recorded continually throughout the test by HR telemetry (Polar, Finland).

Visit 2 (Exercise Trial)

7.2.6 Resting Measures

Participants visited the Human Performance Laboratory again within one week of completing the $\dot{V}O_{2max}$ test. Participants attended the laboratory at 8am after an overnights fast and having refrained from caffeine and alcohol for 24 hours prior to the laboratory visit. They were also advised to refrain from exercise training for 72 hours prior to the laboratory visit.

Participants were measured for height and body mass, from which BMI was calculated (see *3.2 Resting Measures*). BP was measured using an automated blood pressure cuff (Nonin Puresat Avant 2120, Nonin Medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA) after 5 minutes rest in a supine position on the non-dominant arm. BP was measured twice and the average systolic and diastolic measures were recorded.

7.2.7 Blood Sampling and PBNMC Isolation

Blood was taken from participants in a supine position before, immediately post- and 60 minutes post-exercise. Peripheral blood samples were drawn into 6mL vacutainers (BD Biosciences, UK), which were either coated in EDTA to prevent coagulation, serum gel, or contained sodium citrate. EDTA blood was processed for PBMNC as previously described in general materials and methods (*Chapter 3.7*). Isolated PBMNCs were then used for T_{ANG} number and CXCR4 cell surface enumeration (see *7.2.9 Angiogenic T Cell Number and CXCR4 Expression Quantification)*, blood drawn into the citrate tubes was used for the analysis of circulating SDF-1α (see *7.2.10 Platelet-Free Plasma Analysis for Chemotactic Factor SDF-1α*).

7.2.8 10km Running Trial

The exercise trial consisted of a self-paced 10km running effort on a treadmill ergometer. The participant was told to complete the 10km distance in the quickest speed they feel they could maintain for the full distance, however were also told that they could adjust the speed of the treadmill throughout the test if necessary. The participant was unaware of the distance covered or time lapsed during the trial. Participants were notified when they had 1km of the trial remaining. Participants were notified to drink water *ab libitum*.

7.2.9 Angiogenic T Cell Number and CXCR4 Expression Quantification

Isolated PBMNCs ($0.5x10^6$) were labelled with monoclonal antibodies anti-CD3-APC, anti-CD31-FITC, anti-CD4/anti-CD8-PE and anti-CXCR4-PE-Cy5 (all BD Biosciences), and were left to incubate at 4°C for 45 minutes in the dark prior to flow cytometric analysis as detailed in general materials and methods *Chapter 3.9*. CXCR4 cell surface expression was analysed as a surrogate for migratory ability.

7.2.10 Platelet-Free Plasma Analysis for Chemotactic Factor SDF-1α

Potential chemotactic factor SDF-1α was analysed by enzyme-linked immunosorbent assay (ELISA) as described in general materials and methods *Chapter 3.12*. All samples were analysed in duplicate and averaged.

7.2.11 Statistical Analysis

All data were assessed for normal distribution. Not normally distributed data were logarithmically transformed.

To assess the response of total $CD3^+CD31^+$ T-cells (T_{ANG}), $CXCR4^+$ T_{ANG} cells, and CXCR4 cell surface expression (MFI) to acute exercise, several repeated measures analysis of variance (ANOVA) were performed, including the three time points as the dependent factor (pre-exercise, immediately post-exercise and 60 minutes post-exercise). To compare the effect of $CD31^-$ T-cells to their $CD31^+$ counterparts two-way repeated measures ANOVA tests were performed, with time (pre, post- and 1-hour post-exercise) and phenotype ($CD31^{+/-}$) as independent factors. $CD4^+$ and $CD8^+$ T-cell response within the T_{ANG} population were assessed, with one-way and two-way repeated measures ANOVAs performed in order to assess the contribution of these subtypes of $CD3^+$ T-cells to the response seen. The level of ingress and egress of $CD31^+$ cells were compared to their $CD31^-$ phenotype counterpart using paired samples T-test.

Circulating SDF-1α changes were statistically assessed using one-way repeated measures ANOVA. To assess the contribution of SDF-1α to the ingress of $CXCR4^+$ T-cells as a result of exercise, Pearson's coefficient correlations were performed, using percentage changes in circulating SDF-1α and respective $CXCR4^+$ cell changes as the independent factors.

For the various ANOVAs performed, and where necessary, with Bonferonni post-hoc tests undertaken to identify locations of significance.

Data was analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA). Significance was set at $p=0.05$.

7.3 Results

7.3.1 $CD3^+CD31^+$ T-Cell Response to Acute Exercise

There was a large increase in total $CD31^+$ T-cells from pre- to immediately post-exercise (0.514 ± 0.045 cells x $10^9 \cdot L^{-1}$ [0.416-0.612 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 1.040 ± 0.128 cells x $10^9 \cdot L^{-1}$ [0.759-1.320 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.000). The same pattern was observed for both $CD4^+$ (0.224 ± 0.023 cells x $10^9 \cdot L^{-1}$ [0.173-0.275] 95% CI, vs. 0.375 ± 0.047 cells x $10^9 \cdot L^{-1}$ (0.272-0.479], 95% CI, p=0.002) and $CD8^+$ T_{ANG} cells (0.236 ± 0.027 cells x $10^9 \cdot L^{-1}$ [0.177-0.295] 95% CI, vs. 0.505 ± 0.064 cells x $10^9 \cdot L^{-1}$ [0.364-0.645] 95% CI, p=0.001) from pre- to post- exercise (figure 7.1). There was a greater response of $CD8^+$ T_{ANG} cells to the acute exercise stressor than $CD4^+$ T_{ANG} cells (time x phenotype interaction p=0.003; figure 7.2).

CXCR4-expressing T_{ANG} cells displayed a similar pattern, with total CXCR4-expressing $CD31^+$ T-cells (0.089 ± 0.021 cells x $10^9 \cdot L^{-1}$ [0.043-0.135 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.255 ± 0.046 cells x $10^9 \cdot L^{-1}$ [0.153-0.357 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.001), CXCR4-expressing $CD4^+$ (0.038 ± 0.012 cells x $10^9 \cdot L^{-1}$ [0.013-0.63 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.109 ± 0.022 cells x $10^9 \cdot L^{-1}$ [0.060-0.158 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.006) and $CXCR4^+$ $CD8^+$ T_{ANG} cells (0.055 ± 0.014 cells x $10^9 \cdot L^{-1}$ [0.025-0.086 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.137 ± 0.025 cells x $10^9 \cdot L^{-1}$ [0.082-0.192 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.003) (figure 7.1) all demonstrating a significant increase post-exercise.

In the recovery period post-exercise there was a significant drop in total T_{ANG} cells (1.040 ± 0.128 cells x $10^9 \cdot L^{-1}$ [0.759-1.320 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.464 ± 0.039 cells x $10^9 \cdot L^{-1}$ [0.378-0.550 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.000), $CD4^+$ T_{ANG} cells (0.375 ± 0.047 cells x $10^9 \cdot L^{-1}$ [0.272-0.479 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.217 ± 0.021 cells x $10^9 \cdot L^{-1}$ [0.170-0.263 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.002) and $CD8^+$ T_{ANG} cells (0.505 ± 0.064 cells x $10^9 \cdot L^{-1}$ [0.364-0.645 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.218 ± 0.026 cells x $10^9 \cdot L^{-1}$ [0.160-0.276 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.001) from post- exercise to 1 hour post-exercise, to levels similar to pre-exercise (figure 7.1).

$CXCR4^+$ T_{ANG} cells showed a similar egress from the circulation in the post-exercise recovery period, with CXCR4-expressing total $CD31^+$ T-cells (0.255 ± 0.046 cells x $10^9 \cdot L^{-1}$ [0.153-0.357 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.081 ± 0.021 cells x $10^9 \cdot L^{-1}$ [0.035-0.128 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.001), $CD4^+$ T_{ANG} (0.109 ± 0.022 cells x $10^9 \cdot L^{-1}$ [0.060-0.158 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.031 ± 0.009 cells x $10^9 \cdot L^{-1}$ [0.010-0.052 cells x

$10^9 \cdot L^{-1}$] 95% CI, p=0.004) and $CD8^+$ T_{ANG} (0.137 ± 0.025 cells x $10^9 \cdot L^{-1}$ [0.082-0.192 cells x $10^9 \cdot L^{-1}$] 95% CI, vs. 0.057 ± 0.010 cells x $10^9 \cdot L^{-1}$ [0.021-0.093 cells x $10^9 \cdot L^{-1}$] 95% CI, p=0.005) significantly decreased in peripheral circulation from post-exercise to 1-hour post-exercise.

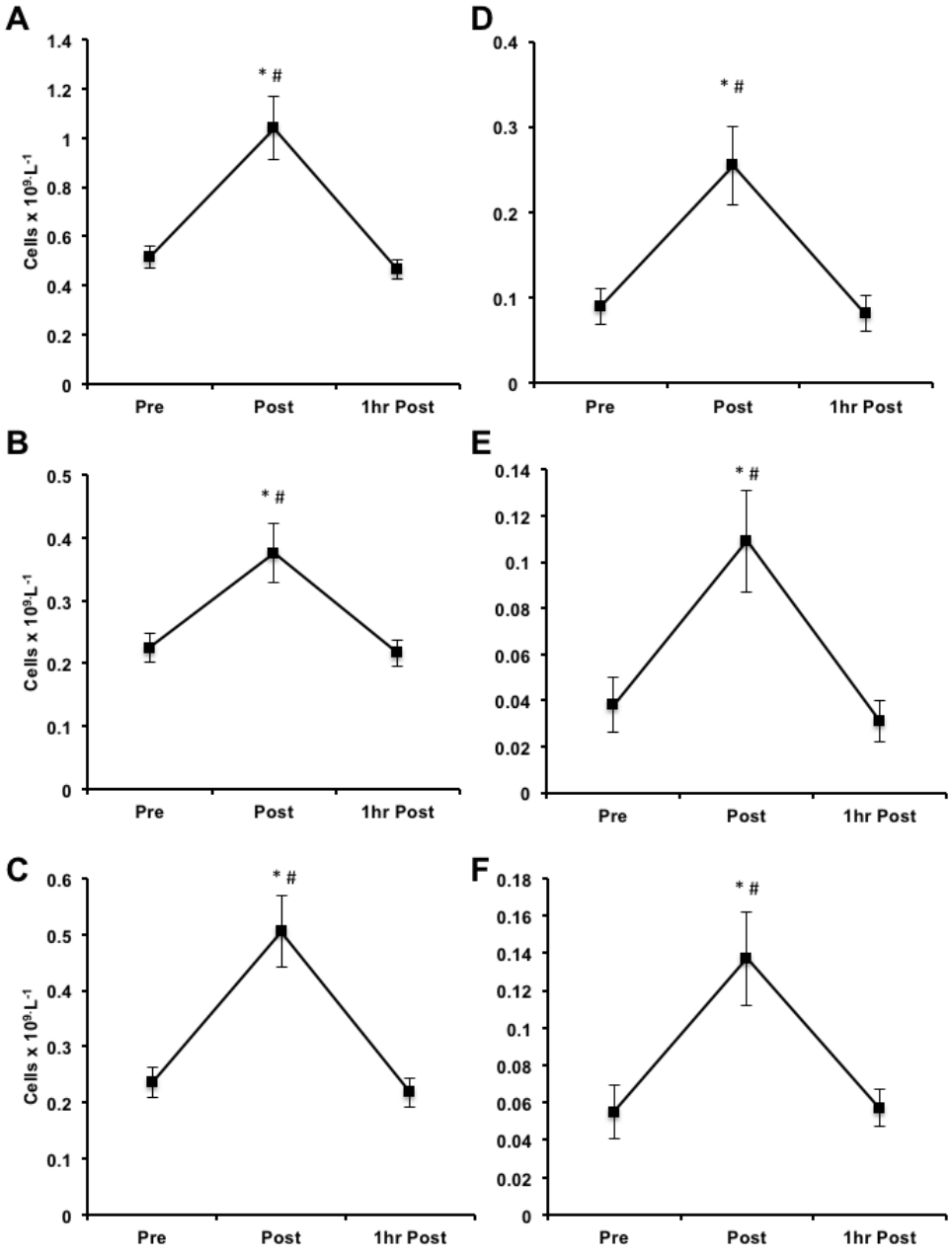

Figure 7.1. Changes in $CD31^+$ T-Cells (A: total $CD31^+$ T-cells; B: $CD4^+$ T_{ANG}; C: $CD8^+$ T_{ANG}) and $CXCR4^+$ T_{ANG} Cells (D: total $CD31^+$ T-cells; E: $CD4^+$ T_{ANG}; F: $CD8^+$ T_{ANG}) with acute exercise (n=12). **p<0.05 vs. Pre-Exercise.* #p<0.05 vs. 1hr Post.*

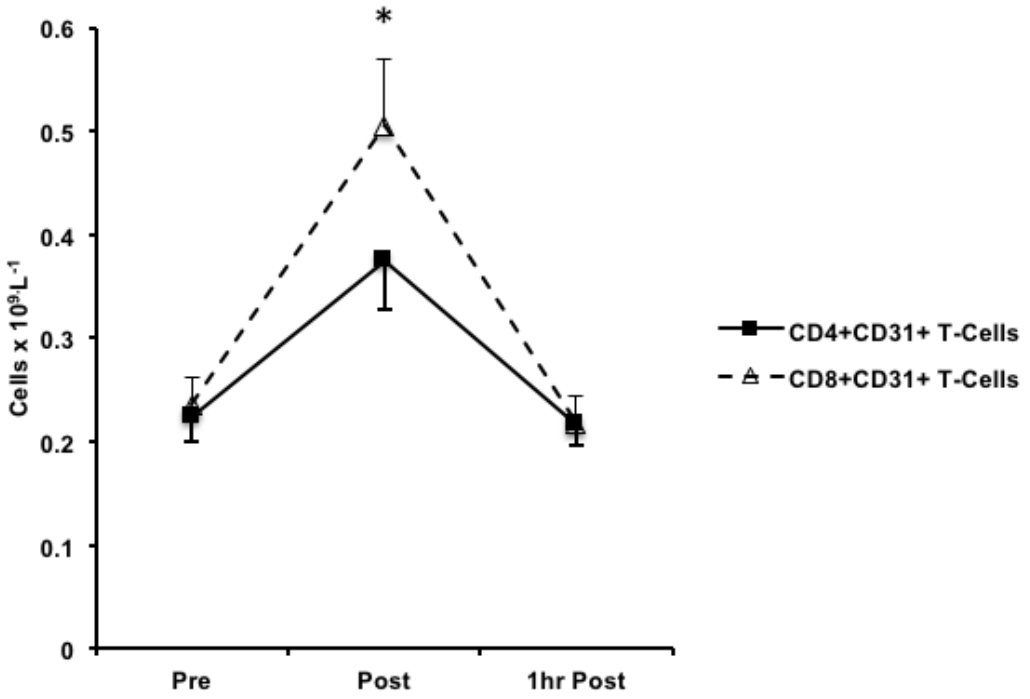

Figure 7.2. Acute exercise and changes in $CD4^+$ and $CD8^+$ T_{ANG} cells (n=12). **p<0.05 time x phenotype interaction.*

Percentage changes in $CD31^+$ T-cells and their $CD4^+$, $CD8^+$ and $CXCR4^+$ subsets with exercise are shown in figure 7.2.

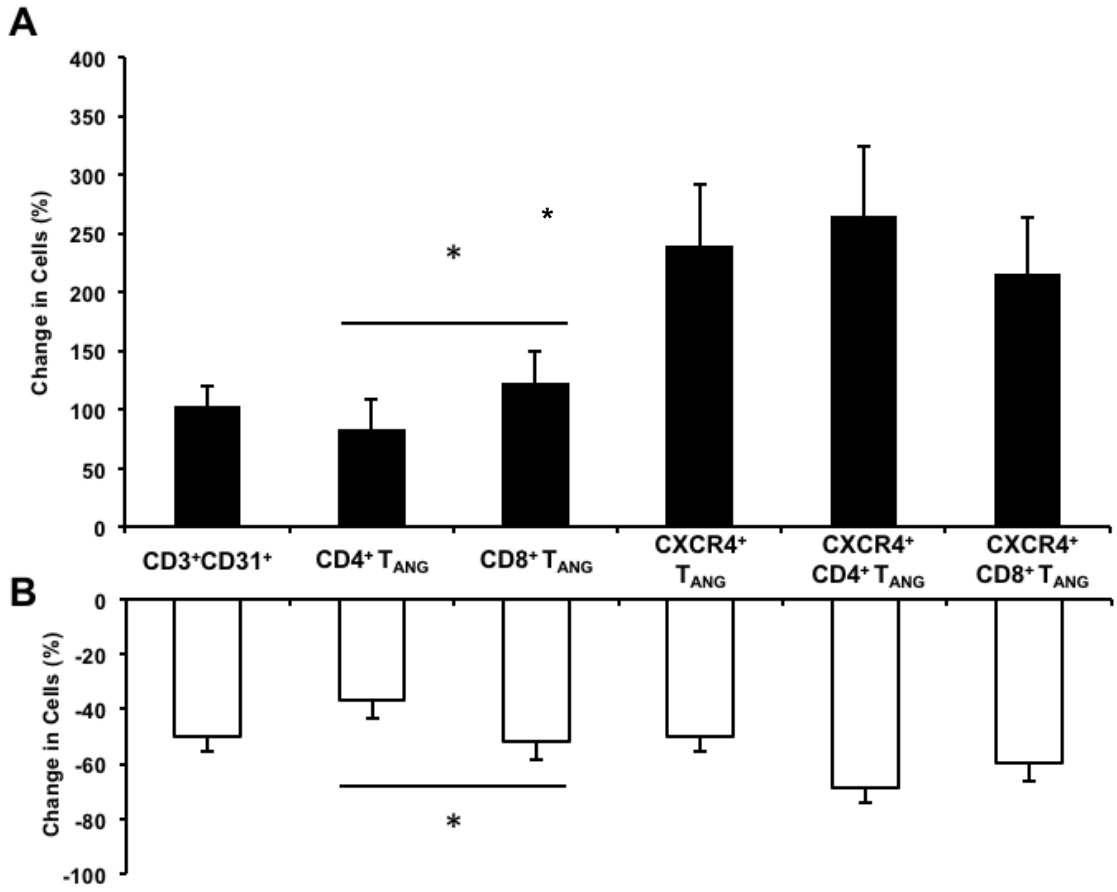

Figure 7.3. Percentage changes in $CD31^+$ T-cells (A: mobilisation into the circulation; B: egress from circulation) with acute exercise (n=12). **p<0.05 vs. $CD4^+CD31^+$ T-cells.*

7.3.2 CXCR4 Cell Surface Expression

CXCR4 cell surface expression on T_{ANG} subsets were compared from pre-exercise to post-exercise and 1-hour post-exercise. As shown in table 7.2 that CXCR4 cell surface expression was unaffected in total T_{ANG} cells and $CD4^+$ subset by exercise. There was an increase in expression of CXCR4 on $CD8^+$ T_{ANG} cells from pre-to post-exercise (10.21 ± 0.83 AU [8.38-12.04] 95% CI, vs. 10.99 ± 0.93 AU [8.95-13.04], p=0.002) and for a decrease in cell surface expression from post-exercise to 1hr post-exercise in these cells (10.99 ± 0.93 AU [8.946-13.04] 95% CI, vs. 10.18 ± 0.86 AU [8.30-12.07], p=0.001).

Table 7.2. CXCR4 cell surface receptor expression (Mean Fluorescence Intensity) in response to acute exercise.

	Pre-Exercise	Post-Exercise	1 hr Post-Exercise	p-value (time)
$CD3^+CD31^+$ (T_{ANG})	11.78 ± 0.44	12.25 ± 0.51	11.97 ± 0.60	0.088
$CD4^+$ T_{ANG}	12.80 ± 0.50	13.08 ± 0.46	12.70 ± 0.51	0.179
$CD8^+$ T_{ANG}	10.21 ± 0.83	10.99 ± 0.93	10.18 ± 0.86	0.000*

*Values shown are mean ± SEM. *p<0.05 effect for time.*

7.3.3 $CD31^+$ vs. $CD31^-$ T-Cell Response to Acute Exercise

To compare the mobilisation patterns of $CD31^+$ and $CD31^-$ T-cells to acute strenuous exercise, two-way repeated measures ANOVAs were performed on total $CD3^+$ T-cells, as well as $CD4^+$ and $CD8^+$ subsets. As shown in figure 7.4A, total $CD3^+$ T-cells phenotypically positive or negative for CD31 showed similar pattern of mobilisation (time x phenotype interaction p=0.521). However, acute exercise had a greater effect on redeployment of $CD4^+$ T cells that did not express CD31 (time x phenotype interaction p=0.010), and this was also the case for $CD8^+$ T-cells lacking CD31 expression (time x phenotype interaction p=0.000) (figures 7.4B and 7.4C).

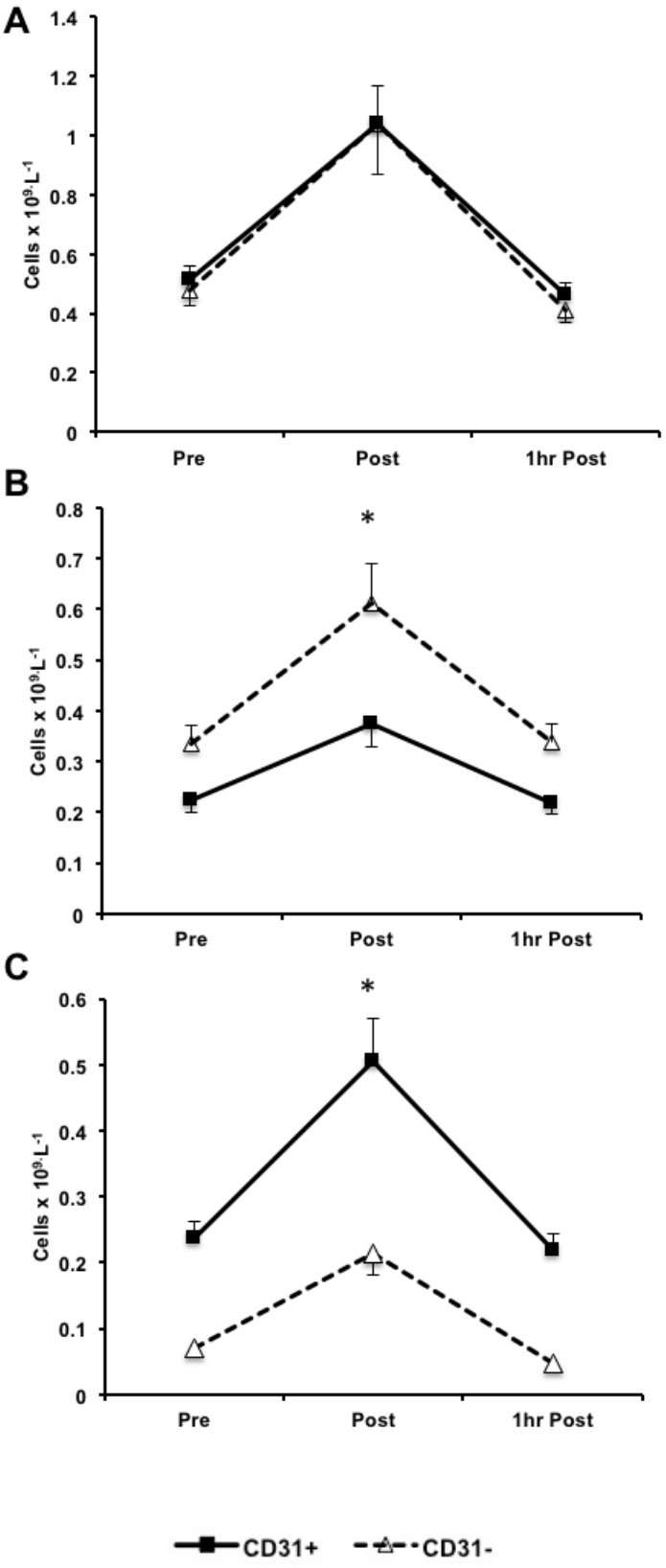

Figure 7.4. Changes in $CD31^+$ and $CD31^-$ T-cells (A: total T-cells; B: $CD4^+$ T-cells; C: $CD8^+$ T-cells) with acute exercise (n=12).

Paired T-test analysis on egress and ingress of $CD31^+$ compared to $CD31^-$ T-cells to acute exercise stimulus showed that $CD31^-$ T-cells displayed greater ingress (119.00 ± 20.30% [74.28-163.66] 95% CI, vs. 102.04 ± 17.95% [62.52-141.55] 95% CI, p=0.041) and egress (56.34 ± 4.17% [47.17-65.51] 95% CI, vs. 49.91 ± 5.65% [37.48-62.35] 95% CI, p=0.047) levels compared to their $CD31^+$ counterparts (figure 7.5). This was due to the significantly greater ingress (200.41 ± 24.60% [146.26-254.55] 95% CI, vs. 122.58 ± 26.72% [63.79-181.37] 95% CI, p=0.005) and egress (76.87 ± 3.01% [70.24-83.50] 95% CI, vs. 52.00 ± 6.35% [38.02-65.98] 95% CI, p=0.002) of $CD8^+CD31^-$ T-cells from the circulation compared to $CD8^+CD31^+$ T-cells (44.78 ± 9.67% [13.99-75.57] 95% CI, vs. 47.33 ± 10.08% [15.24-79.41] 95% CI, p=0.035), whereas there appeared no significant differences in the percentage ingress (89.48± 17.52% [50.92-128.04] 95% CI, vs. 82.75 ± 25.68% [26.23-139.27] 95% CI, p=0.631) and egress (42.25 ± 5.25% [30.70-53.79] 95% CI, vs. 36.63 ± 6.95% [21.33-51.93] 95% CI, p=0.120) between $CD4^+CD31^-$ T-cells and $CD4^+CD31^+$ T-cells.

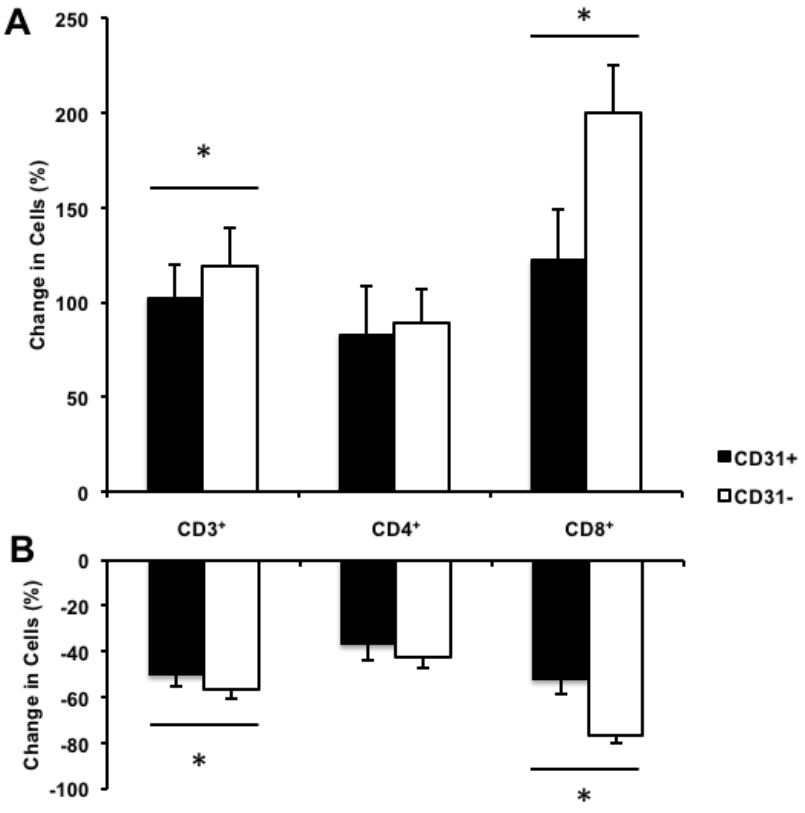

Figure 7.5. Percentage changes in $CD31^+$ and $CD31^-$ T-cells (A: mobilisation into the circulation; B: egress from circulation) with acute exercise (n=12). *$p<0.05$ vs. $CD31^+$.

$CXCR4^+$ T_{ANG} cells are rapidly mobilised in response to exercise. There was a significantly greater response of $CXCR4^+$ T_{ANG} cells compared to $CXCR4^-$ T_{ANG} cells to exercise (p=0.017). This was due to both a greater ingress (239.00 ± 52.61% [123.20-354.79] 95% CI, vs. 81.69 ± 14.33% [50.14-113.23] 95% CI, p=0.005) and a greater egress (69.68 ± 5.42% [57.76-81.61] 95% CI, vs. 44.01 ± 6.66% [29.35-58.67] 95% CI, p=0.004) of CXCR4-expressing T_{ANG} cells compared to their $CXCR4^-$ counterparts, with similar patterns being observed for $CD4^+$ and $CD8^+$ T_{ANG} cells (figures 7.6 and 7.7).

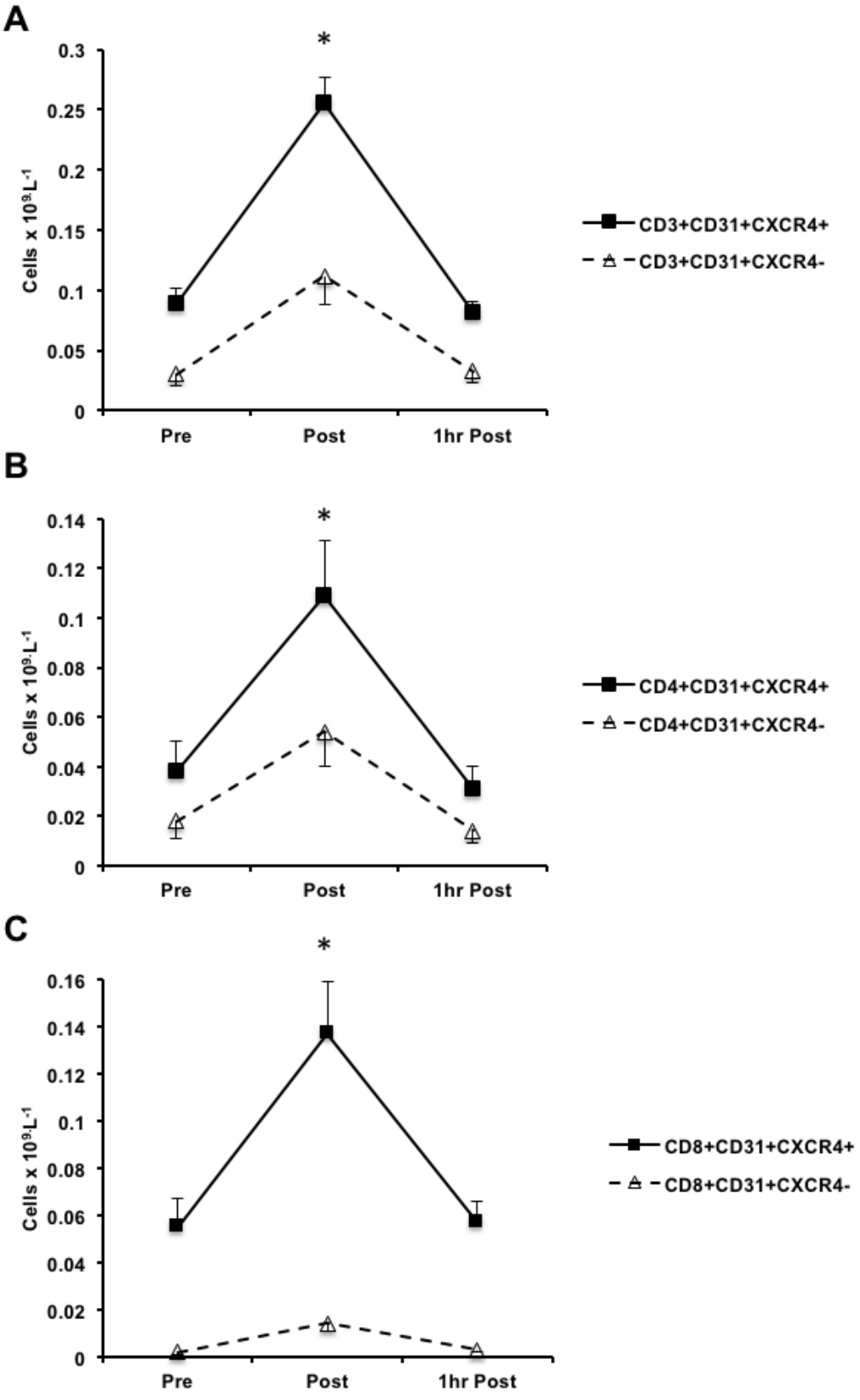

Figure 7.6 Changes in $CXCR4^+$ $CD31^+$ and $CXCR4^+CD31^-$ T-cells (A: total T-cells; B: $CD4^+$ T-cells; C: $CD8^+$ T-cells) with acute exercise (n=12). **p<0.05 time x phenotype interaction*

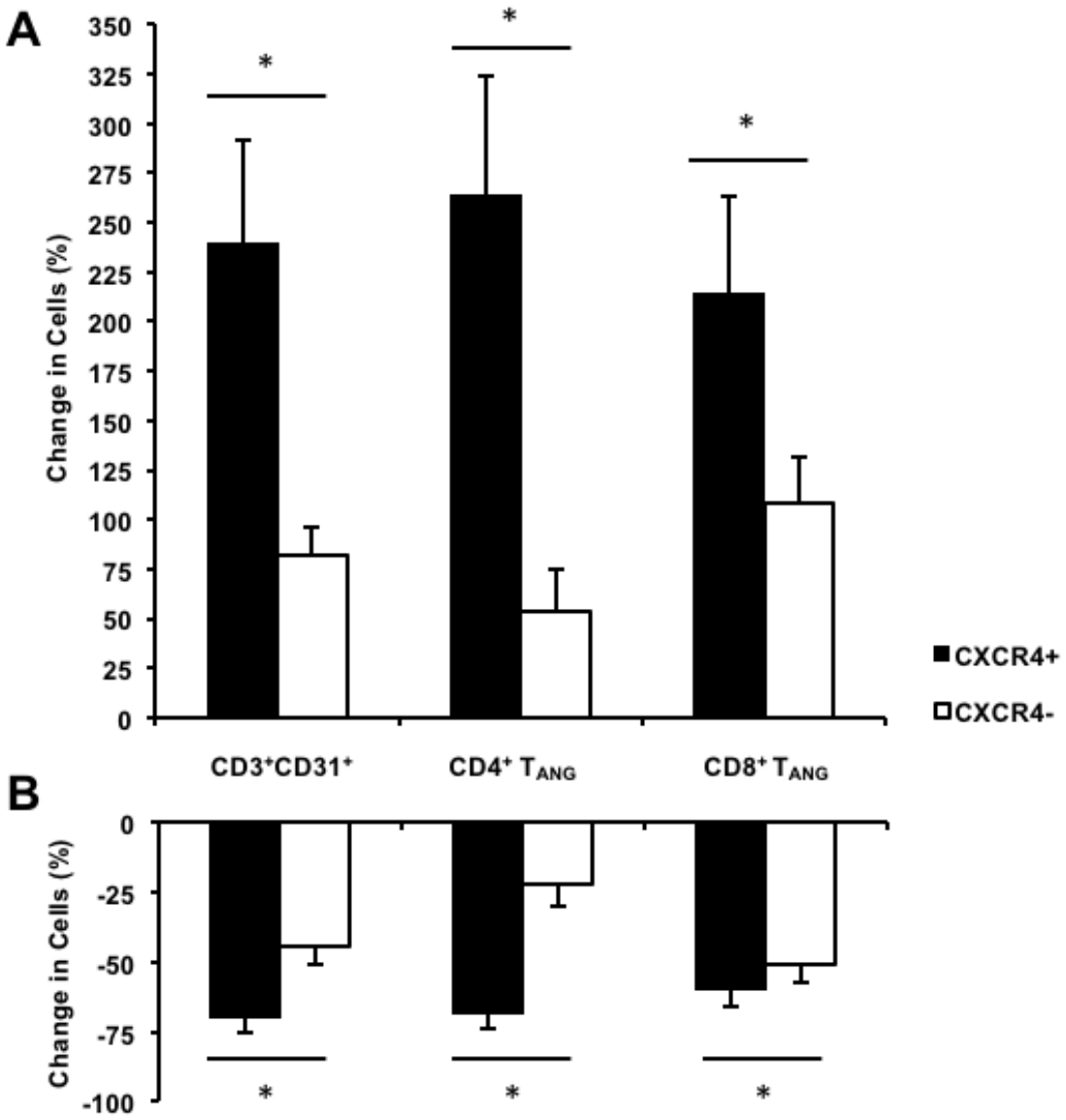

Figure 7.7. Percentage changes in $CXCR4^+$ and $CXCR4^-$ T_{ANG} cells (A: mobilisation into the circulation; B: egress from circulation) with acute exercise (n=12). **p<0.05 vs. $CXCR4^-$*

$CD31^-CXCR4^+$ cells demonstrated a greater response to the acute exercise bout than $CD31^+CXCR4^+$ cells (p=0.005), with greater mobilisation into the peripheral blood compartment post-exercise (381 ± 81.44% [202.54-561.03] 95% CI, vs. 239.00 ± 52.61% [123.20-354.79] 95% CI, p=0.018), and a greater migration from the circulation in the recovery period post-exercise (73.47 ± 4.98% [62.51-84.43] 95% CI, vs. 69.98 ± 5.41 [57.76-81.61] 95% CI, p=0.038). This was due to in part, both increased response of $CD4^+$ and $CD8^+$ $CD31^-$ T-cells (figure 7.8).

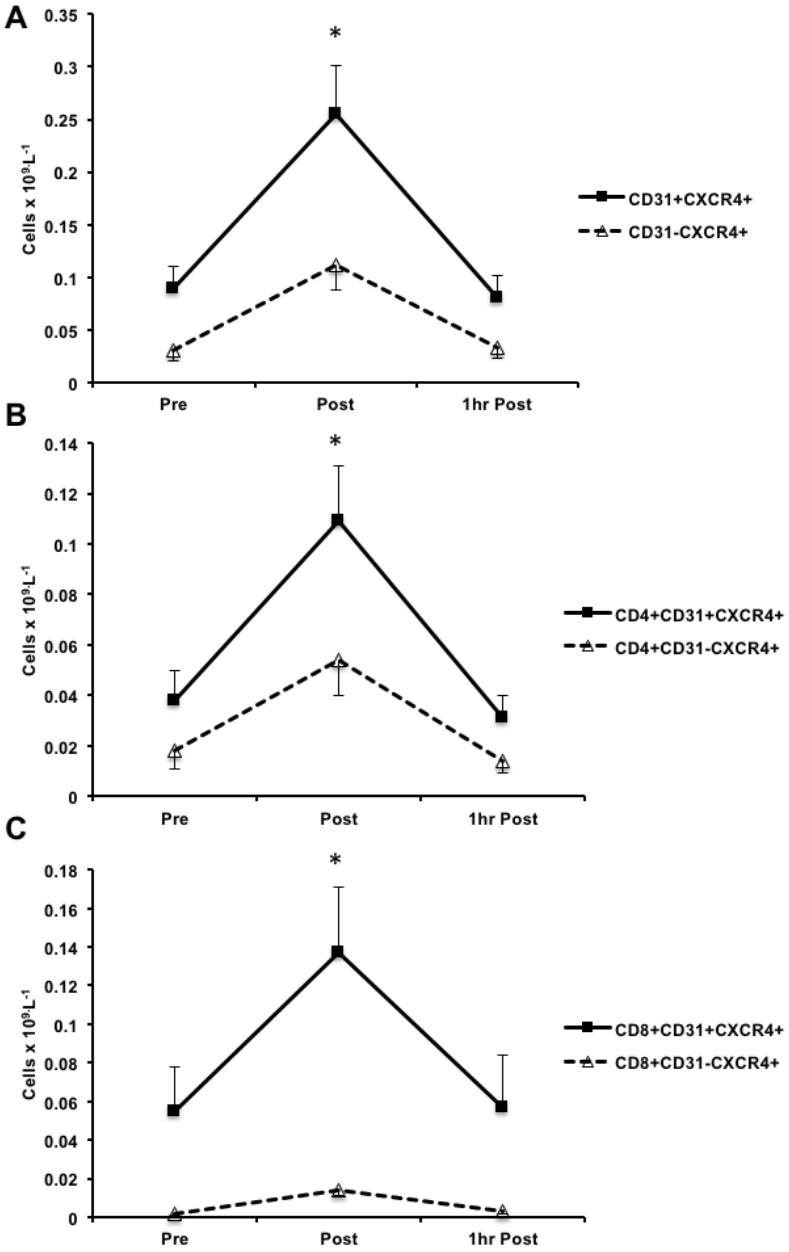

Figure 7.8. $CXCR4^+$ T-cell changes to acute exercise, $CD31^+$ vs. $CD31^-$ phenotypes (n=12). *$p<0.05$ vs. $CD31^+$.

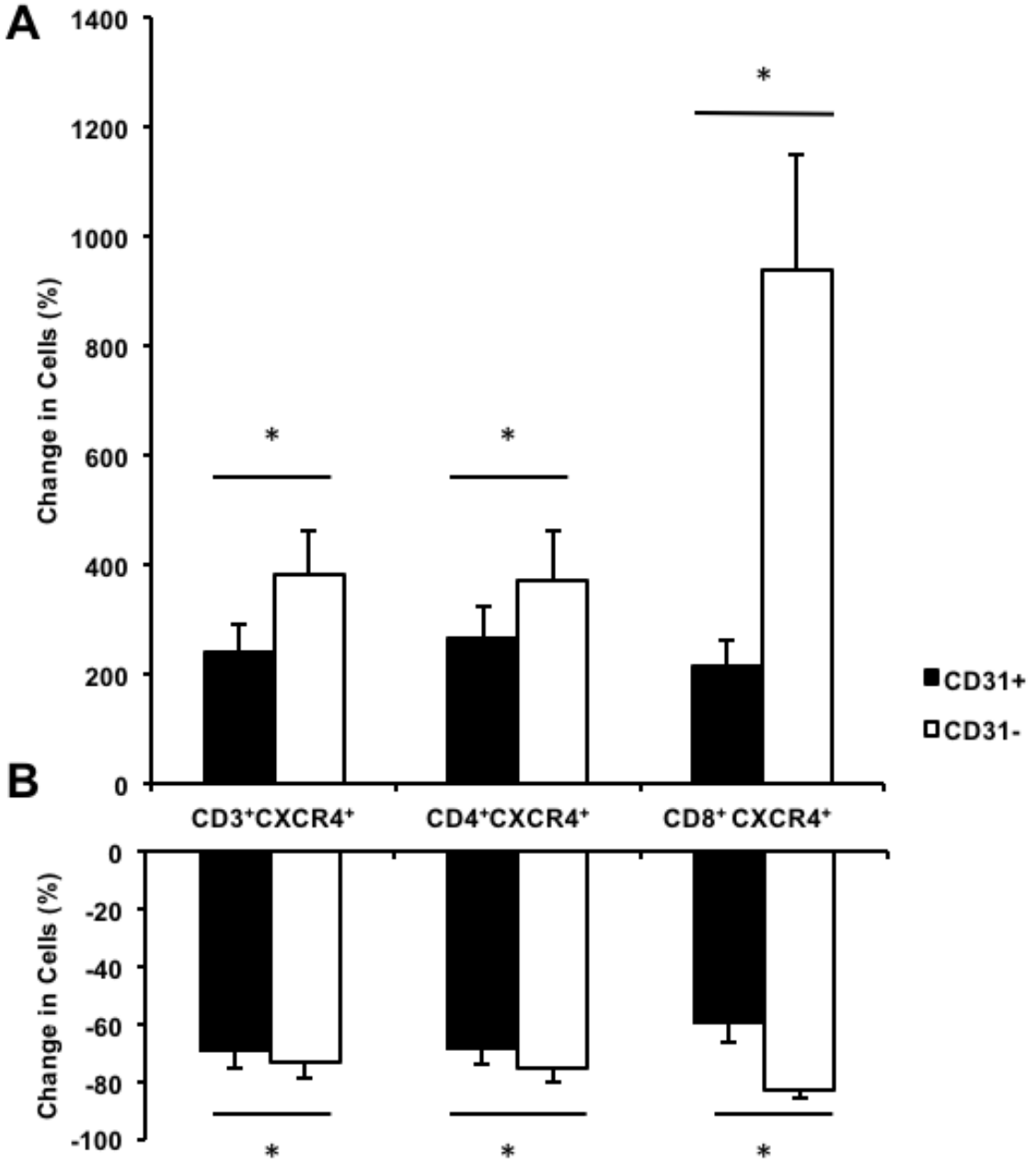

Figure 7.9. Egress and Ingress of CXCR4$^+$ T-cells to acute exercise, comparison between CD31$^+$ and CD31$^-$ T-cells (n=12). **p<0.05 vs. CD31$^+$*.

7.3.4 SDF-1:CXCR4 Axis in the Mobilisation of CD31$^+$ T-Cells in Response to Acute Exercise

CD31$^+$ T-cells can express CXCR4 as shown previously. These CXCR4$^+$ cells are mobilised into the blood in response to acute exercise. The ligand for CXCR4, SDF-1α was measured in platelet-free plasma of individuals undertaking the acute exercise bout pre-exercise, immediately post-exercise and 1 hour post-exercise. One way repeated

measures ANOVA was performed to assess the change in SDF-1α in response to acute exercise.

Circulating SDF-1α significantly increased in the circulation from pre- to immediately post-exercise (1969.53 ± 78.50 pg·mL^{-1} [1796.76-2142.30] 95% CI, vs. 2582.66 ± 102.39 pg·mL^{-1} [2357.31-2808.01] 95% CI, p=0.000). SDF-1α levels recover to pre-exercise levels in the one-hour recovery period post exercise (2582.66 ± 102.39 pg·mL^{-1} [2357.31-2808.01] 95% CI, vs. 2162.13 ± 85.83 pg·mL^{-1} [1973.23-2351.04] 95% CI, p=0.000) (figure 7.10).

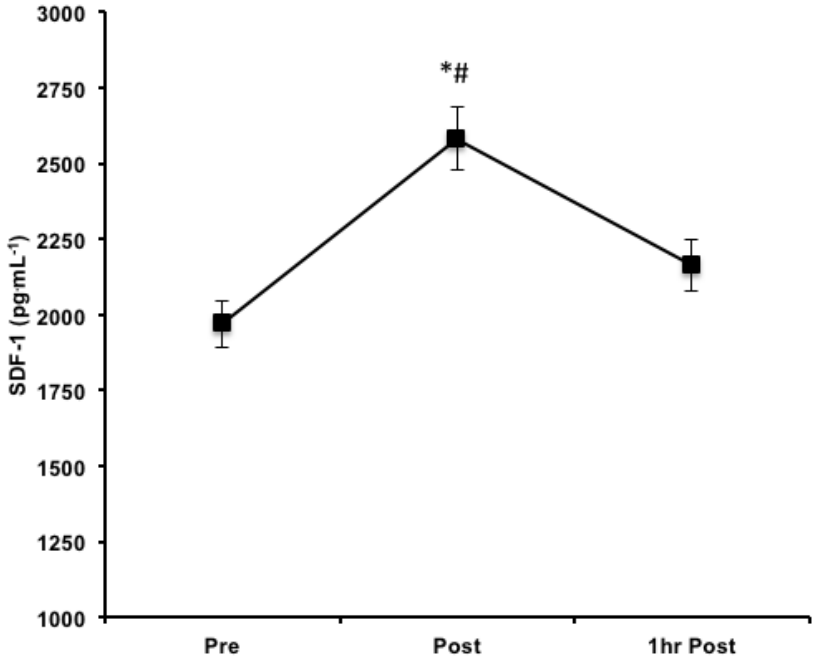

Figure 7.10. The response of circulating SDF-1α to acute endurance exercise (n=12). **p<0.05 vs. pre-exercise, #p<0.05 vs. post-exercise.*

To determine if systemic increases in CXCR4$^+$ T_{ANG} cells are attributable to systemic increases in SDF-1α univariate regression analyses were performed to assess the effect of changes in SDF-1α and CXCR4$^+$ T_{ANG} cells. There was no significant relationship between changes in SDF-1α and changes in CXCR4$^+$ T_{ANG} cells in response to the acute exercise bout (r=0.073, r^2=0.005, p=0.822). Additionally, the change in SDF-1α from pre- to post-exercise was not associated with the increase observed in CD8$^+$ T_{ANG} CXCR4 cell surface expression.

7.4 Discussion

Acute exercise is known to stimulate the mobilisation of T-cells into the peripheral circulation in humans (Simpson *et al.*, 2007; Turner *et al.*, 2010; Witard *et al.*, 2012). In fact, exercise preferentially mobilises specific T-cell subgroups, so that some T-cell groups enter and leave the circulation to a greater extent than others (Simpson *et al.*, 2010). The evidence strongly suggest a preferential mobilisation of senescent and highly differentiated T-cells, and these cells also leave the circulation to a greater extent than naïve T-cells (Simpson *et al.*, 2007; Spielmann *et al.*, 2014). $CD31^+$ T-cells are a group of T-cells which are potentially involved in maintenance of endothelial health (Weil *et al.*, 2011). Data shown in this thesis has shown that these cells are maintained in older age in those with higher levels of CRF than those with lower levels of CRF (*Chapter 5*). Thus far, there is no evidence to show the response of these cells to an acute bout of exercise, therefore the response of these cells were studied in a small cohort of young healthy men (age 24-44yrs) to a 10km running time trial.

The main finding of this study is that $CD3^+CD31^+$ T-cells are mobilised into the circulation in response to an acute exercise bout, and subsequently leave the circulation, mirroring the classic biphasic response of T-cells to exercise. The data also suggests that $CD8^+$ T_{ANG} cells are more responsive to an acute exercise bout than $CD4^+$ T_{ANG} cells. Interestingly, $CD31^-$ T-cells are mobilised to a greater extent than $CD31^+$ T-cells. Additionally, there is a preferential egress from the circulation of $CD31^-$ T-cells in the post-exercise recovery period. This is largely due to a greater mobilisation and subsequent egress of $CD8^+CD31^-$ T-cells observed.

This is the first study to document the response of $CD31^+$ T-cells to an acute exercise bout. Interestingly, $CD31^+$ T-cells are not as highly mobilised as $CD31^-$ T-cells. Previous studies have shown preferential mobilisation of T-cells expressing adhesion molecules (such as integrins and CD56) in response to exercise (Goebel *et al.*, 2000; Shephard, 2003; Simpson *et al.*, 2006) however the data presented in this study suggests this is not the case for CD31-expressing T-cells. $CD4^+$ and $CD8^+$ T-cells expressing senescent and highly differentiated cell surface markers ingress to a greater extent than less differentiated $CD8^+$ T-cells (Campbell *et al.*, 2009). The panel used within this study did not contain cell surface markers for senescence or differentiation status. The exact phenotypic definition of $CD8^+/CD4^+$ T-cells expressing or not expressing CD31 are unknown, and thus cannot

be determined if the greater mobilisation of $CD31^-$ T-cells is due to their senescent or differentiation status.

Potential mechanisms for the increase in circulating $CD31^+$ T-cells as a result of exercise include an increase in shear stress-mediated demargination of T-cells from the vascular endothelium, as well as catecholamine-dependent ß-adrenergic ingress into the circulation of T-cells expressing ß-adrenergic receptors (Schedlowski *et al.*, 1996; Simpson *et al.*, 2007). In addition, increases in circulating SDF-1α may stimulate $CXCR4^+$ T-cell chemotaxis into the peripheral blood compartment. SDF-1α is known to mobilise $CXCR4^+$ progenitors into the circulation (Powell *et al.*, 2005; Prokoph *et al.*, 2012), but as of yet the relevance and contribution of the SDF-1:CXCR4 axis in T-cell lymphocytosis has yet to be addressed. SDF-1α is significantly increased in systemic circulation of the male participants from pre- to post-exercise, and this is concomitantly accompanied by significant increases in $CXCR4^+$ T_{ANG} cells, more so than $CXCR4^-$ T_{ANG} cells, suggesting a role for the SDF-1:CXCR4 axis in this response. SDF-1α also displayed a biphasic response to acute exercise, with a large increase in circulating SDF-1α immediately post-exercise, and a return to baseline levels in the 1-hour post-exercise recovery period. SDF-1α has been shown to be increased in circulation post-acute exercise elsewhere (Van Craenenbroeck *et al.*, 2010b; Chang *et al.*, 2015), and is partly responsible for the acute increases in other CACs in response to an acute exercise stressor, such as EPCs (Chang *et al.*, 2015). Our data also shows that $CXCR4^+$ T_{ANG} cells were more responsive to exercise than T_{ANG} cells which were negative for CXCR4 expression, suggesting that CXCR4-dependent mechanisms may be at play. However, we failed to observe a significant relationship between SDF-1α release and $CXCR4^+$ T_{ANG} ingress into the circulation, suggesting that although $CXCR4^+$ cells are preferentially mobilised in comparison to $CXCR4^-$ cells, there may be other factors associated with increased expression of CXCR4 that may be responsible. As yet, these other factors have yet to be investigated.

Interestingly, CXCR4 expression was largely unchanged in the total $CD3^+$ T_{ANG} pool, with the same observation in $CD4^+$ T_{ANG} cells. However, CXCR4 expression was significantly upregulated on $CD8^+$ T_{ANG} cells, suggesting a differential effect of exercise on $CD8^+$ and $CD4^+$ T-cell receptor expression. CXCR4 expression is mooted to be related to the migratory capacity of these cells, and it appears that exercise either mobilises $CD8^+$ T_{ANG} cells with high levels of CXCR4 cell surface expression, or the circulating environment during exercise stimulates an upregulation of this chemotactic receptor. One

previous study has detailed the effect of exercise-related cortisol levels on CXCR4 expression on T-cells, whereby researchers incubated T-cells with exercise physiological circulating levels of cortisol *in vitro*, and observed this upregulation (Okutsu *et al.*, 2005). However, that study failed to measure any differential effects of the cortisol on $CD4^+$ or $CD8^+$ T-cells. This is the first study to document circulating changes in CXCR4 expression on T-cells. However, the functional significance of this finding is unknown, and whether this upregulation of CXCR4 expression translates to improved migration of this specific subset of CACs is also yet to be observed. However, careful interpretation is advised, as acute exercise may simply mobilise T-cells with an already heightened CXCR4 expression, as indicated by increased mobilisation of $CXCR4^+$ T_{ANG} cells compared to $CXCR4^-$ T_{ANG} cells, rather than any changes occurring at the individual cell level.

In conclusion, $CD31^+$ T-cells are mobilised in response to an exercise stressor, due to both increases in $CD4^+$ and $CD8^+$ T_{ANG} cells. $CD8^+$ T_{ANG} cells were more preferentially mobilised compared to $CD4^+$ T_{ANG} cells, and $CXCR4^+$ T_{ANG} cells mobilise in response to exercise more so than $CXCR4^-$ T_{ANG} cells, suggesting a potential role for the SDF-1:CXCR4 axis in T-cell response to exercise.

Chapter 8: The Impact of Persistent Viral Infection on Circulating $CD31^+$ T-Cells

8.1 Introduction

Human Cytomegalovirus (HCMV) infection is a ß-herpes virus, and between 45% and 75% of the Western population are positive for the virus (as assessed by CMV antibodies in serum/plasma) although this can vary with location and age (Colonna-Romano *et al.*, 2007; Vasto *et al.*, 2007). HCMV can directly infect a wide variety of cell types including fibroblasts, endothelial, epithelial, neuronal, VSMCs, monocytes/macrophages, granulocytes and BM cells (Plachter *et al.*, 1996; Mocarski, 2001). HCMV is asymptomatic in immunocompetent individuals, and once infected an organism it can remain latent for a prolonged period of time within cells before reactivating and multiplying. HCMV can reactivate due to various stimuli, for example inflammation (Humar *et al.*, 1999; Aiello *et al.*, 2008), elevated cortisol levels (Stowe *et al.*, 2012) and physiological adrenergic stress (Prösch *et al.*, 2000). However, in immunocompromised individuals, HCMV may cause disease, for example in AIDS patients, organ and BM transplant patients and in foetal infection. It can also result in death.

HCMV is linked to neutropenia, mononucleosis, thrombocytopenia, and haemolytic anaemia, and reduced immunological function (Weller *et al.*, 1962; Fiala *et al.*, 1973; Jordan *et al.*, 1973; Peterson *et al.*, 1980; Yeager *et al.*, 1983; Frasca *et al.*, 2015) reflecting a significant deleterious impact on the immune system. HCMV exerts a deleterious effect on T-cell-mediated immunity. As HCMV reactivates over periods of years, it can cause a shrinking of the naïve T-cell pool via an expansion of the cytotoxic T-cell pool via T-cells differentiating towards a cytotoxic phenotype. Those who are HCMV seropositive have greater proportion of highly differentiated T-cells than seronegative populations, causing a reduced basal level of naïve T-cells (Spielmann *et al.*, 2011). As a result, antigen virgin T-cells are reduced, leaving the body potentially open to infection by novel viruses that have yet to be experienced by the host. The effects of these recurring reactivation of virus, and consequent clonal expansion of cytotoxic T-cells is termed ‘immunoageing’, and is associated with ‘immunosenescence’- a concept of the T-cells becoming senescent, with reduced telomere length and telomerase activity, and potential for detrimental effect on function (Fulop *et al.*, 2013). $CD8^+$ and $CD4^+$ T-cell subset changes have been documented with prolonged HCMV infection, with an increase in effector memory and terminally differentiated T-cells, with a reduction in proportion of total T-cells that are naïve (Derhovanessian *et al.*, 2011; Spielmann *et al.*, 2011). Those who are CMV seropositive therefore have sustained immune activation profile as

characterised by a chronically elevated level of CRP, IL-18 and IFN-γ (van de Berg *et al.*, 2010) due to the cytotoxic profile of the terminally differentiated cells.

CMV seropositivity has been linked somewhat to the progression of CVD, with an increase in atherosclerotic risk, however, despite a high prevalence, the association between the presence of serum antibodies against CMV and atherosclerosis in humans is still debated (Arasaratnam, 2013; Courivaud *et al.*, 2013; Courivaud and Ducloux, 2013; Haeseker *et al.*, 2013). There is some limited evidence that CMV can accelerate MNC telomere shortening which could promote atherosclerotic plaque development (Spyridopoulos *et al.*, 2009), and that CMV infection could lead to atherosclerotic plaque development in kidney transplant recipients, with CMV reactivation, rather than serostatus being associated with reduced end point of atherosclerotic event or death (Courivaud *et al.*, 2013). Higher circulating CRP levels and increased proportion of effector memory T-cells could promote an atherogenic environment due to the inflammatory profile of CMV-exposed patients (Olson *et al.*, 2013) . Therefore CMV may not be directly associated with the development of atherosclerosis, but instead create a pro-inflammatory environment which can lead to atherosclerotic lesion development.

Murine CMV studies have shown that arteries in CMV-infected mice display vascular dysfunction, both endothelium-dependent and independent (Khoretonenko *et al.*, 2010; Gombos *et al.*, 2013), accompanied by exacerbated leukocyte adhesion to blood vessels (Khoretonenko *et al.*, 2010). These effects are likely due to CMV-infection of endothelial cells (Plachter *et al.*, 1996) as well as the indirect effects via the immune system, as increased production of IFN-γ by cytotoxic T-cells has been shown to reduce VSMC sensitivity to NO and decrease eNOS content (Koh *et al.*, 2004), and consequently result in reduced endothelial-dependent vasodilation. CMV infection of endothelial cells could promote a local inflammatory process which could lead to atherosclerotic plaque formation, as high levels of the virus have been found in carotid plaques in patients undergoing carotid endarterectomy (Yaiw *et al.*, 2013). Despite finding CMV within the atherosclerotic plaques, the source of the CMV, whether it having been initially in resident endothelial cells or potentially macrophages that infiltrate the developing plaque was not assessed.

It could be hypothesised that HCMV infection of BM progenitor cells could be an avenue by which HCMV can be linked with CVD, where the mobilisation and subsequent reactivation of the virus, can transfer the viral DNA to the endothelium where it may have

dysfunctional effects. It may also be that HCMV may cause apoptosis of these BM cells, depleting the BM pool, or may cause functional impairments, such as migration, or mobilisation of these cells. As yet, there is no evidence to support this.

8.2 Materials and Methods

8.2.1 Participants

Male participants (n=50), aged between 18 and 65 volunteered from the study, and consisted of a mixture of students and staff based at Edinburgh Napier University, in addition to general public from the Edinburgh area. Participant information, inclusion and exclusion criteria are detailed in Chapter 4 (*Influence of Age and Cardiorespiratory Fitness on Circulating Endothelial Progenitor Cells and CXCR4 Cell Surface Expression*). Ethical approval for the study was given by the Edinburgh Napier University Research and Ethics Governance Committee.

8.2.2 Resting Measures

On arrival to the Human Performance Laboratory, after informed consent was given, participants were measured for height and body mass, with values used to calculate BMI. Waist and hip circumference were also measured as described in materials and methods *Chapter 3.2*. Resting blood pressure was measured after 5-minute rest in a supine position using an automated sphygmamometer (Nonin Puresat Avant 2120, Nonin Medical Inc, Minnesota, USA; Ultra-Check® Blood Pressure Adult Cuff, Statcorp Medical, Florida, USA). Participant characteristics are shown in table 8.1.

Table 8.1. Participant characteristics.

	All (n=50)	CMV^+ (n=25)	CMV^- (n=25)
Age (years)	39 ± 2	39 ± 3	40 ± 3
Height (m)	1.82 ± 0.02	1.81 ± 0.02	1.83 ± 0.02
Body Mass (kg)	83.04 ± 1.28	83.37 ± 1.93	82.71 ± 1.72
BMI ($kg \cdot m^2$)	26.02 ± 0.34	26.61 ± 0.55	25.43 ± 0.38
SBP (mmHg)	129 ± 2	130 ± 3	128± 3
DBP (mmHg)	79 ± 1	79 ± 2	80 ± 2
MAP (mmHg)	96 ± 1	96± 2	96 ± 2
Waist (cm)	89.79 ± 1.19	90.22 ± 1.81	89.36 ± 1.57
Waist-to-Hip Ratio	0.94 ± 0.01	0.95 ± 0.01	0.94 ± 0.01

BMI- Body Mass Index, SBP- Systolic Blood Pressure, DBP- Diastolic Blood Pressure, MAP- Mean Arterial Pressure. Values shown are mean ± SD.

8.2.3 Blood Sampling and PBMNC Isolation

Blood was taken from participants after a 5-minute supine rest by a certified phlebotomist. Blood samples were drawn into 6mL vacutainers (BD Biosciences, UK), which were either coated in EDTA to prevent coagulation or serum gel. EDTA blood (3 x tubes) was processed for PBMNC as previously described in general materials and methods *Chapter 3.4*. Isolated PBMNCs were then used for T_{ANG} number and CXCR4 enumeration (see *6.2.7 Angiogenic T Cell Number and CXCR4 Expression Quantification)*, and serum was used for analysis of serum for chemotactic factors and selected inflammatory markers (see *6.2.8 Quantification of Serum Inflammatory Markers*).

8.2.4 Angiogenic T Cell Cell Number and CXCR4 Expression Quantification

Isolated PBMNCs ($0.5x10^6$) were labelled with monoclonal antibodies anti-CD3-APC, anti-CD31-FITC and anti-CXCR4-PE-Cy5 (all BD Biosciences), and were left to incubate at 4°C for 45 minutes in the dark prior to flow cytometric analysis as detailed in general materials and methods *Chapter 3.6*. CXCR4 cell surface expression was analysed as a surrogate for migratory ability of T-cells.

8.2.5 Quantification of CMV Serostatus

Serum samples were analysed in duplicates for CMV IgG antibodies by ELISA in accordance with manufacturer's instructions (BioCheck Inc, USA) with the mean values recorded, and CMV IgG index calculated as the mean value of the samples divided by the mean value of calibrator 2. Samples were read at 450nm using a 96 well microplate reader (LT-5000MS ELISA Reader, Labtech International Ltd, UK).

8.2.6 Statistical Analysis

Data was assessed for normal distribution using the Shapiro-Wilk test for normality. Not normally distributed data was logarithmically transformed ($\log_{10}$ or $\log_n$).

Participants were grouped into clusters for age groupings. Clusters were identified by hierarchical cluster analysis using Ward's method for identifying groupings, as well as using the squared Euclidean distance to measure intervals between groups (Mooi and Sarstedt, 2011). Results of the cluster analysis identified 2 age groups. Participants were thus grouped into the following age groups accordingly: 18-40yrs, and 46-65yrs.

CAC levels and CXCR4 cell surface expression, as well as various circulating inflammatory markers were compared between CMV^+ and CMV^- individuals by two-way analysis of variance (ANOVA) with Fisher's Least Significant Differences (LSD) post-hoc test, with age grouping and CMV serostatus as the independent factors.

Univariate linear regression analyses were performed for various T_{ANG} subsets and age for CMV^+ and CMV^- populations respectively to assess the role CMV may play in T_{ANG} ageing.

Data was analysed using SPSS for Macintosh, version 20 (IBM, Chicago, USA). Significance alpha was set at p=0.05.

8.3 Results

8.3.1 CMV Serostatus and CD31$^+$ T-Cells Subsets

Two-way ANOVAs were performed to assess the influence of persistent viral infection on CD31$^+$ T-cells and their subsets (CD4/CD8). Total CD31$^+$ T-cells (expressed as cells·μL^{-1} or % of total CD3$^+$ T-cells) did not differ between CMV seropositive and CMV seronegative individuals, independent of age (figures 8.1 and 8.2). There appeared to be elevated T_{ANG} cells in the younger individuals who are CMV seropositive compared to their age-matched CMV seronegative counterparts, however this was not statistically significant despite a moderate effect size (60.73 ± 2.50% [55.70-65.77] 95% CI, vs. 56.72 ± 2.50% [51.69-61.75], p=0.262, ES= 0.49). CD8$^+$ T_{ANG} cells appeared to be greater in CMV$^+$ compared to CMV$^-$ individuals within the 18-40yrs age group (364 ± 56 cells·μL^{-1} [185-542 cells·μL^{-1}], 95% CI, vs. 272 ± 30 cells·μL^{-1} [196-347] 95% CI), but this was not statistically significant (p=0.060, ES=1.00), but not in the 46-65yrs group (241 ± 30 cells·μL^{-1} [168-314 cells·μL^{-1}], 95% CI, vs. 193 ± 27 cells·μL^{-1} [119-267], 95% CI) (figure 8.1). However, the percentage of CD8$^+$ T-cells expressing the adhesion marker CD31 was significantly greater in the CMV$^+$ group compared to the CMV$^-$ individuals in the 18-40yrs group (87.93 ± 1.24% [83.98-91.88%], 95% CI, vs. 75.86 ± 1.26% [72.61-79.11], 95% CI, p=0.043), but as with total CD8$^+$ T_{ANG} cell number, this was not evident in the 46-65yrs group (71.43 ± 4.31% [60.90-81.97%] 95% CI, vs. 71.57 ± 4.91% [57.95-85.20] 95% CI) (figure 8.3).

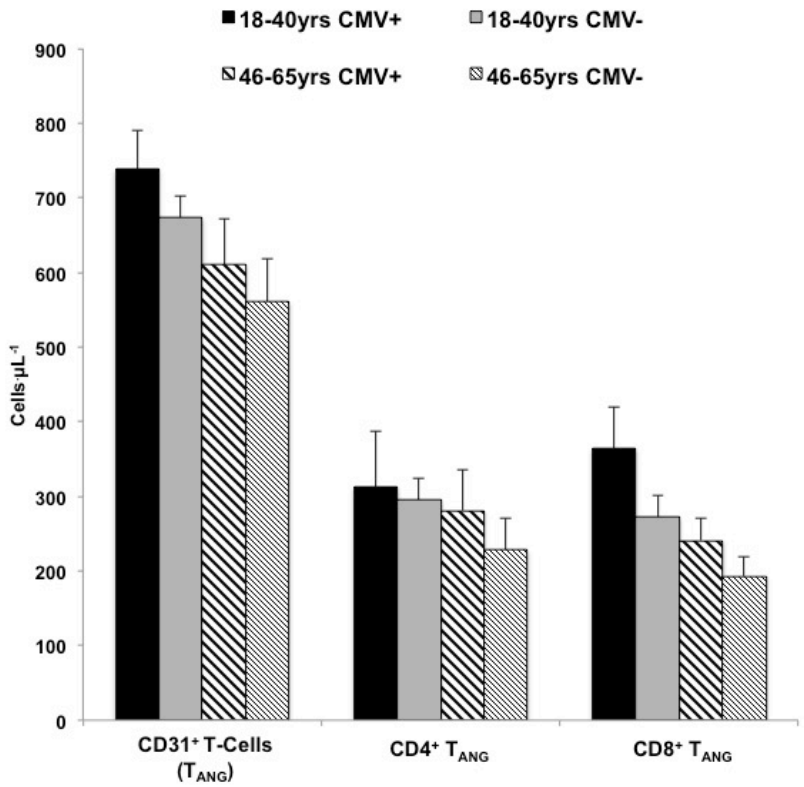

Figure 8.1. Circulating $CD31^+$ T-cells in those aged 18-40yrs and 46-65yrs by CMV serostatus. *Values shown are mean ± SEM, p=NS*

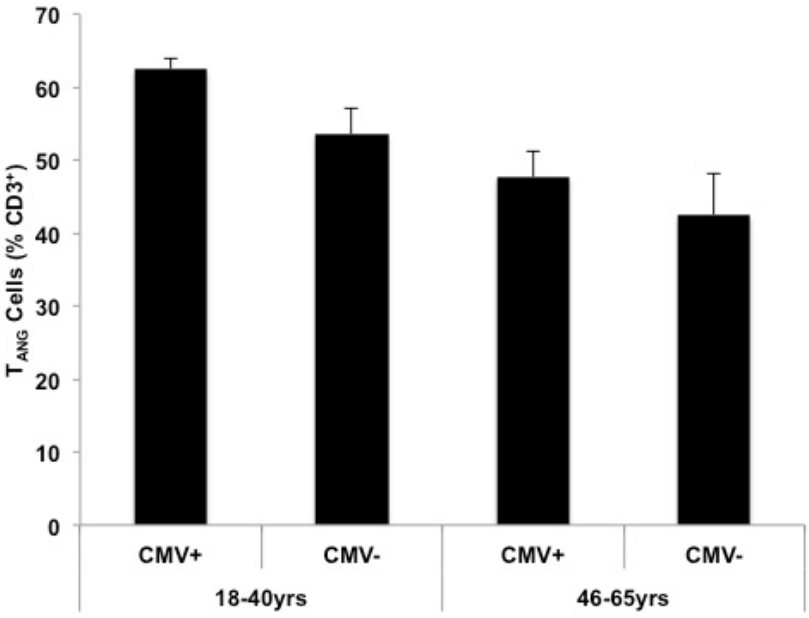

Figure 8.2. Circulating $CD31^+$ T-cells (expressed as percentage of total $CD3^+$ T-cells) in those aged 18-40yrs and 46-65yrs by CMV serostatus. *Values shown are mean ± SEM, p=NS.*

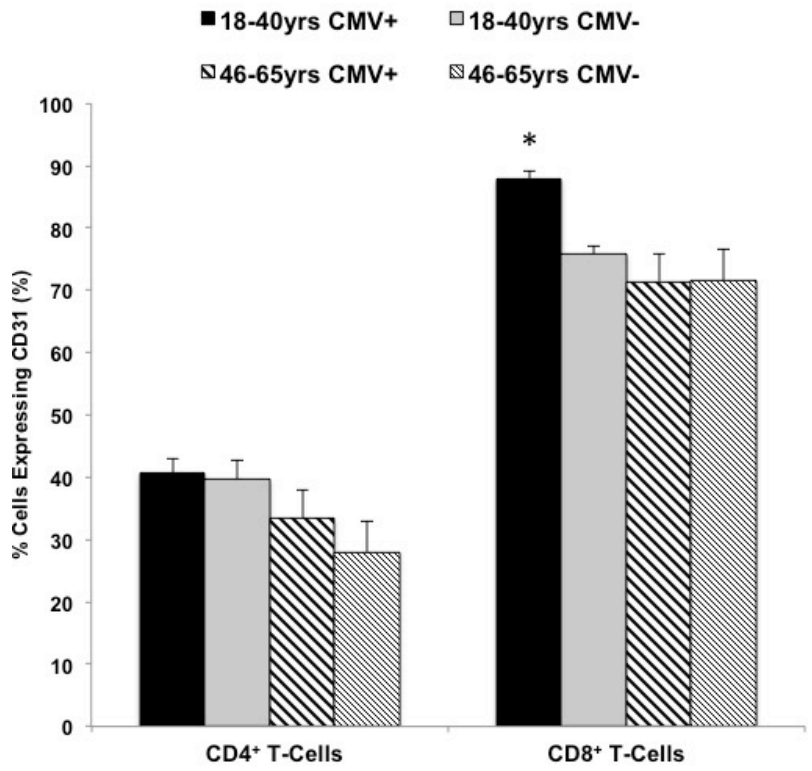

Figure 8.3. Circulating $CD4^+$ and $CD8^+$ T-cells expressing CD31 in men aged 18-40yrs and 46-65yrs by CMV serostatus. *Values shown are mean ± SEM, *p<0.05 vs. 18-40yrs CMV-*.

There was no difference in $CXCR4^+$ T_{ANG} cells (total, $CD4^+$, $CD8^+$) or CXCR4 cell surface expression on these cells (% of T_{ANG} expressing CXCR4) between $CMV^{+/-}$ groups (figure 8.4).

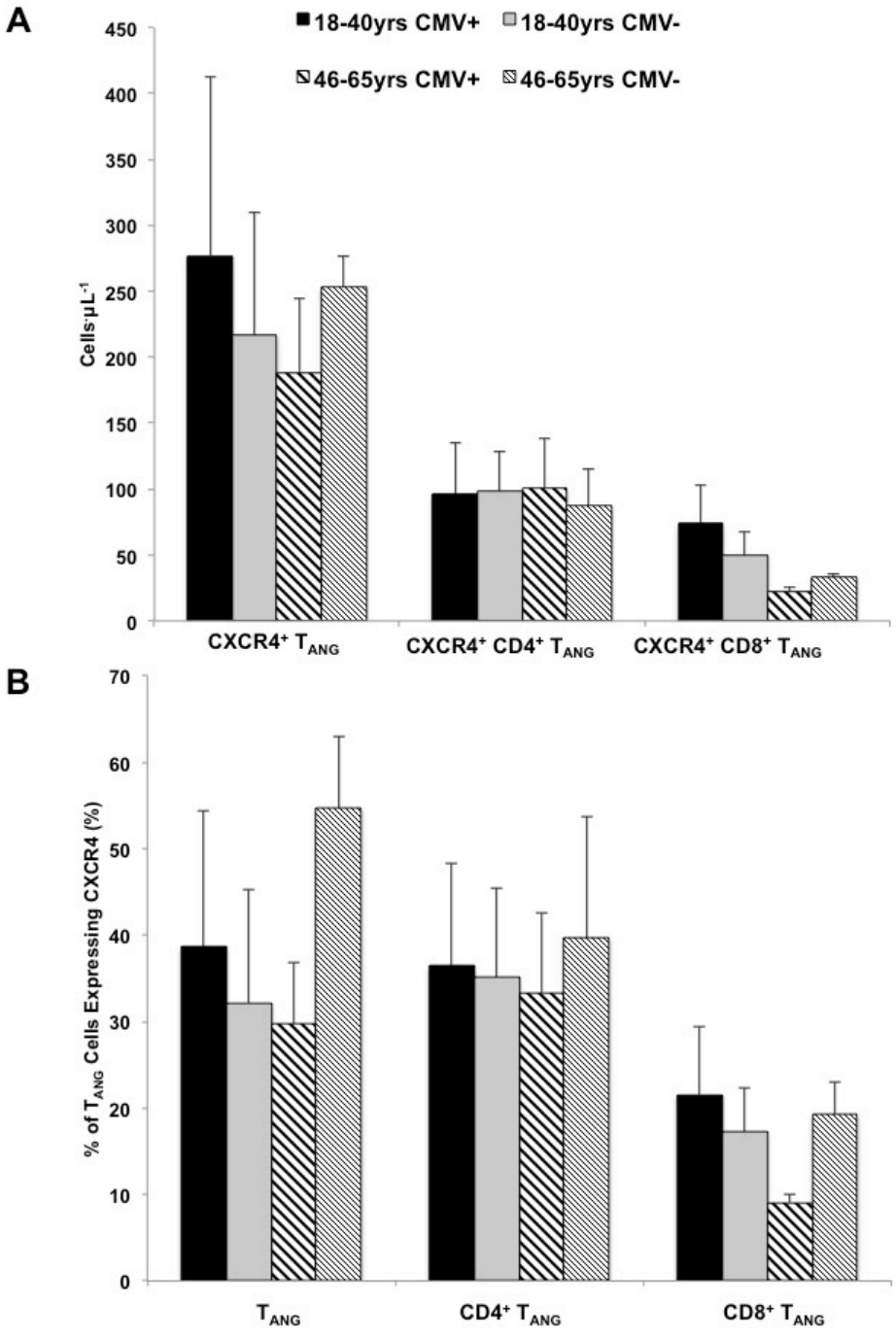

Figure 8.4. Effect of CMV serostatus on CXCR4 cell surface expression on $CD31^+$ T-cells in males aged 18-40yrs and 46-65yrs. *Values shown are mean ± SEM, p=NS.*

To confirm the findings that CXCR4 expression was unaffected by CMV serology, MFI of CXCR4 on $CXCR4^+$ T_{ANG} cells and their subsets (CD4/CD8) was unaffected by CMV serostatus (table 8.2).

Table 8.2. CMV serology and CXCR4 cell surface expression intensity (Mean Fluorescence Intensity) on T_{ANG} cells.

	18-40yrs		**46-65yrs**	
	CMV^+	**CMV^-**	**CMV^+**	**CMV^-**
T_{ANG}	9.20 ± 0.79	13.65 ± 2.65	8.33 ± 0.22	10.79 ± 0.91
$CD4^+$ T_{ANG}	10.98 ± 1.11	10.58 ± 0.95	9.83 ± 0.47	10.08 ± 0.97
$CD8^+$ T_{ANG}	7.97 ± 0.44	7.45 ± 0.35	7.81 ± 0.25	8.15 ± 0.50

Values shown are mean ± SEM

8.3.2 Univariate Regression Analysis. CMV Serostatus and Relationship with $CD31^+$ T-Cell Pool Depletion with Ageing

CMV is associated with immunological ageing, as observed by a depletion of naïve cells and increased proportion of senescent and highly differentiated T-cells (Pawelec and Derhovanessian, 2011; Simpson *et al.*, 2012; Spielmann *et al.*, 2014). Therefore univariate regression analyses were performed for various T_{ANG} subsets and age for CMV^+ and CMV^- respectively to assess if CMV could play a role in accelerate T_{ANG} depletion with age.

Ageing, as shown in *Chapter 5*, is associated with a decline in CD31-expressing T-cells. Figure 8.5 demonstrates the decline in $CD31^+$ T-cells with age being greater in CMV seronegative population (CMV^+: r=-0.360, r^2= 0.129, p=0.077; CMV^-: r=-0.629, r^2=0.396, p=0.001; figure 8.5B).

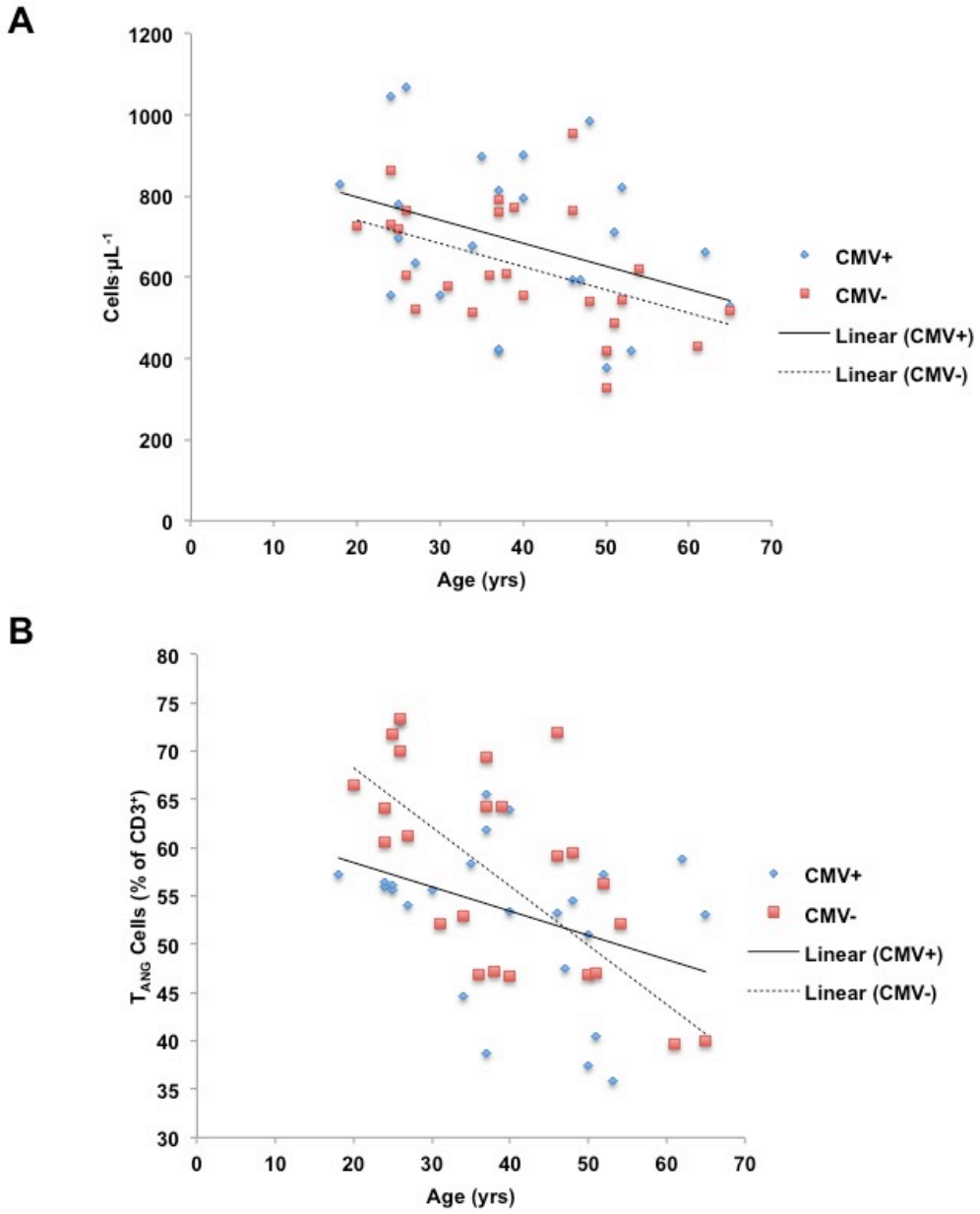

Figure 8.5. Relationship between chronological age and $CD31^+$ T-cells (cells·µL^{-1}; A, % of $CD3^+$ T-cells; B) in CMV seropositive and CMV seronegative males. **A**: CMV^+ r=-0.356, r^2=-0.127 p=0.081, CMV^- r=-0.470, r^2=-0.221, p=0.018, **B**: CMV^+: r=-0.360, r^2= 0.129, p=0.077; CMV^-: r=-0.629, r^2=0.396, p=0.001.

The greater rate of decline in $CD31^+$ T-cells with age in CMV seronegative population may be due to the observed decline in $CD4^+$ T-cells expressing CD31 in a sub cohort of the study (CMV^+: r=-0.331, r^2= 0.110, p=0.320; CMV^-: r=-0.573, r^2=0.329, p=0.065, figure 8.6A). Subsequent analysis of $CD8^+$ T-cell population showed that CMV seropositive individuals display a greater decline in $CD8^+$ T-cells expressing CD31 than CMV seronegative individuals (CMV^+: r=-0.651, r^2=0.423, p=0.030; CMV^-: r=-0.352, r^2=0.124, p=0.288; figure 8.6B).

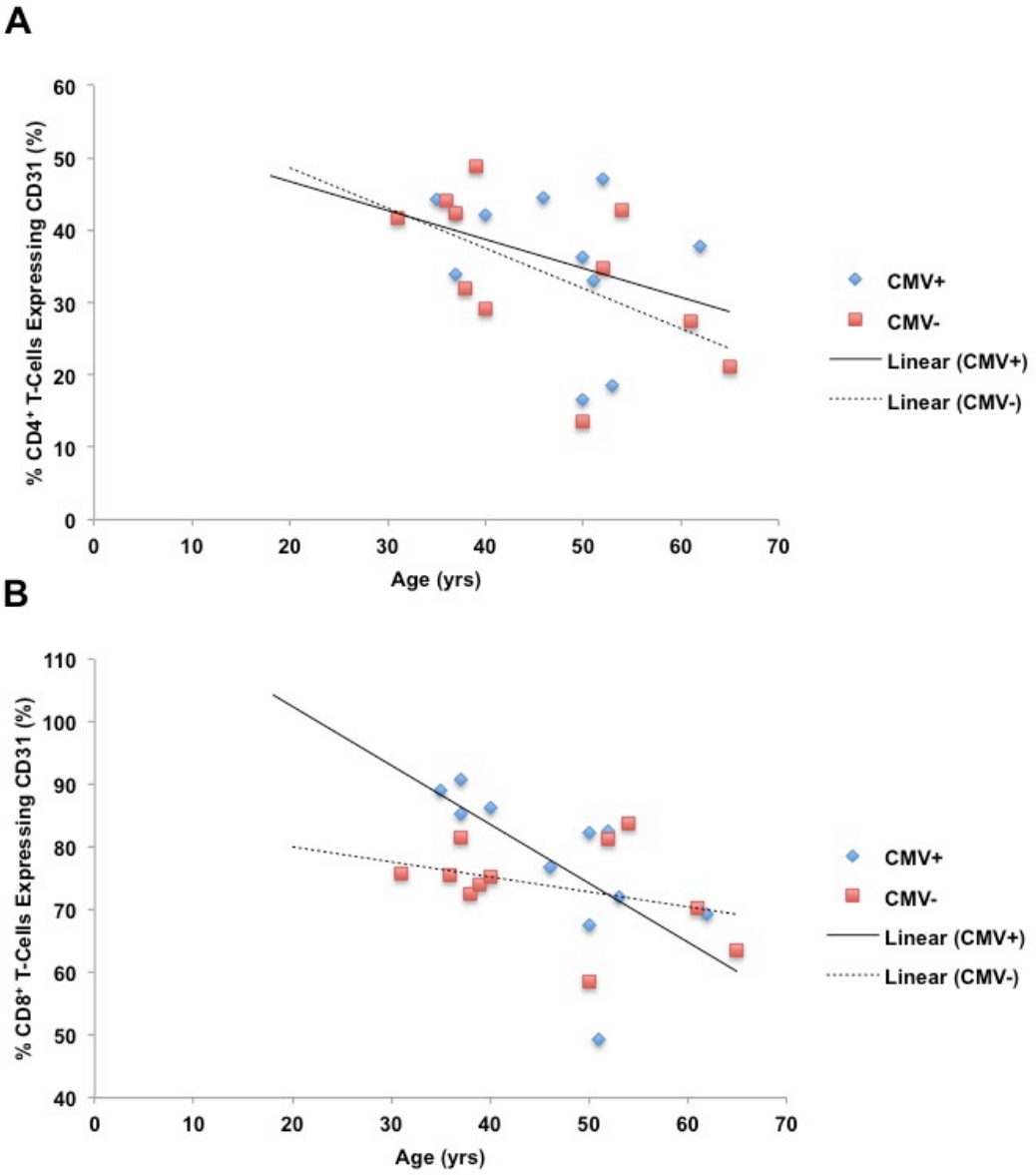

Figure 8.6. CMV serostatus-dependent $CD4^+$ (A) and $CD8^+$ (B) T_{ANG} ageing. **A**: CMV^+: r=-0.331, r^2= 0.110, p=0.320; CMV^-: r=-0.573, r^2=0.329, p=0.065, **B**: r=-0.651, r^2=0.423, p=0.030; CMV^-: r=-0.352, r^2=0.124, p=0.288

8.4 Discussion

CMV seropositivity in a young-middle aged cohort appeared to be associated with a rise in $CD8^+$ T_{ANG} cells in the peripheral circulation of healthy males. This effect of CMV was not seen in the older 46-65yrs group. Additionally, CMV seropositivity actually appeared to be protective in part, against the age-related decline in $CD31^+$ T-cells, through reduced $CD4^+$ T_{ANG} loss. On the other hand, CMV seropositivity was associated with a greater decline in CD31-expressing $CD8^+$ T-cells. The attenuated decline in $CD31^+$ T-cells with chronological age observed is probably due to a reduced loss of $CD4^+$ T_{ANG} cells compared to the loss of $CD8^+$ T_{ANG} cells seen with CMV seropositivity. Furthermore, CMV had no effect on CXCR4 cell surface expression of these cells.

CMV has been strongly implicated in the process of premature immunological ageing, or 'immuno-ageing' (Vasto *et al.*, 2007; Pawelec and Derhovanessian, 2011). CMV, and other persistent viral infections, such as Epstein Barr virus (EBV) are known to be linked with a reduction in the naïve T-cell pool, and an expansion of the senescent and highly differentiated T-cell phenotype in peripheral blood lymphocytes (Ouyang *et al.*, 2003; Simpson, 2011; Simpson *et al.*, 2012; Wang *et al.*, 2014a). However it is CMV which is reported to have the greatest effect on T-cell-mediated immunity (Koch *et al.*, 2007). CMV viral reactivation can stimulate naïve T cells to replicate and differentiate into effector cells, and multiple rounds of division lead to T-cell senescence and differentiation (Simpson, 2011). Consequently CMV-specific cytotoxic T-cells in the blood are elevated which has a large impact on health of the elderly, as numbers of these CMV-specific T-cells are associated with mortality (Pawelec *et al.*, 2010). It is considered so harmful, that it is the main reactivating virus included in the immune risk profile (IRP), which is a wide array of immune markers which can be used to predict mortality in elderly humans (Pawelec *et al.*, 2010; Simpson, 2011).

CMV has also been linked with CVD, with an association between the presence of serum antibodies against CMV and atherosclerosis still being debated (Arasaratnam, 2013; Courivaud *et al.*, 2013; Courivaud and Ducloux, 2013; Haeseker *et al.*, 2013). There is some limited evidence that CMV can accelerate MNC telomere shortening, via excessive rounds of replication, which could promote atherosclerotic plaque development (Spyridopoulos *et al.*, 2009) and that CMV infection could lead to atherosclerotic plaque development in kidney transplant recipients, with CMV reactivation, rather than serostatus being associated with reduced end point of atherosclerotic event or death

(Courivaud *et al.*, 2013). Higher circulating CRP levels and increased proportion of effector memory T-cells could promote an atherogenic environment due to the inflammatory profile of CMV-exposed patients (Olson *et al.*, 2013). Therefore CMV may not be directly implicated with the development of atherosclerosis, but instead create a pro-inflammatory environment which can lead to atherosclerotic lesion development. Murine CMV studies have shown that arteries in CMV-infected mice display vascular dysfunction, both endothelium-dependent and independent (Khoretonenko *et al.*, 2010; Gombos *et al.*, 2013), accompanied by exacerbated leukocyte adhesion to blood vessels (Khoretonenko *et al.*, 2010).

$CD31^+$ T-cells are associated with maintenance of endothelial function (Weil *et al.*, 2011), and lower numbers of these cells are seen in those with vascular disease compared to age-matched healthy controls (Rouhl *et al.*, 2012), and thus may provide to be an easy and reliable biomarker for endothelial function and CVD risk. Data from this study is the first to demonstrate that CMV seropositivity is linked with elevated $CD8^+$ T_{ANG} cells in a young and middle-aged population. CMV seropositivity is also associated with an attenuated decline in $CD31^+$ T-cells with age, via $CD8^+$ T_{ANG} mechanism, thus potentially offering a protective effect on the cardiovascular system. The elevated $CD8^+$ T_{ANG} cell number and attenuated decline of these $CD8^+CD31^+$ T-cells may be due to the observed maintained or elevated $CD8^+$ cytotoxic T-cells within CMV seropositive individuals (Turner *et al.*, 2010; Simpson, 2011), however, without further analysis of cell surface markers on these cells, we cannot confirm this hypothesis.

Interestingly, $CD8^+$ T-cells expressing CD31 displayed a greater decline with age in the CMV seropositive individuals than CMV seronegative individuals. However, it is proposed that the decline in $CD8^+CD31^+$ T-cells with CMV seropositivity may not be so large as to accelerate the decline in total $CD31^+$ T-cells due to the maintenance of the $CD4^+$ subset in this cohort, as it was seen that total $CD31^+$ T-cells in CMV^+ individuals showed an attenuated reduction with age compared to CMV^- individuals.

Pistillo *et al.* (2013) documented similar elevation in γδ T-cells in young individuals who are CMV seropositive compared to seronegative individuals. As addressed in Chapter 5, around 15% of the total $CD3^+CD31^+$ T_{ANG} cells do not express either CD4 or CD8, which would suggest that some of the T_{ANG} cells might in fact belong to the γδ T-cell group. These γδ T-cells rapidly expand upon primary CMV infection (Fornara *et al.*, 2011), and the elevations observed are not apparent in those who have CMV infection for 5yrs+. This

would suggest that the expansion of these γδ T-cells is acute, and would fit with the elevations observed in young individuals, and not in the older group observed by Fornara *et al.* (2011). The lack of difference observed in the older group in both T_{ANG} and γδ T-cells could be attributed to the speculation that older individuals may have harboured CMV for a longer time.

Unsurprisingly CXCR4 expression was unaffected by CMV serostatus. Some reports suggest that cell migration may be affected by CMV in trophoblasts and monocytes (Warner *et al.*, 2012; Varani and Frascaroli, 2013), however no data currently exists for lymphocyte migration. This is an area of research in the future.

Conclusion

CMV seropositivity is associated with an attenuated ageing effect on T_{ANG} cells, with maintained $CD31^+$ T-cells with age compared to CMV seronegative individuals. This may be due to attenuated $CD4^+$ T-cell loss of CD31 expression. However CMV seropositivity is also associated with an accelerated ageing loss of CD31 expression on $CD8^+$ T-cells. CMV serostatus differentially affects $CD4^+$ and $CD8^+$ T-cells expressing CD31. The potential impact of this on the cardiovascular system is unknown.

Chapter 9: General Discussion

The studies that comprised this Ph.D thesis aimed to investigate the effects of chronological age, CRF, sedentary behaviours, exercise and viral serology on various circulating cells shown to be involved in vascular growth and repair, and thus play a very important role in CVD prevention. Data was collected from healthy men of various ages, physical activity and sedentary lifestyle behaviours. The analysis of circulating progenitor cells and $CD31^+$ and $CD31^-$ T-cells were performed using four-colour flow cytometry.

9.1 Main Findings

The aim of this thesis was to address the impact of chronological age, CRF, exercise and sedentary behaviours on circulating progenitor cells and $CD31^+$ T-cells (CACs) and cardiometabolic risk factors. Many novel findings have resulted from the various studies presented within this thesis. The main findings of the studies described within the thesis are:

Aim 1: Examine the effect of chronological age and CRF (as measured by estimated $\dot{V}O_{2max}$) on circulating $CD34^+$ progenitor cells and the cell surface expression of a key migratory and mobilisation factor, CXCR4, in apparently healthy men (*Chapter 4).*

Main Findings:

(1) Chronological age was inversely associated with $CD34^+$ progenitor cells and $CD34^+CD45^{dim}VEGFR2^+$ EPCs. In addition, progenitor cells expressing CXCR4 were also negatively affected by advancing age.
(2) CRF had no effect on these progenitor cell subsets after correcting for age.
(3) The greatest predictor for circulating EPCs were SDF-1α levels in circulation, which itself demonstrated an age-related decline, potentially offering mechanistic effect of advanced chronological age on these progenitor cell subsets.

Hypothesis: advancing age is associated with reduced number of $CD34^+$ progenitors and CXCR4 cell surface expression *(Accept*), which can be attenuated by increasing CRF *(Reject).*

Aim 2: Examine the effect of chronological age and CRF (as measured by estimated $\dot{V}O_{2max}$) on circulating $CD31^+$ T-cells and the cell surface expression of CXCR4 in apparently healthy men (*Chapter 5*).

Aim 3: Investigate any effects of age and/or CRF on $CD4^+$ and $CD8^+$ T-cells expressing CD31.

Main Findings:

(1) Advancing age was associated with a decrease in $CD31^+$ T-cell number and $CXCR4^+$ T_{ANG} cells.
(2) CRF is linked with increased numbers of T_{ANG} cells.
(3) The benefit of higher CRF levels on $CD31^+$ T-cells was due to increased number of $CD4^+$ T_{ANG} cells.

Hypothesis: advancing age is associated with reduced number and proportion of $CD31^+$ T-cells with concomitant decline in CXCR4 cell surface expression. CRF attenuates the effect of age on these cells either through CD4 or CD8-dependent mechanism *(Accept*).

Aim 4: The primary aim of this study was to examine the effect of sedentary behaviours on both $CD34^+$ progenitor cells, $CD31^+$ T-cells and other cardiometabolic risk factors, such as BMI, waist circumference, fasting glucose and inflammatory cytokines (*Chapter 6*).

Aim 5: The secondary aim of this study was to assess if CRF attenuates any deleterious effect sedentary behaviours have on any CAC subset or cardiometabolic risk factors (*Chapter 6*).

Main Findings:

(1) An increase in sitting time was associated with a decrease in $CD31^+$ T-cells.
(2) The decrease in $CD31^+$ T-cells with sedentary behaviours is potentially linked to a decrease in CD31 expression on $CD4^+$ T-cells.

(3) After correcting for CRF, the association between sitting time and $CD31^+$ T-cells no longer existed.
(4) Increased sitting time appears to be also linked with a decreased CXCR4 expression on $CD34^+$ progenitors and $CD34^+CD45^{dim}VEGFR2^+$ EPCs.

Hypothesis: Increasing sitting time and screen time will be associated with reduced number of $CD34^+$ progenitor cells and $CD31^+$ T-cells, which will be attenuated by increasing CRF levels *(Accept)*.

Aim 6: The primary aim of this study was to evaluate the changes in $CD31^+$ and $CD31^-$ T-cells to an acute bout of strenuous exercise (*Chapter 7*).

Aim 7: The secondary aim of this study was to assess the relationship between changes in circulating SDF-1α and the changes in $CXCR4^+$ T-cells expressing CD31 (*Chapter 7*).

Main Findings:

(1) Strenuous exercise mobilised $CD31^+$ T-cells into the blood.
(2) $CD31^-$ T-cells were preferentially mobilised into circulation as a result of acute strenuous exercise.
(3) This preferential mobilisation is due to greater ingress of $CD8^+CD31^-$ T-cells.
(4) $CXCR4^+$ T_{ANG} cells mobilise to greater extent than $CXCR4^-$ T_{ANG} cells as a result of acute endurance exercise, suggesting a role for SDF-1:CXCR4 axis in the T_{ANG} cell response to exercise.

Hypothesis: Strenuous exercise causes an ingress of $CD31^+$ T-cells *(Accept)*, more so than $CD31^-$ T-cells *(Reject)* and this ingress is associated with CXCR4 expression on the cell surface of these cells *(Accept)*.

Aim 8: The primary aim of this study was to evaluate the impact of persistent viral infection (cytomegalovirus; CMV) on $CD31^+$ T-cells (*Chapter 8)***.**

Aim 9: The secondary aim of this study was to assess the $CD4^+$ and $CD8^+$ T_{ANG} cell differences between CMV seropositive versus seronegative individuals (*Chapter 8)***.**

Aim 10: The final aim of this study was to assess the impact CMV plays in the age-related decline in $CD31^+$ T-cells (*Chapter 8)***.**

Main Findings:

(1) CMV seropositivity is associated with attenuated ageing of T_{ANG} cells
(2) CMV seropositive individuals appear to have greater circulating number of $CD8^+CD31^+$ T-cells compared to CMV seronegative individuals.
(3) CMV seropositive individuals have reduced CD31 expression on cell surface of $CD4^+$ T-cells compared to CMV seronegative individuals.

Hypothesis: CMV is linked with reduced number of $CD31^+$ T-cells as a result of $CD4^+$ and $CD8^+$ T-cell changes within CMV seropositive individuals. In addition, CMV will be linked with accelerated ageing of the $CD31^+$ T-cell pool *(Reject)*.

9.2 Ageing

Advanced chronological ageing is associated with increased CVD risk (Lozano *et al.*, 2012). Reduced endothelial function (Taddei *et al.*, 2001; Muller-Delp, 2006; Soucy *et al.*, 2006; Black *et al.*, 2008; Black *et al.*, 2009) and angiogenic capacity (Rivard *et al.*, 1999; Sadoun and Reed, 2003; Reed and Edelberg, 2004; Wang *et al.*, 2011; Gunin *et al.*, 2014) of individuals with advancing age are reported to be two factors contributing to the age-related risk of CVD onset and/or progression. Muscle capillary content is also reduced in metabolic syndrome (Frisbee *et al.*, 2006) indicating that angiogenesis may play a role in attenuating disease processes. CACs play an important role in maintenance of tissue capillarity and endothelial function (Hur *et al.*, 2007; Sibal *et al.*, 2009; Kushner *et al.*, 2010b; Weil *et al.*, 2011; Bruyndonckx *et al.*, 2014), through promotion of angiogenesis, either by paracrine means or by differentiating into mature endothelial cells. EPCs (Heiss *et al.*, 2005; Thijssen *et al.*, 2006; Hoetzer *et al.*, 2007; Thum *et al.*, 2007; Xia *et al.*, 2012a; Xia *et al.*, 2012b; Williamson *et al.*, 2013; Yang *et al.*, 2013) and T_{ANG} (Kushner *et al.*, 2010c) have been shown to be reduced or dysfunctional with increasing age. The reduced number and function of these cells may contribute to the increased CVD risk with age.

Data from *Chapter 4* and *Chapter 5* show that a selection of these CACs, namely $CD34^+$ progenitor cells, EPCs and T_{ANG} cells are reduced with advancing age. Additionally, CXCR4-expressing CACs were also reduced with advancing age, which may be linked to the reduced circulating SDF-1α, which was found in older individuals compared to their younger counterparts. The data presented confirms reports by previous studies showing reduced number of EPCs (Thijssen *et al.*, 2006; Thum *et al.*, 2007) and T_{ANG} (Kushner *et al.*, 2010c) cells with advancing age, however, this is the first study showing reduced number of $CXCR4^+$ CACs ($CD34^+$, $CD34^+CD45^{dim}VEGFR2^+$, $CD3^+CD31^+$). Although many studies have shown reduced EPCs with age, this is also the first study to show that $CD45^{dim}$ EPCs are diminished with age. As these are the cells reported to directly participate in endothelial repair via differentiating into mature endothelial cells (Case *et al.*, 2007; Timmermans *et al.*, 2007), these cells may reflect endothelial repair capacity more specifically than total $CD34^+VEGFR2^+$ cells that have been reported previously in the literature.

We failed to observe any effect of age on $CD4^+$ or $CD8^+$ T_{ANG} subset, probably due to these cell surface markers of T-cells only being measured in participants within the 31-50yrs and 51-65yrs group, and as we observed significant reductions in total T_{ANG} in the 31-50yrs and 51-65yrs group vs. 18-30yrs, and not between the 31-50yrs and 51-65yrs groups, then we would expect to find no changes. Junge *et al.* (2007), Gomez *et al.* (2003) Kilpatrick *et al.* (2008) demonstrated that $CD4^+$ cells reduce their CD31 expression with age in humans, and the loss of CD31 expression was greater from birth to age 20 compared to 21-80 yrs, yet there was still a trend of CD31 loss between 20-80 yrs (Junge *et al.*, 2007). These cells were predominantly naïve $CD4^+$ T-cells with high telomerase activity, indicating a low replicative history. The loss of CD31 on these $CD4^+$ T-cells may be explained in part due to the increased activation of $CD4^+$ T-cells with age. Further studies are required to elucidate the effect of ageing in both $CD4^+$ and $CD8^+$ T_{ANG} cells.

The reduced CXCR4 cell number within the $CD34^+$ progenitor and T_{ANG} cell subsets may reflect an impaired function of this cell pool. CXCR4 cell surface expression is linked to migratory capacity of various cell subtypes, including CACs (Walter *et al.*, 2005; Hur *et al.*, 2007; Bryant *et al.*, 2012; Xia *et al.*, 2012a; Xia *et al.*, 2012b; Adams *et al.*, 2013; Mao *et al.*, 2014), emphasising its importance in CAC function. However Xia *et al.* (2012b) failed to observe a reduction in CXCR4-expressing cells or CXCR4 cell surface expression intensity, but rather found a reduction in CXCR4:JAK-2 signalling, as

identified by reduced phosphorylation of JAK-2 under stimulation with SDF-1α with older men compared to younger men. The reduction in $CXCR4^+$ progenitor cell subsets may be a direct result of the overall reduction in the progenitor cell pool, rather than specific $CXCR4^+$ cells. Yet our data further shows that the proportion of these progenitor cells expressing CXCR4 also reduced progressively from 18-30yrs to 51-65yrs. In addition, the CXCR4 expression intensity, although not affected by age in the total $CD34^+$ progenitor cell group, was significantly reduced in the 51-65yrs group compared to the 18-30yrs and 31-50yrs group, further suggesting a negative impact of age on these cells. Interestingly, the percentage of $CD8^+$ T_{ANG} cells expressing CXCR4 was significantly reduced in the 51-65yrs vs. 31-50yrs group, with no change in the $CD4^+$ population. This is the first study to demonstrate this, and may imply a selective negative functional impact of age on $CD8^+$ T_{ANG} cells.

The exact mechanisms for the age-related decline in CAC number and potentially function are yet to be fully elucidated. The data presented shows that the reductions in circulating SDF-1α with age may contribute to the reduced progenitor cell number, however it is highly likely that this is not the single causative factor. Many studies have demonstrated the role that oxidative stress may play in age-related decline in progenitor cells and their functional capacities (Mandraffino *et al.*, 2012; Rimmelé *et al.*, 2014; Wang *et al.*, 2015). High circulating levels of ROS are greater in aged humans and this is accompanied by a reduction in EPC SIRT1 content (Mandraffino *et al.*, 2012). SIRT1-depletion in endothelial cells causes reduction in vasculogenic ability of endothelial cells, as well as reduced gene expression of CXCR4 (Potente *et al.*, 2007). SIRT1 protects against ROS-induced cellular apoptosis (Donato *et al.*, 2011; Wang *et al.*, 2015), and SIRT1 expression and activity is reduced in aged endothelial cells (Donato *et al.*, 2011), therefore there may be a potential loss of SIRT1 activity/content within these progenitor cells with age. In addition to oxidative stress, shortening of telomeres (Kushner *et al.*, 2009) and an increased susceptibility to apoptosis (Kushner *et al.*, 2011) may also account for the reduction in progenitor cell number observed in the study presented (*Chapter 4*).

Reductions in the T_{ANG} population are most likely due to several factors, such as declining thymic output of T-cells (Fagnoni *et al.*, 2000; Simpson, 2011). However our data shows that the percentage of T-cells expressing CD31 reduced also, thus other mechanisms must be responsible. As with EPCs, telomere shortening has been observed in these cells (Kushner *et al.*, 2010c), but may or may not be linked with CD31 loss. CD31 expression

on T-cells has been linked with non-activated status of the T-cells (Zehnder *et al.*, 1992), and so with increasing age and exposure to viruses over the lifespan, it would not be surprising that we get an expansion of activated T-cells with age (Simpson, 2011).

Some of these populations ($CD34^+$, EPCs) have previously been shown to be affected by exercise, both acute (Rehman *et al.*, 2004; Van Craenenbroeck *et al.*, 2008; Ross *et al.*, 2014) and long-term exercise training (Ajijola *et al.*, 2009; Choi *et al.*, 2014), yet no investigations have been performed thus far into how exercise modulates T_{ANG} cells, although there is clear evidence for exercise attenuating expansion of cytotoxic T-cells with age (Spielmann *et al.*, 2011) Therefore the role of CRF in age-related changes in CACs were investigated.

9.3 Cardiorespiratory Fitness

Regular exercise is heavily associated with reduced mortality rates in those with CVD (Wisløff *et al.*, 2006; Aijaz *et al.*, 2008; Sakamoto *et al.*, 2009). This could be due to, in part, to the maintenance or improvement in endothelial function seen with exercise training (Black *et al.*, 2009; Ades *et al.*, 2011; Ashor *et al.*, 2015). The improvements in endothelial function are as a result of direct effects on the endothelium, such as increased NO bioavailability (Hambrecht *et al.*, 2003; Miyaki *et al.*, 2009) and reduced oxidative stress (Goto *et al.*, 2003; Moien-Afshari *et al.*, 2008; Durrant *et al.*, 2009; Mitranun *et al.*, 2014), as well as reported improvements in CAC number and/or function (Laufs *et al.*, 2004; Steiner *et al.*, 2005; Hoetzer *et al.*, 2007; Van Craenenbroeck *et al.*, 2010a; Schlager *et al.*, 2011; Sonnenschein *et al.*, 2011; Fernandes *et al.*, 2012; Xia *et al.*, 2012a). CACs are important for the maintenance of endothelial health (Sibal *et al.*, 2009; Bruyndonckx *et al.*, 2014), and reductions in CAC circulating number and function are observed in CVD patients compared to age-matched healthy controls (Hill *et al.*, 2003; Fadini *et al.*, 2005; Walter *et al.*, 2005; Fadini *et al.*, 2006; Xiao *et al.*, 2007; Sibal *et al.*, 2009; Rouhl *et al.*, 2012; Shantsila *et al.*, 2012; Teraa *et al.*, 2013; Barsotti *et al.*, 2014; Castejon *et al.*, 2014), implicating a loss of CAC number and/or function in the CVD process. As shown by data presented within the thesis (*Chapter 4* and *Chapter 5*) several subpopulations of CACs are reduced with age, and this has been corroborated by previous studies (Thijssen *et al.*, 2006; Thum *et al.*, 2007; Kushner *et al.*, 2010c). CRF, through improving the

number and function of these cells may help attenuate the ageing decline in these cells, and thus provide an 'anti-ageing' effect on the vasculature, thus reducing CVD risk.

Data from *Chapter 4* and *Chapter 5* show that those with higher levels of CRF display greater number of T_{ANG} and CXCR4-expressing T_{ANG}, but appeared to have no effect on circulating progenitor cells. Those with higher levels of CRF displayed on average 7% greater circulating T_{ANG} cells, and 6% higher percentage of T-cells expressing CD31 than those with low CRF. However this appeared to be an age-dependent effect, as our data showed that those in 31-50yrs group were not affected by CRF in terms of T_{ANG} cells. The young group appeared more sensitive to the effects of higher CRF levels, with those individuals in the 18-30 yrs group reporting 24% greater circulating number of T_{ANG} cells in those with high CRF than low CRF levels. The beneficial effect of CRF on these cells in the older age group was potentially due to the greater number of $CD4^+$ T-cells expressing CD31, and there was no effect on the CD8 subpopulation, or interestingly, CXCR4 expression on these cells.

The effect of CRF on T_{ANG} observed (specifically $CD4^+$ T_{ANG}), may be a result of maintenance of a high proportion of naïve $CD4^+$ T-cells through the lifespan. CD31 is reported to be lost upon T-cell activation (Zehnder *et al.*, 1992) and differentiation (Demeure *et al.*, 1996), and thus may be used as a marker for unactivated T-cells with a low replicative history. CRF has previously been shown to attenuate the age-related increase in senescent $CD4^+$ T-cell pool, as well as having a beneficial impact on $CD8^+$ naïve T-cells with increasing age (Spielmann *et al.*, 2011). Therefore by maintaining levels of $CD4^+$ naïve T cells through regular exercise training may subsequently maintain $CD4^+$ T_{ANG} cells throughout the lifespan.

With no changes in CXCR4 expression on these T_{ANG} cells, it may be postulated that exercise affects these T_{ANG} cells by maintaining their number through the ageing process, rather than modulating their function. However, function may not be related to CXCR4 cell surface expression, as migratory function in EPCs in previous studies were associated with CXCR4:JAK-2 signalling rather than CXCR4 cell surface expression (Xia *et al.*, 2012a; Xia *et al.*, 2012b). The effect of CRF on potential effects on T_{ANG} function, such as migration and pro-angiogenic cytokine release are yet to be investigated, therefore further studies are needed.

Interestingly, the data shown in *Chapter 4* shows no effect of CRF on $CD34^{+}$ progenitor cells or EPCs, either age-dependent or independent. Previous studies have shown the beneficial impact of regular exercise and CRF on these CACs (Laufs *et al.*, 2004; Steiner *et al.*, 2005; Hoetzer *et al.*, 2007; Sarto *et al.*, 2007; Manfredini *et al.*, 2009; Van Craenenbroeck *et al.*, 2010a; Sonnenschein *et al.*, 2011; Fernandes *et al.*, 2012; Xia *et al.*, 2012a), yet our data shows no effect. This however, is in line with several studies demonstrating no effect of an exercise training program, or CRF on these cells (Thijssen *et al.*, 2006; Witkowski *et al.*, 2010; Luk *et al.*, 2012). The study presented within this thesis is the first study to investigate the role of CRF on the $CD34^{+}CD45^{dim}VEGFR2^{+}$ population of EPCs, reported to have endothelial differentiation capacities (Case *et al.*, 2007), whereas the $CD45^{bright}$ population do not (Case *et al.*, 2007; Timmermans *et al.*, 2007), and may instead exert beneficial effects on the endothelium by acting in a more paracrine manner by secreting pro-angiogenic cytokines (Hur *et al.*, 2004). Van Craenenbroeck *et al.* (2013b) published guidelines on measuring and quantifying endothelial precursors in circulating blood, and promoted the use of $CD45^{dim}$ within the flow cytometric panel, and such the data presented is in line with these recent guidelines.

The study, being cross-sectional in nature, may not be ideal for such investigations into rare cell populations, and thus requires more controlled longitudinal studies. The clear effects of BMI (Bellows *et al.*, 2011; Graziani *et al.*, 2014; Ruszkowska-Ciastek *et al.*, 2015), smoking history (Lamirault *et al.*, 2013), epigenetics (Rajasekar *et al.*, 2015), stress (Rocha *et al.*, 2015), and diet (Yue *et al.*, 2011; Vafeiadou *et al.*, 2012; Turgeon *et al.*, 2013; Bruyndonckx *et al.*, 2015) on these rare cell populations mean that it is difficult to compare effects of lifestyle factors in a cross-sectional study.

Rather than maintaining high levels of CRF through regular exercise bouts, it has been proposed that simply refraining from sedentary behaviour, and living an active lifestyle may promote endothelial health. Therefore, the effect of sedentary behaviours on these CACs was investigated.

9.4 Sedentary Behaviours

Sedentary behaviour, such as prolonged periods of sitting, is linked to increased CVD risk (Laufs *et al.*, 2005b; Hamilton *et al.*, 2007; Katzmarzyk *et al.*, 2009; van der Ploeg *et al.*, 2012; Wilmot *et al.*, 2012; Stamatakis *et al.*, 2013; Chau *et al.*, 2014; Gibbs *et al.*,

2014; Staiano *et al.*, 2014; Young *et al.*, 2014; Chau *et al.*, 2015). Prolonged periods of inactivity are also linked to an increase in systemic pro-inflammatory cytokines (Fischer *et al.*, 2007). Sitting time is negatively related to endothelial function (Nosova *et al.*, 2014), however the mechanism is unclear. These effects may be attributable to direct effects on the endothelium through the observed increase in pro-inflammatory cytokines and/or oxidative stress, or reduced eNOS activity. Long periods of inactivity may also exert a deleterious effect on CACs, which may have a knock-on effect on the endothelium.

Data presented in *Chapter 6* suggests that sedentary behaviour is associated with $CD31^+$ T-cells, with an increase in sitting time being correlated with a loss of T_{ANG}. As with CRF, the effect of this lifestyle behaviour appears to be attributable to its effects on $CD4^+$ T-cells, with a loss of CD31 expression with increasing sitting time. The data also suggests that increasing sitting time may have a negative impact on CXCR4 expression on $CD34^+$ progenitor cells and EPCs. However, due to the dichotomy that exists, with those reporting high levels of sitting time may also be regular exercisers, the effects of sedentary behaviour on these cells were assessed after controlling for CRF, as data from *Chapter 5* strongly suggests that CRF beneficially modulates these T_{ANG} cells. After correcting for participants CRF levels, the negative impact of sitting time no longer existed. This data suggests that the negative impact of sedentary lifestyle may be attenuated by regular exercise training and maintaining high levels of $\dot{V}O_{2max}$. This is the first study to show the impact sitting time may have on CACs. However further study is required to elucidate the *in vitro* and *in vivo* function of these cells as a result of physical inactivity.

The data presented represents self-reported sitting time, and thus is limited in its accuracy. Although the questionnaire used to quantify sedentary behaviour has been recently validated in a large cohort (Wijndaele *et al.*, 2014), the use of accelerometry and global positioning systems data would provide clearer results, with the potential to further determine domain-specific sedentary behaviour and their effects on vascular health. In addition, interventions used to either increase or decrease physical activity can be utilised in further controlled investigations.

9.5 Acute Exercise and Mobilisation of $CD31^+$ vs. $CD31^-$ T-Cells

As data presented within this thesis demonstrates a positive effect of CRF on $CD31^+$ T-cells (*Chapter 5* and *Chapter 6*), it was hypothesised that these cells are also responsive to an acute bout of strenuous exercise. T-cells typically respond to exercise, demonstrated by a large ingress into the peripheral circulation (lymphocytosis) followed by a large egress from the circulation (lymphocytopenia) (Simpson *et al.*, 2007; Turner *et al.*, 2010; Witard *et al.*, 2012; Ingram *et al.*, 2015). Although a large proportion of the exercise-induced ingress is due to both shear stress and $ß_2$-adrenergic mechanisms (Simpson *et al.*, 2007), the ingress could be also due to chemotactic factors within the blood which are known to be increased in the circulation with exercise, such as SDF-1α (Van Craenenbroeck *et al.*, 2010b; Wang *et al.*, 2014b). SDF-1α is known to mobilise $CXCR4^+$ CACs into the blood (Aiuti *et al.*, 1997; Moore *et al.*, 2001) as well as play a role in the homing of these cells to ischaemic tissue (Yamaguchi *et al.*, 2003). SDF-1:CXCR4 axis may play an important role in the chemotaxis of T-cells into the blood as a result of strenuous exercise. This has yet to be investigated.

T-cells expressing adhesion molecules, such as integrins (Shephard, 2003; Simpson *et al.*, 2006) and CD56 (Goebel *et al.*, 2000; Simpson *et al.*, 2006) have been shown to be preferentially mobilised into the circulation during exercise. The preferential ingress of T-cells expressing adhesion molecules may be explained by the demargination of T-cells from the endothelium. The response to exercise of T-cells expressing CD31, however, has yet to be documented.

Data from *Chapter 7* shows that $CD31^+$ T-cells are mobilised into the peripheral circulation in response to a strenuous exercise bout. The observed increase was greater for $CXCR4^+$ T_{ANG} cells compared to $CXCR4^-$ T_{ANG}, implicating the potential for the SDF-1:CXCR4 axis in mobilisation of T-cells to exercise. Our data also shows an increase in systemic circulating SDF-1α, which may be responsible for the greater ingress of CXCR4-expressing cells compared to CXCR4-deficient cells. However, in contrast to several studies which found a preferential mobilisation of T-cells expressing adhesion molecules (Goebel *et al.*, 2000; Shephard, 2003; Simpson *et al.*, 2006), the $CD8^+$ T-cells which did not express CD31 showed a greater ingress and egress pattern in response to the exercise stressor compared to $CD8^+$ T-cells expressing CD31 (233% vs. 162%). $CD8^+$

T-cells which express markers of senescence and differentiated status show similar preferential response to exercise as the $CD31^-$ subset (Campbell *et al.*, 2009). The significance of this preferential ingress of $CD8^+CD31^-$ T-cells to exercise is not yet known, but may lie in the exact phenotypic definition of these $CD31^+$ and $CD31^-$ T-cells.

The data shows that there was a 140% ingress of $CD3^+CD31^+$ T-cells into the peripheral circulation of healthy active males as a result of the strenuous exercise bout. The vasculogenic nature of these cells could contribute to post-exercise vascular adaptation through the secretion of pro-angiogenic growth factors and cytokines (Hur *et al.*, 2007; Kushner *et al.*, 2010b). Regular exercise may promote the benefits associated with improved endothelial health through regular mobilisations of these vasculogenic cells.

The acute exercise bout did not affect CXCR4 cell surface expression on these $CD31^+$ T-cells. This is in contrast to reports suggesting that the exercise-induced increase in cortisol can augment the T-cell CXCR4 expression (Okutsu *et al.*, 2005), or data showing increased CXCR4 cell surface expression on NK cells post-exercise (Okutsu *et al.*, 2014). Interestingly, participants may turn out to be cortisol responders or non-responders to an exercise stressor (Shinkai *et al.*, 1996). Our study did not include measure of cortisol response to the exercise bout, so different cortisol responders and non-responders to exercise may explain the lack of change in CXCR4 cell surface expression in this small cohort. There was a trend for increased CXCR4 expression on the $CD8^+CD31^+$ T-cells but this failed to reach statistical significance. Therefore the possibility of increased CXCR4 cell surface expression on these CACs due to acute exercise cannot be discounted until statistically more powerful data arises and circulating cortisol is taken into account.

9.6 Viral Serostatus and $CD31^+$ T-cells

Cytomegalovirus (CMV) exerts significant effects on the human T-cell pool, with an expansion of senescent and highly differentiated T-cell subsets (Ouyang *et al.*, 2003; Simpson, 2011; Simpson *et al.*, 2012; Wang *et al.*, 2014a; Di Benedetto *et al.*, 2015). CMV has therefore been strongly linked to premature 'immuno-ageing'. However, recent evidence may suggest that CMV may simply be a bystander rather than participant. CMV has also been tenuously linked with CVD (Arasaratnam, 2013; Courivaud *et al.*, 2013;

Courivaud and Ducloux, 2013; Haeseker *et al.*, 2013). Some studies have linked CMV with endothelial dysfunction in mice (Khoretonenko *et al.*, 2010; Gombos *et al.*, 2013) but as yet no evidence for the link in humans. With the previous data chapters (Chapters 5-7) focusing on various lifestyle factors on T-cells expressing CD31, we sought to investigate the role CMV may have in regulating the number of these cells. Young CMV^+ individuals (aged 18-40 years) appeared to display greater levels of $CD31^+$ T-cells (however not significant) and greater percentage of $CD8^+$ T-cells expressing CD31 compared to their age-matched CMV seronegative counterparts. This effect was not observed in the older (41-65 years) individuals. These results for these $CD31^+$ T-cells are similar to those reported for γδ T-cells (Pistillo *et al.*, 2013), and with a small percentage of these $CD31^+$ T-cells not expressing CD4 or CD8, there is a high likelihood that the CMV infection may be driving not just increases in $CD8^+$ T_{ANG} cells seen in the data presented, but also total $CD31^+$ T-cells. This has yet to be investigated and is a limitation of the study (see *9.7.2 Phenotypic Quantification of Circulating Angiogenic Cells*). The significance of our data has yet to be addressed, but CMV may drive an increase in $CD8^+CD31^+$ T-cells and total $CD31^+$ T-cells (some of which are likely to be γδ T-cells) after primary infection (Fornara *et al.*, 2011), an effect which appears to dampen in later life as evidenced by no difference in our T-cell subsets between CMV seropositive and seronegative individuals within the 41-65 year age bracket.

Our data also implies that if there is a link between CMV and CVD and endothelial dysfunction, then it is likely not due to any effect on T_{ANG} number, however function has yet to be assessed in these cells from CMV seropositive and seronegative individuals. In addition, there appears to be subset-specific effects, with the $CD8^+$ T-cell subset appearing most affected. This research investigating CMV and 'angiogenic' T-cells is at a preliminary stage, with much scope for future research.

9.7 Limitations of the Studies Presented in this Thesis

9.7.1 Submaximal Exercise Test for $\dot{V}O_{2max}$ Prediction

In *Chapters 4*, *5* and *6*, CRF was estimated using a submaximal graded exercise protocol (YMCA) (Golding *et al.*, 1989). Submaximal exercise tests have been used widely to estimate or predict $\dot{V}O_{2max}$ (Akalan *et al.*, 2008; Faulkner *et al.*, 2009; Spielmann *et al.*,

2011). Gellish *et al.* (2007) demonstrated that using HR_{max} predictions can underestimate HR_{max}, and thus using these equations can be a limiting factor when using submaximal exercise tests to predict $\dot{V}O_{2max}$. The YMCA submaximal cycling graded exercise tests used in *Chapters 4, 5* and *6* has been validated by Garatachea *et al.* (2007), who showed that in healthy men, $\dot{V}O_{2max}$ predicted from YMCA submaximal exercise test was not significantly different from, $\dot{V}O_{2max}$ measured during a maximal exercise test to exhaustion. Unpublished data from our laboratory confirm that the YMCA submaximal exercise test is a suitable test to use for predicting $\dot{V}O_{2max}$ in a healthy male population, however the spread of age groups was narrow.

It cannot be discounted however, that there may be population specific accuracies and inaccuracies when using the test utilised in these chapters, with the effects of training status and age (Grant *et al.*, 1999) needing to be considered for further validation studies.

9.7.2 Phenotypic Quantification of Circulating Angiogenic Cells

Quantification of CACs measured in this thesis has been performed using a 4-colour flow cytometer (FACSCalibur, BD Biosciences, USA). Due to the measurement of CXCR4 on the T_{ANG} panel, it was not possible to measure expression of typical naïve ($CD45RA^+$, $CD28^+$) and senescent ($CD57^+$, $KLRG1^+$, $CD28^-$) T-cell markers (Simpson, 2011; Spielmann *et al.*, 2011) to fully identify the $CD31^+$ T-cell subset further. With regard to previous changes in $CD31^+$ T-cell subsets with age and exercise, it was suggested that the changes seen were likely due to changes in the $CD4^+$ naïve T-cell population (*Chapter 5* and *Chapter 6*), as CD31 is reported to be lost upon T-cell activation (Zehnder *et al.*, 1992) and differentiation (Demeure *et al.*, 1996). However this cannot be confirmed by the data shown. The use of larger panels within flow cytometry are required to measure the co-expression of naïve and senescent markers to fully explain the changes seen within the data shown.

Flow cytometric analysis of EPCs can be problematic, and is littered with limitations (Brandes and Ushio-Fukai, 2011; Van Craenenbroeck *et al.*, 2013b; Rose *et al.*, 2014). The rare nature of these cells (0.0001-0.01% of MNCs) means flow cytometric detection is difficult, and can result in as few events as <5 being detected within the flow cytometric assay performed. Further expression of CXCR4 on these progenitor cells is therefore

difficult, and even more difficult to interpret. The flow cytometric assay utilised within the thesis for quantification of circulating EPCs was that used and recommended by Van Craenenbroeck *et al.* (2013b), and the $CD45^{dim}$ phenotype is reported to represent the endothelial precursors within the $CD34^{+}VEGFR2^{+}$ population (Case *et al.*, 2007; Timmermans *et al.*, 2007). Some have previously isolated MNCs by density gradient centrifugation to quantify EPCs (Liu and Xie, 2012), yet this could result in the loss of some progenitor cells through human error or through the density gradient (Van Craenenbroeck *et al.*, 2013b). Due to the 4-colour limit on the panel, apoptosis was not assessed by using the labelling with Annexin-V, therefore it is likely that the resulting EPCs quantified may contain non-viable cells. In addition, there is a high risk of false positives due to difficulty with isotype control. There was no isotype control used in the methodology detailed in *Chapter 4* and *Chapter 6*, with negative samples for both VEGFR2 and CXCR4 instead being used (CD34 and CD45 did not require negative or isotype controls due to high relative expression of these proteins on the MNCs of interest). Future studies investigating the effects of exercise or lifestyle on these cells should instead be focused on isolating these cells from whole blood, using magnetic beads and measuring EC-CFU and *in vitro*/*in vivo* cellular functions.

9.7.3 CXCR4 Cell Surface Expression as Putative Marker of Cell Function

Analysis of EPCs, although problematic, has still been repeatedly shown to be a risk factor for CVD (Fadini *et al.*, 2005; Fadini *et al.*, 2006; Barsotti *et al.*, 2014; Berezin and Kremer, 2014; Castejon *et al.*, 2014) and endothelial function (Sibal *et al.*, 2009; Bruyndonckx *et al.*, 2014), as have the $CD31^{+}$ T-cells (Weil *et al.*, 2011; Rouhl *et al.*, 2012). These cells are clearly reduced with age, as seen with other reports (Thijssen *et al.*, 2006; Thum *et al.*, 2007; Kushner *et al.*, 2010c), but may also be dysfunctional with age and disease states, as suggested by previous reports (Kushner *et al.*, 2010c; Xia *et al.*, 2012b). CXCR4, although reduced with age, may not accurately or sensitively reflect functional properties of these cells, despite being heavily linked with migratory function. Functional impairments have been shown irrespective of CXCR4 expression on EPCs isolated from young and old human participants (Xia *et al.*, 2012a; Xia *et al.*, 2012b) and thus *in vitro* and *in vivo* experiments of cellular function, for example cell migration, pro-angiogenic cytokine release, gene expression and tube formation assays, need to be performed to fully understand how CAC function changes with age and exercise.

9.7 Future Areas of Research

Data presented within this thesis have shown that chronological age can negatively impact on circulating CAC number ($CD34^+$ progenitor cells, EPCs, and T_{ANG}), and maintaining high level of CRF may attenuate certain subsets of these cells (*Chapter 4* and *Chapter 5*).

In respect to these observations, it is suggested that future studies address the following research questions:

1. What are the *in vitro* and *in vivo* effects of age and regular exercise on T_{ANG} cells?
2. The $CD31^+$ T-cells contain a heterogenous T-cell pool, with ~43% (6-69%) are $CD4^+$ T-cells and ~42% (16-67%) are $CD8^+$ T-cells. What are the specific phenotypes of these $CD4^+$ and $CD8^+$ T-cells? Are $CD4^+$ or $CD8^+$ T-cells expressing CD31 predominantly naïve phenotype as reported previously? What are the subset-specific pro-angiogenic functions of these cells, if any? Are there a proportion of these $CD31^+$ T-cells which are γδ T-cells?
3. Does age and CRF have a similar impact on biomarkers of endothelial damage as measured by circulating endothelial microparticle (EMP)?

In *Chapter 6*, we have demonstrated that sedentary behaviour may negatively affect CAC number. Some of these effects can be lost when correcting for CRF, indicating that regular exercise may negate the harmful effects of sedentary behaviour.

In light of these observations, further study is required to address the following research questions:

1. What are the effects of prolonged periods of bed rest on CAC number and function, and does any effect correspond to changes in endothelial function?
2. Can regular breaks in sitting time attenuate the harmful effects of sitting time on CAC number and/or function?
3. Are older individuals at greater risk of the harmful effects of sedentary behaviour than younger individuals, and if so can targeting these individuals with interventions to reduce sitting time affect the observed deleterious effects on CACs and endothelial function?

In *Chapter 7*, we have demonstrated that acute strenuous exercise is a powerful stimulus for mobilising $CD31^+$ T-cells into the peripheral circulation, thus offering a window for T-cell-mediated vascular adaptation. It appears that the SDF-1:CXCR4 axis may play an important role in mobilising T-cells as a result of an increase in circulating SDF-1α from ischaemic contracting muscle.

Future research should aim to address the following research questions:

1. What are the phenotypes of these $CD31^+$ T-cells that mobilise as a result of acute exercise?
2. Are circulating number changes accompanied by functional changes, such as increased pro-angiogenic cytokine release or expression?
3. Viral serology plays an important role in determining the lymphocyte response to exercise. Thus does viral serostatus affect $CD31^+$ T-cell kinetics with acute strenuous exercise?

In *Chapter 8*, we detected differences in $CD8^+CD31^+$ T-cell population between CMV seropositive and seronegative young individuals. There was also a trend for increased total $CD31^+$ T-cells in the CMV seropositive group. Future research should aim to address the following research questions:

1. Are the changes in $CD8^+CD31^+$ population seen in our cohort as a result of differentiation or senescence status as typically observed in CMV seropositive individuals?
2. Are changes seen in these T_{ANG} cells accompanied by changes in function (for example, pro-angiogenic growth factor and cytokine content/secretion, migration)?

Conclusions

The findings of this thesis demonstrate that CACs are reduced with chronological ageing and sedentary lifestyle, but may be partly rescued by regular exercise or maintaining high levels of CRF. Indeed acute exercise can briefly increase the number of T_{ANG} cells

offering a potential window of vascular adaptation. In addition, viral serology affects the number of a subpopulation of CACs, with potential significance not yet known.

With an increasing aged population, the beneficial effects of regular and acute exercise and understanding of the mechanisms driving these effects will prove to be useful for care givers and clinicians in the prevention of age-related increased CVD risk. This may have large implications for reduction in CVD risk, morbidity and mortality associated with CVD and vascular-related disorders.

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Appendix 1: Information Sheet

Edinburgh Napier UNIVERSITY

Information Sheet for Potential Participants

The Influence of Age, Aerobic Fitness, Cardiovascular Risk Factors and Cytomegalovirus Infection on Angiogenic T Cells and Endothelial Progenitor Cells

My name is Mark Ross and I am a postgraduate PhD student from the School of Life, Sport and Social Science at Edinburgh Napier University. As part of my degree course, I am undertaking a research project for my PhD thesis. The title of the project is: The Influence of Age, Aerobic Fitness, Cardiovascular Risk Factors and Cytomegalovirus Infection on Angiogenic T Cells and Endothelial Progenitor Cells.

The endothelium makes up the inner layer of all blood vessels and is now know to perform a range of crucial functions related to cardiovascular health. Endothelial progenitor cells (a type of stem cell) and angiogenic T cells (a subset of immune cells) aide in the process of cardiovascular repair. Thus these cells are very important for cardiovascular health. Age, aerobic fitness, cardiovascular risk factors and cytomegalovirus infection may all impact on the level of these cells circulating in the blood, as well as their function to help grow new blood vessels and repair damaged endothelium.

This research is being funded by Edinburgh Napier University.

I am looking for volunteers to participate in the project. Inclusion criteria are:

Males and females between ages of 18 and 65 years old

The exclusion criteria are:

- Body Mass Index of more than 35 (measured in the laboratory through height and weight measurements) and smokers
- Alcohol intake >14 drinks per week
- Taking medication affecting the immune system
- Routinely using ibuprofen and/or aspirin, anti-depressants, and/or medications designed to alter blood pressure or cardiovascular function and hormone replacement therapy will be excluded from the participant population, as these may affect results of the study
- Participants also reporting major affective disorders, HIV infection, hepatitis, chronic/debilitating arthritis, central or peripheral nervous disorders, previous stroke or cardiac events, were bedridden in the past 3 months, suffer from known cardiovascular disease or autoimmune diseases will all be excluded from the study.
- Participants must also be free from infectious disease for 6 weeks prior to study.

As a participant in this project, you will be asked to refrain from any strenuous exercise 1 day prior to any exercise testing. You will also be asked to refrain from ingesting any caffeine and alcohol the day prior to exercise testing. You will also be asked to attend the exercise laboratory in the morning after an overnights fast

You will be asked to give informed consent prior to any procedures. Once this is obtained, you will be asked to give blood sample from your arm. You will also be asked to fill out a questionnaire regarding health and family disease history, through which we will determine whether you can take part in the study (see inclusion and exclusion criteria). You will also be asked to complete a set of questionnaires designed to assess how much time you spend sitting. You will also be asked to take part in a 9-12 minute light-to-moderate exercise test to determine aerobic fitness on a cycle ergometer.

There is a risk that you will experience muscle discomfort during the exercise test, however the procedures will all take place in a controlled environment, and first aid-trained personnel will be in attendance to ensure safety, as well as a defibrillator in the laboratory at all times. You may also experience some fatigue as well as some delayed soreness in the muscle as a result of the exercise bout. There is also a risk of some slight bruising where blood will have been taken. However every caution will be taken to ensure minimal bruising. The whole procedure should take no longer than 1 hour. You will be free to withdraw from the study at any stage; you will not have to give a reason.

All data will be anonymised as much as possible. Your name will be replaced with a participant number or a pseudonym, and it will not be possible for you to be identified in any reporting of the data gathered. All data collected will be kept in a secure place (data will be stored on a password protected laptop and pc) to which only the main investigator (Mark Ross) has access. These will be kept until the end of the research process, following which all data that could identify you will be destroyed.

The results may be published in a journal or presented at a conference, again with your results being completely anonymous.

If you would like to contact an independent person, who knows about this project but is not involved in it, you are welcome to contact Dr Mick Rae. His contact details are given below.

If you have any questions or concerns regarding the research project, you may contact one of the following:

Principal Researcher

Mark Ross

[redacted]

Director of Studies

Dr. Geraint Florida-James

[redacted]

[redacted]

Independent Advisor

Dr Mick Rae

[redacted]

Appendix 2: Informed Consent Form

Edinburgh Napier UNIVERSITY

Informed Consent Form

The Influence of Age, Aerobic Fitness, Cardiovascular Risk Factors and Cytomegalovirus Infection on Angiogenic T Cells and Endothelial Progenitor Cells

Project Title:

Influence of age, aerobic fitness, cardiovascular risk factors and cytomegalovirus infection on angiogenic T cells and endothelial progenitor cells.

Introduction to this study:

The endothelium makes up the inner layer of all blood vessels and is now know to perform a range of crucial functions related to cardiovascular health. Endothelial progenitor cells (EPCs; a type of stem cell) and angiogenic T cells (a subset of immune cells) are circulating cells which aide in the process of new blood vessel growth and vascular repair. Thus these cells are very important for cardiovascular health. Age, aerobic fitness, cardiovascular risk factors and infection may all impact on the level of these cells circulating in the blood, as well as their function to help grow new blood vessels and repair damaged blood vessels.

I am being asked to participate in this research study. The study has the following purposes:

1. To determine the influence of different factors (age, aerobic fitness, cardiovascular risk, sedentary behaviours and CMV infection) on circulating levels cells involved in maintaining cardiovascular health in the general population.

2. To assess these factors' influence on the biological aging of these cells.

This research study will take place at Edinburgh Napier University, Sighthill Campus, Sighthill, Edinburgh

This is what will happen during the research study:

1. You will be asked to give a blood sample, which will be analysed for circulating angiogenic T cells, EPCs, cardiovascular risk factors, and to determine CMV infection status. Approximately 30mL of blood will be taken (equivalent to 2 tablespoons).
2. You will also be asked to fill out a questionnaire regarding health and family disease history.
3. You will undertake initial measurements in the exercise laboratory that include height, weight, waist and hip measures, as well as assessment of aerobic fitness capacity.
4. The assessment of aerobic capacity involves a moderate bout of exercise on a laboratory bicycle. The exercise will last approximately 9-12 minutes.
5. You will be asked to refrain from exercise training 1 day prior to these assessments, and not to consume alcohol on the day prior to each exercise session.

There are certain risks and discomforts associated with participation in the study

1. Strenuous exercise carries with it a very small risk of heart attack. This risk only exists for a small number of individuals with pre-existing heart problems. Every effort will be made through pre-exercise screening to identify individuals with heart and other conditions that could be made worse with exercise. A defibrillator is always on site in the laboratory as well as there being first aid-trained individuals in attendance.

2. Fatigue will be experienced during the exercise sessions. In addition, muscle soreness may be experienced for a day or two after exercise, particularly after the exercise test.

3. A small amount of localized bruising can occur after a blood sample is taken. Every effort will be made to avoid this by the individual taking the blood sample. The individual taking the blood sample will be a trained phlebotomist.

My confidentiality will be guarded:

Edinburgh Napier University will protect all the information about me and my part in this study. My identity or personal information, will not be revealed, published or used in future studies. The study findings will form the basis for preparation of a postgraduate thesis, academic publications, conference papers and other scientific publications.

If I have questions about the research project, I am free to contact the following:

Principal Researcher:

Mr Mark D. Ross

[redacted]

[redacted]

Director of Studies:

Dr. Geraint Florida-James

[redacted]

[redacted]

Independent Advisor:

Dr. Mick Rae

[redacted]

I have read and understood the information sheet and this consent form. I have had an opportunity to ask questions about my participation.

I understand that I am under no obligation to take part in this study.

I understand that I have the right to withdraw from this study at any stage without giving any reason.

I agree to participate in this study.

Name of participant: _____

Signature of participant: _____

Signature of researcher: _____

Date: _____

Contact details of the researcher

Name of researcher: Mark Ross

Address: Graduate Teaching Assistant and PhD Student,

Faculty of Life, Sport and Social Science

Edinburgh Napier University

Sighthill Campus

[redacted]

Email / Telephone: [redacted]

Appendix 3: Physiological Screening Questionnaire

Physiology Screening Questionnaire

Please read the following carefully and answer all the questions truthfully. Information will be treated with the strictest confidence.

This is a strictly private confidential document.

Name: _____ Gender: _____

Date of Birth: _____ Age: _____

Have you ever had a heart problem such as a heart attack, hypertrophic cardiomyopathy, congenital abnormality, heart valve defect, heart failure or heart rhythm disturbance?

Yes/No

Have you ever received treatment for a heart problem such as heart surgery, the fitting of a pacemaker/defibrillator, coronary angioplasty or heart transplantation?

Yes/No

Are you currently taking medication for your heart? (Please indicate if so, what you are taking)

Yes/No

How many units of alcohol do you take a week? _____

N.B One alcohol unit is measured as 10ml or 8g of pure alcohol. This equals one 25ml single measure of whisky (ABV 40%), or a third of a pint of beer (ABV 5-6%) or half a standard (175ml) glass of red wine (ABV 12%).

Do you currently or have you ever suffered from any of the following:

Arthritis, osteoporosis or any other bone or joint problem	Yes/No
Asthma, bronchitis or any other respiratory problem	Yes/No
Coagulation disorders	Yes/No
Diabetes (Type I or Type II)	Yes/No
Epilepsy	Yes/No
Hypertension (High Blood Pressure)	Yes/No
Liver or gastrointestinal problems	Yes/No
Kidney problems	Yes/No
Infectious disease such as HIV, hepatitis or glandular fever	Yes/No
Autoimmune disease	Yes/No
Any peripheral or central nervous system disease (e.g. Alzheimer's, Meningitis, Huntington's, Parkinson's, Tourette's)	Yes/No

Do you experience any of the following:

Chest discomfort with exertion	Yes/No
Unreasonable breathlessness	Yes/No
Dizziness, fainting, blackouts	Yes/No
Palpitations or skipped beats	Yes/No
Unusual levels of fatigue	Yes/No

Please indicate if any of the following are true

You have a close blood male relative (father or brother) who has had a heart attack before the age of 55 or a close female relative (mother or sister) who has had a heart attack before the age of 65	Yes/No
You have elevated levels of cholesterol or are on lipid lowering medication	Yes/No
You are a cigarette smoker	Yes/No
You have elevated levels of blood glucose	Yes/No
You are completely inactive (do not take part in 20 minutes of moderate physical activity such as walking, 3 times per week)	Yes/No
You have suffered a stroke or major cardiac event	Yes/No
You have been bedridden in the past 3 months	Yes/No
You have suffered from an infectious disease in last 6 weeks	Yes/No
About how many hours in each 24-hour day do you usually spend sitting?	_____

Are you currently taking any medications?

If Yes please give details:

_____

Have you any other conditions that may be relevant to an individual undertaking strenuous exercise?

If Yes please give details:

_____

Declaration:

I have understood all of the questions put to me and that my answers are correct to the best of my knowledge. I understand that this information will be treated with the strictest confidence.

Signed: _____ Date: _____

(Participant)

Signed: _____ Date: _____

(Tester)

Appendix 4: SIT-Q -7d Questionnaire

MRC | Epidemiology Unit

SIT-Q-7d

Questionnaire

These questions are about the amount of time that you spent **sitting or lying down** in the **last 7 days**. This questionnaire is organised into five sections, each asking about sitting or lying down in different settings.

Please first answer the question below and read the instructions underneath, which will help you to complete this questionnaire.

*Please tick (✓) **one** box only*

Compare your amount of sitting time over the **last 7 days** with a **typical week for you**. **In the last 7 days**, my amount of sitting was …				
Much less than normal ☐	A little less than normal ☐	About the same ☐	A little more than normal ☐	A lot more than normal ☐

Instructions:

1. Please complete the following sections by thinking about the **last 7 days**.
2. Each period of sitting down **should only be entered once** on this questionnaire. For example, if you spent one hour sitting on the sofa reading a book while you were listening to music, count this time as one hour reading if this was your main focus. Do not also count this as one hour listening to music.
3. If there is a big difference between different weekdays or between different weekend days for some answers, then tick the box which is nearest to the **average** for those weekdays or weekend days **in the last 7 days**.
4. The focus of this questionnaire is **sitting and lying** while doing the activities specified below. If some of these activities also involved standing or walking around, please try to only include the time spent sitting and lying during these activities.
5. If you tick the wrong box, please put a large cross through it and then tick the correct box.

Please try to answer every question as accurately and honestly as possible. Your answers will be treated as strictly confidential.

Thank you for your help!

SECTION 1 – SLEEPING AND NAPPING

Sleeping

Think about what time you went to sleep and got up in the last 7 days. If you had variable sleeping patterns (e.g. you did shift work), please record the average time you went to bed and got up on weekdays and on weekend days.

➔ **DO NOT** INCLUDE:

- reading or watching TV before falling asleep or after waking. This is part of section 5.

	1. **In the last 7 days,**	
	at what time did you go to sleep?	at what time did you get up?
Weekday	____ (pm ☐ / am ☐)	____ (am ☐ / pm ☐)
Weekend day	____ (pm ☐ / am ☐)	____ (am ☐ / pm ☐)

Napping

A nap is a brief period of sleep, often during the day. A nap can be taken on a sofa as well as in a bed.

Please tick (✓) **one** box only per line.

	2. **In the last 7 days,** on average, how long did you nap per day?					
	No daily napping	1-15 min	15-30 min	30-45 min	45 min-1 hour	More than 1 hour a day
Weekday	☐	☐	☐	☐	☐	☐
Weekend day	☐	☐	☐	☐	☐	☐

SECTION 2 – MEALS

Please think about the amount of time you spent sitting for breakfast, lunch and dinner, on average in the last 7 days.

➔ **DO NOT** INCLUDE:
- time spent eating while watching TV. This is part of section 5.

➔ **DO** INCLUDE:
- time spent sitting for breakfast, lunch and dinner (at home, work,…), also when you were reading, chatting to other people or listening to the radio. For example if you spent 30 minutes sitting for breakfast while reading the newspaper, or for lunch while working, then include this in this section.

Please tick (✓) **one** box only per line.

	3. **In the last 7 days,** on average, how long did you sit for each of these meals per day?						
	None	1-10 min	10-20 min	20-30 min	30-45 min	45 min-1 hour	More than 1 hour a day
Weekday							
Breakfast	☐	☐	☐	☐	☐	☐	☐
Lunch	☐	☐	☐	☐	☐	☐	☐
Dinner	☐	☐	☐	☐	☐	☐	☐
Weekend day							
Breakfast	☐	☐	☐	☐	☐	☐	☐
Lunch	☐	☐	☐	☐	☐	☐	☐
Dinner	☐	☐	☐	☐	☐	☐	☐

SECTION 3 – TRANSPORTATION

This section refers to the time you spent sitting during transportation (travelling in a car, bus, train, on a motorbike, etc.) in the last 7 days. The questions are about travelling to and from your occupation, travelling as part of your occupation, and getting about apart from your occupation.

"Occupation" refers to three different types of activities: work, study and volunteering. "Work" refers to all tasks done to earn money. "Study" refers to educational activities. "Volunteering" refers to work that you do for no pay, such as helping in a sports club. Please think about all three of these categories for the following questions.

➔ **DO NOT** INCLUDE:
- cycling on a pedal bicycle

4. Have you been working, studying or volunteering (referred to as "occupation") **in the last 7 days**?	Yes ☐ No ☐

➔ If you did not have an occupation in the last 7 days, please skip to the **"Getting about – apart from your occupation" section** below.
➔ If you did have an occupation, please answer the questions below. There is space for two different occupations ("Occupation 1" and "Occupation 2").

Travelling to and from your occupation

5. **In the last 7 days**, how many days a week did you sit while travelling to and from your occupation? (in a car, bus, train, on a motorbike, etc.; do not include cycling on a pedal bicycle)	**Occupation 1**: _____ days **Occupation 2**: _____ days

Please tick (✓) **one** box only per line.

	6. **In the last 7 days,** on average, how long did you sit while travelling to and from your occupation on such a day? (in a car, bus, train, on a motorbike, etc.; do not include cycling on a pedal bicycle)													
	None	1-15 min	15-30 min	30-45 min	45 min-1 hour	1-1.5 hours	1.5-2 hours	2-2.5 hours	2.5-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	More than 7 hours
Occupation 1	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Occupation 2	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Travelling as part of your occupation

Now think about the time you sit while travelling as part of your occupation, for example driving from one customer to another, driving a taxi, etc.

7. **In the last 7 days**, how many days a week did you sit while travelling as part of your occupation? (in a car, bus, train, on a motorbike, etc.; do not include cycling on a pedal bicycle)

Occupation 1: _____ days

Occupation 2: _____ days

Please tick (✓) **one** box only per line.

	8. **In the last 7 days,** on average, how long did you sit while travelling as part of your occupation on such a day? (in a car, bus, train, on a motorbike, etc.; do not include cycling on a pedal bicycle)													
	None	1-15 min	15-30 min	30-45 min	45 min-1 hour	1-1.5 hours	1.5-2 hours	2-2.5 hours	2.5-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	More than 7 hours
Occupation 1	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Occupation 2	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Getting about – apart from your occupation

Now think about the time you sit while getting about apart from your occupation, for example when going to the supermarket, going to visit friends, etc. Please include time spent sitting to and from your destination.

Please tick (✓) **one** box only per line.

	9. **In the last 7 days,** on average, how long did you sit for getting about apart from your occupation per day? (in a car, bus, train, on a motorbike, etc.; do not include cycling on a pedal bicycle)													
	None	1-15 min	15-30 min	30-45 min	45 min-1 hour	1-1.5 hours	1.5-2 hours	2-2.5 hours	2.5-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	More than 7 hours
Weekday	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

SECTION 4 – WORK, STUDY AND VOLUNTEERING

This section is about the time you spent sitting during your occupation, which refers to work, study and volunteering. Please think about all three of these categories for the following questions.

10. Did you have an "occupation" **in the last 7 days**? Yes ☐ No ☐

➔ If you did not have an occupation in the last 7 days, please skip to **section 5**.
➔ If you did have an occupation, please complete this section. There is space for two different occupations ("Occupation 1" and "Occupation 2").

Occupation 1

11a. Type of occupation 1 Work ☐ Study ☐ Volunteering ☐

12a. Name of occupation 1 (e.g. receptionist) _____

13a. How many days did you do occupation 1 **in the last 7 days**? _____ days

14a. **In the last 7 days**, on average, how much time per day did you spend sitting while doing occupation 1?

➔ **DO NOT** INCLUDE:

- time spent sitting for transportation (in a car, bus, train, on a motorbike, etc.) either for travelling to and from this occupation, or as part of this occupation. This was part of section 3.
- breakfast, lunch or dinner. This was part of section 2.

Please tick (✓) **one** box only

None	1-15 min	15-30 min	30 min-1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	7-8 hours	More than 8 hours
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

15a. **In the last 7 days**, on average, how many times per day did you interrupt your sitting time while doing occupation 1, for example by standing up, walking somewhere, or getting a coffee? _____ times

16. Did you have a second occupation **in the last 7 days**? Yes ☐ No ☐

➔ If you did not have a second occupation in the last 7 days, please skip to **section 5**.
➔ If you did have a second occupation in the last 7 days, please answer the questions below.

Occupation 2

11b. Type of occupation 2 Work ☐ Study ☐ Volunteering ☐

12b. Name of occupation 2 (e.g. receptionist) _____

13b. How many days did you do occupation 2 **in the last 7 days**? _____ days

14b. **In the last 7 days**, on average, how much time per day did you spend sitting while doing occupation 2?

➔ **DO NOT** INCLUDE:

- time spent sitting for transportation (in a car, bus, train, on a motorbike, etc.) either for travelling to and from this occupation, or as part of this occupation. This was part of section 3.
- breakfast, lunch or dinner. This was part of section 2.

Please tick (✓) **one** box only

None	1-15 min	15-30 min	30 min-1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	7-8 hours	More than 8 hours
☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

15b. **In the last 7 days**, on average, how many times per day did you interrupt your sitting time while doing occupation 2, for example by standing up, walking somewhere, or getting a coffee? _____ times

SECTION 5 – SCREEN TIME AND OTHER ACTIVITIES

This last section refers to the time you spent sitting or lying down during other activities in the last 7 days. Remember, each period of sitting down should only **be entered once**. For example, if you spent one hour sitting on the sofa reading a book while you were listening to music, count this time as one hour reading if this was your main focus. Do not also count this as one hour listening to music.

Screen time

Please tick (✓) **one** box only per line.

	17. **In the last 7 days,** on average, how long did you spend sitting or lying down in the following activities per day?										
		None	1-15 min	15-30 min	30 min-1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	More than 7 hours
Watching TV, dvds/videos ➔ **DO include meals while sitting and watching TV**	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Using computer apart from work *(internet, e-mail, chat, networking (Facebook,…))*	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Playing computer games *(Playstation, Xbox,…)* ➔ **DO NOT include non-sitting games**	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Now think about the total time you spent **watching TV** in the last 7 days.
➔ If you did not watch TV in the last 7 days, please skip to the **"Other activities" section** below.
➔ If you did watch TV in the last 7 days, please answer the questions below.

18. **In the last 7 days**, on average, how many times per day did you interrupt your sitting time while watching TV for example, by standing up, walking somewhere, or getting a drink? _____ times

Snacking while watching TV

This is about how often you had snacks or drinks while watching TV in the last 7 days in addition to your usual meals. Only think of snacks which are not part of your breakfast, lunch or dinner.

Please tick (✓) **one** box only per line.

	19. **In the last 7 days,** on average, how often did you have the following snacks or drinks while watching TV in addition to your breakfast, lunch or dinner?									
	None	1-2 times a week	3-4 times a week	5-6 times a week	Once a day	2 times a day	3 times a day	4 times a day	5 times a day	More than 5 times a day
Savoury snacks (e.g. crisps, salted nuts)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sweets, chocolate(s) (bars), cakes, biscuits	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Ice cream, chocolate mousse	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Yoghurt, rice pudding	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Soda (e.g. coke)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Fruit juice	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Squash	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Milk, milkshake, hot chocolate	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Tea or coffee	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Alcoholic drinks (e.g. beer, wine, spirits)	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Other:_____	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Other activities

Please remember that each period of sitting down should only **be entered once**.

Please tick (✓) **one** box only per line.

		20. **In the last 7 days** on average, how long did you spend sitting or lying down in the following activities per day?										
		None	1-15 min	15-30 min	30 min-1 hour	1-2 hours	2-3 hours	3-4 hours	4-5 hours	5-6 hours	6-7 hours	More than 7 hours
Sitting while reading (book, magazine, newspaper,…)	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting while doing household tasks (cooking, ironing,…)	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting while caring for children, grandchildren, elderly or disabled relatives	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting for hobbies (playing piano, cards, doing crosswords,…)	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting for socializing (visiting friends, pub, cinema, sporting event,…)	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting while listening to music (radio, CD, MP3, iPOD,…)	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
Sitting for other activities (write below): _____	**Weekday**	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Weekend day	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

Thank you for your help!

Appendix 5: Published Papers and Communications Produced From Studies Presented Within Thesis