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Patient-derived endothelial progenitor cells as a potential ex vivo model of vascular health and disease

Northern Clinical School

A thesis submitted to fulfil requirements for the degree of Master of Philosophy

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DECLARATION

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes.

I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in the preparation of this thesis and sources have been acknowledged.

Christine Yu

ABSTRACT

Introduction: Ischaemic heart disease (IHD) is the leading cause of death in Australia. Its onset is due to abnormal pathology such as blockages or narrowing in the arteries resulting in poor perfusion. This is usually caused by atherosclerotic plaque development and can result in a myocardial infarction (MI). Through exploration of molecular pathways, we hope to identify novel biomarkers and/or therapies for cardiovascular disease (CVD). I have approached this by utilising patient-derived EPCs from the BioHEART study for molecular and functional characterisation.

Aims:

1. To develop methodology to isolate EPCs from participants in the BioHEART study.
2. To functionally characterise EPCs and compare them to standard vascular endothelial cells.
3. To collate molecular and functional data from EPCs with participant coronary artery disease (CAD) and CVD risk profile.

Methods: Patients were recruited to the BioHEART study at North Shore Private Hospital, and data was collected on age, sex, medication and cardiovascular risk factors. Blood samples were collected in heparin-coated vacuettes and peripheral blood mononuclear cells were extracted via Ficoll gradient centrifugation and cultured in 0.1% gelatin-coasted flasks in endothelial cell growth medium. Following the emergence of EPCs, cells were passaged 3 times and cryopreserved. Cells were stained with fluorescent tagged antibodies and surface marker expression was detected through flow cytometry. Angiogenesis was assessed by migration and tubule formation

assays. Redox signalling was assessed by superoxide generation in live cells and expression of reactive oxygen species (ROS) and other proteins by immunoblotting.

Results: The spontaneous development of EPCs was assessed in BioHEART participants. Body mass index (BMI) of participants affected the capacity for EPCs to develop in culture. Samples where EPCs spontaneously appeared came from patients with BMI of 25.9 ± 0.3, (n=215) whereas samples where EPCs failed to grow were from patients with BMI 30.6 ± 0.4, (n=728; p<0.0001). Having a BMI ≥ 30, diabetes and taking beta-blockers negatively affected growth, while taking statins positively contributed to growth. EPCs had an expression and functional profile similar to human umbilical vein endothelial cells (HuVECs). NADPH oxidase (Nox)-2 protein expression was increased two-fold in EPCs of participants that were classified to have CVD based on their coronary calcification measure by computer tomography (n=13-14, p<0.05). Interestingly, I observed disparity in between EPCs extracted from males and females. In females with CVD there was elevated eNOS expression in comparison to EPCs from healthy females, but this was not evident in male EPCs. This correlated with increased tubule formation in females with CVD but not males. However, I found that migration of EPCs were not affected by CVD in females, yet slightly accelerated in males. These functional changes in capacity for angiogenesis were seemingly not caused by the common angiogenesis signalling pathways, as expression and phosphorylation of AKT and ERK were not altered in EPCs from CVD patients.

Conclusion: Patient-derived EPCs have characteristics similar to vascular endothelial cells and are a robust source of molecular information relevant to CVD. They may be useful in predicting disease and/or developing personalised therapies in the future.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ACE	Angiotensin-converting enzyme
AKT	Protein kinase B
AngII	Angiotensin II
ANOVA	Analysis of variance
ARB	Angiotensin II receptor blockers
BH4	5,6,7,8-tetrahydrobiopterin
BMI	Body mass index
Ca$_{2+}$	Calcium
CABG	Coronary artery bypass graft
CAD	Coronary artery disease
CECs	Circulating endothelial cells
CHD	Coronary heart disease
CTCA	Computed tomography coronary angiogram
CVD	Cardiovascular disease
DHE	Dihydroethidium
DVT	Deep vein thrombosis
ECG	Electrocardiogram
EDHF	Endothelium-derived hyperpolarising factor
EGM+	Endothelial Growth Media plus
EGM-2	Endothelial growth media-2
eNOS	Endothelial nitric oxide synthase
EPCs	Endothelial progenitor cells
ERK	Extracellular signal-regulated kinases
ET-1	Endothelin-1
FGF	Fibroblast growth factor
Grx-1	Glutaredoxin 1
H$_2$O$_2$	Hydrogen peroxide

HBSS	Hank's balanced salt solution
HF	Heart failure
HuVECs	Human umbilical vein endothelial cell
IHD	Ischaemic heart disease
iNOS	Inducible nitric oxide synthase
iPSC	Induced pluripotent stem cell
MI	Myocardial infarction
MK2/MAPK	Mitogen-activated protein kinase
MnTMPyP/MN	Manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
NOAC	Non-vitamin K antagonist oral anticoagulants
NOS	Nitric oxide synthase
NOX	NADPH oxidase
Nox-2	NADPH oxidase 2
Nox-4	NADPH oxidase 4
$O_2^{-\bullet}$	Superoxide
PAD	Peripheral artery disease
pAKT	phosphorylated AKT
PBMC	Peripheral blood mononuclear cell
PE	Pulmonary embolism
pERK	phosphorylated ERK
PI3K	Phosphoinositide 3-kinase
PPI	Proton pump inhibitors
ROS	Redox oxidative signalling
SEM	Standard error mean
SMuRFs	Standard modifiable cardiovascular risk factors
SOD	Superoxide dismutase
TGF-β	Transforming growth factor-beta
TIA	Transient ischaemic attack
TNF-α	Tumour necrosis factor-alpha

TxA$_2$	Thromboxane A2
VEGF	Vascular endothelial growth factor
VEGFR2[+]	Vascular endothelial growth factor receptor 2

1 INTRODUCTION

Ischaemic heart disease (IHD) accounted for 12% of deaths in 2016, making it the leading cause of death in Australia (Statistics, 2017a). An increasing lifespan and ageing population have resulted in an $18.3 billion financial burden in Australia (Statistics, 2017b). IHD begins with abnormalities in the vasculature, resulting in narrowed or blocked arteries reducing blood flow. This leads to a reduced supply of oxygen to heart tissue, causing angina and in severe cases, necrosis. In coronary heart disease (CHD), the development of atherosclerotic plaque can result in rupture leading to a myocardial infarction (MI). While there are many treatment options for CAD in modern medicine such as; angioplasty, stents or coronary bypass surgery, early detection of disease would significantly reduce the burden on a patient, their families, and the healthcare system.

Standard **m**odifiable cardiovascular **r**isk **f**actors (SMuRFs) such as diabetes, hypertension, hyperlipidaemia and smoking contribute significantly to the development of atherosclerosis and are currently entered into algorithms to predict a patient's risk of a CVD event. However, in recent years, our laboratory team has been working to understand the substantial number of heart attack patients who are "SMuRFless" who get "missed" by such algorithms. Dr Vernon and other members of our team have identified for the first time, an increasing proportion of patients presenting with a myocardial infarction (MI) and no SMuRFs (Vernon, 2017). This highlights the need to identify new prognostic markers, and a deeper understanding into the mechanisms behind disease that will be broadly relevant to all patients susceptible to atherosclerosis.

One of the key contributors to CVD is endothelial dysfunction, which is mechanistically downstream and thus reflective of known risk factors such as hyperlipidaemia, hypertension, smoking and diabetes. Endothelial progenitor cells (EPCs) have been identified as cells that may potentially help regrow blood vessels post-MI and improve cardiovascular outcomes for patients. They may also have other roles in improving vascular function in pathological conditions. However, their exact role remains to be elucidated and controversies in their origins and characteristics still exist. In this thesis, I aimed to further characterise patient-derived EPCs and their potential relationship with clinical history and risk factor profiles. I also examined potential differences in molecular and cellular signalling within two groups; healthy controls and patients with CAD.

1.1 OVERVIEW OF VASCULAR PATHOLOGY

1.1.1 Coronary Artery Disease

Over time, our understanding of the pathophysiology of CAD has changed remarkably. While it was once considered a cholesterol storage disease, current understanding identifies this disease as a complex interaction of risk factors between the artery wall and circulating factors (Libby & Theroux, 2005). Inflammation and immune responses are prominent factors in the development of atherosclerosis and have become a key focus in the research field (Libby, 2002). Diagnosis of CAD usually begins with chronic stable angina, caused by an obstruction in at least one major coronary artery by plaque, resulting in a mismatch of oxygen supply and demand (Cassar, Holmes et al., 2009). Stratification scores have been created for all aspects of the disease [Figure 1.1] to help clinicians make informed decisions about patient care (Buccheri, D'Arrigo et al., 2018). Treatment of CAD includes lifestyle changes, medication interventions and revascularisation procedures where patient

expectations, short- and long-term benefits and drawbacks are considered by the treating physician (Russ, Werdan et al., 2009).
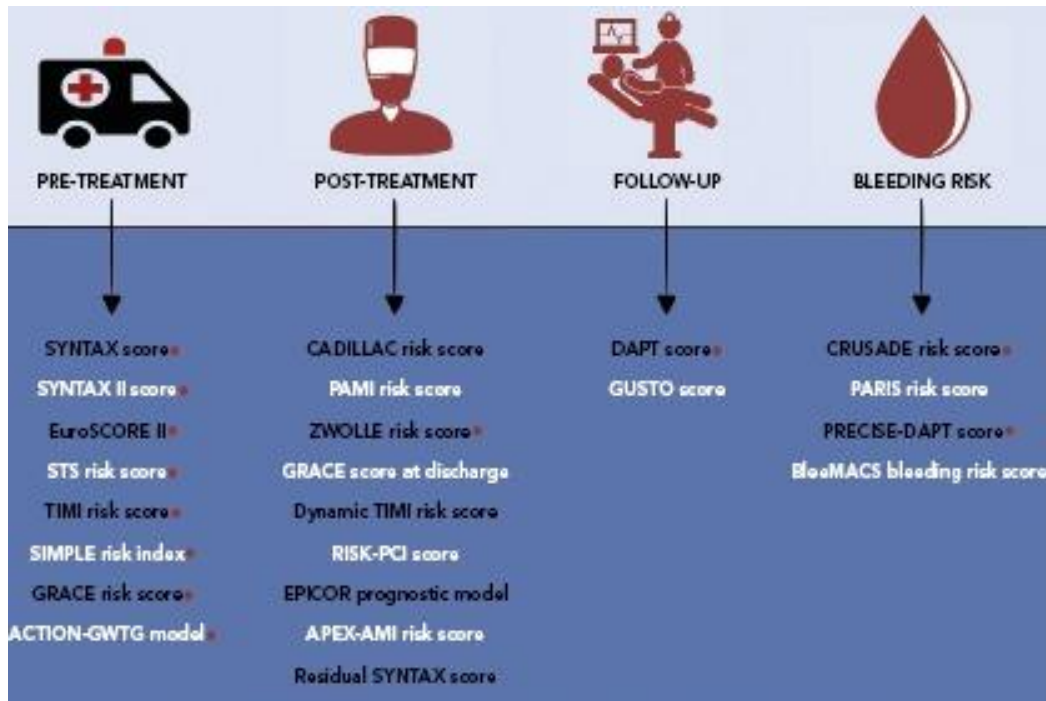
Figure 1. 1 Risk stratifications for patients triaged in the field (Buccheri et al., 2018).

1.1.2   Atherosclerosis

The vascular system exchanges oxygenated and deoxygenated blood with all organs and tissues through a network of arteries, capillaries, and veins [Figure 1.2]. The endothelium consists of a monolayer of endothelial cells (Sandoo, van Zanten et al., 2010), providing a critical physical barrier with the lumen which also serves a functional role. A poor functioning endothelium has been associated with atherosclerosis, and this often coincides with hypertension and/or diabetic complications (Ross, 1993). Vascular injury results in inflammation, leading to increased permeability to lipids and adherence of monocytes. An inflamed endothelium recruits monocytes, resulting in the build-up of cholesterol filled macrophages (foam cells) in the sub-endothelium. This creates a fatty streak inside the coronary arteries which is a precursor to plaque formation (Lusis, 2000). This continues with smooth muscle cells migrating from

the tunica media and synthesizing extracellular matrix (Bennett, Sinha et al., 2016).
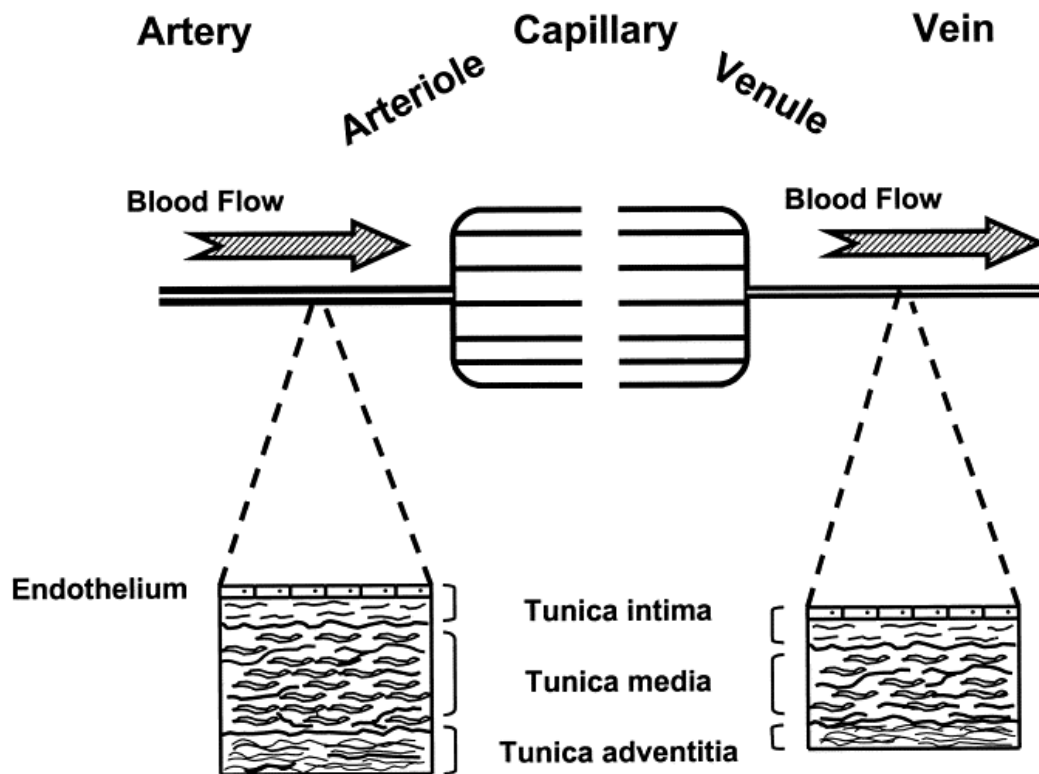
Figure 1. 2: The vascular system comprises of arteries, capillaries which supply oxygen-rich blood to organs and tissues in the body. The arteries are compo1sed of four layers, endothelium, tunica intima, tunica media and tunica adventitia (Pugsley & Tabrizchi, 2000).

Over time, more macrophages invade the fatty streak resulting in the formation of a lipid-core which eventually becomes necrotic as the atheroma grows (Bentzon Jacob, Otsuka et al., 2014). At the same time, calcification occurs within the plaque and a fibrous cap forms, which can be spotty or fragmented (Otsuka, Sakakura et al., 2014) [Figure 1.3]. As the atheroma increases in size, it encroaches on the lumen of small arteries, disrupting the flow and causing ischemia. Clinical disease can manifest from a slow expansion of the atheroma, or when this fibrous cap ruptures. When this occurs in the coronary artery it can result in an acute MI (Insull, 2009).
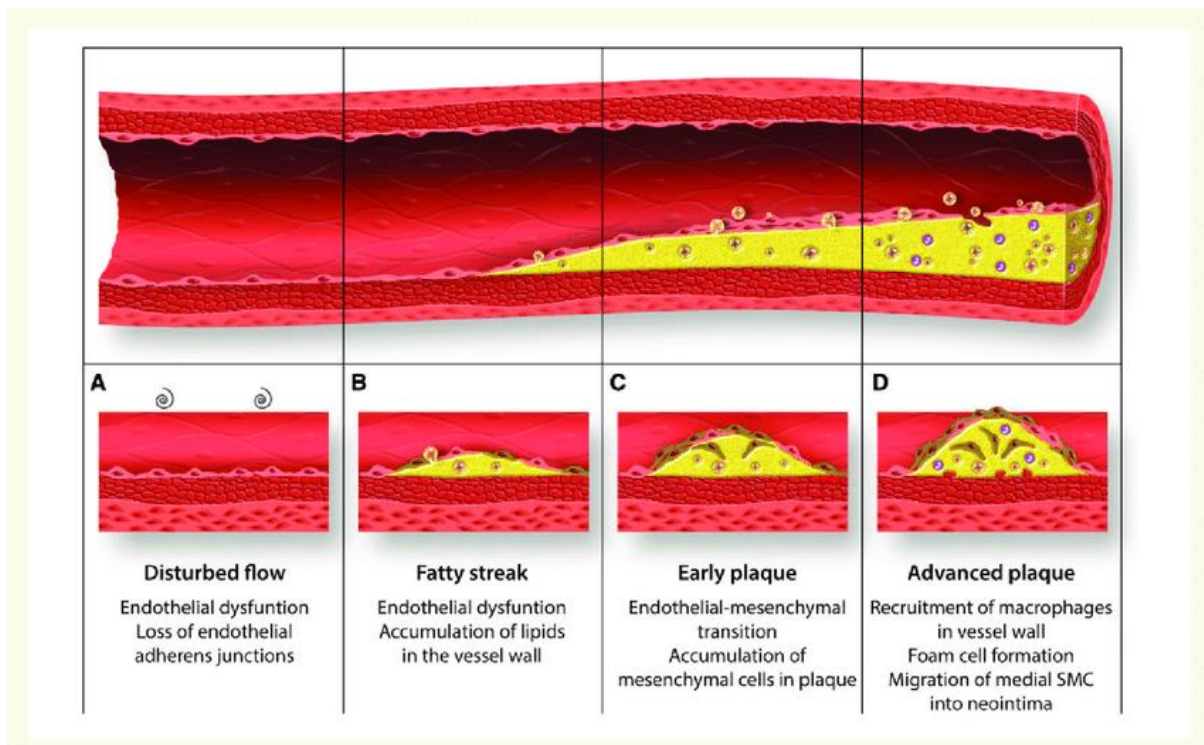
Figure 1. 3: Formation of atherosclerotic plaque over time (Souilhol, Harmsen et al., 2018)

1.1.2.1 Epidemiological factors driving atherosclerosis

Epidemiological studies have suggested that atherosclerosis is a complex aetiology impacted by many risk factors. These 'modifiable' and 'non-modifiable' risk factors impact endogenous factors influencing vascular function, and contributing to the pathogenesis of CVD. Modifiable risk factors include hypercholesterolemia, smoking, hypertension, and diabetes (Yusuf, Hawken et al., 2004). Major non-modifiable risk factors are age, sex and family history of coronary disease (Tabei, Senemar et al., 2014). Other external factors include lack of exercise, obesity, emotional stress and diet (Hajar, 2017). Longitudinal studies like the Framingham study have revealed that risk factors act in synergy when present in one individual, increasing the risk of cardiovascular disease by 2-3 fold with different co-morbidities (Mahmood, Levy et al., 2014).

1.1.3 Factors controlling vascular function

A monolayer of endothelial cells line the entire vasculature, where an impaired endothelial function is associated with hypertension, atherosclerosis, and diabetic vascular dysfunction. Endothelial inflammation and oxidative stress play a key role in mediating the effects of known and unknown risk factors driving CVD [Figure 1.4]. Maintenance of healthy vascular function is critical for regulation of vascular tone via vasodilators and vasoconstrictors, prevention of plaque formation, and homeostasis of antiplatelet and anticoagulation status.
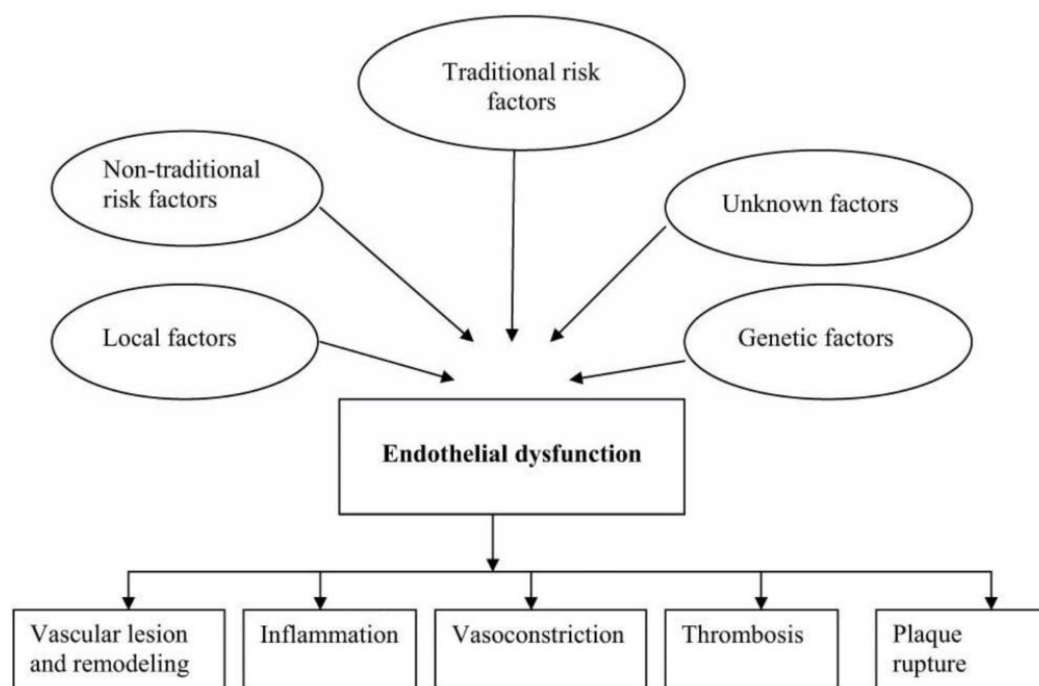
Figure 1. 4, Factors controlling vascular function. Endothelial dysfunction is seen in many of the standard modifiable risk factors of cardiovascular diseases, such as diabetes, obesity, hypertension, and dyslipidaemia (Hadi, Carr et al., 2005).

1.1.3.1 Vascular tone

Vascular tone is essential to vascular health as it determines blood flow to the body and organs. Vascular tone dysfunction can lead to a vasovagal episode or the development of hypertension due to the stiffening of the arteries. While there are many factors involved in the maintenance of vascular health, exercise is a behavioural modification that is believed to preserve and restore vascular health that is easily accessible to all. A study showed that trained elderly patients had improved vascular relaxation upon stimulation of the endothelium in comparison to sedentary elderly patients, highlighting the benefit of increased endothelial function training (Taddei, Galetta et al., 2000)[Figure 1.5].
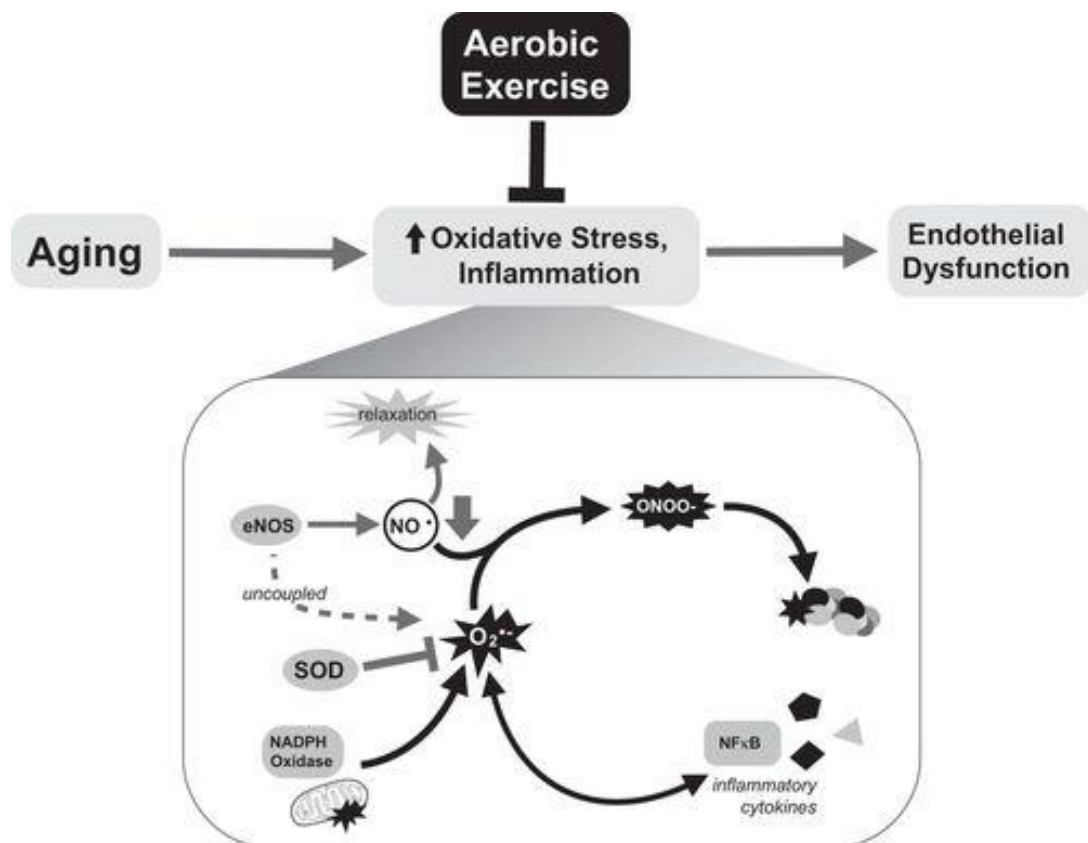
Figure 1. 5   Aerobic exercise suppresses arterial stiffening that occurs with age and is responsible for cardiovascular risk factors like hypertension(Santos-Parker, LaRocca et al., 2014); eNOS, endothelial nitric oxide synthase; NO, nitric oxide; O$_2$., superoxide; SOD, superoxide dismutase; ONOO-, peroxynitrate; NADPH, nicotinamide adenine dinucleotide phosphate; NF-κB, transcription factor .

1.1.3.1.1 Nitric Oxide – a key regulator of vascular tone

Maintenance of appropriate vascular tone requires both vasodilators and vasoconstrictors. Nitric Oxide (NO) is a well-known endothelial-derived vasodilator. NO is produced enzymatically by NO synthases (NOS) of which there are three isoforms. Neuronal NOS (nNOS) is found in central and peripheral nerves that control smooth muscle relaxation, blood pressure and vasodilation. Inducible NOS (iNOS) can influence blood pressure and inflammation but is also connected to the immune system (Lirk, Hoffmann et al., 2002). Lastly, there is endothelial NOS (eNOS) which regulates blood pressure, is vasoprotective and anti-atherosclerotic (Förstermann & Sessa, 2012). eNOS is the primary isoform responsible for vascular NO bioavailability, its structure contains a C-terminal reductase domain and an N-terminal oxidase domain, which binds heme and tetrahydrobiopterin (BH4) [Figure 1.6]. Oxygen and L-arginine bind to the reductase domain while NADPH, flavin adenine dinucleotide and favin mononucleotide cofactors bind to the C-terminal domain (Maron & Michel, 2012). The ferrous-dioxygen complex in the reductase and oxygenase domains in eNOS is responsible for producing NO (Förstermann & Münzel, 2006). Disrupted eNOS function can occur due to 'uncoupling' of the ferrous-dioxygen complex, resulting in increased superoxide ($O_{2-\bullet}$) production.
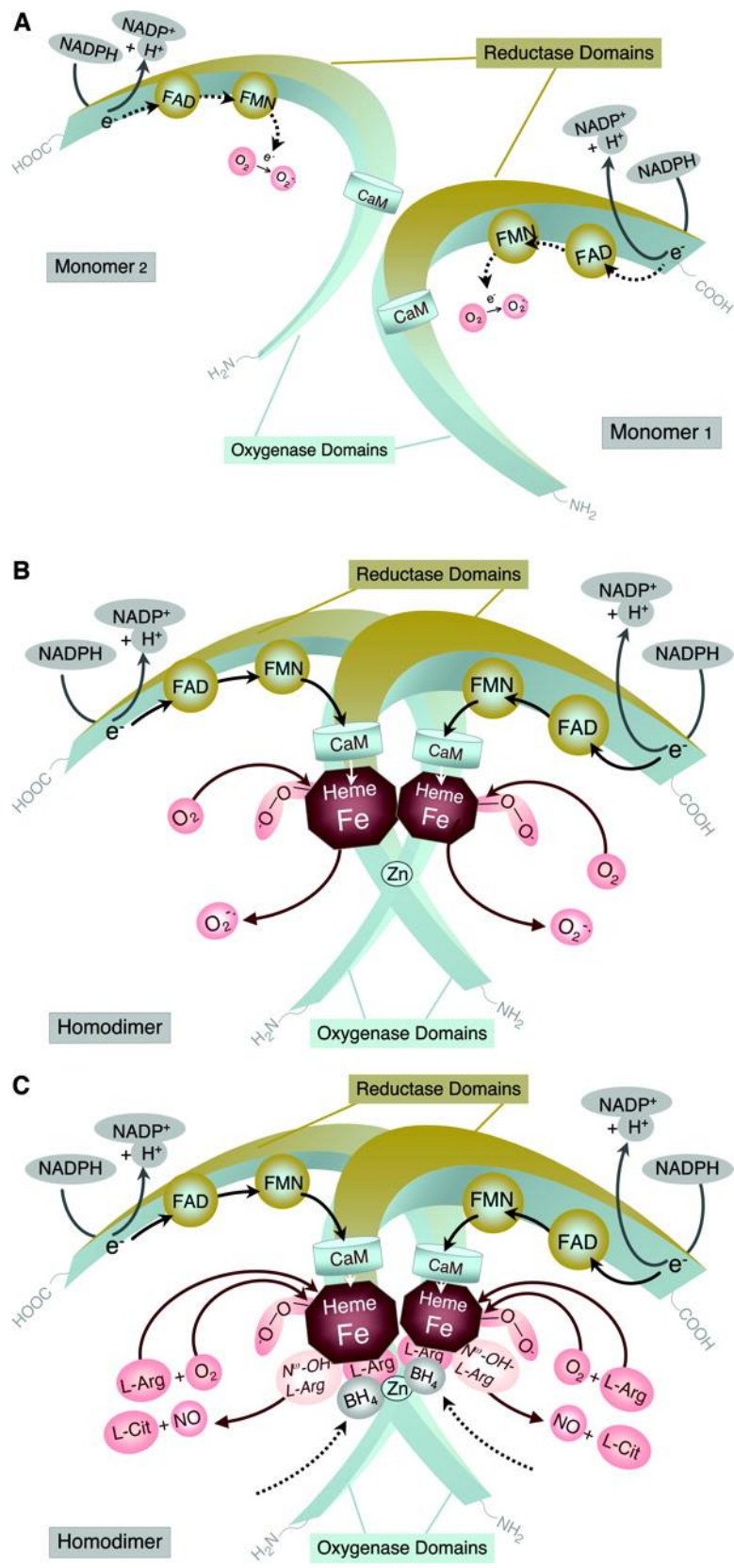
Figure 1. 6: eNOS uncoupling; A) eNOS structure, B) Ferrous dioxygen complex allowing NOS dimerization, C) In the presence of L-arginine(L-Arg) and BH4, ferros-dioxygen complex produced NO instead of superoxide (Förstermann & Münzel, 2006). FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; CaM, calmodulin; NADPH, nicotinamide adenosine dinucleotide phosphate; L-Cit, L-citrulline

NO has multiple beneficial roles in vascular function. These include dilation of vessels, counteracting inflammation, preventing platelet and leukocyte adhesion to vessel wells and being a vital part of the immune system (Wink, Hines et al., 2011). NO dilation is achieved by the activation of guanylate cyclase (Chen, Pittman et al., 2008), making it an important molecule in the regulation of vascular homeostasis. One mechanism that may increase stimulation for NO production is exercise. Shear stress on the endothelium brought on by increased blood flow through main muscle groups may regulate endothelial NO bioactivity (Maiorana, O'Driscoll et al., 2003). The inhibition of eNOS may result in vasoconstriction, emphasizing the importance of NO-dependent vasodilation in the maintenance of blood pressure and its overall effect on vascular health (Philipp, Georg et al., 2002).

NO is a free radical which is an inflammatory mediator that is mobilised in antimicrobial defence via macrophages (Korhonen, Lahti et al., 2005). Inversely, NO can also increase inflammation within the body but the pathogenesis of the inflammation is not well understood (Tripathi, Tripathi et al., 2007). eNOS produces most of the NO in vasculature. However, during oxidative stress, 5,6,7,8-tetrahydrobiopterin (BH4) availability, which regulates eNOS, may be impaired. This can lead to production of superoxide at the expense of eNOS (Hajar, 2017). Alternatively, NO may react with excess free radicals resulting in production of peroxynitrate, and thereby lower NO bioavaliability (Pacher & Szabó, 2006) [Figure 1.7]. The consequences are pathological, due to lack of bioavailable NO causing increased inflammation, and resulting in an atherosclerosis-prone state (Förstermann & Sessa, 2012).
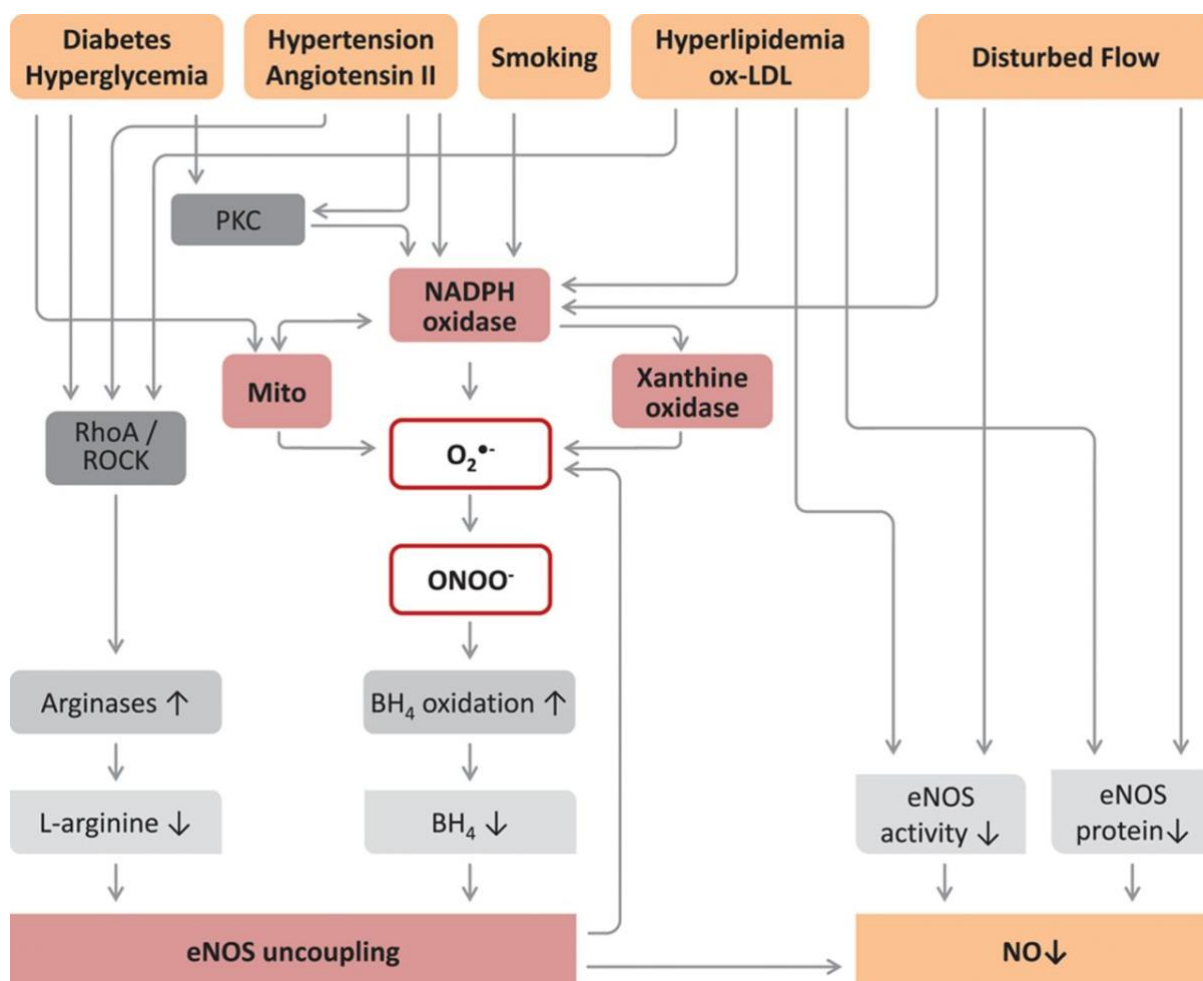
Figure 1. 7: A number of factors contribute the oxidative stress, which results in an increase in eNOS uncoupling and a reduction of NO production, leading to an atherosclerosis-prone state (Förstermann, Xia et al., 2017). PKC, protein kinase C; ROCK, rho-associated protein kinase; O_2^{-} •superoxide; ONOO-, peroxynitrate.

Risk factors such as hyperlipidaemia affect NO production, often due to the increase in free radical production. In a pre-clinical model, hypercholesterolaemia in rabbits resulted in impaired endothelial-dependent relaxation, however, when supplemented with polyethylene superoxide dismutase to quench free radicals, maximum contraction intensity was restored (Mügge, Elwell et al., 1991). Another study showed increased NADPH oxidase activity in cholesterol-fed rabbits, a key driver of superoxide. This correlated with reduced endothelial-dependent vasodilation with the

perseverance of endothelial-independent vasorelaxation (Warnholtz, Nickenig et al., 1999).

1.1.3.2 Other regulators of vascular tone

Aside from NO, there are a number of other key pathways that regulate vascular tone. These are briefly reviewed below, but are not a major focus of this thesis.

1.1.3.2.1 Prostacyclin

Prostacyclin is a part of the prostanoid family which are derived from arachidonic acids. Family members include phospholipase A_2, cyclooxygenase and specific prostaglandins (Moncada & Vane, 1979). Endothelial cells release prostacyclin which acts as a potent vasodilator of arteries via smooth muscle relaxation and inhibits platelet aggregation (Ruan, Dixon et al., 2010). It is more prominently active in certain vascular beds, in particular the pulmonary circulation (Ruan et al., 2010)

1.1.3.2.2 Endothelium-derived hyperpolarising factor

Another vasodilator of interest is an endothelium-derived hyperpolarising factor (EDHF) which has a defined action of causing membrane hyperpolarisation, thereby increasing potassium ion conduction and causing relaxation (Ozkor & Quyyumi, 2011). EDHF is often able to compensate when NO production is impaired by atherosclerosis and other vascular diseases, particularly in the microvasculature (Luksha, Agewall et al., 2009) and may be more active in women (Villar, Hobbs et al., 2008).

1.1.3.2.3 Endothelin

Endothelin-1 (ET-1) is a vasoactive peptide that works as a counterpart to NO to maintain vascular health. In disease when NO production is subdued, there is often also augmented production of ET-1. This can lead to the development of hypertension (Marasciulo, Montagnani et al., 2006). ET-1 is known to cause long-lasting contractions leading to the dysfunction of the endothelium, producing superoxide and increasing oxidative stress (Kowalczyk, Kleniewska et al., 2015). This was seen in patients who underwent percutaneous coronary interventions or thrombolytic therapy who had increased levels of ET-1, and a decrease in NO and eNOS expression (Zhaoying, Jinliang et al., 2013). The combination of high ET-1 levels and low NO and eNOS was also seen in patients present with acute MIs and diabetes (Li, Yang et al., 2012).

1.1.4 Role of NO and redox signalling in vascular health

1.1.4.1 Anti-platelet and anti-coagulation

Thromboxane A2 (TxA$_2$) is also a member of the prostanoids family but is a vasoconstrictor, as well as contributing to platelet adhesion and aggregation in cardiovascular disease. Often when endothelial dysfunction occurs, there is an increase in TxA$_2$ release which is the primary event in thrombus formation. This can exacerbate endothelial dysfunction by inhibiting NO and prostacyclin (Ellinsworth, Shukla et al., 2014). It has been shown in a pre-clinical model that TxA$_2$ blocks dilation of vessels through the EDHF signalling pathway, which can be restored by blocking thromboxane/prostanoid receptors (McNeish & Garland, 2007). This is further supported in patient studies, where inhibition of TxA$_2$ with low dose aspirin is often used to protect against thrombotic events in CVD (Smyth, 2010).

Atherosclerosis initiation requires activated platelets or activated endothelial cells or both, which is a result of pathological stimuli such as hypertension, diabetes or smoking (Hamilos, Petousis et al., 2018). It is shown that CAD patients have significantly increased expression of platelet activation markers (Corinaldesi, 2011). Endogenous inhibitors of platelets are prostaglandin and NO, which under normal conditions are produced by the endothelium. They inhibit platelet activation, aggregation and the recruitment of platelets into a thrombus (Willoughby, Holmes et al., 2002), which in the case of vascular dysfunction are stimulated by thrombin, adenosine diphosphate and TxA_2 (Freedman Jane, 2008).

1.1.5 Dysregulated redox-signalling in the unhealthy artery

With ageing and pathological changes, free radicals are often increased. This leads to increased inflammation and decreased elasticity in vessels (Khanna RD, 2014). NADPH oxidase is one of the significant drivers of free radical generation. It is composed of five NOX complexes that have different roles in redox-signalling (see section 1.1.5.1). NOX 1, 2, 4 and 5 have all been shown to be involved in some way in CVD (Griendling, 2004). Reactive Oxygen Species (ROS) which includes superoxide ($O_2\text{-}\bullet$), hydrogen peroxide (H_2O_2) and hydroxyl radicals, are a by-product of NADPH oxidase and are responsible for the development of disease. Other factors influencing NADPH oxidase in producing ROS are the renin-angiotensin system and anti-oxidants that counter-balance the oxidative stress.

Oxidative stress is the term used to describe the damage caused by ROS. It is normally attenuated by NO in a functional vasculature. In dysfunctional conditions, ROS contributes to vascular remodelling and the development of atherosclerosis (Taniyama & Griendling, 2003). $O_2\text{-}\bullet$ is typically generated from the endothelium and vascular smooth muscle and is utilised by the body to target and kill invading pathogens (Munzel & Harrison, 1999). However, a

surplus in $O_2\text{-}\bullet$ production is harmful and causes protein denaturation and lipid peroxidation (Hayyan, Hashim et al., 2016). In cardiovascular disease, an increase in $O_2\text{-}\bullet$ leads to a decrease in the bioavailability of NO, inhibits proliferation of vascular smooth muscle cells (VSMCs) and aggregation of platelets which are the first stages of atherosclerosis development (Fukai, Folz et al., 2002).

H_2O_2 is a vital component in the immune system as an anti-microbial (Zubko & Zubko, 2013), however, under pathological conditions such as hypertension, hyperlipidaemia, and diabetes, an overproduction of H_2O_2 leads to macrophage infiltration contributing to lesion development (Leopold & Loscalzo, 2008). H_2O_2 is generated by specific isoforms of NADPH oxidase. Nox1, 2, and 4 are generally implicated in the progression of CVD (Brandes, Weissmann et al., 2010), although in a complicated pattern where Nox-4 is sometimes shown to be protective (Hakami, Ranjan et al., 2017). Superoxide dismutase (SOD) is an antioxidant that catalyses $O_2\text{-}\bullet$ into H_2O_2, reducing oxidative damage and the build-up of $O_2\text{-}\bullet$ (Bayr, 2005). It is present in 3 forms in the human body; SOD1 which is present within the cytoplasm, SOD2 is found within the mitochondria, and lastly, SOD3 which is extracellular (Fukai & Ushio-Fukai, 2011). When stress occurs, ROS may be up-regulated, increasing $O_2\text{-}\bullet$ and lowing bioavailability of NO contributing to increased cell apoptosis and diabetic complications (Li & Shah, 2004).

1.1.5.1 NADPH Oxidase isoforms in vascular disease

NADPH oxidase is generally found on the membrane of cells, and in humans there are seven isoforms; Nox1-5 and Duox 1/2 (Panday, Sahoo et al., 2014). NADPH oxidase is an integral part in the generation of ROS, including O_2-•, NO, and H_2O_2 [Figure 1.8]. It also affects cell adhesion, hypertrophy, proliferation, apoptosis, inflammation and matrix remodelling (Griendling, Sorescu et al., 2000). The inhibition of NADPH results in a decreased survival and proliferation of endothelial cells (Li, Zhang et al., 2017).
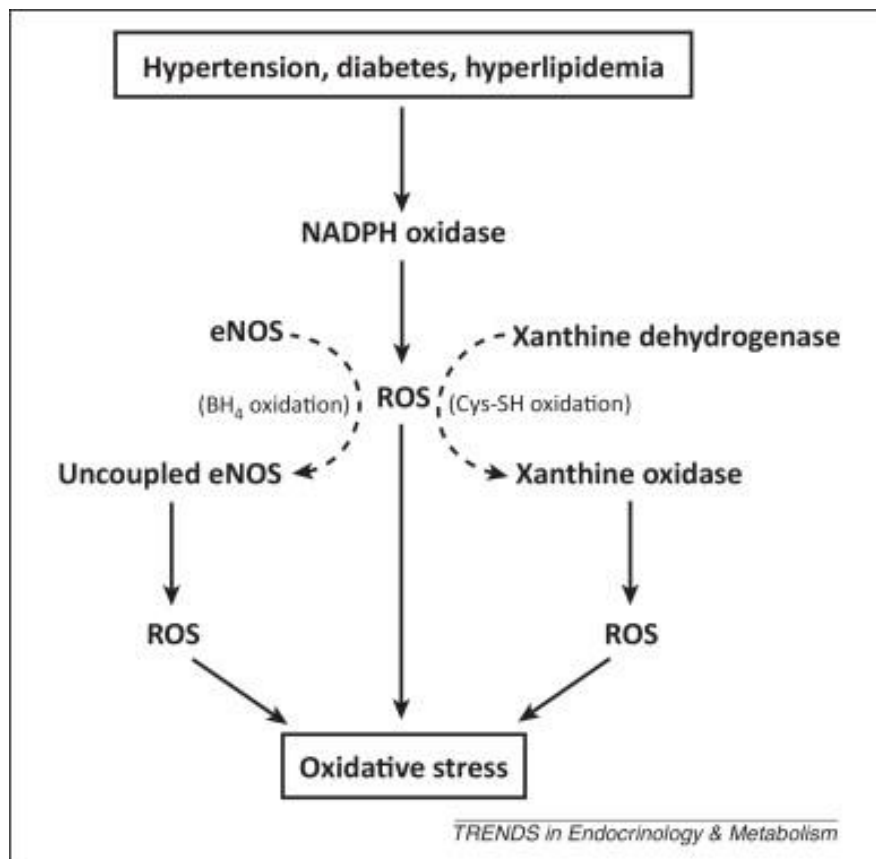
Figure 1. 8: Due to various conditions, NADPH oxidase are upregulated, promoting endothelial dysfunction (Drummond & Sobey, 2014).

1.1.5.1.1 Nox-2

Nox-2 is an isoform of NADPH oxidase, which is also known as gp91$_{phox}$. It is found in endothelial cells, adventitial fibroblasts and circulating macrophages (Madamanchi & Runge, 2010). Out of all the NADPH oxidase isoforms, Nox-2 is one of the main drivers of CAD, causing increased monocyte/macrophage infiltration (Quesada, Lucero et al., 2015). Nox-2 also contributes to vasoconstriction and aggregation properties that is associated with platelet-related thrombosis. Inhibition of Nox-2 in animal models showed a delayed atherosclerotic progression which suggests it may be a potential target in delaying CAD (Violi, Carnevale et al., 2017). However, there has been contradicting studies in mice that suggest that while Nox-2 is responsible for initial recruitment of inflammatory cells, it did not alter plaque progression over time (Douglas, Bendall et al., 2012). Nox-2 produces ROS which is a cause of oxidative stress, however, up-regulation of ROS is used as an antimicrobial; playing a role in immunity (Lam, Huang et al., 2010).

1.1.5.1.2 Nox-4

Conversely, Nox-4 produces H_2O_2 instead of $O_2{-}\bullet$ (Schürmann, Rezende et al., 2015). In pre-clinical models, Nox-4 deficient mice had significant protection against atherosclerosis development (J. Kim, Seo et al., 2016). However, there is evidence of increased plaque progression and vascular remodelling in knockout Nox-4 mice (Gray, Di Marco et al., 2016). It has also been shown the Nox-4 preserves eNOS function, promotes angiogenesis and reduces inflammation (Fulton & Barman, 2016). Interestingly, Nox-4 localises in the mitochondria (Block, Gorin et al., 2009), where angiotensin II (AngII) also stimulate NADPH oxidases (Dikalov & Nazarewicz, 2013). Despite this apparent protection mechanism of Nox-4, some studies have suggested that Nox-4 can have a damaging role (Thallas-Bonke, Jandeleit-Dahm et al., 2015; Vendrov, Vendrov et al., 2015)

1.1.5.2 Renin-angiotensin system

Diabetes, hypertension, and hypercholesterolaemia are associated with increased arterial oxidative stress. One of the pathways responsible is the renin-angiotensin system, which binds AngII via the protein kinase C (PKC) dependent mechanism to promote fibrosis and oxidative stress influencing the remodelling of the vasculature (Van Thiel, Van der Pluijm et al., 2015). In animal studies, mice supplemented with Ang II had increased $O_2\text{-}\bullet$ production and mitogen-activated protein kinase (MK2) which increased blood pressure (Ebrahimian, Li et al., 2011). In our own laboratory we have found AngII to promote oxidative stress in endothelial cells via a specific post-translational event that impairs eNOS and reduce NO (Galougahi, Liu et al., 2014; Karimi Galougahi, Antoniades et al., 2015)

1.1.6 Angiogenesis

Angiogenesis is the growth of new blood vessels throughout all phases of life [Figure 1.9]. It is a pathological process from which new blood vessels are formed from pre-existing vessels through sprouting and splitting (Chung & Ferrara, 2011). Uncontrolled angiogenesis can lead to cancer or retinopathies, while insufficient angiogenesis can lead to CAD (Gupta & Zhang, 2005). It was found that neovascularization may play a role in unstable angina (Tenaglia, Peters et al., 1998), but remains a highly contested theory. Conversely, angiogenesis can also be used as a therapeutic strategy to re-vascularise ischaemic areas (Simons & Ware, 2003).
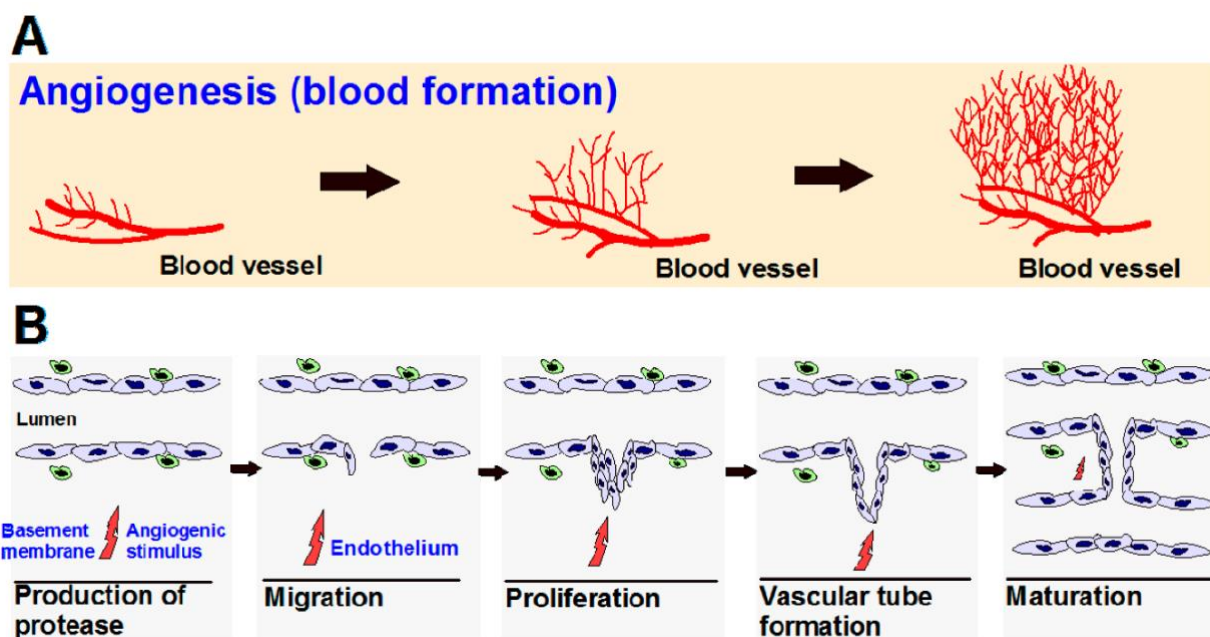
Figure 1. 9: Formation of new blood vessels via angiogenesis (Rajabi & Mousa, 2017).

1.1.6.1 Factors involved in regulating angiogenesis

Commonly identified angiogenic growth factors and cytokines are vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta (TGF-β), and the angiopoietins (Ang). VEGF enhances endothelial functions which result in increased synthesis of NO and prostacyclin, resulting in VEGF-dependent protection (Zachary, Mathur et al., 2000). FGF is stored in the vascular basement membrane and is up-regulated in angiogenesis. The binding of FGF to receptor tyrosine kinases FGFR-1 leads to an increase in endothelial cell migration and capillary morphogenesis (Ucuzian, Gassman et al., 2010). There are 4 different types of angiopoietin isoforms identified where Ang-1 is a critical part of vessel maturation and facilitates migration, adhesion, and survival of endothelial cells. Conversely, AngII disrupts the endothelium, promotes cell apoptosis and vascular regression (Fagiani & Christofori, 2013).

TNF-α appears to have different effect on angiogenesis, dependent on where it is activated. It negatively impacts endothelial cells through the inhibition of cell proliferation, while stimulating vessel growth in the cornea (Fajardo, Kwan et al., 1992). Another molecular modulator with dual roles in angiogenesis is TGF-β. It plays crucial roles in vasculogenesis and angiogenesis which can either promote or suppress endothelial migration, proliferation, permeability and sprouting (Viloria-Petit, Richard et al., 2013). All these factors influence angiogenesis upstream through various signalling pathways.

1.1.6.2 NO in angiogenesis

Therapeutic angiogenesis is often needed in patients with coronary and peripheral artery disease to attenuate ischaemic symptoms and re-supply blood to affected areas (Cooke, 2003). NO is pro-angiogenic, acting through eNOS and cyclic guanosine monophosphate, triggering cell growth and differentiation (Morbidelli, Donnini et al., 2003). In HuVECs, stimulation with VEGF resulted in a significantly higher production of NO (Hood, Meininger et al., 1998). With basic fibroblast growth factor, HuVECs and pulmonary artery endothelial cells showed increased NO mRNA expression and extensive capillary structure formation (Babaei, Teichert-Kuliszewska et al., 1998). This is further shown in ex-vivo cell modelling where an increase in NO resulted in capillary structures being formed, whereas reduced NO resulted in attenuated angiogenesis (Cooke John & Losordo Douglas, 2002) [Figure 1.10]. While this is promising for therapeutics in the cardiovascular field, it also identified NO as a potential target in tumor angiogenesis. NO can have promoting and tumoricidal effects in cancer depending on timing, location, and concentration (Choudhari, Chaudhary et al., 2013)
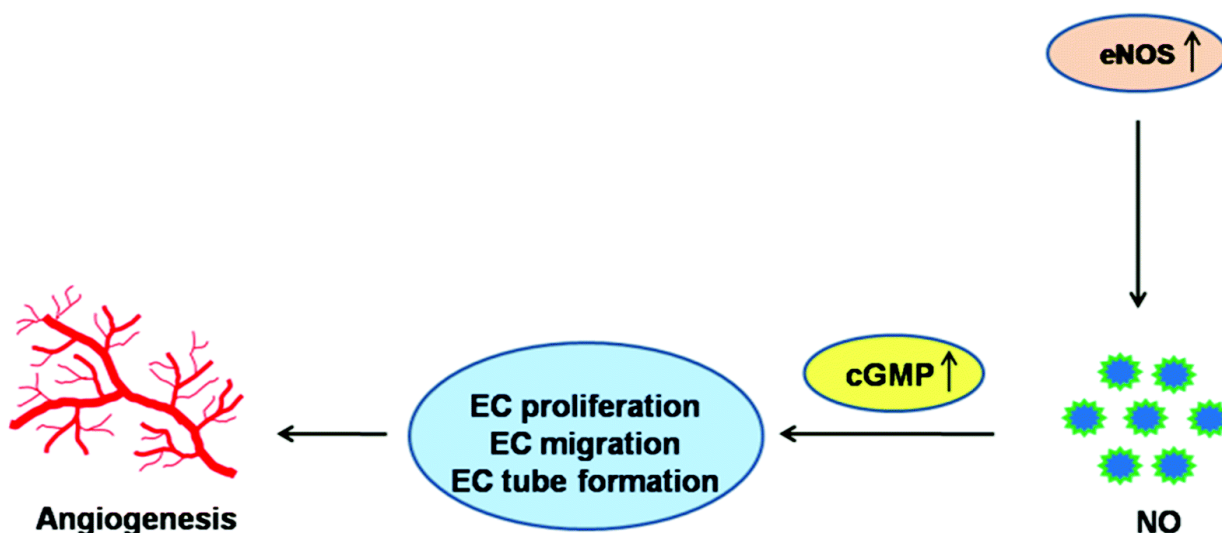
Figure 1. 10: The role of NO in angiogenesis (Barui, Nethi et al., 2017)

1.1.6.3 AKT

PI3K is a family of enzymes that phosphorylate the 3'-OH and downstream phosphatidylinositol 3,4,5-triphosphate activates AKT. Phosphatase and tensin homolog opposes this action and reduces the activation of AKT which is an important part of cell survival, proliferation, tumour growth and angiogenesis (Jiang & Liu, 2008). Activation of AKT is achieved through the phosphorylation of the mammalian target of rapamycin and also through NO regulation (Karar & Maity, 2011), which is triggered by VEGF and fibroblast growth factor (Somanath, Razorenova et al., 2006) [Figure 1.11]. In pre-clinical models, deletion of AKT1 affect angiogenesis, confirming its purpose in physiological angiogenesis (M. Y. Lee, Luciano et al., 2014). Activation can also be achieved with statins, shown in a pre-clinical model which resulted in the promotion of angiogenesis in the limbs of rabbits (Kureishi, Luo et al., 2000). AKT signalling not only affects vascular endothelial cells, but also act on tumours in leukemia, making it a potential therapeutic target (Naoko Okumura, 2012).
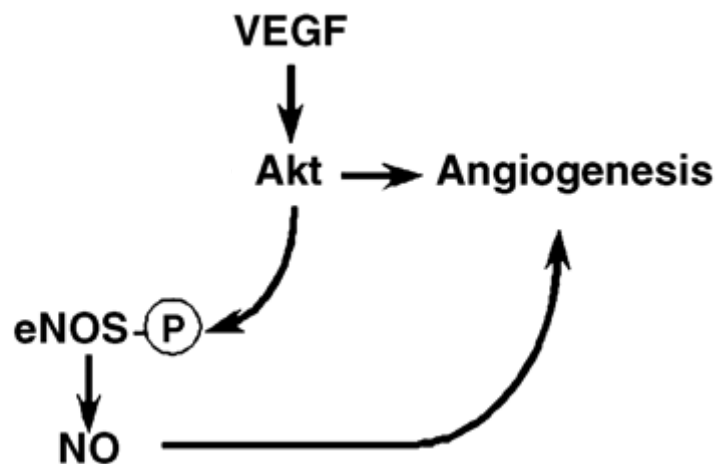
Figure 1. 11: VEGF activates AKT to contribute to angiogenesis (Nagata, Mogi et al., 2003)

1.1.6.4 MAP Kinases

Mitogen-activated protein kinase (MAPK) is grouped into three families; ERK (extracellular-signal-regulated kinase), JNK (jun amino-terminal kinases) and p38/SAPKs (stress-activated protein kinases) (Morrison, 2012). ERK is activated by a large number of stimuli and plays a central role in proliferation, differentiation, development, and migration of endothelial cells (Shaul & Seger, 2007). VEGF is one of the stimuli that was seen to induce angiogenesis in zebrafish, which was lost in VEGF signalling mutants (Shin, Beane et al., 2016a) [Figure 1.12]. However, shear stress has also been shown to cause MAPK phosphorylation which then induces angiogenesis (Gee, Milkiewicz et al., 2010). Within the vasculature, inhibition of the ERK pathway resulted in the prevention of endothelial sprouting but not artery differentiation (Shin, Beane et al., 2016b).
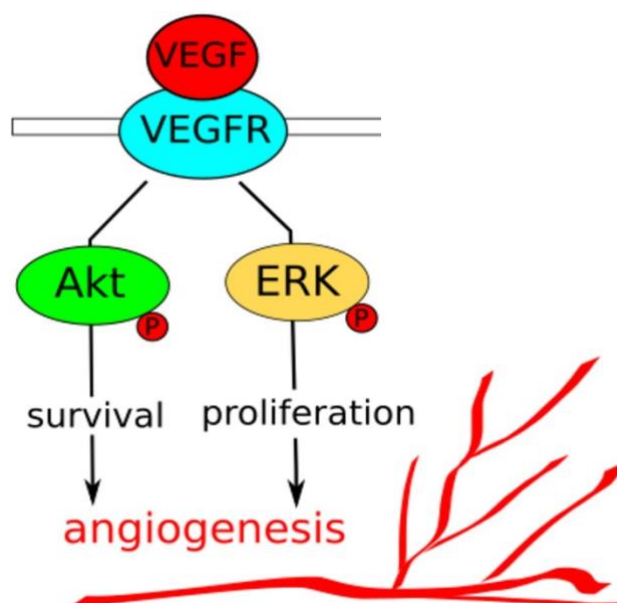
Figure 1. 12: VEGF activation of ERK pathway resulting in angiogenesis (Hara, Monguchi et al., 2017).

All of these signalling pathways are active within endothelial cells and are a crucial part of promoting angiogenesis in the vascular system. EPCs are a particular endothelial cell of interest due to their ability to move around the

body and to sites that are damaged in the heart. Their origins and characteristics are still disputed, but they could be a useful tool in the diagnosis and/or treatment of cardiovascular disease.

1.2 ENDOTHELIAL PROGENITOR CELLS (EPCs)

There is continued interest in EPCs due to their potential to identify cardiovascular risk in a variety of different clinical settings. Their ability to migrate, proliferate and develop microvasculature allows it to be a potential therapeutic target (Aragona, Imbalzano et al., 2016).

1.2.1 Origins

EPCs are still poorly characterised and defined, making it difficult to determine the exact role that they play in the recovery of patients' post-myocardial infarction (MI). There is still no firm consensus on the definition of an EPC, but there is potential for clinical use in re-endothelialisation in ischemia, tissue engineering and delivery of proangiogenic factors (Xu, 2005) It is thought that EPCs originate from bone marrow as mesodermal stem cells before differentiating into haemangioblasts (Rae, Kelly et al., 2011) [Figure 1.13]. However, it has also been shown that myeloid cells, which are CD14 positive, acquire a functional endothelial phenotype when cultured in angiogenic conditions (Chopra, Hung et al., 2018). This finding was substantiated using mesenchymal stem cells (Oswald, Boxberger et al., 2004).
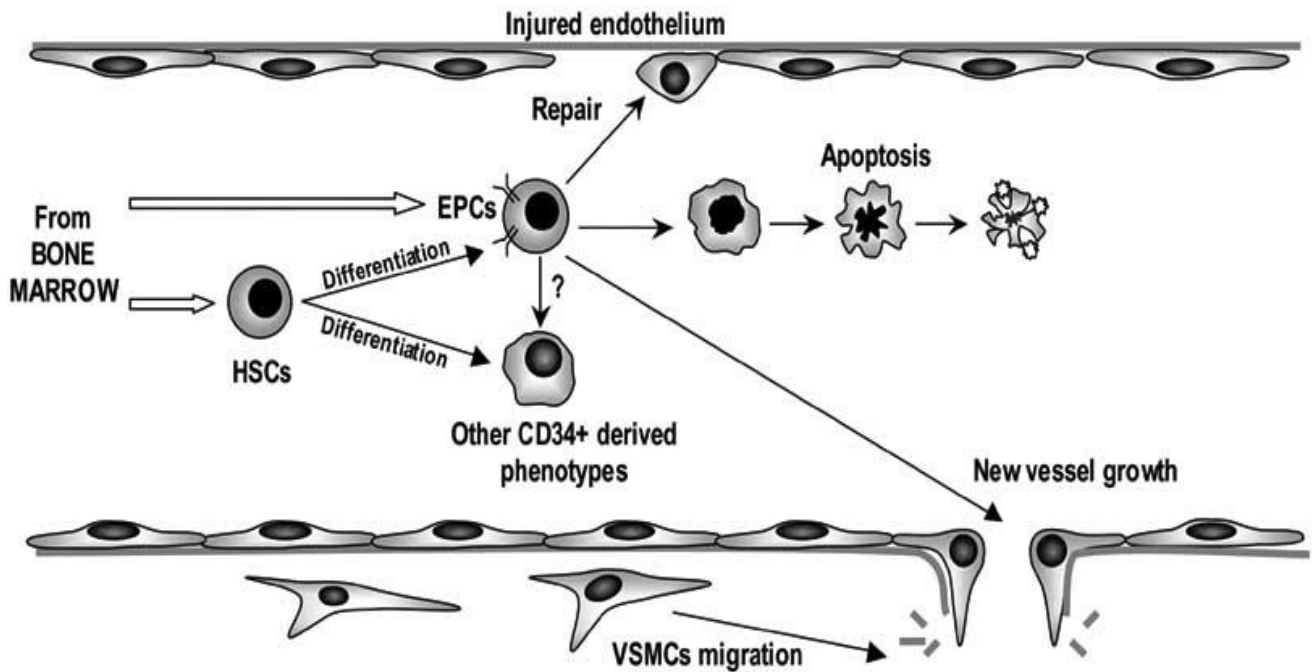
Figure 1. 13: Possible origins of EPCs and their impact on vascular health (Gian Paolo Fadini, Agostini et al., 2005)

There are two types of EPCs; early and late growers which are identified by their distinct morphology. Early growers are generally seen in small colonies with spindle-shaped cells appearing within the first week of culture (Yoo, Ahn et al., 2005). Late growers exhibit the more traditional cobblestone morphology that is characteristic of vascular endothelial cells (Tagawa, Nakanishi et al., 2015). Late growers appear in culture at 2-3 weeks and have slightly different surface markers, however, the vasculogenic capacity between the two types of EPCs remain the same (Yoo et al., 2005).

1.2.1.1 Surface markers

Identification of EPCs generally involves assessing surface markers expression. CD133+ and VEGFR2+ are thought to represent immature cells, and/or the cells from the bone marrow (Urbich & Dimmeler, 2004). However, there is debate over how accurate and distinct surface markers expression is on EPCs. For example, CD34+ and VE-Cadherin are often seen on myeloid cells ex vivo, suggesting that no surface marker can be used in isolation to determine the type of cell (Reyes, Dudek et al., 2008) [Figure 1.14]. Questions are also raised as to the origin of circulating EPCs which may affect the role that the cells undertake (Gian Paolo Fadini, Losordo et al., 2012). A concern in using EPCs is the requirement for many hormones and antibiotics to be used during cell culturing likely causes modification to EPCs.
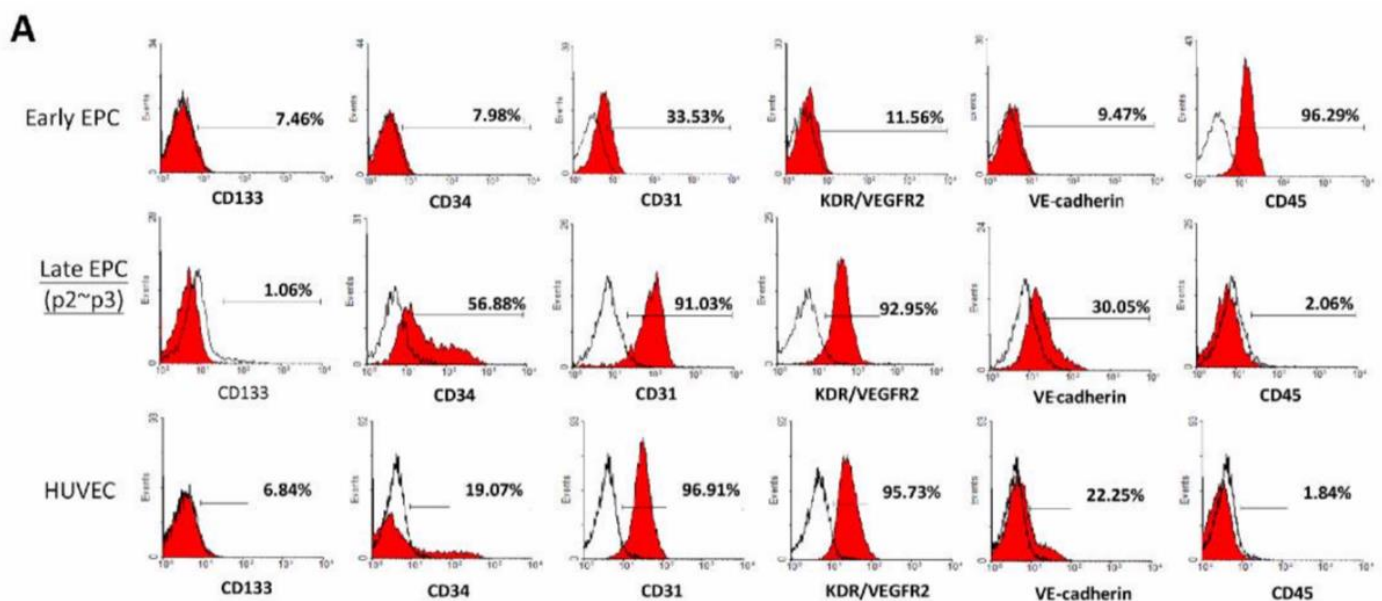
Figure 1. 14: Though there is some consensus on surface markers, there is certainly a need to use several different surface markers and other functional assays to determine the EPC (Cheng, Chang et al., 2013a).

Studies	Markers													
	CD45	CD14	CD31	VE-Cadherin	CD 34	CD133	VEGFR2 /KDR	Flk 1 and Flt 1	CD105	CD144	CD146	UEA-1	CD115	CD106
(Rehman, Li et al. 2003)	+	+	+	+										
(Urbich and Dimmeler 2004)		+			+	+	+							
(Reyes, Dudek et al. 2008)				+	+			+						
(Yoder, Mead et al. 2007)	−	−	+						+	+	+	+	−	
(Cheng, Chang et al. 2013)			+	+	+	+	+							
(Povsic 2009)					+	+	+							
(Anjum, Lazar et al. 2012)		+	+		+	+	+				+			+
(de la Torre, Fernandez-Durango et al. 2015)		+	+				+							

Table 1. 1: Surface markers used in different studies.

1.2.2 Circulating EPCs in CVD

Individuals at a higher risk of cardiovascular disease may have a decreased number of circulating EPCs (Zampetaki, Kirton et al., 2008). This is further supported by a study which identified that those with high CAD risk and low EPC count had higher rates of senescence as circulating EPCs were repairing damaged vessels (Hill , Zalos et al., 2003). Medications commonly used in cardiovascular disease may also alter EPC numbers. For example, statin use is correlated with an increased number of circulating EPCs (Liu, Wei et al., 2012a). There may be specific changes in EPCs in relation to CVD co-morbidities as discussed below.

1.2.2.1 Hyperlipidaemia

Hyperlipidaemia negatively influences EPC function by up-regulating NADPH oxidase activity. This leads to downstream effects of decreased migration and adherence (Li et al., 2017). Hyperlipidaemia patients have previously displayed lower circulating EPC numbers and proliferation capacity, however, after lipid apheresis treatment, CECs numbers returned to normal (Patschan, Patschan et al., 2009). Low-density lipoprotein (LDL) causes endothelial dysfunction when oxidise. This down-regulates eNOS and its ability to produce NO, thus up-regulating activity of other ROS (Mehta, Chen et al., 2006)[Figure 1.15]. Oxidised LDL causes inactivation of AKT through the PI3K pathway which increased EPC apoptosis (Tie, Yan et al., 2010).
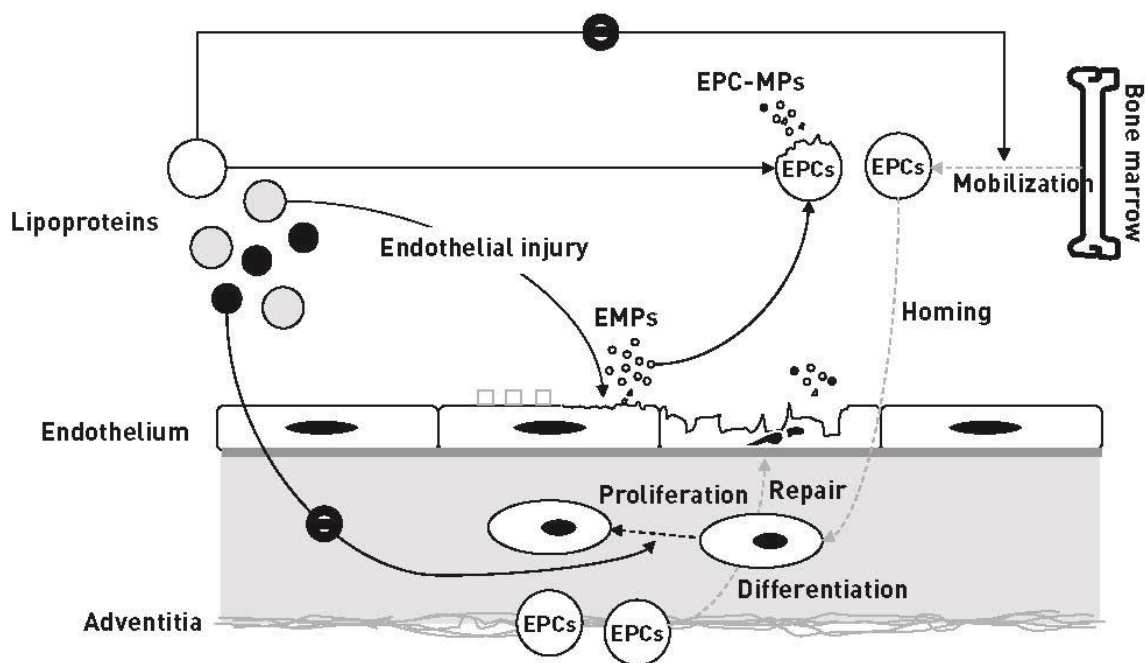
Figure 1. 15: Effects of plasma lipoproteins on EPCs and their micro-particles (MP) (Pirro, Bagaglia et al., 2008)

1.2.2.2 Heart failure

In heart failure (HF), the balance between bioavailability of NO and oxidative stress is lost, affecting mobilisation of EPCs from the bone marrow (Hare & Stamler, 2005) [Figure 1.16]. It has been shown that a low EPC count is associated with HF, independent of clinical characteristics and also predicted a 1.6 fold increase in mortality (Samman Tahhan, Hammadah et al., 2017). A biphasic response has been noted in the EPC numbers during HF, where there is an increase at the onset of HF, and numbers decreasing in advanced phases (Valgimigli, Rigolin Gian et al., 2004). This identifies the EPCs as a potential diagnostic tool for HF. An observation of preserved left ventricular ejection fraction over a year was observed in-patients with higher EPCs at time of MI (Wyderka, Wojakowski et al., 2012), suggesting a therapeutic advantage of EPCs as a prognostic indicator.
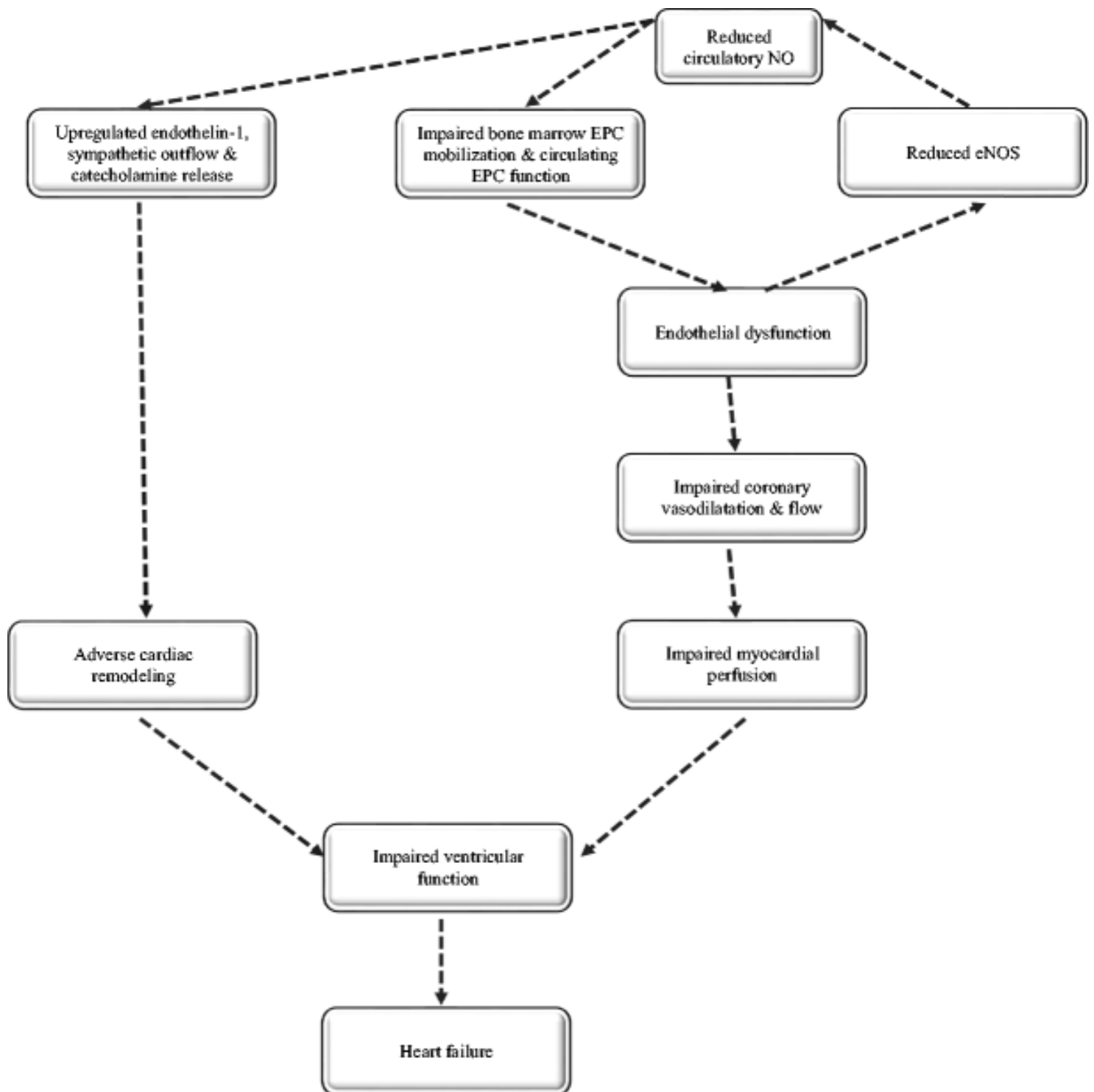
Figure 1. 16: Interactions between various mechanisms contributing to downstream effects such as impaired coronary vasodilation, and myocardial perfusion which leads to heart failure (Djohan, Sia et al., 2018)

1.2.2.3 Hypertension

In hypertension, the renin-angiotensin system increases EPC senescence and oxidative stress (Du, Zhou et al., 2012). In contrast, inhibitors of the renin-angiotensin system reversed the depressed EPC numbers (Takashi Umemura, Soga et al., 2008b). It was also identified that hypertension affects EPC function, which is restored with anti-hypertensive medication. Furthermore, the effect of hypertension on late EPC's capacity for proliferation was more significant in comparison to other types of EPCs (S. Luo, Xia et al., 2016). It has been identified that during early phases of hypertension, there is an increase in circulating endothelial cells (Mandraffino, Sardo et al., 2011), serving as a prognostic marker. This is also seen in pulmonary arterial hypertension, where EPCs numbers are reduced (Diller, van Eijl et al., 2008). It has also been shown in a pilot study in pulmonary hypertension, that EPC transplantation improved exercise tolerance and pulmonary haemodynamics (G. P. Fadini, Avogaro et al., 2010).

1.2.2.4 Diabetes

Early detection of endothelial dysfunction can be identified through assessing numbers and function of EPCs, circulating endothelial cells (CECs) or endothelial micro-particles (Burger & Touyz, 2012). In the 10 patients with diabetes, there is a reduced number of EPC counts (Ambasta, Kohli et al., 2017). Hyperglycemia appears to have a detrimental effect on the yield of EPCs and the EPCs that are generated are less angiogenic and more pro-inflammatory (Loomans, van Haperen et al., 2009). In diabetes, ROS is normally up-regulated, resulting in a loss of BH4 which has been identified as a required co-factor for the synthesis of NO (Alp, Mussa et al., 2003). However, in pre-clinical models, BH4 supplementation appears to restore BH4 to normal levels in diabetic rats (Gangula, Mukhopadhyay et al., 2010).

1.2.2.5 Obesity

In obesity, there is a positive correlation between the number of EPCs and body mass index, waist circumference, and insulin (Graziani, Leone et al., 2014). EPCs from obese patients also displayed reduced adhesion, migration and angiogenic properties (N. M. Heida, Muller et al., 2010). Interestingly, there appears to be a distinction between EPCs enumeration between overweight and obese individuals, with a lower number observed only in obese individuals, and not the overweight individuals (Jung, Fritzenwanger et al., 2009). Similarly, another study found the EPCs from obese and overweight patients had a reduced colony-forming capacity, but in this case EPCs from overweight patients were also affected (MacEneaney, Kushner et al., 2009).

1.2.2.6 Smoking

Smoking has previously been identified as a modifiable risk factor in cardiovascular disease. Smoking results in elevation of ROS activity, which is a main contributor to the development of atherosclerosis. There are variable reports on the effects of smoking in EPC numbers and function. It has been previously reported in 15 chronic smokers, a reduced number of EPCs, which is rescued when cessation of smoking (Kondo, Hayashi et al., 2004). A reduction in EPC differentiation was also observed (Tang, Lu et al., 2008). However, a recent study showed acute smoking significantly increased EPCs and micro-particles (Mobarrez, Antoniewicz et al., 2014). With an increase in the use of e-cigarettes, a recent study found that 10 puffs from an e-cigarette promoted a significant increase in EPC numbers similar to cigarette smoke (Heiss, 2016). However, second-hand smoking has been shown to depress mobilisation of EPCs and increase endothelial dysfunction (Heiss, Amabile et al., 2008).

Endothelial progenitor cells remain a source of debate, from their origins (Chopra et al., 2018), to their identification through different surface markers which is dependent on whether they were early or late growers (Tagawa et al., 2015). Their potential use as a prognostic marker of CVD has mostly been explored through circulating EPCs, however, various diseases and medications can affect circulating EPCs. There has been evidence to suggest a reduction in circulating EPCs in hyperlipidaemia (Y. Luo, Yan et al., 2018), heart failure (Nonaka-Sarukawa, Yamamoto et al., 2007) and many other conditions. These factors are important when considering the possible use of patient-derived EPCs as a model to study new mechanisms and markers of susceptibility in atherosclerosis.

1.3 HYPOTHESIS AND AIMS

Our laboratory is focused on identifying new markers and mechanisms of atherosclerosis. Blood samples are more feasible to obtain than tissue and can be collected on a large scale. Circulating EPCs can be extracted from blood and used to explore distinct molecular and cellular differences in patients with CAD versus healthy controls. I hypothesised that these cells would exhibit characteristics of vascular endothelial cells expected in patients with CVD and could therefore be used as a model for performing mechanistic studies to understand cell signalling. To investigate this, I have worked with the Figtree laboratory to establish a method of culturing patient-derived EPCs. I have recruited >1000 patients undergoing a computed tomography coronary angiogram (CTCA) for suspected coronary artery disease, collected their clinical and imaging data, prepared and aliquoted blood and cultured EPC's.

The key aims of my thesis are:

1. To determine whether clinical and demographic factors influence the spontaneous growth of EPCs;

2. To establish whether EPCs culture ex vivo retain molecular or signalling features that are reflective of their burden of coronary artery atherosclerosis;

These patients make up the initial core of the expanding BioHEART study (Clinical Trials No. ACTRN12618001322224), which has recently been supported by NSW Office of Health and Medical Research to be transferred to the new State Biobank for board research open access.

2 GENERAL METHODS

2.1 PATIENT RECRUITMENT

Patients undergoing a clinically indicated CTCA were recruited from North Shore Radiology (North Shore Private Hospital, St Leonards). Consent was obtained via recruitment criteria and established protocol which conforms to the Helsinki declaration and approved by on-site human research ethics committee (HREC/17/HAWKE/343) and governance (ACTRN12618001322224). Medical history was obtained by a questionnaire (Figure 2.1), including their medical and demographic details.

Figure 2. 1: BioHEART questionnaire, administered to patients at the time of consent.

2.1.1 Assessment of coronary calcification from CTCA

Analysis of scans from CTCAs was undertaken by qualified, experienced cardiologists. The routinely reported Agaston score was used to quantitate coronary calcification. Coronary calcification was determined as an Agaston score >0 (i.e. any coronary artery calcification). A more comprehensive semi-quantitation of total and non-calcified plaque was also studied based on the previously established Gensini score (Niccoli, Giubilato et al., 2012).

2.1.1.1 Gensini Scoring

Gensini scoring is widely used to quantify atherosclerosis on CTCA scans, taking into proximity and degree of narrowing when calculating a final score, which predicts cardiovascular outcomes (Niccoli et al., 2012). A modified Gensini score, gives different multiplication factors to calcified, mixed and non-calcified plaque, with non-calcified plaque having the highest factor due to the high-risk of rupture (Braganza & Bennett, 2001). I have examined the characteristics of patients with a modified Gensini score of zero, which indicates no calcified or soft plaque.

2.1.2 Comparative analysis of patient history data

Using SPSS, prevalence of cardiac risk factors and medications trends were calculated. Chi-squared statistics test were used to determine whether clinical or demographic features and medications were able to predict spontaneous appearance of EPCs. Student t-tests were also performed to determine significant differences between patient profiles of those who spontaneous grew EPCs, and those who did not.

2.2 BLOOD COLLECTION

20 mLs of venous blood was drawn from the cannula at the time of insertion for the CTCA. For EPC culture, 4 mLs of blood was immediately put into a lithium heparin vacuette (Greiner Bio-one (AT), 4mL Vacuette, 018495) and inverted several times.

2.2.1   PBMC isolation

Blood was mixed with equal parts of Hank's Balanced Salt Solution (HBSS) with calcium, magnesium and no phenol red (Life Technologies (US), 14025134). The mixture was pipetted on top of 14 mLs of Ficoll-paque plus ((GE Healthcare (US), 17-1440-03), forming two layers which were then centrifuged at 22°C at 1462 x g for 20 mins with no brake. The buffy coat containing the peripheral blood mononuclear cell (PBMC) layer was extracted [Figure 2.2] and added to 14 mLs of HBSS and centrifuged at 4°C at 1462 x g for 10 mins with the brake on high. The supernatant was removed, and the pellet was re-suspended in 10 mLs of HBSS and a sample was extracted to determine the number of cells before being centrifuged at previous parameters. Cell count was conducted using a cell haemocytometer after cells were further diluted with equal parts of Trypan blue. The supernatant was discarded, and the pellet was resuspended at 10 x 10^6/mL of Gibco Bovine Serum, Heat Inactivated (Life Technologies (US), New Zealand, 26170043).
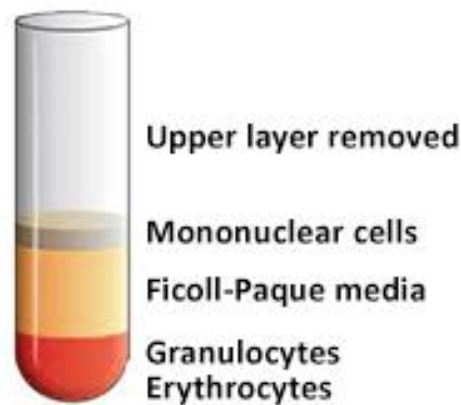
Figure 2. 2: Ficoll-paque preparation to extract PBMCs

2.2.2 Cell culture

0.6×10^6 of PBMCs were plated onto EasY T25cm$_2$ Flasks (Thermofisher Scientific (AU), NUN156367). Initially, the cells were plated into 12 wells plates, but due to frequent infection and difficulties in expanding colonies, I transitioned to T25s . 5mLs of Endothelial Growth Medium-2 (EGM-2) (Lonza (CH), CC-3162) was added to each T25 using standard cell culture conditions. Culture media was changed every 2-3 days and culture flasks were monitored closely for the appearance of EPCs colonies. In the case of spontaneous appearance of EPCs, culture flasks were maintained until a monolayer of ~80% confluence was obtained. EPCs were then passaged T75s for use in experiments. Once reaching passage 3, sufficient EPCs were obtained to freeze down in the biobank. Using 10% dimethyl sulfoxide (DMSO, Sigma Aldrich (US)) and Bovine serum, heat-inactivated (26170043, Life Technologies, AU). Any samples that did not show appearance of EPCs by 30 days in culture were discarded.

2.3 IMMUNOBLOTTING

Cells in an EasY T75cm$_2$ flask (Thermofisher Scientific (NSW, AU), NUN156367) were washed with ice-cold Phosphate Buffered Saline (PBS) twice, with 600 µL of lysis buffer containing 150 nmol/L NaCl (Sigma Aldrich (US), 793566), 1% Igepal CA-630 (Sigma Aldrich (MO, US), I3021), and 50 mmol/L Trizma base pH 8.0 (Sigma Aldrich (MO, US), T1503) which was supplemented by 1 tablet of PhosSTOP (Sigma Aldrich (MO, US), 04906837001) and cOmplete ULTRA (Sigma Aldrich (MO, US), 05892970001). Cells were mechanically removed via scraping, vortexed for 30 seconds and centrifuged at 4$_o$C at 19722 x g for 15 mins. Protein concentration was determined using a MicroBCA Kit (Thermofisher scientific (NSW, AU), 23235) and 10 µg of protein lysate was denatured and run under reducing conditions on SDS-PAGE in 4-12% Tris-bis pre-cast gels (Thermofisher Scientific (NSW, AU), NW04122BOX). Gels were then transferred onto Immobilon polyvinylidene fluoride membrane (Sigma Aldrich (MO, US), IPFL10100) and incubated in primary antibodies to determine the protein expression of various redox markers. Secondary fluorescent antibodies specific to primary antibodies were then used (Donkey anti-rabbit IRDye 680LT, LCR-926-32210, Goat Anti-Mouse 800CW, LCR-926-68023; Millenium Science (VIC, AU)). Membranes were probed using the Odyssey Imaging Platform (Licor (NE, US)).

2.4 SURFACE MARKERS

Cells in an EasY T75cm$_2$ flask (Thermofisher Scientific (AU), NUN156367) were washed with room temperature sterile PBS then lysed with 7 mLs of 2 x 2.5% Trypsin with no phenol red (LifeTech (US), 15090046). Cells were disaggregated with trypsin, neutralised with media before being centrifuged and pelleted down at 350 x g for 4 mins. The supernatant was removed, and cells are re-suspended in 15 mLs of cell staining buffer (Australian Biosearch (AU), 420201)

and spun for 5 mins at 50 x *g*. The supernatant was removed and cells were resuspended in 5 x 10_6/mL in cell staining buffer. 5 µL of Human TruStain FcX™ was added (Fc Receptor Blocking Solution, Australian Biosearch (AU), 422301) per 100 µL of re-suspended cells and incubated at room temperature for 10 mins. Fluorochromes were added to a final concentration of 1:100 and incubated in the dark on ice for 20 mins. This was then washed with 100 µL of cell staining buffer, centrifuged at 350 x g for 5 mins, twice. Cells were resuspended in 0.5 mL of buffer and pipetted into flow cytometry tubes and probed using BD LSRFortessa cell analyser (BD Science, (NJ, US). Spectral compensation was done prior using HuVECs.

2.5 CONFOCAL MICROSCOPY

50k cells were seeded onto glass coverslips 24 hours in a 12-well plate (CLS3513-50EA, Sigma Aldrich (US)) before the experiment, to reach 90-95% confluency. Wells receiving Angiotensin II (AngII) were washed with warm KREBS buffer which consisted of; 0.126 M NaCl, 2.5 mM KCl, 25 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$ with a pH of 7.2, before AngII at 100 nmol/L was added 60 mins before DHE. Wells pre-incubated with manganese (III) tetrakis (1-methyl-4-pyridyl) porphyrin (MnTMPyP/MN) at 5 µmol/L, were pre-washed with KREBS at 15mins before DHE was added. Remaining wells were pre-washed and DHE was added to all the wells except for the control well at 5 µmol/L, then the plate was incubated in the dark for 30 mins. Each well was washed 3 x with KREBs buffer and 0.5 mLs of PFA/Formalin 4% was added to each well and incubated for 15 mins. All wells were re-washed with three times with KREBs buffer and mounted onto slides with Prolong Diamond Anti-fade Mountant containing DAPI (P36962; Life Technologies (CA, US)). Slides were left at room temperature overnight in the dark. Coverslips were sealed the next day with clear nail polish and fluorescent images taken with the Leica TCS SP5 Confocal (Leica Camera, (DE)). A negative well was used to determine

autofluorescence before analysis was performed by measuring intensity of channel 647 and 405. DHE was normalised to DAPI to determine the ratio of superoxide produced.

2.6 MITOSOX

Cells are seeded onto a 6-well plate (CLS3506-100EA, Sigma Aldrich (US)) at a final density of 80% on the day of flow cytometry. Cells were stained with 2.5 μM Mitosox and 10 μM Antimycin A (A8674-25MG, Sigma Aldrich (US)) was added as a positive control in HBSS with added Mg and Ca. Cells were incubated in the dark for 20 mins at 37°. Each well was washed three times with HBSS and cells were disaggregated using trypsin (59428C-100ML, Sigma Aldrich (US)) and spun down at 5 mins at 50 x g three times. Cells were then re-suspended in 450 μL of HBSS and pipetted into flow cytometry tubes and probed using BD LSRFortessa cell analyser (BD Science, (NJ, US). Spectral compensation was done prior using HuVECs. This experiment was performed by Nicole Seebacher.

2.7 MIGRATION ASSAY

20 x 10$_3$ cells were seeded into a 96 well plate (CLS3599, Corning [USA]) with each patient sample in triplicate and incubated overnight to reach 90-100% confluence. Cell media was removed, and a denuded zone was made with a 10 μL pipette tip. 200 μL of EGM-2 (CC-3162, Lonza (CH)) was added back into wells, and images were obtained at 4 x magnification using Evos FL Auto (AMAFD1000, ThermoFisher Scientific, MA; USA) at 0, 8, 16, 24, 32, 40 and 48 hours.
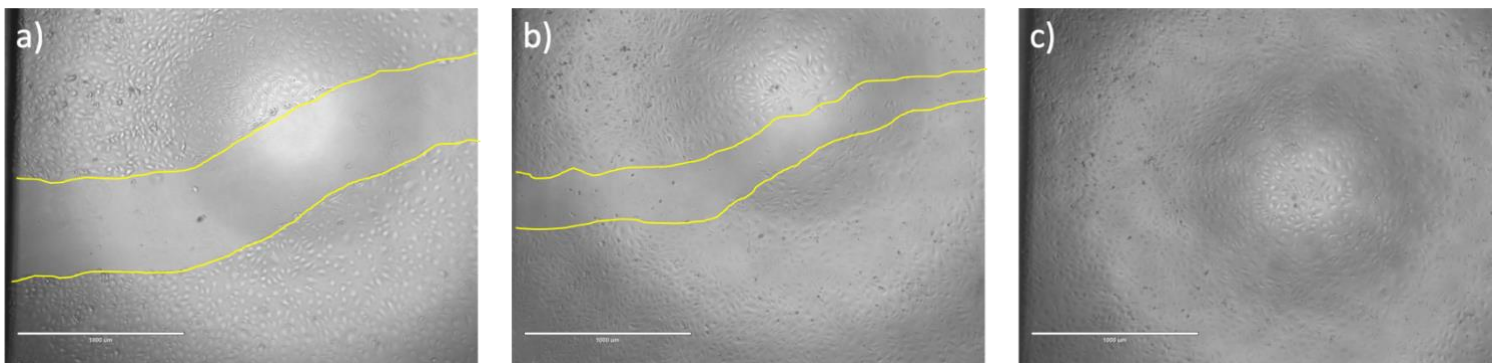
Figure 2. 3: Migration Assay performed on patient-derived EPCs. a) EPCs at 0 hrs with denuded zone, b) EPCs at 8 hrs, c) EPCs at 24 hrs. Magnification bar represents 1000 μm.

2.8 TUBULE FORMATION

50 μL of Cultrex PathClear 3-D Culture Matrix reduced growth factor basement membrane extract (Matrigel) (3433-005-01, R&D Systems [USA]) was added into a 96-well plate (CLS3599, Corning [USA]) with care to avoid bubbles. Matrigel was then incubated at room temperature for 15 minutes, followed by 37° for a further 15 minutes to set. 15 x 10$_3$ of EPCs from patients were added per well (in duplicates) in addition to HuVECs as a positive control. EGM-2 was added to wells for EPCs and EGM+ for HuVEC, up to 200 μL. Images were 5obtained every hour for up to 16 hours on the EVOS FL Auto (AMAFD1000, ThermoFisher Scientific, MA; USA) which was maintained at a temperature of 37°C with carbogen to maintain culture conditions at 5% carbon dioxide.

2.8.1 Statistical Analysis

Data were analysed and graphed using Graphpad software, Microsoft Excel and SPSS. Mean ± SEM is presented for each group and a student's t-test, Chi-squared, one/two-way ANOVAs were used to determine statistical significance which was accepted at $p < 0.05$.

3 Patient-derived endothelial progenitor cells: a platform for unravelling new mechanisms of coronary disease susceptibility and resilience

3.1 Introduction

Coronary artery disease (CAD) is a major cause of death, with many factors including genetics, traditional risk factors, and other unknown factors (Hadi et al., 2005). A recent study identified a 16% increase in ST Elevated Myocardial Infarction (STEMI) patients over the last 8 years, presenting to emergency without any traditional risk factors (Vernon, 2017). This highlights the importance in establishing the unknown risk factors for these patients. However, it is also interesting to understand patients who present with very extensive risk factors and yet are resilient to disease. To discover new markers and therapeutic targets to disease, understanding an individual's susceptibility is vital.

A CTCA provides a non-invasive quantification and characterisation of plaque in the coronary arteries. It gives superiority over the traditional invasive angiography as it can visualise the lumen and vessel walls, helping to identify patients who truly have no CAD. Patients undergoing a CTCA at North Shore Private Hospital (NSPH) consented to participate in the BioHEART research study (ACTRN12618001322224). Blood was obtained from patients and processed for storage in biobanks, and for extraction of EPCs. Corresponding patient medical history was collected. Whilst circulating markers, immune and red cells from the serum of patients are crucial in the study of CVD, ultimately patient-derived vascular cells will likely provide more accurate molecular information, particularly if paired with a detailed clinical and imaging phenotype of the patient.

Cultured patient-derived endothelial cells provide a research platform to study differences in expression under baseline conditions, specific signalling

pathways, and resilience or susceptibility to disease-relevant challenges. Obtaining sufficient functioning vascular endothelial cells from patients for laboratory culture experiments is extremely difficult, thus establishing a source of endothelial cells accessible from a patient's circulation would be greatly beneficial. Two main approaches are taken to study endothelial and vascular signalling: Induced pluripotent stem cells (iPSC) derived cardiovascular cells or EPCs. iPSCs are created via genetic mutations and closely resemble human embryonic stem cells and can be used to personalise drug treatment options and model a specific patient's disease state (Musunuru, Sheikh et al., 2018). However, the "reprogramming" can result in a difference in DNA methylation in the iPSC in comparison to fertilised embryonic stem cells (K. Kim, Doi et al., 2010), thus has the potential to remove epigenetic modifications. EPCs are selectively cultured from PBMCs, and may, therefore, have the advantage of retaining an adult phenotype.

Dyslipidaemia, diabetes, hypertension, age and smoking have long been recognised as risk factors for CVD (Fried, Kronmal et al., 1998). These factors negatively impact our cardiovascular health at a community level; however, it is not well known what determines the individual's response at a cellular and system level. Endothelial cell function is well recognised to be central to arterial health. In particular, inflammatory and redox signalling are known to be early markers of arterial disease and increase susceptibility to atherosclerosis (Sandoo et al., 2010). EPCs are a vital part of the regeneration of vessels post-MI, thus the identification of factors that influences their ability to migrate and grow are important in predicting outcomes in the event of a cardiovascular event.

In this chapter, I examined on the hypothesis that EPCs derived from BioHEART patients provide a feasible and valid model to explore endothelial signalling and molecular differences that may be marker of mechanisms of coronary disease. Specific aims of the chapter are:

Aim 1: Establish and optimise the protocol for growing EPCs in culture that have similar properties to commercially available endothelial cell lines;

Aim 2: Identify clinical and demographic predictors of EPC growth that may influence the ability to study factors driving individual patient susceptibility or resilience to atherosclerosis;

Aim 3: Examine redox signalling in patient-derived EPCs from the BioHEART study, and establish if signalling is associated with a coronary disease phenotype

3.2 METHODS

3.2.1 EPC isolation and assessment

Blood was collected from patients enrolled in the BioHEART study using methods as outlined in Chapter 2.2. PBMCs were isolated from patient samples using a Ficoll gradient separation (see 2.2) and was further cultured for the appearance of EPCs (see 2.2.1). Immunoblotting was performed to examine expression of redox signalling proteins such as NADPH oxidase isoforms, eNOS, and glutaredoxin as detailed in section 2.3. Primary antibodies were used are as follows: (Anti-Nox 2, ab129068 Abcam (UK), 1:5000, Anti-eNOS/NOS Type III, 610297 BD Science (US), 1:1000, Anti-Nox 4, ab13303 Abcam (UK), 1:5000, Anti-Glutaredoxin 1, ab45953 Abcam (UK), 1:1000). 4-12% gradient Bis-Tris gels were used for all proteins and probed using the Licor Odessey imaging platform.

In order to establish an endothelial phenotype, EPCs from 31 patients with a variety of risk factors and CAD profiles detailed in section 2.1.1, were cultured and underwent functional test using the Matrigel assay to examine the tubule formation ability of the cells which was then compared to HuVECs. Duplicates were completed for all patients and are detailed in section 2.8. A subset of EPCs were examined using flow cytometry for endothelial surface marker expression; CD34 (BioLegend, (Ca, US), 343605), CD14 (BioLegend, (Ca, US), 367715), CD31 (BioLegend, (Ca, US), 303115), and CD133 (BioLegend, (Ca, US), 372807) and compared to HuVECs. Further details are in section 2.4. Analysis of O_2-• generation in live cells, EPCs from 6 healthy and 8 CAD patients were probed for O_2-• production under normal conditions, and after pre-incubation with AngII, detailed in section 2.5. Mitochondrial superoxide staining (Mitosox), was performed on 12 EPC cell lines to determine mitochondria superoxide generation between EPCs from healthy patients, and those with disease detailed in section 2.6.

3.2.2 Statistical Analysis

Data were analysed and graphed using Graphpad software, Microsoft Excel and SPSS. Mean ± SEM is presented for each group. In section 3.3.3.1, comparisons were made between EPCs and HuVECs and in 3.3.4.1 - 3.3.4.3 a student's t-test was used to analyse redox signalling data between patients EPCs with CAD and those without. Section 3.3.2.1 uses a student's t-test or Chi-squared to determine statistical significance between patient risk factors in those who grew EPCs and those who did not. To further determine oxidative response, a one-way ANOVA was used to determine statistical significance, which was accepted at $p < 0.05$.

3.3 RESULTS

3.3.1 Clinical characteristics and atherosclerosis phenotype in BioHEART patients

Patients were clinically indicated to have CTCA performed for suspected coronary artery disease due to a range of factors such as family history, abnormal electrocardiogram/echocardiography, suggestive symptoms and/or general cardiovascular assessment [Figure 3.1]. Symptoms of chest pain and shortness of breath were the key indications for the CTCA scans. Within the cohort, there was an average age of 61.3±17.0 years, with slightly more males (55.1%) than females (43.1%) in addition to the patients where sex was not recorded (1.8%). The most common risk factor was hypercholesterolaemia which was present in more than half the cohort, 36.5% of the cohort were ex-smokers and 6.1% were current smokers. Arthritis was also present in a large proportion of the cohort, at 38.9% [Table 3.1].

CLINICAL INDICATIONS FOR CTCA
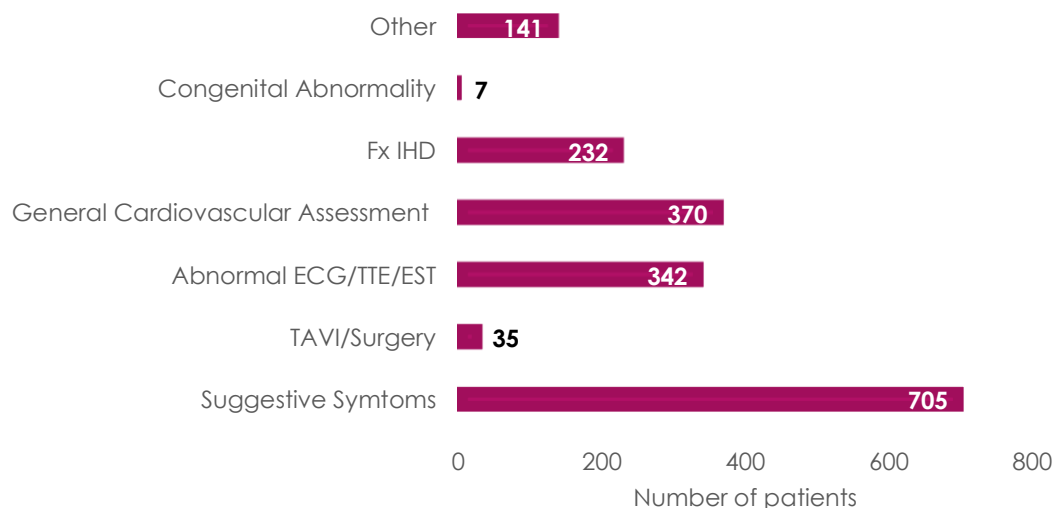
Figure 3. 1: Clinical indications in BioHEART cohort including family history of ischaemic heart disease (Fx IHD, electrocardiogram (ECG), transthoracic echocardiogram (TTE), exercise stress test (EST), and transcatheter aortic valve implantation (TAVI).

With increasing age, it is expected that participants develop at least one traditional risk factor and are at higher risk of CVD. However, we were interested in exploring how the presence of traditional factors does not necessarily correlate with presence of CAD. We addressed this by examining an older population with a zero modified gensini score, which is suggestive of resilience to CAD. Interestingly, when looking at the number of traditional risk factors in patients over the different age groups, there is a slow increase in risk factors with age as may be expected [Figure 3.2]. Out of the 362 patients with 0 modified Gensini score, 103 patients are in the 50 years and below age group, where 79% have ≥1 traditional risk factor. In 200 patients aged between 50 and 70 years, the proportion with ≥1 traditional risk factor has dropped to 63%. For the 59 patients aged 70 years and above, with a modified Gensini Score of 0, there remains 51% of patients who have ≥1 traditional factors.
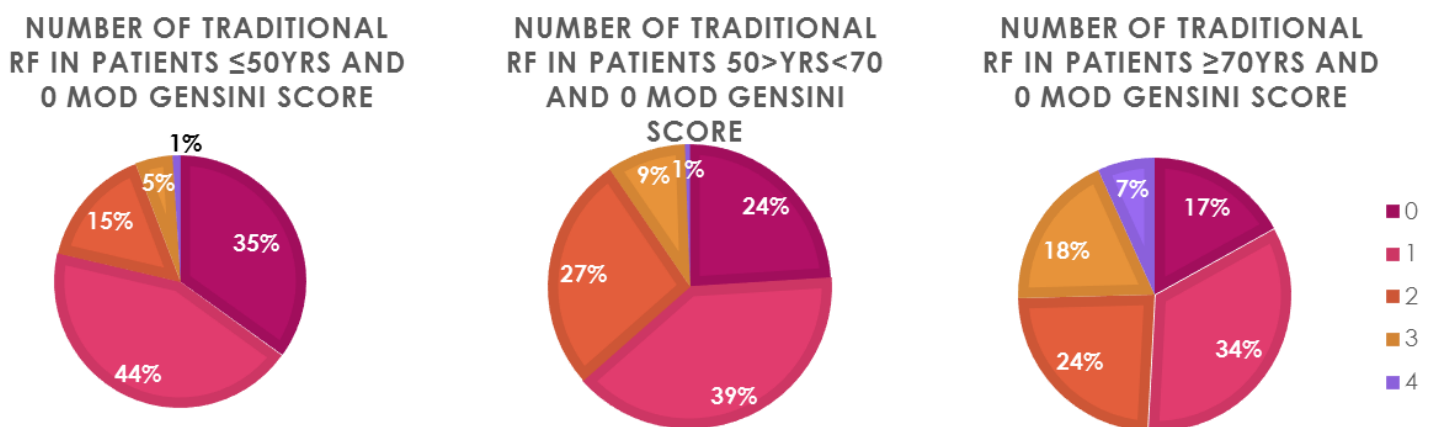
Figure 3. 2: Number of traditional risk factors for patients in BioHEART who have a zero Modified Gensini Score at different age ranges

Inversely looking at a positive modified Gensini score, there is consistently a larger proportion of patients with ≥2 risk factors. It appears the risk factors are unable to predict disease accurately in the ≤50 years group (n=38), with 26% of patients with no risk factors and a modified Gensini score. In the ≥70 years group, there is still a large proportion (40%) of the group with ≥1 risk factor and a positive Gensini score. This further confirms the need for new biomarkers to risk factors to be identified as prognostic markers in order to improve early risk identification.
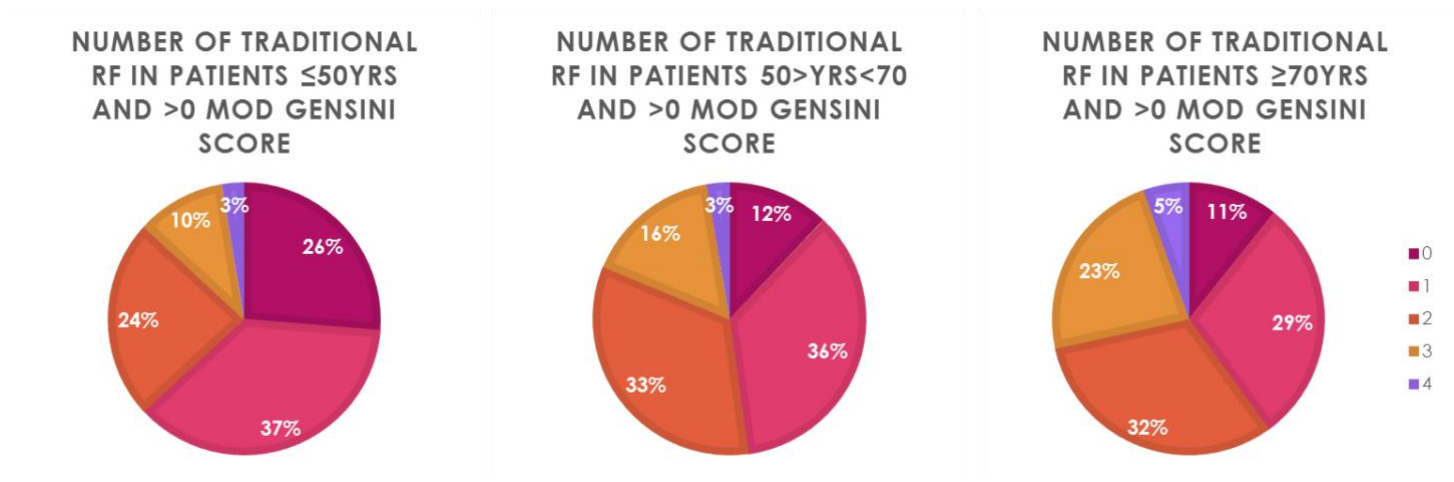
Figure 3. 3: Number of traditional risk factors in different age groups in the BioHEART cohort, in patients with >0 modified Gensini score.

Patients with new cardiovascular symptoms are often referred to CTCA's as the first investigation, and dependent on this result further testing is requested. This may explain the large proportion (40%) of patients with zero disease. The median percentile is 42% [Figure 3.4].

Histogram of Ca Percentile
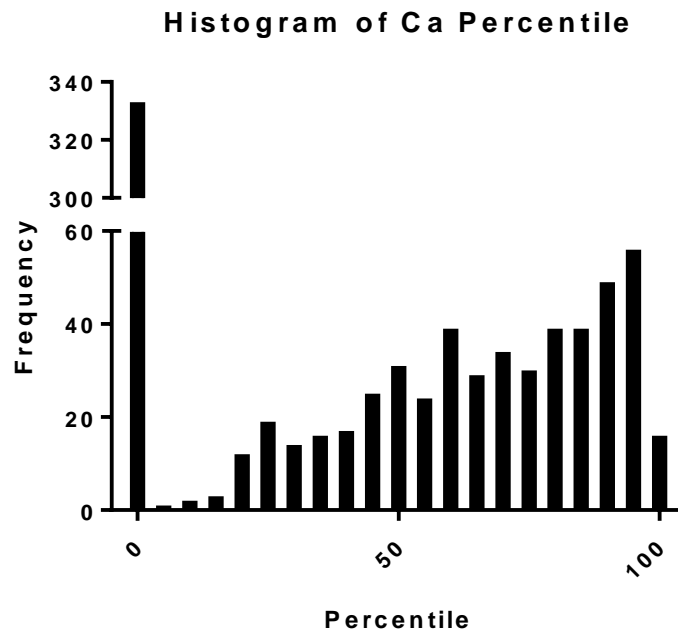
Figure 3. 4: Age and sex adjusted calcium percentile of participants who were indicated for a CTCA in the BioHEART cohort (n=828).

Unsurprisingly 19.1% of the cohort were prescribed aspirin, but there was only 0.2% of the cohort taking the newer generation of platelet aggregation inhibitors; Ticagrelor and Prasugrel. 32.9% and 31.3% were prescribed statins and angiotensin-converting enzyme (ACE)/Angiotensin II receptor blockers (ARB) inhibitors respectively [Table 3.1].

	Clinical demographics and medication		BioHEART Cohort
Characteristics	Total Patients		1157
	Age (Years)		61.3
	Sex (%)	Female	43.1
		Males	55.1
Risk Factors (%)	**Cardiac**	Diabetes	8
		Hypertension	38.5
		Hypercholesterolemia	53.8
		Smoking	Current	6.1
			Ex-smoker	36.5
		Angina	4.3
		MI	3.5
		Stent	2.9
		Coronary artery bypass graft (CABG)	1.6
	Other	Arthritis	38.9
		Osteoporosis	6.1
		Stroke/Transient ischaemic attack (TIA)	4.8
		Peripheral artery disease (PAD)	1
		Deep vein thrombosis (DVT)/Pulmonary embolism (PE)/Thrombus	0.4
		Kidney Disease	2.1
Medication (%)	Aspirin		19.1
	Clopidogrel		1.8
	Ticagrelor		0.2
	Prasugrel		0.2
	BetaBlocker		14.9
	Calcium Channel Blocker		9.7
	Statin		32.9
	ACE/ARB		31.3
	Diuretic		6.6
	Warfarin		1.7
	Non-vitamin K antagonist oral anticoagulants (NOAC)		6.6
	Other Anti-Arrythmics		4.8
	Proton Pump Inhibitors (PPI)		13.8
Coronary Score	Calcium (Median)	Raw Score	12 [0-183.5]
		Percentile (%)	42 [0-77]
		Gensini	0 [0-7]

Table 3. 1: Patient characteristics of BioHEART. The RF and medication profile of the cohort.

3.3.2 Predicting spontaneous appearance of EPCs from BioHEART cohort using clinical and demographic features

Of the 1152 patients enrolled to date in BioHEART, I attempted EPC derivation and culture in 815 of them. Figure 3.3 schematically illustrates the selection of EPCs from BioHEART-CT, based on spontaneous growth. Within 3 weeks of culture, there was spontaneous appearance of EPCs in 164 out of the 815 PBMCs. However, due to technical difficulties in expanding the colony, only 51 of the patient-derived EPCs were suitable for further functional experiments.

Figure 3. 5: Flow chart of patient-derived EPC spontaneous appearance and use in further experiments. 51 patient-derived EPCs were used in functional/expression experiments, while
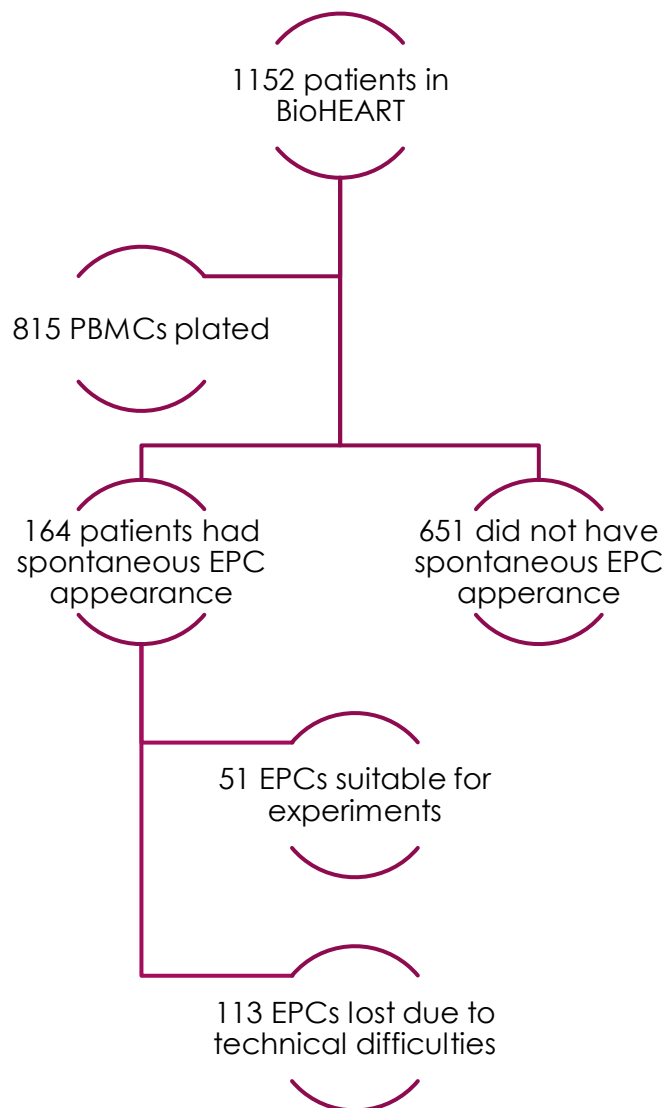
all 164 patients were included in risk factor analysis.

The median length of time until EPC appearance was 14±0.4 days, which would suggest that our patient-derived EPCs are mostly considered late-growing EPCs. Looking further into whether disease severity affected the days of appearance [Figure 3.6], it does not appear to have any influence. It is evident from this figure that there is a wide-range in time to appearance amongst the EPC colonies. So further characterisation was undertaken to ensure all colonies were of similar phenotype.
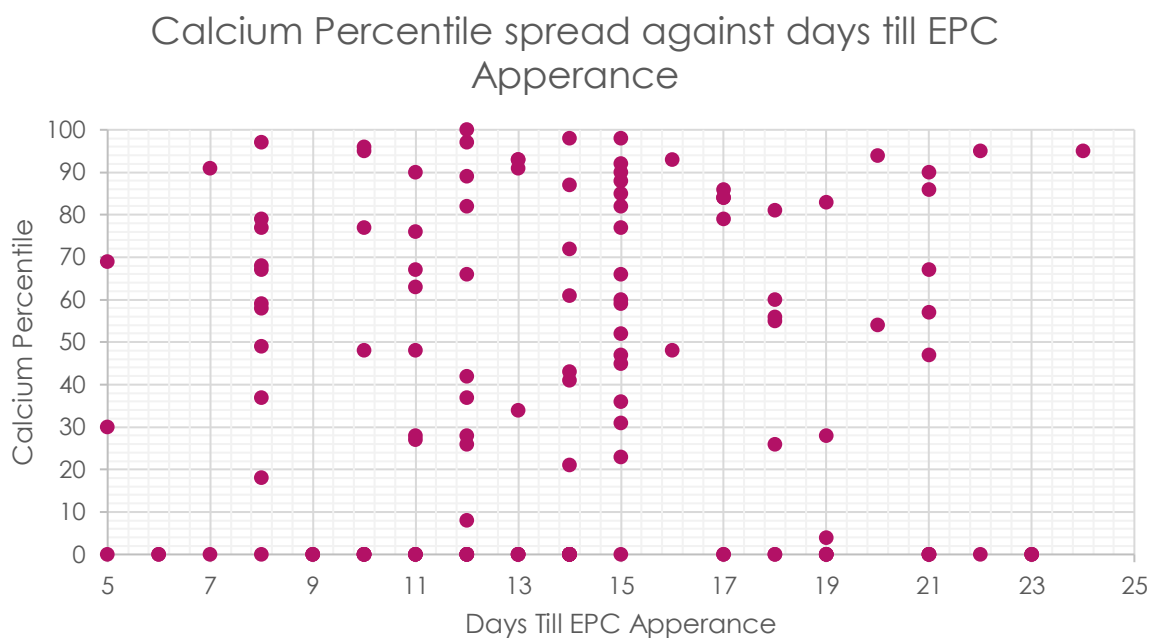
Figure 3. 6: Calcium percentile compared to days till EPC appearance.

3.3.2.1 *Predicting growth of EPCs using clinical or demographic features*

We examined whether clinical or demographic features were associated with spontaneous growth of EPCs. Patients with diabetes and/or BMI≥30 as a risk factor, had significantly lower spontaneous EPC appearance (patients without these conditions had a 2-fold increase in spontaneous EPC appearance) (p<0.05). However, even with a reduction in EPC appearance from patients with these risk factors, it did not influence overall study. Female sex tended to increase the chance of appearance (p=0.07). Other clinical factors; CAD, age over 70, smoking, hyperlipidaemia and hypertension did not influence the growth of EPCs. Patients who took beta-blockers had a significantly lower chance of EPC appearance (12.3%) compared those who did not (20%; p<0.05); while inversely those who took statins, had a significantly higher chance of EPC appearance (22.9%) in comparison to those who did not (16.7%; p<0.05) [Table 3.2]. Whilst there were significant differences, the absolute number of EPC culture successes per subgroup was moderate and would permit further study.

Clinical or demographic feature	Feature yes (%)	Feature no (%)	P value

Female		80/372 (21.5)	76/457 (16.6)	0.07
CAD		98/498 (19.7)	57/330 (17.3)	0.39
Age >70		43/196 (21.9)	111/624 (17.8)	0.19
Body mass index (BMI)≥30		**21/186 (11.3)**	**126/618 (20.4)**	**<0.05***
Diabetes		**7/70 (10)**	**149/759 (19.6)**	**<0.05***
Smoking	Current	9/56 (16.1)	147/769 (19.1)	0.74
	Ex-smoker	59/313 (18.8)	97/512 (18.9)	
	Non-smoker	88/456 (19.3)	68/369 (18.4)	
Hyperlipidaemia		94/466 (20.2)	62/363 (17.1)	0.26
Hypertension		67/325 (20.6)	89/504 (17.7)	0.29
Aspirin		123/667 (18.4)	33/162 (20.4)	0.57
Beta-blocker		**16/130 (12.3)**	**140/699 (20.0)**	**<0.05***
Calcium Channel blocker		16/84 (19.0)	140/745 (18.8)	0.96
Statin		**65/284 (22.9)**	**91/545 (16.7)**	**<0.05***
ACE/ARB		56/263 (21.3)	100/566 (17.7)	0.21
Diuretic		16/60 (26.7)	140/769 (18.2)	0.12
Warfarin		4/14 (28.6)	152/815 (18.7)	0.35
PPI		20/123 (16.3)	136/706 (19.3)	0.43

*Table 3. 2: Comparison of Medication between growers and non-growers. Out of 186 patients with a BMI≥30, 21 of the patients had spontaneous EPC growth (11.3%) in contrast to 618 patients who did not have a BMI≥30, 126 had spontaneous EPC appearance (20.4). *p<0.05 with Chi-squared test.*

To establish patient-derived EPCs as a potential model to discover mechanisms behind susceptibility of coronary disease and resilience, it is vital to understand the potential impact of clinical and demographic factors on spontaneous growth. After exploring the potential association, we then moved on to examine each factor in the grower versus non-grower group. First, we explored the influence of age, BMI, sex, coronary disease, blood pressure and smoking on the capacity for EPCs to develop in culture. BMI of participants were associated with the capacity for EPCs to develop in culture. Patients with a lower BMI were more likely to provide samples where there was successful development of EPCs than their counterparts with higher BMI ($p < 0.0001$). There was no significant difference in age, sex, coronary calcium score, blood pressure, heart rate, smoking pack-years, or alcohol consumption [Table 3.3].

Clinical or demographic feature		Growers	Non-growers	P value
Age (mean +/- sem)		62.7 ± 1.0	60.9 ± 0.5	0.09
BMI		**25.9 ± 0.3**	**30.6 ± 0.4**	**<0.0001***
Sex N (%)	Females	80 (51.3)	292 (43.4)	0.07
	Males	76 (48.7)	381 (56.6)	
Calcium (mean ± sem)	Raw Score	230.9 ± 52.3	234.9 ± 26.5	0.90
	Percentile (%)	40.32 ± 3.0	38.7 ± 1.3	0.61
Heart Rate (bpm)		65.9 ± 1.0	66.9 ± 0.5	0.36
Blood Pressure (mmHg)	Systolic	130.9 ± 1.4	131.7 ± 0.7	0.58
	Diastolic	74.8 ± 0.9	75.6 ± 0.4	0.40
Alcohol Consumption		7.4 ± 0.6	7.0 ± 0.3	0.57
Smoking Pack Years		14.5 ± 1.8	14.2 ± 1.0	0.88

*Table 3. 3: Comparison of clinical and demographic features of growers and non-growers. *p<0.05 with student t-test.*

Univariate logistic regression was used to assess association of spontaneous growth with the covariates; sex, BMI, diabetes, age, beta-blocker and statin use, were $p<0.01$ and were included in the multivariate logistic regression to predict spontaneous EPC appearance. BMI, diabetes, beta-blocker and statin use statistically significantly predicted EPC spontaneous appearance, however, the model was not statistically significant, $\chi_2(6) = 8.53$, $p=0.38$. The model explained 4.9% (Nagelkerke R_2) of the variance in spontaneous EPC appearance and correctly classified 81.7% of cases. Absence of beta-blocker use resulted in a 1.75 times likelihood of spontaneous EPC appearance, and similarly, EPC appearance in patients without diabetes were 2.65 times more likely [Table 3.4]. Absence of statin use and a higher BMI, was associated with a lower likelihood with EPC appearance.

Variables	Sig.	Exp (B)	95% Confidence Interval for B	
			Lower Bound	Upper Bound
Sex (Female)	0.35	1.20	0.82	1.74
BMI	0.04*	0.96	0.92	1.00
Beta Blocker	0.06*	1.75	0.99	3.10
Statin	0.03*	0.65	0.44	0.95
Diabetes	0.03*	2.65	1.11	6.33
Age	0.20	1.00	0.99	1.03

*Table 3. 4: Multiple logistic regression to identify through clinical and demographic factors, which are driving the spontaneous growth of EPCs. *p<0.05 using Chi-squared test.*

The average calcium percentile score between patient samples with EPCs appearance and those without, was not different. However, 40% of patients enrolled in BioHEART had no evidence of calcification on the CTCA scan, despite being referred due to the presence of modifiable risk factors of CVD. The median percentile of from patients with no spontaneous EPC growth and those with patient-derived EPCs are 43% and 42% respectively.
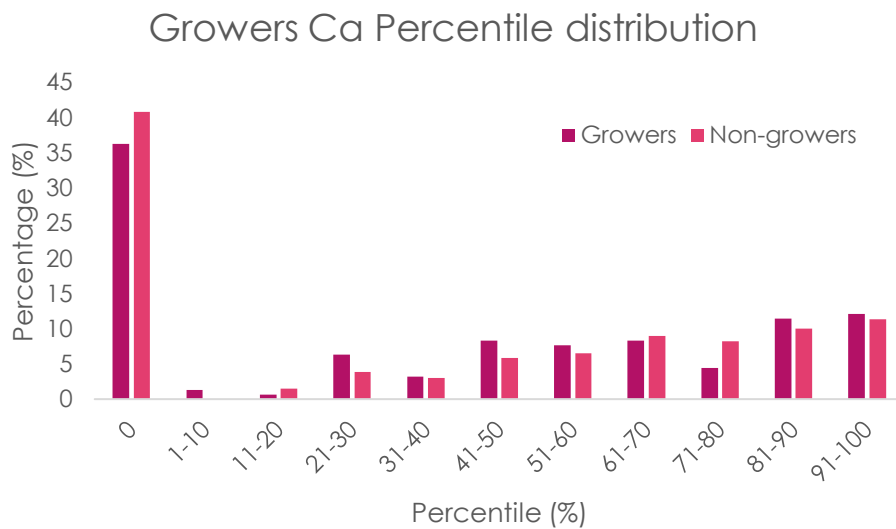
Figure 3. 7: Calcium percentile of participant samples with EPC appearance, and those without demonstrated similar distributions.

3.3.3   Characterisation of endothelial-like features of EPCs

Considerable debate on the true nature of cells grown in this manner, their "purpose" and utility exists in the literature (Shantsila, Watson et al., 2007). We aimed to test if these patient-derived cells bore sufficient endothelial-like characteristics to reflect patient disease susceptibility which would aid in the discovery of new mechanisms. Hence, we tested their morphology, expression of key proteins and function.

3.3.3.1 Biomarkers and functional characteristics of an endothelial phenotype

A cobblestone morphology was observed in our patient-derived EPCs [Figure 3.8a], which is similarly reported in vascular endothelial cells (H. Wu, Riha et al., 2005). We then wanted to confirm whether the patient grown EPC supported an endothelial phenotype. Endothelial Nitric Oxide Synthase (eNOS) expression and NO production are proteins that confirm an endothelial phenotype. There was no difference in the expression of eNOS between the EPCs and HuVECs (HuVEC 1.9±0.8 vs. EPC 1.8±0.6; p=0.92) [Figure 3.8b]. HuVECs are a widely available human-derived endothelial cell line commonly used to examine the vascular endothelial function and molecular parameters. The HuVECs we use are commercially produced and selected for their high eNOS expression. Our patient-derived EPCs showed near identical eNOS expression to these HuVECS.
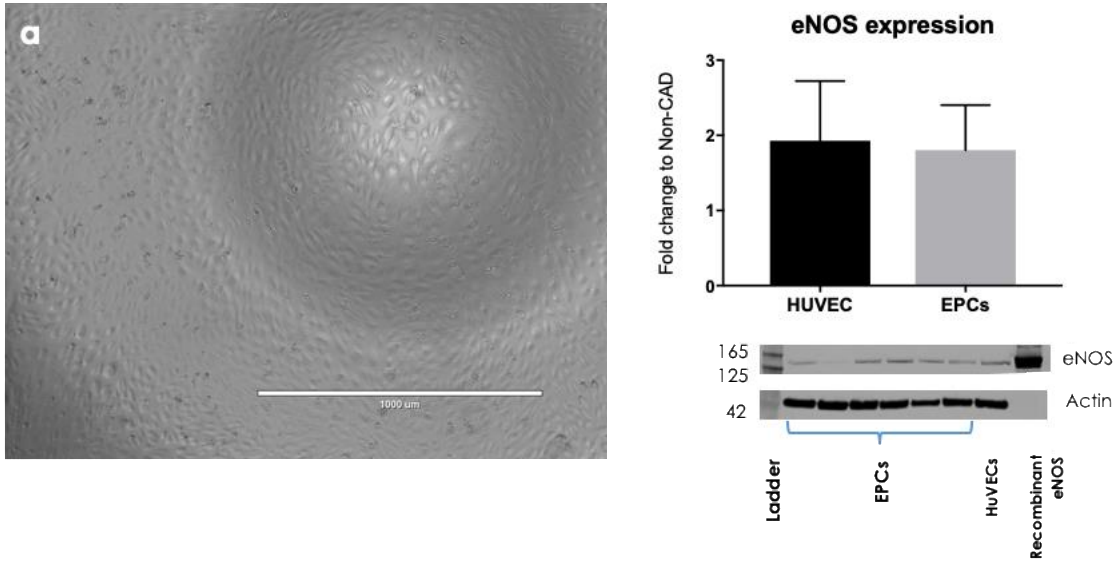
Figure 3. 8: eNOS expression in EPCs and HuVECs. PBMCs were plated into gelatin-coated flasks with EGM-2 media, a) Cobblestone morphology in late growing EPC b) EPC (n=14) and HuVECs (n=4 experiments; cells passaged from single pooled source) have a similar expression of eNOS. Data are shown as mean ± SEM

With a similar expression in eNOS indicative of an endothelial cell phenotype, we next examined next the angiogenic capabilities of the EPCs using the tubule formation assay to determine the cells ability to form tubules. While a direct comparison between the different cell lines is not possible, there is tube formation in both HuVECS and EPCs [Figure 3.9], and surface marker expression [Figure 3.11]. This continues to confirm that the patient-derived EPCs have an endothelial phenotype similar to commercial lines.
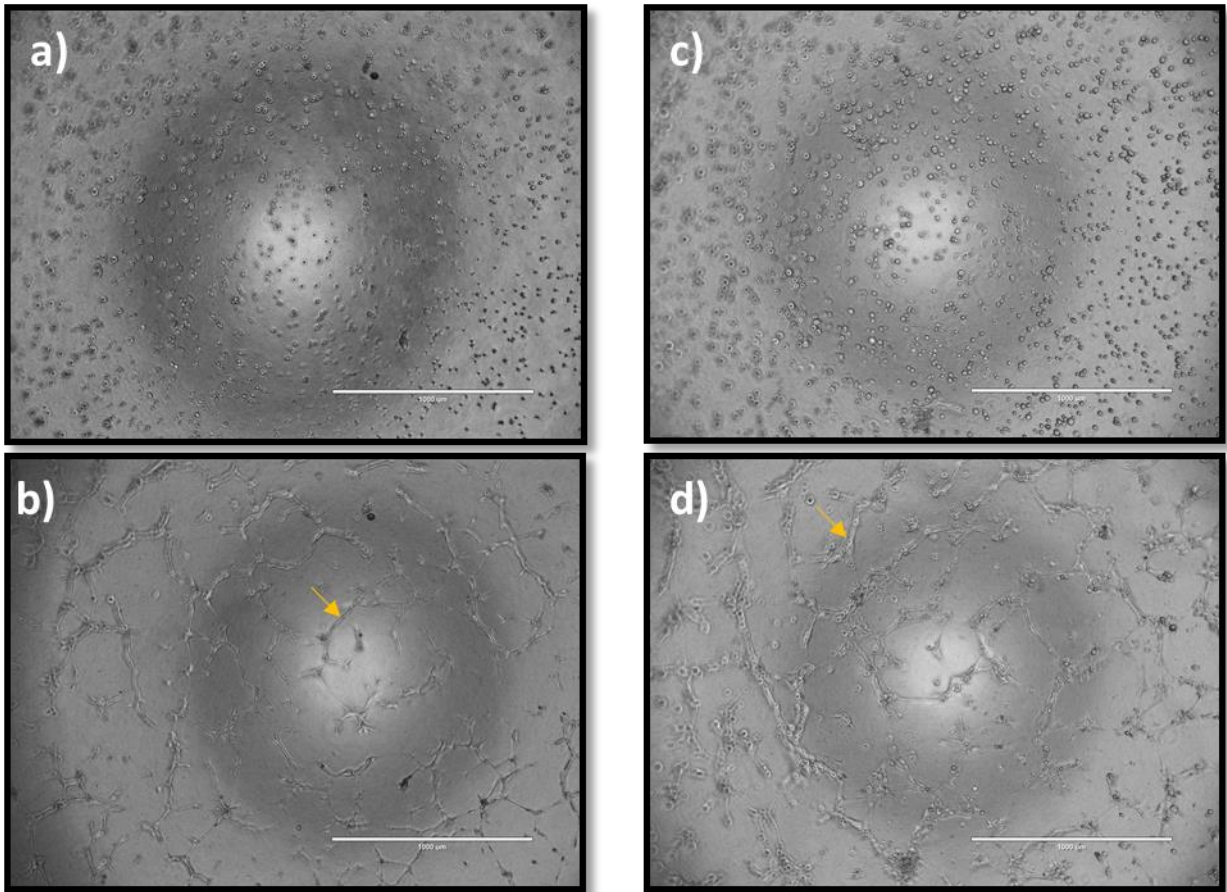
Figure 3. 9: Tube Formation in EPCs and HuVECs at 0hrs and 6hrs. Matrigel was applied to wells and incubated for 30 mins before cells were added a) EPC at 0hrs, b) EPCs at 6hrs, c) HuVECs at 0hrs, d) HuVECs at 6hrs. Images were taken at 10x magnification on EVOS FL Auto, and the magnification bar represents 1000μm, arrows indicate tubules.

3.3.3.2 Surface markers of endothelial progenitor cells

Aside from measuring time to appearance (after more than a week in culture) and observing cobblestone morphology (Hur, Yoon et al., 2004). It is possible to categorise cells as late EPCs by examining surface marker expression. Using surface markers were selected based on previous studies, we performed flow cytometry (Cheng, Chang et al., 2013b; W. Qiao, Zhou et al., 2015). We detected for positive expression of CD133, CD34, CD31 and a negative expression of CD14 in the patient-derived EPCs and compared them to HuVECs. Of the viable HuVECs [Figure 3.10] and EPCs [Figure 3.11], there was positive staining for CD133, 34 and 31 and all were negative for CD14.
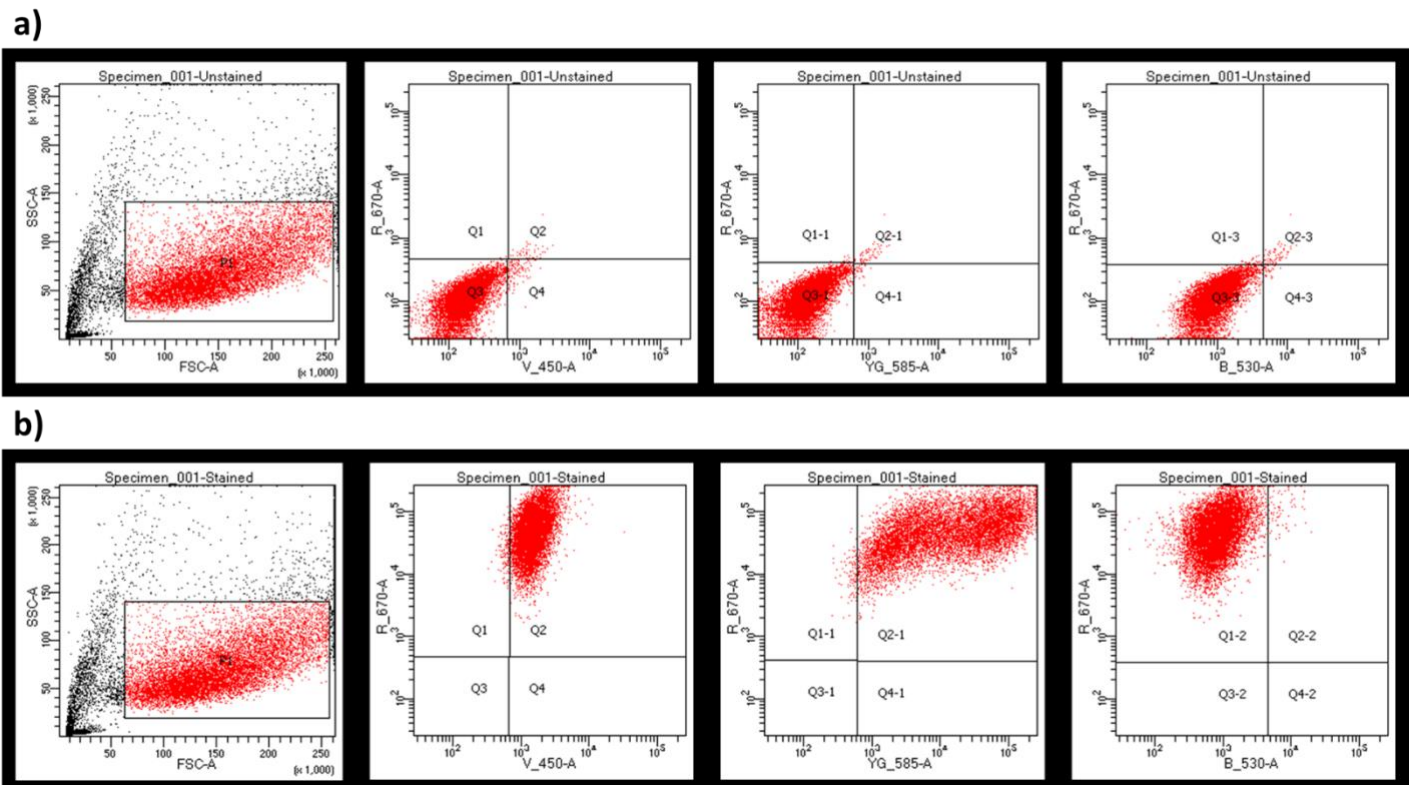
Figure 3. 10: Surface marker expression of HuVECs a) unstained cells, b) stained for the markers (from left to right) CD133, CD34, CD31 and CD14

a)

b)
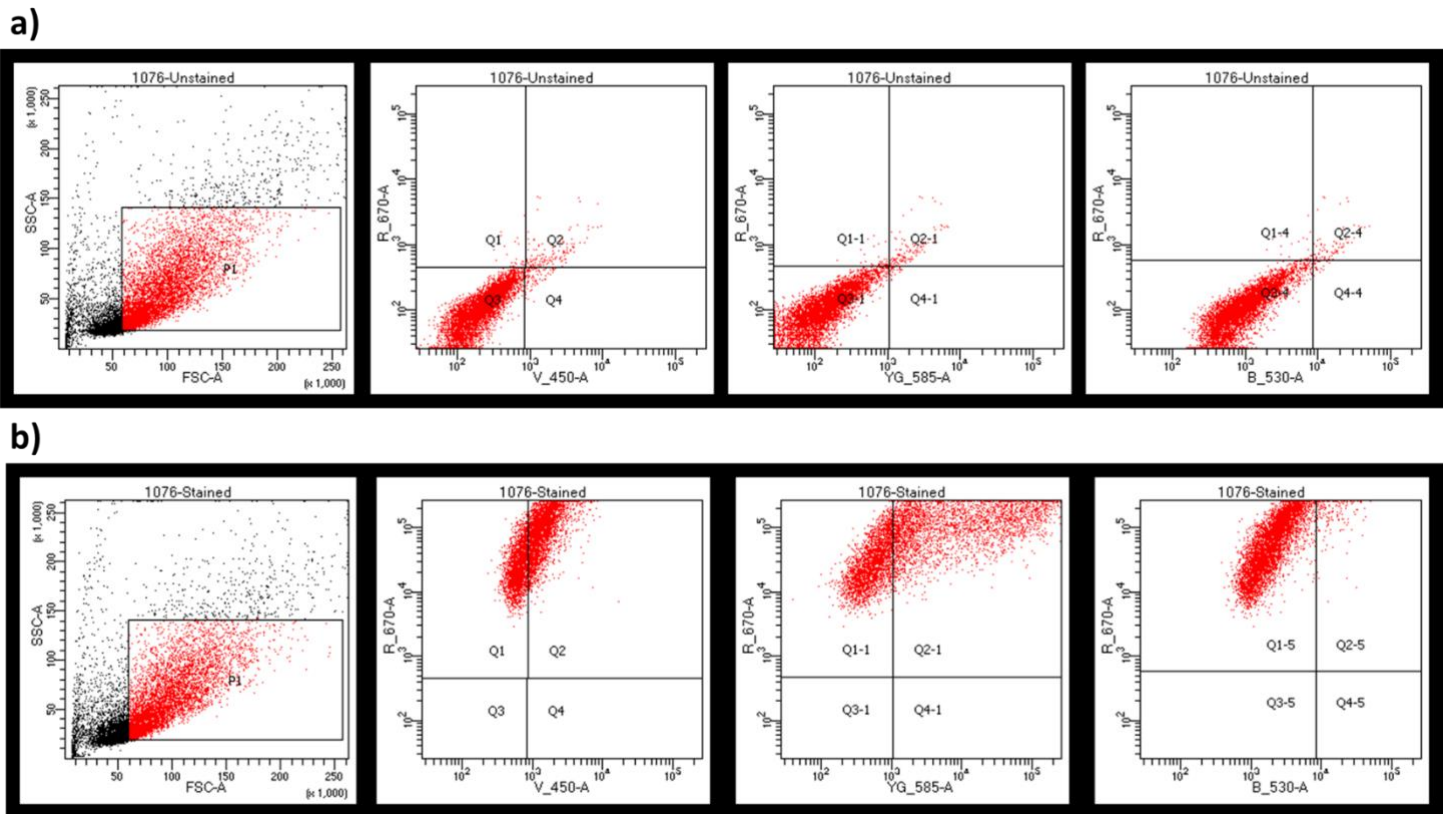
Figure 3. 11: Surface marker expression of EPCs a) unstained cells, b) stained for the markers (from left to right) CD133, CD34, CD31 and CD14

However, there was some difference in the expression in EPCs, CD31 is still strongly expressed, however, there is a reduced expression of CD34 and a much lower expression of CD133 in comparison to HuVECs [Table 3.5]. Nevertheless, the expression profiles gave confidence that EPC samples were of endothelial phenotype and consistent.

	CD133/V_450 (%)	CD34/YG_585 (%)	CD31/R_670 (%)	CD14/B_530 (%)
HuVEC	77.6	84.3	92.6	0.8
EPCs	3.5 ± 3.1	26.1 ± 3.6	90.3 ± 9.4	1.8 ± 1.7

Table 3. 5: Surface marker expression. Breakdown of surface marker expression in percentages between the patient-derived EPCs compared to expression in HuVECs.

3.3.4 Prediction of disease using redox signalling

Whilst traditional clinical risk algorithms consider factors driving vascular dysfunction as hypertension, diabetes and hyperlipidemia and smoking, we have very little in the "tool box" to measure an individual's response to these factors. Given dysregulated oxidative signalling is recognised as an early downstream abnormality in the development of atherosclerosis, measurements of oxidative stress in patient-derived biological samples may provide insights into disease susceptibility. This may have relevance to the development of new markers of disease (Ho, Karimi Galougahi et al., 2013). We next examined whether molecular markers of oxidative stress in patient-derived EPCs was associated with burden of CAD and, thus, whether they may be an appropriate model to explore novel mechanisms of disease.

3.3.4.1 Nox Isoforms

Nox is a major contributor to ROS, and is a key ROS produced in vascular diseases (Bedard & Krause, 2007) which is linked to the pathophysiology of coronary artery disease (Panth, Paudel et al., 2016). Nox enzymes modulate vital parts of cell signalling and contribute to CAD through redox-sensitive signalling pathways (Libby & Theroux, 2005). We examined cellular signalling characteristics of patient-derived EPCs from those with CTCA-defined CAD and those with no CAD. Nox-2 is ~2x higher in the diseased group (Non-CAD 1.02 ± 0.13, vs. CAD 1.97 ± 0.37; $*p<0.05$) indicating an up-regulation of inflammation in CAD patient-derived cells. Nox-4 expression appeared slightly elevated in the CAD group but was not significantly different when compared to the Non-CAD group (Non-CAD 1 ± 0.25 vs. CAD 1.65 ± 0.37; $p=0.17$).
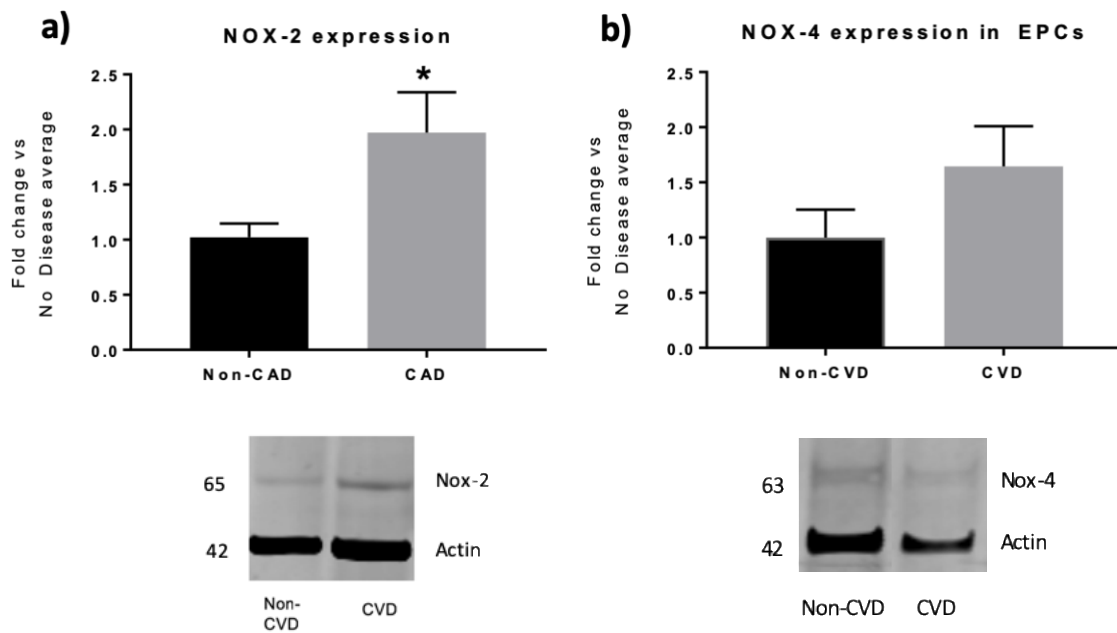
Figure 3. 12: NOX isoform expression in the EPCs. Protein is extracted from EPCs and fluorescent immunoblotting was done a) Nox-2 expression in Non-CAD (n=14) in comparison to CAD patients (n=13); d) NOX-4 expression in the EPCs Non-CAD (n=13) vs. CAD (n=15).

3.3.4.2 eNOS expression between Non-CAD and CAD patients

eNOS is responsible for enzymatic production of NO, which in the body contributes to the reduction of cell apoptosis, inhibition of smooth muscle proliferation and regulating blood pressure (Abolhalaj, Amoli et al., 2013). Our results showed that eNOS expression of EPCs did not differ between patients from the Non-CAD and CAD group (Non-CAD 1±0.34 vs. CAD 1.70±0.66; p=0.34). This shows that eNOS expression is not associated with disease status.
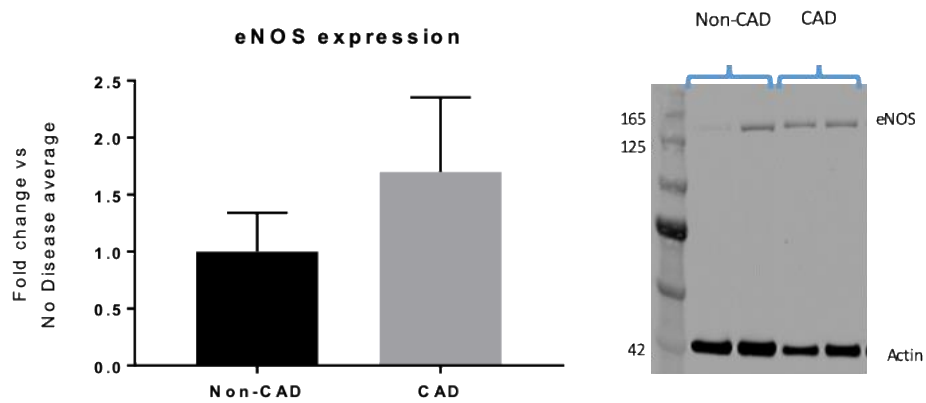
Figure 3. 13: eNOS expression compared between Non-CAD (n=14) vs. CAD group (n=13). Protein is extracted from EPCs and fluorescent immunoblotting was completed.

3.3.4.3 Glutaredoxin-1 expression

Variation in levels of cellular antioxidant protective mechanisms is a potential candidate to explain individual difference in susceptibility to cardiovascular disease. Glutaredoxin is an antioxidant enzyme which has been previously shown to increase expression in the presence of ROS (Okuda, Inoue et al., 2001). In our patient-derived EPCs, we found that Grx-1 expression was not significantly associated with CAD (patients with a raw calcium score <0) (Non-CAD 1 ± 0.27 vs. CAD 1.16 ± 0.21; n=12-12; p=0.66).
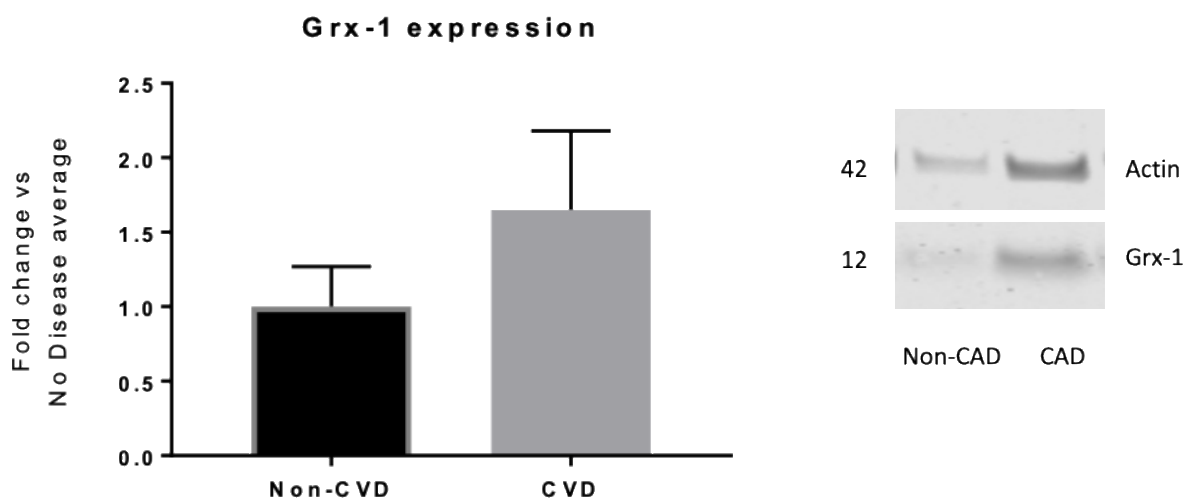
Figure 3. 14: Grx-1 expression between Non-CAD (n=13) vs. CAD (n=12).

3.3.4.4 Oxidative response in EPCs

With an increase in Nox-2 observed in EPCs from patients with CAD, we examined potential impact on cellular superoxide levels. In EPCs loaded with O_2-• sensitive dye dihydroethidium (DHE), we found a clear signal in the 647 nm channel not observed in control cells [Figure 3.15a]. O_2-• was partially, but no significantly quenched down by O_2-• dismutase mimetic, MnTMPyP (DHE 28.1±12.2 vs. DHE+MN 13.4±6.0; where p=0.33) [Figure 3.15b].
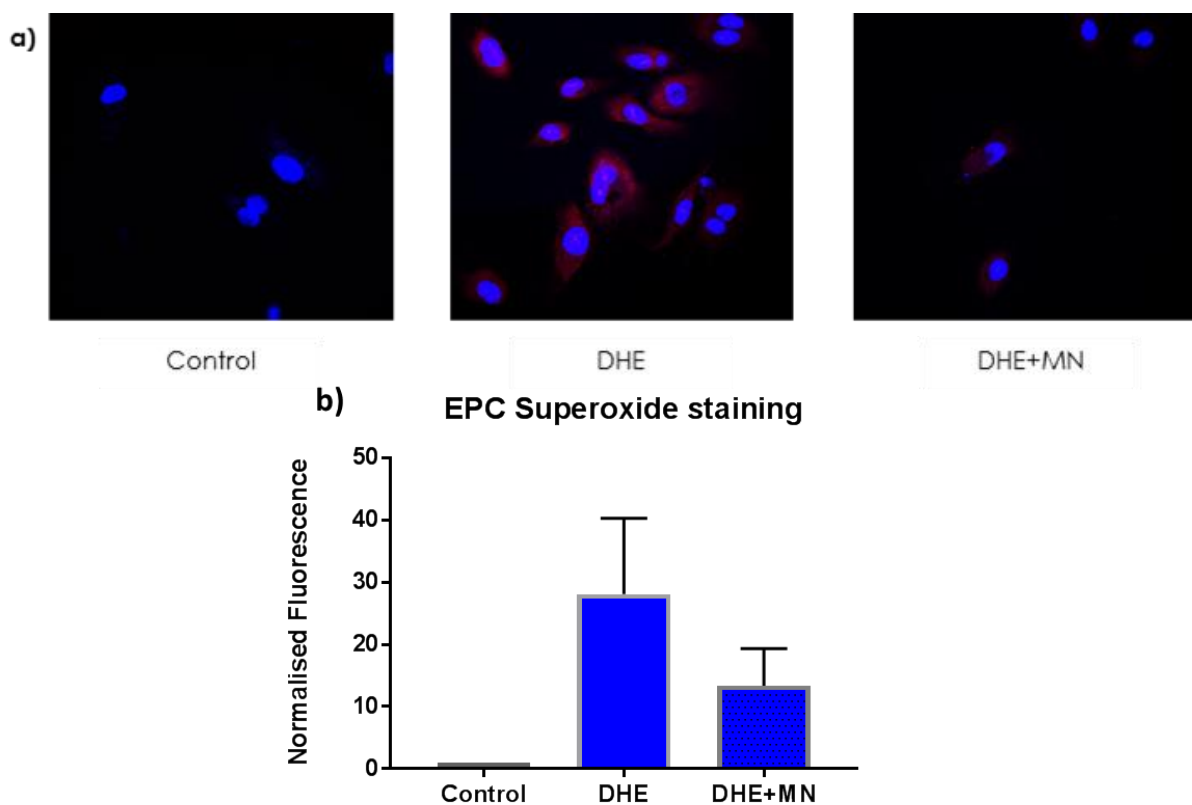
Figure 3. 15: O_2-• expression with MnTMpyp quenching. Examing superoxide levels using DHE fluorescence. Cells were seeded onto coverslips and pre-treated with ANGII, before being mounted ProLong diamond Antifade mountant with DAPI. Representative confocal images are shown from cells without DHE loading (a)l with DHE (n=14 patient-derived EPCs; b) and DHE+MN (n=11).

When comparing cells from BioHEART patients with CAD and non-CAD, there was no significant difference in $O_2\text{-}\bullet$ generation (Non-CAD 20.2±8.6 vs. CAD 34.07±20.8; where p=0.6), noting the substantial inter-patient variability. After Angiotensin II stimulation, there was no significant difference in DHE-fluorescence observed between the Non-CAD and CAD group (p=0.3).
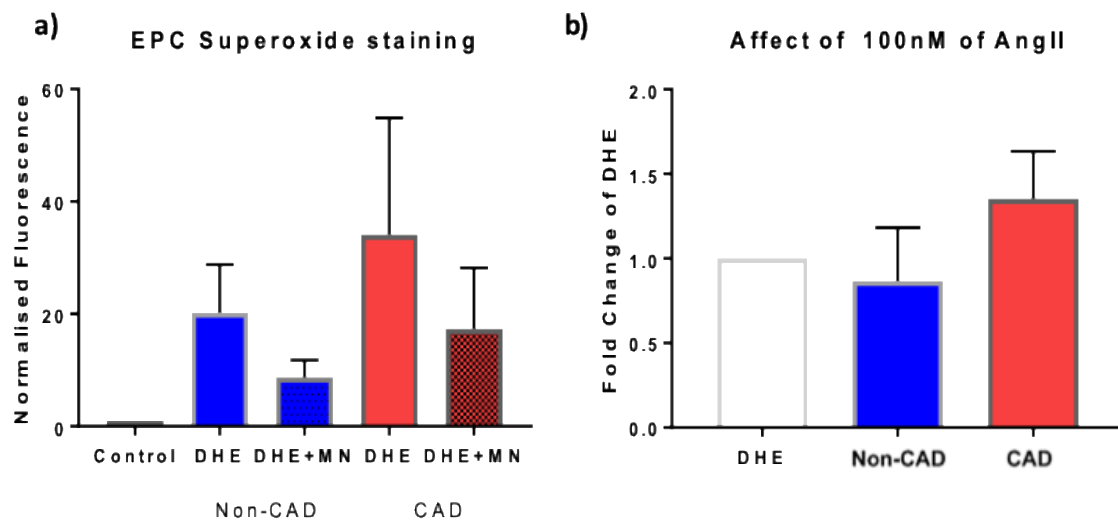
Figure 3. 16: Normalisation was achieved by division of each patient's average DHE fluorescence by the average of the control. a) Normalised DHE fluorescence signal intensity in EPCs from patients with no CAD (n=6) vs. EPCs from patients with CAD (n=8) which is then scavenged by MnTMPyP (MN), b) DHE-fluorescence in the pressure of 100 nM Ang II in EPCs from Non-CVD (n=4) and CVD patients (n=5).

3.3.5 Mitochondria superoxide generation

While there was no significant difference in total superoxide production of EPCs from disease patients, there was a trend towards greater superoxide in EPCs from CAD patients. Therefore, we decided to investigate compartmentalised superoxide production. The mitochondria is a source of superoxide and mitochondrial superoxide production is often elevated in endothelial cells in CVD (Chistiakov, Shkurat et al., 2018). Therefore, working with an MD student (Nicole Seebacher), utilising the patient-derived EPCs that I had cultured for the BioHEART study, we found a strong association between calcium percentile and mitochondrial superoxide expression (R_2=0.90; n=18) [Figure 3.17].
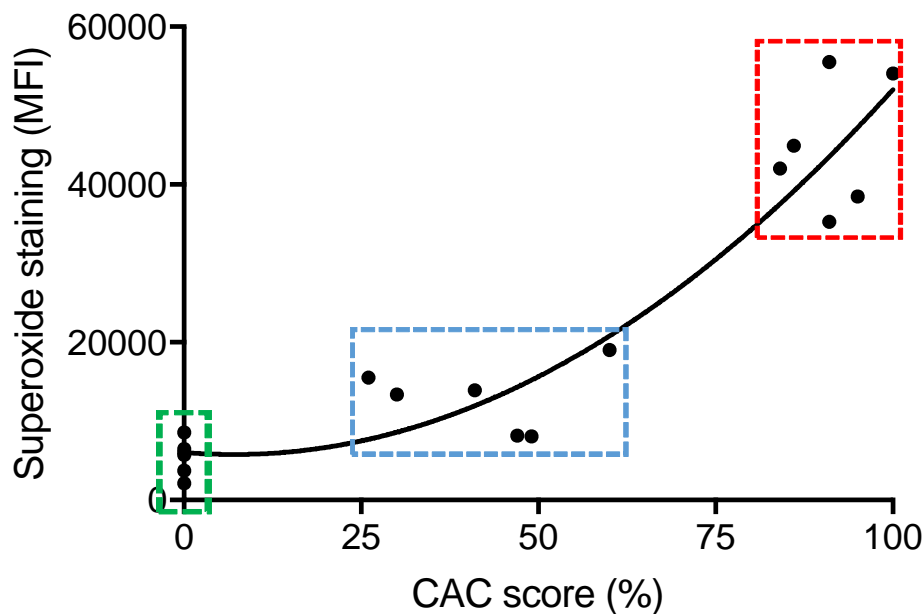
Figure 3. 17: Association between calcium percentile and mitochondrial superoxide specific fluorescence signal in patient-derived EPCS loaded with mitosox ((mean fluorescence intensity (MFI). Statistical tests were completed using a nonlinear fit. (Mitosox studies were performed by MD student Nicole Seebacher)

When comparing superoxide expression between EPCs from healthy patients and those with CAD, there was a significantly higher superoxide expression in the EPCs from patients with disease (p<0.05, n=6). This suggests that superoxide expression and oxidative stress of the EPCs ex vivo, may be primarily driven by the mitochondria and reflects the disease phenotype of the individual.
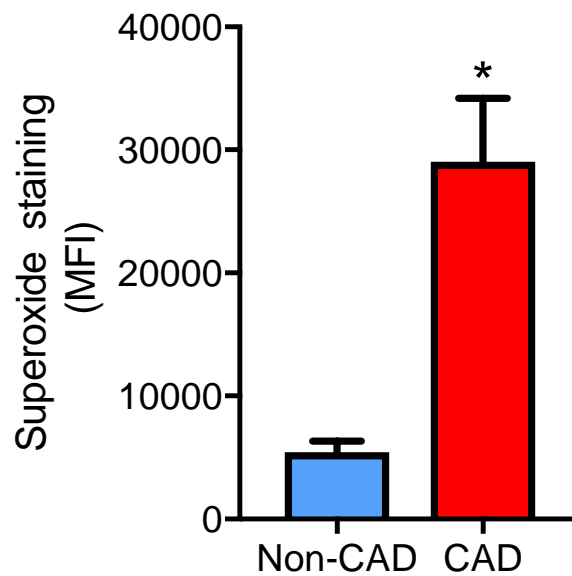
Figure 3. 18: Superoxide staining in mitochondria between EPCs loaded with Mitosox from healthy patients (n=6) and patients with CAD (n=6; p<0.05), statistical test was performed using a paired t-test. (Mitosox studies were performed by MD student Nicole Seebacher.)

3.4 DISCUSSION

In this Chapter, I demonstrate the feasibility of using patient-derived endothelial progenitor cells as a cellular model to explore mechanisms of individual patient susceptibility to coronary artery disease. Firstly, cells that spontaneously appeared, demonstrated endothelial-like features including eNOS expression and angiogenic capabilities. There are potential limitations related to the ~20% growth rate from plated PBMCs. We demonstrated that the presence coronary disease was not a predictive factor for spontaneous EPC growth. However, demographic and clinical factors such as BMI, diabetes, and medications such as beta-blockers and statins, had a small but significant impact on the probability of spontaneous growth. Our demonstration of elevated Nox-2 expression and elevated mitochondrial reactive oxygen species levels in EPCs derived from patients with CAD support the potential opportunity in this model. It poses as a prominent oxidative stress source and EPCs as an appropriate model of cardiovascular disease.

The potential for socioeconomic bias should be considered (Phillips & Klein, 2010), given our method of recruitment from an outpatient radiology practice in a relatively affluent area of Sydney. 40% of the patients enrolled into BioHEART had no evidence of CAD. Participants are often referred to a CTCA scan in an outpatient setting due to the presentation of CAD symptoms and a clinical suspicion of atherosclerosis. Higher risk patients with suspected acute coronary syndrome are more likely to be assessed in an Emergency Department of the local hospital and are therefore not represented in this cohort. Limitations of the BioHEART cohort clinical data is the reliance on patient responses, rather than acquisition via medical records. Participants were asked to complete a questionnaire with the researcher at the time of consent, and there is a chance of recall bias at this point. Often patients were unable to accurately recall the name of drugs they were taking (Coughlin, 1990) especially in an older cohort, who may struggle to recall in general.

Therefore, our records may not accurately capture medication and risk factors for every subject.

The prevalence of various risk factors also differs from our general population statistics. For example, 4.9% of the population has diabetes (Davis, Peters et al., 2018), however 8% of the BioHEART cohort had diabetes. Interestingly, there is a 10% chance of EPCs developing from samples obtained from participants with diabetes, in comparison to 19.6% in participant samples without diabetes. It has been shown previously that diabetic patients have a reduced number of circulating EPCs (Churdchomjan, Kheolamai et al., 2010), which may explain the decreased rate of spontaneous appearance. BMI is another factor that affects the appearance of patient-derived EPCs, where a higher BMI is associated with lower spontaneous growth. This is further supported by a study that observed reduced adhesion, migration and angiogenic capabilities in EPCs from obese patients (N.-M. Heida, Müller et al., 2010b). Obesity is often associated with several other comorbidities such as hypertension, hyperlipidaemia, and diabetes. These all lead to endothelial dysfunction which is one of the first steps in the development of atherosclerotic plaque. These conditions negatively affect EPCs, which are part of vascular regeneration and may impact cardiovascular event outcomes (Tobler, Freudenthaler et al., 2010). However, despite the significant difference in EPC spontaneous growth between patients with or without diabetes and elevated BMI, the number of EPC cultures from each group was adequate to study.

Within the cohort, statin use was documented in 32% of patients. Statins are one of the most studied medications and have consistently been shown to reduce the incidence of heart attack and stroke (Ble, Hughes et al., 2017; W. Wang & Zhang, 2014). Hyperlipidaemia is present in over 50% of the BioHEART cohort which has been shown to negatively affect migration and adhesion of EPCs (T.-B. Li, Zhang et al., 2016). In the BioHEART cohort, there is a significant increase in EPC spontaneous growth in patients who take statins in comparison

to those who do not. This is not surprising as statins have been consistently proven to improve EPC function (P. S. S. Lee & Poh, 2014). It has been shown that statins can affect the differentiation of EPCs through the phosphoinositide 3-kinase pathway (Y. Liu, Wei et al., 2012b). This may demonstrate the mechanisms behind the increased appearance of EPCs in patients who take statins. The beneficial effect of statins on EPC growth and function may have relevance for patients with chronic ischaemic heart disease or peripheral vascular disease. Potential difference across the class of statins and relevance to myocardial and limb perfusion independent on the effect on conduit vessel atherosclerosis burden warrants further exploration.

Another medication seen to affect the spontaneous appearance of EPCs are beta-blockers. Beta-blockers can be used for a variety of conditions, but in particular, they are used in hypertension. It has been previously shown, with hypertensive subjects that there is increased EPC senescence (Imanishi, Moriwaki et al., 2005). In the BioHEART cohort, beta-blockers significantly reduce the chance of EPC appearance, however, the mechanisms behind this are unknown. The number of circulating endothelial cells and angiogenic capacity are not thought to be affected by the medication (Stati, Musumeci et al., 2014; T. Umemura, Soga et al., 2008a). Whether b-blocker therapy effects the development of collateral formation in ischaemic limbs or myocardium of patients in vivo is worth clinical investigation.

Whilst a number of other studies have examined clinical and demographic factors affect EPC growth and function, few studies have included the large numbers I have recruited- over 800. In this large cohort, I have clearly identified factors that impact on the appearance of EPCs. I have confirmed that elevated BMI and diabetes are associated with impaired spontaneous growth, but also identified the use of beta blockers as a novel factor inhibiting spontaneous growth. My data supports a positive effect of statins on EPC growth, similar to previous findings. We also did not find statins to significantly

counteract the EPC appearance; 8 out of 17 patients taking beta-blockers, were also taking statins. However, shown in the multiple logistic regression, through all the complex demographic and clinical factors, diabetes, beta blockers and statins all influenced the spontaneous appearance of EPCs.

The characterisation of EPCs has been controversial since they were discovered (T. Asahara, Murohara et al., 1997). Methods in enumeration and culture protocol vary significantly and are highly debatable as EPCs themselves can express different surface markers and thus can represent different types of EPCs. We observed cobblestone morphology of late EPCs which normally appear in culture greater than one week after PBMC plating, while early growers are spindle-shaped and appear within a few days of culture (Tagawa et al., 2015). They demonstrated tubule formation when the cells were applied to Matrigel, which is largely unique to endothelial cells (Francescone, Faibish et al., 2011). The tubule formation was also seen in HuVECs, which are endothelial cells extracted from the umbilical cord. It is an embryonic cell line that has not been influenced by various environmental factors that occur over a lifetime. The patient EPCs had a very similar expression of eNOS to HuVECs which further reinforce an endothelial phenotype (Wei Qiao, Niu et al., 2010).

Surface markers commonly used to distinguish an endothelial progenitor lineage are CD34, a marker of human haematopoietic stem cell from the bone marrow, however it has been shown that CD34 is often lost in an in vitro environment with passaging (Sidney, Branch et al., 2014). CD133 a glycoprotein in the cell membrane which is expressed in hematopoietic stem cells, endothelial progenitor cells and various cancer cells (Z. Li, 2013). Another marker is CD31, a platelet endothelial cell adhesion molecule which makes up a large proportion of endothelial cell intercellular junctions (Cheng et al., 2013a; Hager, Holnthoner et al., 2013). However, some disagree (Case, Mead et al., 2007), arguing that cells with all the above markers have not been tested

on endothelial cell clonogenic assays. While this is true, we also don't expect all the cells to uniformly express all three markers in all cells which are seen throughout the literature. Our EPCs showed a high expression of CD31, which is consistent with HuVECs, however, CD133 expression was very low. Though it has been previously shown that CD133 is not present in mature endothelial cells (Erdbruegger, Haubitz et al., 2006), and as the EPCs are passage 5 at the time of the experiment, they may be matured and no longer have CD133 as a surface marker.

Nox-2 is responsible for antimicrobial host defence, but overexpression can result in chronic inflammation that can result in atherosclerotic development (Singel & Segal, 2016). A ~2-fold increase in Nox-2 was observed in the diseased group which presents the patient-derived EPCs as a potential model for cardiovascular disease. eNOS is a vital part of producing NO in the vascular system and reducing oxidative stress in the vascular system (Förstermann et al., 2017). However, there was no significant difference between the healthy and diseased patients (Abolhalaj et al., 2013). Nox-4 which also plays a part in the production of ROS (Petry, Djordjevic et al., 2006), was not significantly different in the disease patients. Grx-1 is an enzyme responsible for removing glutathione adducts from proteins and is a participant in redox signalling (Swain, Kiley et al., 2017). However, difference in Grx-1 signalling was not observed in our EPCs. This suggests that while the EPCs can model some CAD phenotype and act as an appropriate proxy to explore mechanisms, it does not encapsulate all dysregulation associated with CAD.

Lastly, to test the EPCs response to an oxidative stimulus, we exposed the cells to AngII, a potent activator of ROS (Hitomi, Kiyomoto et al., 2007). There was no increased O_2-• generation when stimulated with AngII in diseased patients in comparison to the healthy. Our EPCs may not have seen this increased O_2-• generation due to the short incubation time of AngII (1.5 hrs) in comparison to

the 48hr incubation other ex-vivo cell models used (Imanishi, Hano et al., 2005; L. Y. Wu, Dang et al., 2009). However, the EPCs did express angiotensin II receptor 1 (data not shown) suggesting they should be responsive to exogenous AngII. It may also be due to patients taking ACE/ARB inhibitors which may have a lingering role in suppressing any endogenous AngII activity even after cells were cultured ex-vivo (Ferrario, 2006). I also observed substantial variability between experiments which may be under-powered to detect a difference. However, when we specifically measured superoxide generation in the mitochondria we found it was increased in EPCs from patients with CAD. These recent findings show the excellent potential of EPCs to be used for investigating specific mechanisms. We now are planning further experiments in the future using selective mitochondria preparations. This could uncover a novel mechanism and may also offer a new therapeutic angle. This allows patient-derived EPCs to be an appropriate disease model for further study of the underlying mechanisms of CAD.

In conclusion, the EPCs extracted from patients enrolled in BioHEART appear to be of endothelial origin and express surface markers consistent with the consensus of late growing EPCs. A detailed risk profile was created for all patient-derived EPCs which allowed the identification of new factors affecting the growth of EPCs, which could be used as early identification of patients with potentially poorer cardiovascular outcomes. It also appears that these EPCs have some characteristics of a CAD phenotype, and could, therefore, be used as an ex-vivo model to further unravel mechanisms contributing to the development of atherosclerosis.

4 THE POTENTIAL OF ENDOTHELIAL PROGENITOR CELLS TO AID IN DISCOVERY OF MECHANISMS DRIVING SEX DIFFERENCES IN ANGIOGENESIS

4.1 INTRODUCTION

Cardiovascular disease is the leading cause of death in women and men in the world (2018). However, women are less likely to recognise symptoms of cardiovascular disease, due to beliefs that women are at a lower risk of cardiovascular events, and lack of awareness that women may experience different MI symptoms (Gallagher, Marshall et al., 2010). This may result in a late presentation to a hospital resulting in limited treatment options, and worse cardiovascular outcomes. Women are also less likely to receive lipid-lowering therapy and therapeutic life changes (Garcia, Mulvagh et al., 2016). The differences in medication prescription may be due to gender bias from treating physicians who see men to be at a higher risk than women (Abuful, Gidron et al., 2005; Mosca, Linfante Allison et al., 2005).

Multiple factors contribute to the pathophysiology of cardiovascular disease, some of them are modifiable; smoking and diet, while others are not; ethnicity, sex and access to medical care (Miller, 2014). Risk factors for men and women are mostly similar, however, smoking has a greater negative effect on CVD in women (Yahagi, Davis et al., 2015). Differences in pathology were observed through 70% of young child-bearing females presenting with spontaneous coronary artery dissection (Shamloo, Chintala et al., 2010) in a non-atherosclerosis cardiovascular disease, however, the importance of estrogen in stabilising plaque in premenopausal women was identified (Burke, Farb et al., 2001). This leads to an inherent need to understand the mechanisms

underlying the pathophysiology of disease in women and men, to effectively identify and treat cardiovascular disease.

In the event of a MI with medical intervention, angioplasty or stents are often used to restore blood flow to heart tissue. However, not all patients are eligible for this procedure due to microvascular dysfunction(van der Laan, Piek et al., 2009). Hence, angiogenesis is a vital part of recovery post-MI to restore blood flow to areas of the heart affected by the blockage (Cochain, Channon et al., 2013). Angiogenesis is a fundamental process involved in crucial physiological processes throughout life. It is not only part of the revascularisation process post-MI but is also involved in wound healing and tissue remodelling. Unregulated angiogenesis however, can result in tumour growth, metastasis and visual impairment (Gupta & Zhang, 2005). EPCs are an essential part of recovery post-MI due to their ability to migrate to the area of infarct to construct collateral blood vessels and re-supply blood to areas of the infarcted heart. Patients with cardiovascular disease often have reduced EPC numbers and function (Lin, Lin et al., 2013) thus, migration of the EPCs is important to re-vascularize the heart.

Angiogenic processes such as endothelial cell activation, proliferation, tube formation, and migration require VEGF. A tyrosine kinase receptor VEGF-2 is important for vascular development through the recruitment of signalling pathways ERK and MAPK (Chrzanowska-Wodnicka, Kraus et al., 2008). The ERK pathway is associated with cell growth and proliferation (Y. Wang, 2007) and may also be an appropriate marker of CVD, as ERK is up-regulated when macrophages are treated with oxidised low-density lipoprotein (Muslin, 2008). Signalling cascade for ERK starts through the activation of small G proteins, which then signal through MAP3K phosphorylating MAPK/ERK kinases (Shaul & Seger, 2007). Pre-clinical studies suggest that ERK-2 is protective (Lips, Bueno et al., 2004) where heterozygous ERK-2 knockout mice had markedly

decreased heart function post ischaemic injury. While phosphorylated ERK (pERK) is the active form of ERK, it is not known whether the expression of ERK1/2 or pERK is responsible for a cellular response (Mutlak & Kehat, 2015).

Phosphoinositide 3-kinase (PI3K) affects cell growth and survival (Hers, Vincent et al., 2011) and is able to affect downstream regulation of calcium ion flux through Protein Kinase B (AKT) (Ghigo, Laffargue et al., 2017) which mediates endothelial migration and angiogenesis (Yu, Littlewood et al., 2015). Calcium signalling is vital to the contraction of cardiac skeletal and smooth muscles, where dysregulation can result in arrhythmias and contribute to hypertension (Marks, 2013). AKT has three isoforms; AKT1 which is expressed throughout the body, AKT2 is found in adipose tissues, liver and skeletal muscle and AKT3 is found in the brain (Yu et al., 2015). In AKT1 knockout (KO) mice, there was increased aortic lesion expansion and coronary atherosclerosis (Fernández-Hernando, Ackah et al., 2007) which suggests that it is cardio-protective. AKT1 mediates the activation of eNOS and subsequently the production of NO (Dimmeler, Fleming et al., 1999) therefore, deficiency of AKT1 in mice results in reduced eNOS production which is essential in fighting inflammation and inducing vasodilation (Di Lorenzo, Fernández-Hernando et al., 2009).

In this chapter, I focus on the hypothesis that EPCs derived from female patients will have different angiogenic capabilities than males. I aimed to:

1. Examine migration capacities of EPCs between males and females, considering CAD burden;
2. Explore angiogenic pathways that may explain potential differences in poor outcomes in women with disease.

4.2 METHODS

4.2.1 EPC culture and experiments

Using methods outlined in Chapter 2.1, blood was collected from patients in the BioHEART study. PBMCs were extracted from patient blood using a Ficoll-plaqe density gradient (see 2.1.1) and were cultured for 21 days for the spontaneous appearance of EPCs (see 2.1.2). Functional assays were used to determine angiogenic capabilities between different disease and sex groups. The tube formation assay (see 2.8) was performed using 31 EPC cell lines. Each sample was used in duplicate in each assay and an average was taken. For the assessment of EPC migration, 26 EPC cell lines were used and prepared in triplicates, with an average result used per sample. For this assay, a denuded zone was created, and images were obtained over 48 hours (see 2.7). To further examine mechanisms driving angiogenesis, immunoblotting was performed. Protein lysate was obtained, and probed for ERK1/2, pERK, AKT and pAKT, primary anti-bodies are as follows: ERK 1+2 (1:1000), 137F5; phospho ERK (1:2000), 4370S; AKT (1:1000), 9018S; phospho AKT (1:1000), 2938S; Cell Signalling Technology (US) and anti-eNOS/NOS Type III, 610297 BD Science (US). 4-12% gradient Bis-Tris gels were used for all proteins and probed using the Licor Odessey imaging platform (see 2.2).

4.2.2 Statistical Analysis

Data were analysed and graphed using Graphpad software, Microsoft Excel and SPSS. Mean ± SEM is presented for each group. In section 4.3.1, comparisons were made between EPCs from patients with CAD and those without before being divided further on the basis of sex using a two-way ANOVA. In 4.3.2 and 4.3.3.1-4.3.3.3 a student's t-test was used to analyse tubule formation capabilities and protein expression between patients EPCs with CAD

and those without, a further analysis on the basis of sex using a two-way ANOVA was performed. Significance for all results was accepted at $p<0.05$.

4.3 RESULTS

4.3.1 Clinical and demographics of EPC cell lines used for experiments

Though 164 of the cultured samples resulted in the appearance of EPCs, only 51 of these were able to be utilised in functional and exploratory experiments. Demographics and clinical factors were examined in patients from which the EPC cell lines were used [Table 4.1]. Interestingly, when these factors were further examined on the basis of sex, significant differences were noted including age and blood pressure. EPC cell lines were successfully obtained from a similar proportion of males and females (see Table 3.3), yet female participants were significantly older than the males. Systolic blood pressure was similar for both sexes, but females had significantly lower diastolic blood pressure [Table 4.2]

Clinical or demographic feature		Females	Males	P value
Age (mean ± sem)		**66.0 ± 3.0**	**58.0 ± 2.6**	**<0.05**
BMI		26.0 ± 1.0	27.0 ± 0.8	0.39
Calcium (mean ± sem)	Raw Score	159.7 ± 51.6	191.9 ± 77.0	0.75
	Percentile (%)	48.4 ± 8.2	33.0 ± 7.3	0.17
Heart Rate (bpm)		65.5 ± 4.2	67.3 ± 2.3	0.70
Blood Pressure (mmHg)	Systolic	133.5 ± 5.1	132.6 ± 3.5	0.88
	Diastolic	**70.32 ± 2.3**	**78.4 ± 2.1**	**<0.05**
Alcohol Consumption		8.0 ± 1.3	8.9 ± 1.5	0.64
Smoking Pack Years		12.1 ± 3.7	14.9 ± 3.6	0.63

Table 4. 1: Clinical and demographics on the basis of sex

		Clinical demographics and medication		Patient EPC Used (%)
		Total Patients (n)		51
Characteristics		Age average (Years)		61.5
		Sex	Female	43.1
			Males	56.9
Risk Factors	Cardiac	Diabetes		3.9
		Hypertension		58.8
		Hypercholesterolemia		58.8
		Smoking	Current	2
			Ex-smoker	41.2
		Angina		5.9
		MI		5.9
		Stent		5.9
		CABG		0
	Other	Arthritis		39.2
		Osteoporosis		7.8
		Stroke/TIA		7.8
		PAD		0
		DVT/PE/Thrombus		0
		Kidney Disease		0
Medication		Aspirin		21.6
		Clopidogrel		2
		Ticagrelor		0
		Prasugrel		0
		Beta-Blocker		9.8
		Calcium Channel Blocker		21.6
		Statin		37.3
		ACE/ARB		49
		Diuretic		15.7
		Warfarin		3.9
		NOAC		9.8
		Other Anti-Arrythmics		5.9
		Proton Pump Inhibitors		13.7
Coronary Score		Calcium (Median)	Raw Score	16.5 [0-188.8]
			Percentile	35.5 [0-80]
			Gensini	2.5 [0-8]

Table 4. 2: Clinical and demographic factors of EPCs used in experiments throughout this thesis.

When separating the cohort of patients from which EPCs were used, the clinical and demographic data were largely similar aside from a difference in proportion of EPCs obtained from older-age patients [Table 4.3]. There were significantly more EPC samples available from female participants that are older than 70 and this further supports the age difference observed in the EPCs in Table 4.1. However, there is a trend towards more EPC samples available from females with hypertension, where more than 70% of EPC colonies from females were from hypertensive patients compared with less than 50% in males. Also, approximately twice as many samples from females were from patients taking statins compared with males.

Clinical or demographic		Female (%)	Male (%)	P value
CAD		15/22 (68.2)	14/27 (51.9)	0.25
Age >70		**12/22 (54.5)**	**7/29 (24.1)**	**<0.05***
Diabetes		0/22 (0)	2/29 (6.9)	0.19
Smoking	Current	0/22 (0)	1/29 (3.4)	0.53
	Ex-smoker	8/22 (36.4)	13/29 (44.8)	
	Non-smoker	14/22 (63.6)	15/29 (51.7)	
Hyperlipidaemia		16/22 (72.7)	14/29 (48.3)	0.15
Hypertension		**17/22 (77.3)**	**13/29 (44.8)**	**0.05**
Aspirin		5/22 (22.7)	6/29 (20.7)	0.86
Beta-blocker		2/22 (9.1)	3/29 (10.3)	0.88
Calcium Channel blocker		4/22 (18.2)	7/29 (24.1)	0.61
Statin		11/22 (50)	8/29 (27.6)	0.10
ACE/ARB		13/22 (59.1)	12/29 (41.4)	0.21
Diuretic		3/22 (13.6)	5/29 (17.2)	0.73
Warfarin		0/22 (0)	2/29 (6.9)	0.21
PPI		3/22 (13.6)	4/29 (13.8)	0.99

*Table 4. 3: Clinical and demographic factors assessed on the basis of sex, where *p<0.05*

4.3.2 Migration capabilities of EPCs in healthy and diseased patients

EPCs from a sub-set of these patients were used in angiogenesis assays. The samples were separated into four groups, EPC cell lines from males and females and from patients categorised to have CAD on the basis of calcium score (Agaston score >0) or no CAD (Agaston score = 0). Migration of EPCs, assessed using a scratch assay over 32 hours was not significantly different between samples from the healthy or CAD patients. However, there was a slight trend for a faster scratch closure in those with CAD (p=0.09) [Figure 4.1a]. Females had very similar migration rates regardless of CAD status (p=0.75). Interestingly however, samples from males with CAD had a marked increase in migration compared to samples from healthy males (p<0.05) [Figure 4.1b]. The increase in CAD males suggests increased capacity for angiogenesis by circulating EPCs under diseased conditions. This is in contrast with the lack of effect of CAD on spontaneous EPC growth in either males or females seen in Chapter 3.
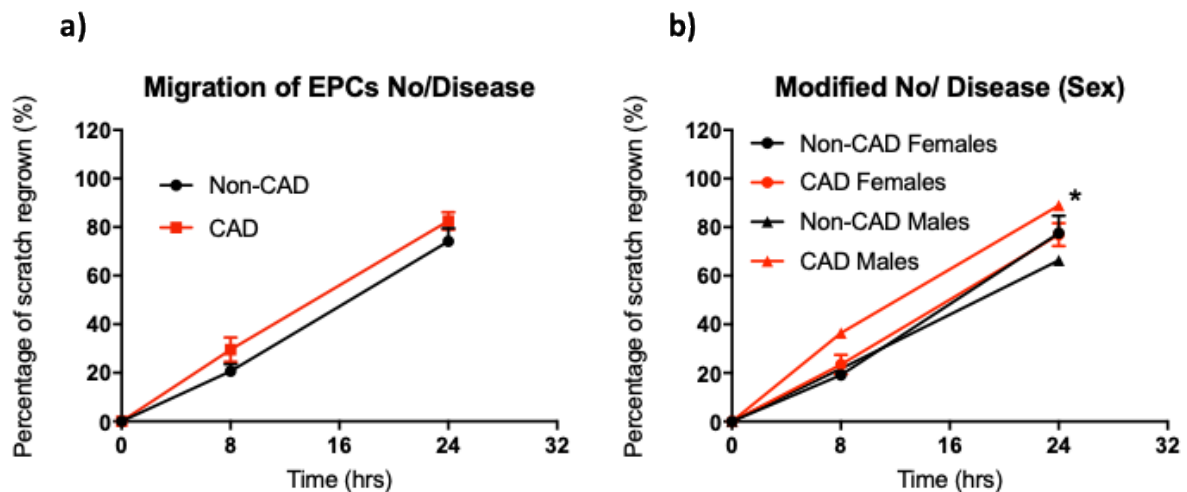
Figure 4. 1: Migration assay between sexes. Cells seeded onto a 96-well plate and a denuded zone created, images were obtained over 48 hours. a) Migration between Non-CAD (n=8) vs. CAD (n=17), b) Non-CAD (n=3) vs. CVD (n=9) migration in females, and Non-CAD (n=5) vs. CAD (n=9) migration in males. All data are shown as SEM, statistical tests using two-way ANOVA where *p<0.05.

4.3.3 Tubule formation capabilities in EPCs

EPCs can be vital in the formation of new blood vessels and they can contribute to neovascularisation (Takayuki Asahara, Masuda et al., 1999). Capacity for tubule formation can be assessed by plating cells on extracellular matrix-like substance and measuring the formation of endothelial cell tubules. This was compared between samples from healthy and CAD patients. There was a trend towards a higher branch number in the CAD patients (Non-CAD 1.64 ± 0.24 vs. CAD 2.42 ± 0.33; p=0.07) [Figure 4.2].
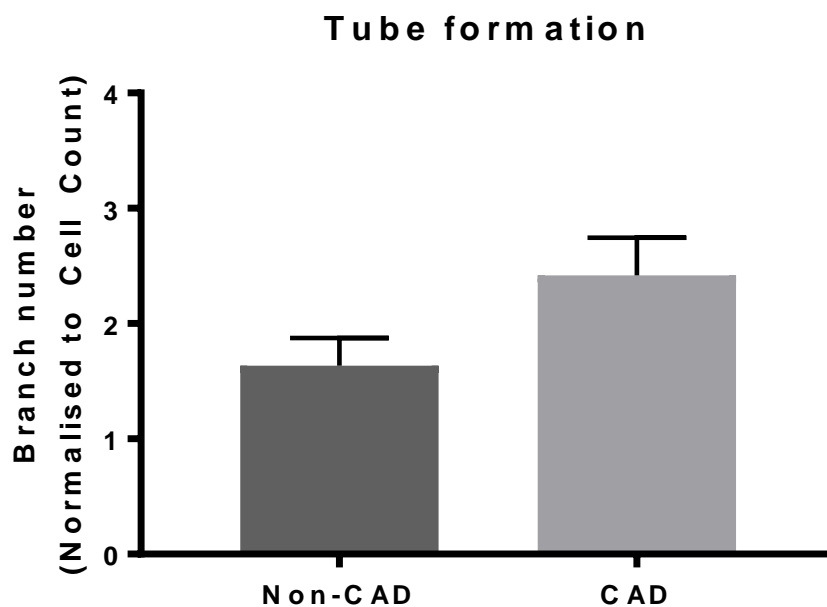
Figure 4. 2: Tube formation between healthy and diseased. 15x10$_3$ cells seeded onto Matrigel and images were taken hourly for 16 hours. a) A trend is present in tubule formation between Non-CAD (n=15) and CAD (n=16; p=0.07).

When comparisons were made between sexes, it became evident that the EPCs from females with CAD have significantly greater number of branches in comparison to the healthy patients (p<0.05). This was not evident in the males (p=0.1) [Figure 4.3]. Contrasting the migration of EPCs, this suggests that females with CAD have a greater capacity to grow new vessels in comparison to healthy females and males.
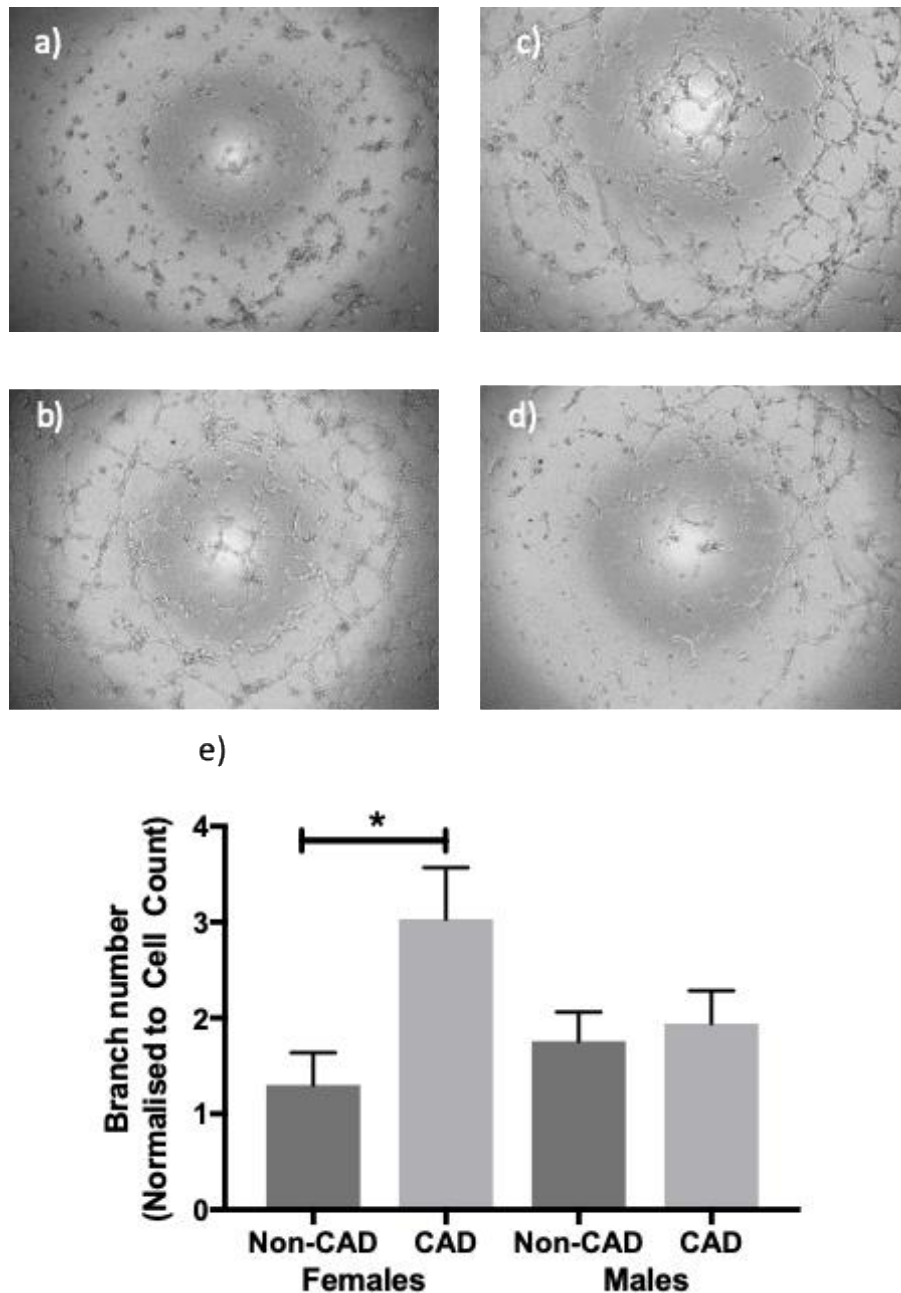
*Figure 4. 3: Tube formation further broken down by sex. a) Female Non-CAD, b) Female CAD, c) Males Non-CAD and d) Male CAD, e) Comparison of normalised tubule formation Non-CAD (n=4) vs. CAD (n=7) females, and Non-CAD (n=11) vs. CAD (n=9) in males. *p=0.05 by ANOVA, all data shown as SEM.*

4.3.4 Expression of angiogenesis signalling intermediates

4.3.4.1 *AKT and pAKT expression in EPCs*

With opposing differences in migration and tube formation between males and female EPC's from patients with CAD, I further investigated the expression profiles of signalling molecules that promote angiogenesis in EPCs. AKT1 and phosphorylated AKT1 were probed in the EPCs. There was no significant difference in the expression of AKT1 between healthy and diseased patients (Non-CAD 1 ± 0.44, vs. CAD 0.88 ± 0.26; p=0.8) [Figure 4.4a]. When assessed based on sex, there was also no significant difference (p=0.77) [Figure 4.4b].

pAKT1 is the active component of AKT1 which indicates the activation of the PI3K pathway and the phosphorylation at the site Ser473. Further exploration into the ratio of pAKT1 to AKT1 also found no significance (Non-CVD 1 ± 0.23 vs. CVD 1.15 ± 0.48; p=0.9) [Figure 4.4d] and when assessed based on sex, there was also no difference either (p=0.72) [Figure 4.4e]. AKT1 signalling is not apparently responsible for angiogenic differences seen in the migration and tubule formation assays, however the other isoforms; AKT2/3 may have a role and will need further investigation.
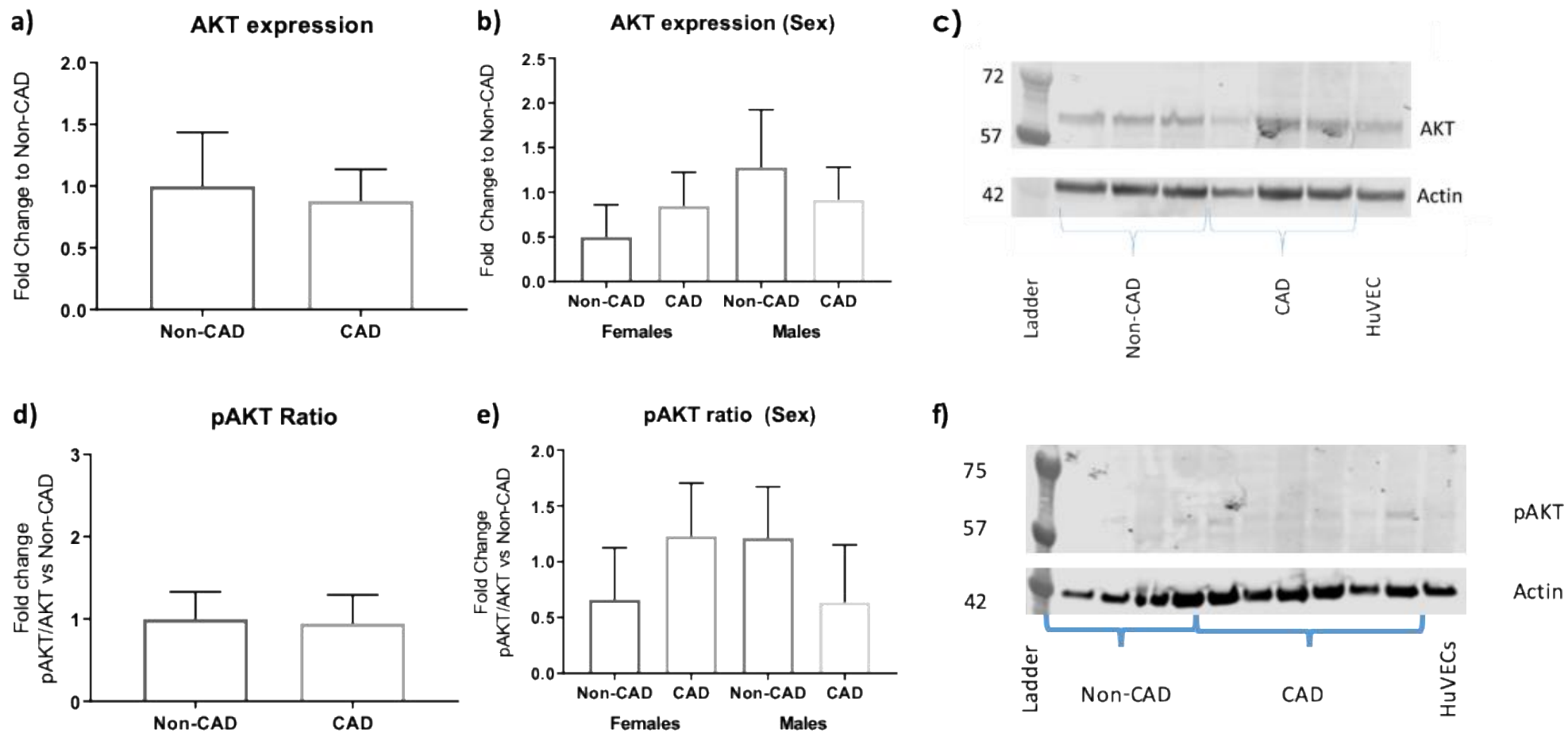
Figure 4. 4: AKT and pAKT expression between healthy and diseased and by sex. Protein is extracted from EPCs and immunoblotting was performed a) AKT expression between Non-CAD and CAD (n=14-19; p=0.8), b) AKT expression divided by sex; females Non-CAD (n=5), CAD (n=10); both males groups (n=9). c) Representative AKT immunoblotting. Ratio of pAKT/AKT to determine the active AKT d) pAKT in Non-CVD vs. CVD (n=13-19; p=0.9) e) pAKT divided into sex; females Non-CVD (n=5), CVD (n=9); Males Non-CVD (n=10), CVD (n=9; p=0.72) f) Representative immunoblotting for pAKT. All data shown as SEM, statistics test were student t-test and two-way ANOVA.

4.3.4.2 eNOS

eNOS is an important enzyme responsible for the generation of NO to maintain vascular homeostasis. Upstream signalling like AKT can affect eNOS expression and the angiogenic capabilities of EPCs. However, AKT expression did not show any significance, there may be up-regulation downstream driving angiogenesis. AKT Kinase which is activated upstream by PI3K, phosphorylates eNOS which then produces NO resulting in vasodilation (Huang, 2009). EPCs from CAD and non-CAD patients showed no difference in eNOS expression [Figure 4.5].
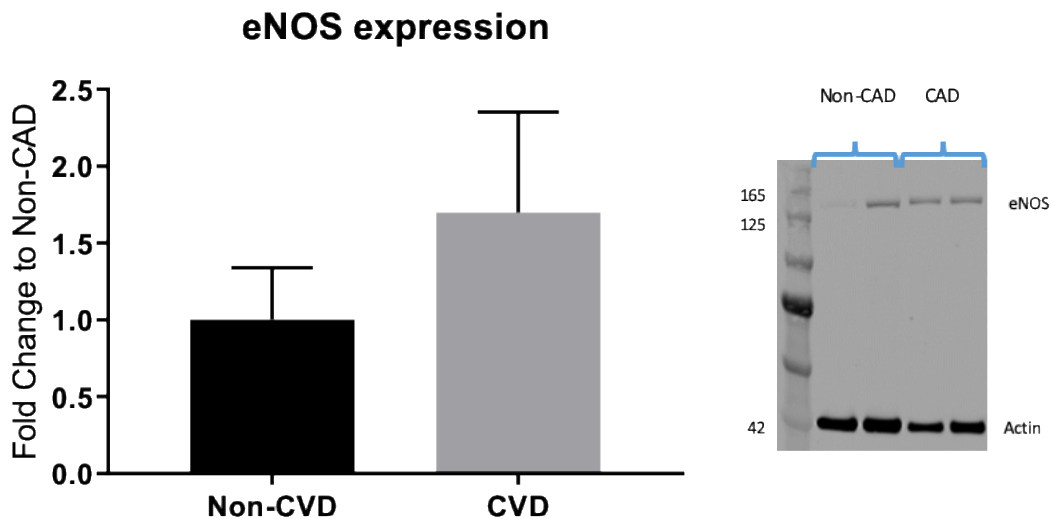
Figure 4. 5 Protein is extracted from EPCs that spontaneously appeared and fluorescent immunoblotting was performed. eNOS protein expression between EPCs from participants who had Non-CAD (n=16) and CAD (n=13).

EPCs from CAD women showed an ~3-fold increase in eNOS expression compared Non-CAD (p=0.04). eNOS expression compared between CAD women and CAD men was also significantly elevated (p=0.02) [Figure 4.6]. eNOS appears to be elevated without an associated increased in AKT signalling.
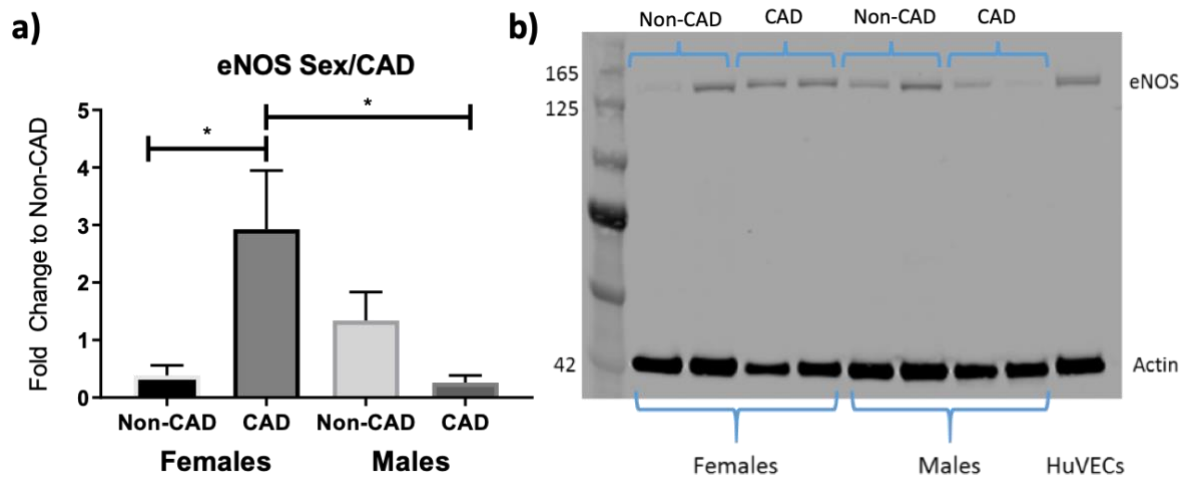
Figure 4. 6: eNOS expression divided based on sex. a) Sex is separated by Non-CAD (n=7) vs. CAD (n=7) in females and Non-CAD (n=9) vs. CAD (n=6), b) representative blot of eNOS expression. Statistical tests were performed using student t-test and two-way ANOVA where *p<0.05.

Given the parallel increase in eNOS expression with the angiogenic assay shown in Figure 4.3, we explored the relationship of eNOS expression with this

tube formation in a scatterplot [Figure 4.7]. There is a trending association between eNOS expression and tube formation ($R^2 < 0.05$; $p = 0.44$).
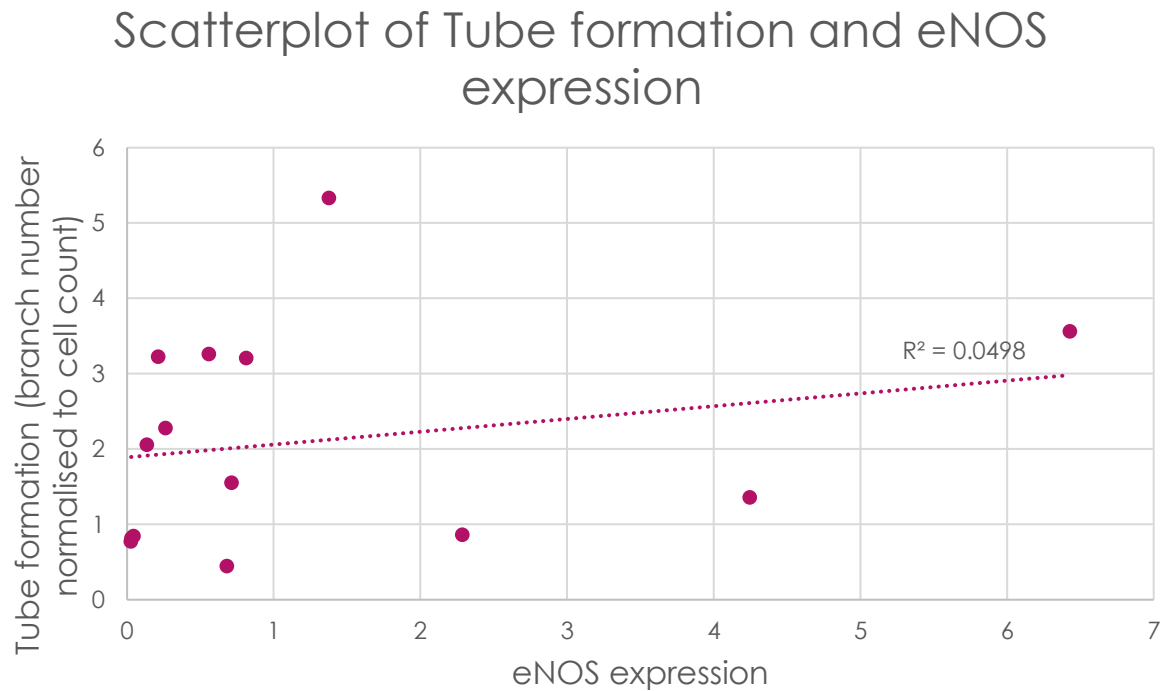
Figure 4. 7: Association between eNOS expression and tube formation in EPCs derived from patients in BioHEART (n=14, $R^2 < 0.05$; $p=0.44$). Statistical test performed using a linear regression.

4.3.4.3 ERK and pERK expression

ERK is an alternative pathway to which cell growth and proliferation can occur, as AKT was not up-regulated in the EPCs, I investigated whether the differences observed in angiogenesis was a result of the ERK pathway. ERK expression between healthy and diseased patients were unchanged (Non-CVD 1 ± 0.13 vs. CVD 1.31 ± 0.26; p=0.4) [Figure 4.8a] and remained that way when observed based on sex (p=0.38) [Figure 4.8b].

With no changes in ERK expression, I further examined the active form of ERK to determine its role in the observed angiogenic differences. Phosphorylated ERK expression was not significantly different between the Non-CAD and CAD patient-derived EPC's (Non-CAD 1 ± 0.33 vs. CAD 0.95 ± 0.35; p=0.92) [Figure 4.10a]. When the ratio is further examined by sex (p=0.73) [Figure 4.8b], there was no significant difference between any of the groups. Significant change in ERK expression between sexes was not observed, therefore, ERK pathways may not be responsible for differences observed in angiogenesis.
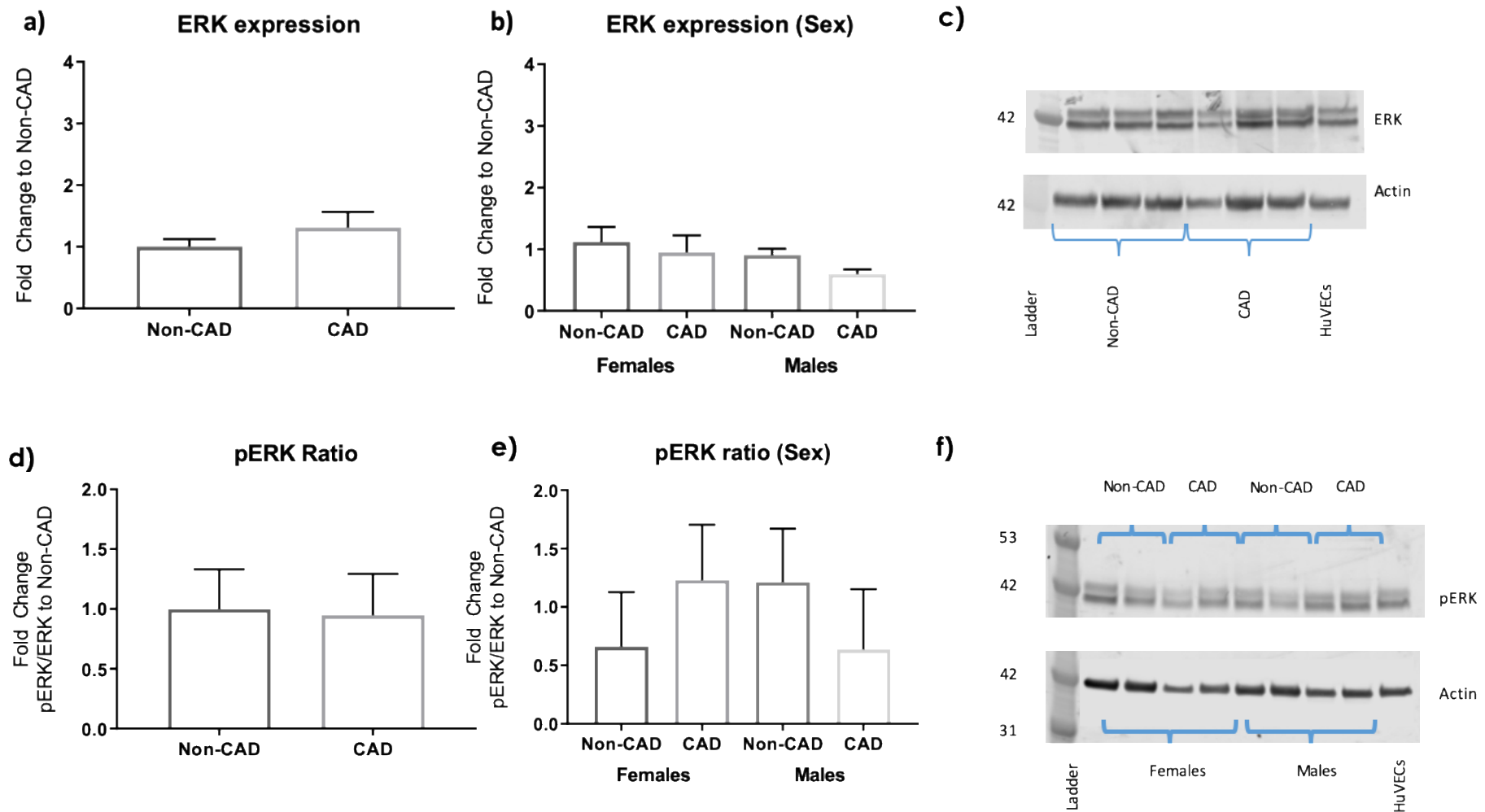
Figure 4. 8: ERK and pERK expression in EPCs. Protein is extracted from EPCs and fluorescent immunoblotting was performed a) ERK expression in Non-CAD (n=13) vs. CAD (n=19) b) ERK expression divided into female Non-CVD (n=6), CVD (n=10); males Non-CVD (n=7), CVD (n=9) c) Representative immunoblotting of ERK in EPCs. pERK ratios d) pERK expression between Non-CAD (n=13) vs. CAD (n=19), e) pERK expression divided into female Non-CVD

(n=5), CVD (n=10); Males Non-CVD (n=8), CVD (n=9), f) Representative immunoblotting. All data is shown as SEM, statistical tests were completed using student t-test and two-way ANOVA.

4.4 DISCUSSION

In this chapter, the main finding was that EPCs are more likely to be pro-angiogenic when derived from patients with CAD, but there was a complex relationship to the sex of the patients and the mechanisms of angiogenesis. Males with CAD have a significantly increased ability to migrate compared with their healthy male counterparts with no CAD, while EPCs from females with CAD have an increased ability to form tubules compared with their healthy counterparts. Further exploration into the common angiogenic signalling pathways was performed but I was unable to elucidate the mechanisms behind these differences by looking at the key known angiogenic signalling pathways. However, it was observed that eNOS is significantly up-regulated in CAD females, and that eNOS expression is associated with tubule formation assay, suggesting a potential mediation role. The effect of this increased tube formation on cardiac outcomes in ischaemic conditions in vivo (e.g post-MI or in peripheral vascular disease) is also unknown, and further research is required.

EPC migration is a vital part of re-endothelialisation, tissue repair and neovascularisation in ischemia-reperfusion (Favero, Paganelli et al., 2014). A cell migration model using HuVECs showed that senescent cells had impaired wound healing, which suggests that an impaired endothelium resulted in a reduction of cell migration (Alique, Bodega et al., 2019). While our results showed a trend in increased migration towards EPCs from patients with disease, sex segregation revealed that males had a significantly faster migration capacity compared to females who displayed no difference in migration between healthy and disease cohorts. It is interesting that males have an increased migration rate, when it has been previously shown that CAD

impairs the migration of endothelial progenitor cells and reduces circulating EPCs, however, the results of the migration were not further divided into sex, and all the CAD patients had not been previously treated with statins (Vasa, Fichtlscherer et al., 2001). This may explain why males have better outcomes post-MI in comparison to females.

It has been recently shown in diabetic models that there is reduced tube formation (Bitar, 2019) which also significantly reduced eNOS expression. In CAD we expect a reduced expression of eNOS due to increased inflammation. Isoprostanes are deemed a marker of oxidative stress (Montuschi, Barnes et al., 2004) and have been shown to inhibit tubule formation (Benndorf Ralf, Schwedhelm et al., 2008), which suggest that when oxidative stress is present, there is a reduced ability to form tubules. However, EPCs from females had an expression of eNOS and an associated increased ability to form tubules. In an ex-vivo co-culture model, it has been previously shown that eNOS overexpression, and an associated increase in NO production results in a significant increase in capacity for tube formation (Babaei & Stewart, 2002). Females have been shown to demonstratea higher basal eNOS availability than males, affecting migration and proliferation of the ECs, where female angiogenesis is dependent on eNOS expression, while males are independent (Cattaneo, Vanetti et al., 2017). The increased tube formation observed in females may have been driven by the inherent increased basal eNOS, however, inherent increase in eNOS and tube formation appears to be independent of the outcomes. The significant increase in tubule formation in females with CAD is surprising considering their often-poorer outcomes post-MI (Graham, 2016; Khan, Brieger et al., 2018; Shih, Chen et al., 2019).

The PI3K/AKT pathways play a key role in proliferation, adhesion, migration, and angiogenesis. It is thought that angiogenesis is affected by the AKT pathway through its regulation of NO (Karar & Maity, 2011) as eNOS has an

important role in vascularisation. This is demonstrated by the decreased vascularisation in eNOS knockout mice in comparison to the WT (Fukumura, Gohongi et al., 2001). It was interesting to see that AKT1 expression was not significantly up-regulated to match the significantly higher expression of eNOS, however, this may be explained by a higher endogenous eNOS expression in females in comparison to males (Cattaneo et al., 2017). It may also been other isoforms of AKT regulating eNOS. Premenopausal women had significantly higher expression of pAKT1 in comparison to men and postmenopausal women suggesting it may have an impact on cardiovascular outcomes (Sugden Peter & Clerk, 2001). An ex vivo model in EPCs showed a significant reduction in EPC proliferation when the AKT1 signalling pathway was suppressed (W. Li, Wang et al., 2012). It may also be the reason that we see significantly increased tubule formation in females with disease.

ERK is an important part of endothelial cell proliferation and migration. In an ERK1/2 null embryonic mouse model, it was shown that endothelial cells, had reduced proliferation and migration (Srinivasan, Zabuawala et al., 2009). Further, in a pre-clinical model, ERK1/2 was up-regulated in females wildtype mice in comparison to males (Mahmoodzadeh, Fliegner et al., 2013). In EPCS, the inhibition of ERK1/2 resulted in decreased proliferation, migration and angiogenesis (Song, Yu et al., 2009). In our EPCs, there was a significantly increased rate of closure in the scratch assay in the males, however, ERK was not up-regulated to match this increased migration. eNOS and AKT also do not explain the increased migration in the EPCs derived from CAD males. Further exploration into other factors driving migration, such as angiopoietin-2 (Gill & Brindle, 2005) may explain the increased angiogenesis observed in the males with CAD.

It is not surprising that there are sex differences in the angiogenic capabilities of the patient-derived EPCs, but it leads to the question of what drives these

changes. While the driver of angiogenesis in females was explained by up-regulated eNOS which has been previously reported in the literature (Cattaneo et al., 2017) and the increased migration explained through hormonal levels in males (Foresta, Zuccarello et al., 2008). This reinforces the idea that males and females have very different pathology and is also demonstrated by their different angiogenic functions, which may be driven by angiogenic signalling pathways. In this thesis, the main pathways investigated were MAPK and PI3K which did not appear to be the driver behind the sex differences. However, many other signalling pathways may have contributed to these differences (Bicknell & Harris, 2004), such as sprouted-related protein 1, VEGFR-2 and focal adhesion kinase. This reinforces the concept that males and females have different cardiovascular responses to traditional factors and disease development as well as angiogenic reaction.

A limitation of this study was the wide-range of variation in clinical and demographic features of the BioHEART patients. As only 51 cell lines were available for experiments, it was difficult to limit the degree of CAD or to determine contribution of certain risk factors. Nevertheless, assessing the angiogenesis capacity in this number of cell lines was an enormous undertaking and has given valuable insight into the basal capacity for angiogenesis in this type of cells. Only around a third of the EPC cell lines originally appearing were suitable for functional and immunoblotting experiments due largely to their viability after passaging. A greater proportion of EPC cell lines were viable from samples obtained from women aged over 70 years. This may be due to the fact that females normally present with CAD at an older age (Hemal, Pagidipati et al., 2016).

In conclusion, I found that EPCs derived from patients with CAD demonstrate enhanced angiogenic capabilities, but this occurred in a complex, sexually dimorphic way. EPCs from males with CAD showed faster migration, but

females had greater tubule formation. This is suggestive of a sex-dependent vessel repair role in patient-derived EPCs.

5 GENERAL DISCUSSION AND CONCLUDING REMARKS

With an increasing proportion of patients presenting without traditional risk factors (Vernon, 2017), the need to find new biomarkers and treatments are required. Such markers would have board relevance to the broader population at risk of CAD. In this thesis I aimed to explore whether markers and function of circulating EPCs from individual patients reflect their CAD status and the potential opportunity of using these EPC as an ex vivo model to unravel new mechanisms of disease susceptibility. The key findings were that EPCs displayed similar endothelial-phenotypes to widely used vascular cells and exhibited some aspects of a disease phenotype consistent with patient CAD profile. The large cohort allowed us to identify clinical and demographic predictors of EPCs growth as well as different angiogenic properties of the EPC in CAD patients which was dependent on sex.

Endothelial cells are a vital part of the cardiovascular system, due to their barrier and signalling roles including controlling vascular tone, leukocyte adhesion and inflammation (Favero et al., 2014). When homeostasis of the endothelium is disrupted, there is a predisposition to vasoconstriction, pro-oxidation, and vascular inflammation leading to the development of atherosclerosis (Verma & Anderson Todd, 2002). With endothelial cell dysfunction initiating the development of atherosclerosis, there is increased interest in using them as biomarkers for disease (Verma, Buchanan Michael et al., 2003). Endothelial cells, however, are not easily obtained from patients and thus EPCs, cultured from peripheral blood, are a potential proxy in studying disease development.

It has been previously reported that patients with CAD have a lower number of circulating EPCs (Sen, McDonald et al., 2011). EPCs have been identified as

a potential diagnostic tool in cardiovascular function and regenerative medicine (Sen et al., 2011). This is supported by our patient-derived EPCs showing disease characteristics, consisting of increased expression of Nox-2 in patients with CAD. NADPH isoforms, such as Nox-2 have been identified as the main producer of O_2-• contributing to an increase in oxidative stress in patients with CAD (Guzik, Sadowski et al., 2006). It has also been previously shown Nox-2 expression was up-regulated in EPCs from patients with hyperlipidaemia which was also positively correlated with an increase in ROS production (Li, Zhang et al., 2018). In these studies, Nox-2 expression was increased in EPCs from diseased patients indicating their potential as a biomarker. This shows that the EPCs exhibit oxidative stress signalling, which can be used to further uncover mechanisms driving CAD. High mitochondria superoxide generation was also identified in EPCs from patients with CAD, which suggest that the mitochondria is driving oxidative stress in CAD, which may allow for EPCs to be used as a model for drug testing and to uncover underlying mechanisms of CAD. Further investigation into these findings, may include possible mitochondrial-specific drug therapies (i.e MitoTEMPO (Bubb, Drummond et al., 2019)) to reduce superoxide generation and therefore oxidative stress in patients with CAD.

Traditional factors have long been used as predictors of cardiovascular disease and as the basis of many CVD risk scores. The Framingham risk score (Dahlöf, 2010), was developed through following large generational cohorts to determine what risk factors could predict cardiovascular outcomes. With age, 90% of the population will gain at least one traditional risk factor; hyperlipidaemia, hypertension, diabetes or smoking. These factors are all known to contribute significantly to the development of atherosclerosis which is a substantial financial burden to health institutions and the patient. The development of the risk score allowed for high-risk individuals to be monitored more closely identified early for medical therapy and the encouragement of lifestyle changes as a preventative measure (Berry, Lloyd-Jones et al., 2007).

These factors, however, have never been applied to EPC's spontaneous growth. This is driven primarily by research design, with many researchers looking at the effect one condition may have on functional aspects of EPCs. The method of which EPCs are sought is often through selective targeting of surface markers, which differs from our approach of spontaneous appearance. In this thesis, BMI, diabetes, and medications like beta-blockers and statins were all identified as factors that affect the growth of EPCs.

Diabetes is associated with a 2 to 4-fold increase in CAD mortality from heart disease and is identified as a traditional risk factor of CVD. Patients with diabetes in the BioHEART cohort had a significantly reduced ability to grow spontaneous EPCs, which has been identified for the first time in this study. The implications of this have a noteworthy impact on diabetic complications such as peripheral vascular disease. There is an increased interest in the use of stem cells such as EPCs, to be used in the treatment of diabetic complications due to their ability to increase endogenous NO and reduce inflammation produced by ROS (Bernardi, Severini et al., 2012). BMI was another factor that had an interesting effect on the spontaneous growth of EPCs. There was a negative association between increasing BMI and EPC spontaneous appearance. This contrasts with the previously published finding that patients with a BMI >30 kg/m_2, have a five-fold increase in circulating EPCs compared with healthy weight individuals (Bellows, Zhang et al., 2011). Our observation regarding impaired spontaneous growth is consistent with observation of Heida and colleagues regarding functional impairment of EPC's from obese individuals (Heida, Müller et al., 2010a). It is important to note that while there have been many investigations into circulating endothelial cells, but not spontaneous appearance of EPCs from these cohorts. This making this thesis novel in its investigation into spontaneous growth of EPCs in these cohorts.

When patients have a multitude of comorbidities and medications to help with managing a variety of symptoms and diseases, there is a potential for drug interactions. In our BioHEART cohort, both statins and beta-blockers affected the growth of EPCs. From my analysis, I discovered that beta-blockers significantly reduced the spontaneous appearance, however, do not affect angiogenic capacity (data not shown). It has been previously shown that beta-blockers can improve EPC numbers and function in hypertensive rats (Yao, Fukuda et al., 2008) however, this is not a direct comparison to the spontaneous growth of EPCs. Beta-blockers are widely used for a variety of conditions such as hypertension, arrhythmias and heart failure, and have been proven to improve outcomes in patients (Lazarus, Jackevicius et al., 2011). With the well-accepted clinical benefits of beta-blockers demonstrated in large clinical trials, a reduction in the spontaneous appearance of EPCs appears to be potentially contradictory. One potential reason for this discrepancy is drug adherence and recall in other patients may not be reliable and thus our data may not be an accurate representation. Our demonstration of impaired EPC spontaneous growth in patients on beta-blocker therapy is worth of clinical study in specific subgroups of patients with cardiovascular disease. For example, in situations where collateral formation may be key to a patient outcome such as a critically ischaemic diabetic foot, or in a patient with a chronic total occlusion of a major coronary artery that is unsuitable for a surgical or percutaneous intervention, a trial of beta-blocker withdrawal may be warranted.

Statins are another medication that has been identified as a significant promoter of EPC spontaneous growth from my analysis. Almost a third of BioHEART participants use statins which are used to lower cholesterol and reduce cardiovascular mortality. It has been shown that statins can improve endothelial function (Fichtlscherer, Schmidt-Lucke et al., 2006), however, there are some side effects to the medication such as myopathy, rhabdomyolysis, and polyneuropathy (Moosmann & Behl, 2004). It has also been shown

previously that statins can increase EPC differentiation (Dimmeler, Aicher et al., 2001). Therefore, statins could be considered as an appropriate medication to prescribe patients who would benefit from pro-angiogenesis therapy, even if they do not have hyperlipidaemia.

The use of EPCs as a potential novel biomarker for cardiovascular disease is advantageous, however, they can also be used to understand the mechanisms behind the disease. It has been shown previously that EPCs from patients with peripheral artery disease have a reduced ability to form colonies and adhere (Fadini Gian, Sartore et al., 2006). However, the angiogenic capacity of patient-derived EPCs has not often been explored and was found in this thesis that males and females with CAD displayed different angiogenic capabilities. EPCs from males were able to migrate at a faster rate, while EPCs from females were able to form more tubules. This could mean that male EPCs are better are homing into areas in need of microvasculature, while female EPCs are better in forming new blood vessels. Males and females have displayed different pathologies in disease with women exhibiting a higher number of non-obstructive disease than men (Herscovici, Sedlak et al., 2018). They also have varying cardiovascular outcomes post-MI (Okunrintemi, Valero-Elizondo et al., 2018), so it was important to explore whether EPC capacities would differ. As there were functional differences in the EPCs between sexes, it may partly explain the differences between the sexes in cardiovascular outcomes. Differences in the angiogenesis between sexes further support the differences in disease pathologies between males and females, which hopefully can lead to tailored strategies in prevention and treatment between the sexes.

5.1 LIMITATIONS

Upon culturing patient-derived PBMCs, approximately 20% of samples resulted in spontaneous appearance of EPCs. This is still quite a low cell yield in comparison to other methods of stem cell production such as iPSCs. However, iPSCs do not display epigenetic modifications that are preserved with the spontaneous appearance of EPCs (Omole & Fakoya, 2018). Epigenetic modifications are reversible modifications that affect gene expression and with the various stages of iPSC programming, these individual modifications between patients are likely to be lost. Therefore, while iPSCs may be useful therapeutically, from a biomarker perspective spontaneous EPCs are superior. However, to further optimise EPC isolation, a viable option to ensure a high yield of patient-derived EPCs would be cell sorting the PBMCs via the surface marker CD34, as it still maintains epigenetic modifications without the need for cell reprogramming. Another method be to further investigate the use of growth factors and cytokines such as VEGF, which has been previously shown to improve the angiogenic capacity of EPCs (Peplow, 2014).

A further limitation of culturing EPCs is the need the culture the PBMCs within a few hours. During the development of my protocols, I also attempted culture of PBMCs from patients undergoing MI with very limited success. This appeared to be due to the time between the blood sample being taken and isolation of PBMCs commencing. This was often delayed by MI patient samples during the night due to in an inability to consent and begin culture until the following morning. To try and overcome delays, I also attempted to culture EPCs from PBMCs that had been immediately frozen down. This method would have major advantages in multi-centre studies like BioHEART where the resources at each site may not be in place to culture EPCs. However, success was limited. Improving the protocol to enhance growth of EPC from frozen PBMCs would broaden the potential application of patient-derived EPC for the study of CAD susceptibility.

Comprehensive data were collected from patients as they were recruited, and this was vital in identifying predictors of growth. Patients often have multiple risk factors. Whilst our study has the advantage of large numbers compared with many previous studies of EPCs and associated clinical factors, the study is limited in its ability to identify the specific role of individual factors. Although long term follow-up will be undertaken in the BioHEART patients, the time frame of my thesis did not allow for me to examine the potential association of EPC markers and signalling with cardiovascular events and outcomes. This will be possible in the future and will be undertaken by colleagues in our laboratory.

It was demonstrated that EPCs displayed some aspects of a disease phenotype and functional sex differences. However, there are still limitations to their use in a wider population. Risk factor profiles uncovered in this study have only been tested in a population of patients who were specifically referred for a CTCA, therefore, further validation in a wider cohort inclusive of healthy people is vital to ensure that these profiles can be used in the general population. While only approximately 20% of patient-derived PBMCs developed into spontaneous EPC cell lines, they displayed characteristics of a disease phenotype which allows them to be used as a proxy to further uncover mechanisms driving CVD. The effect of various risk factors also allowed patient-derived EPCs to be a potential biomarker of disease, allowing for early management of disease.

5.2 FUTURE DIRECTIONS

BioHEART is aiming for total recruitment of 5000 patients, which would mean access to approximately 1000 cultured EPCs. This allows for a much more thorough risk factor assessment. Further exploration into potential signalling pathways contributing to the different angiogenic capacities between the

sexes would be particularly interesting; especially considering the impact it may potentially have on cardiovascular outcomes. Colleagues in the BioHEART team are working on performing board genomic, metabolomic, proteomic and transcriptomic studies as well as comprehensive immunophenotyping on peripheral blood from the cohort. This data, along with the data from my EPC characterisation, will be assembled in an open biorepository for the benefit of a board range of researchers from across the globe. The EPCs will also be stored in liquid nitrogen in the NSW state-wide Biobank for potential access of researchers. The cells may be useful for further mechanistic studies as well as testing of new therapeutic options.

In summary, this body of work has thoroughly investigated the potential for EPCs to be used as patient-derived model of vascular endothelial cell function and mechanisms of disease susceptibility. The culturing process of the EPCs was moderately labour-intensive process with a relatively low yield rate. However, we have demonstrated the feasibility and potential advantages of using them as a patient-derived model for the study of endothelial signalling in patients with unexplained CAD. Whilst some clinical factors influenced the probability of spontaneous growth of EPC, ample numbers of EPC cultures were obtained to reflect a broad range of patients and disease states. Some molecular and signalling differences in EPC from patients with CAD versus healthy disease-free control subjects was demonstrated, further investigations into the mechanisms not explained in this thesis will be valuable to current literature. However, the bank of EPCs will be an important component for future studies that also incorporate other molecular phenotyping.

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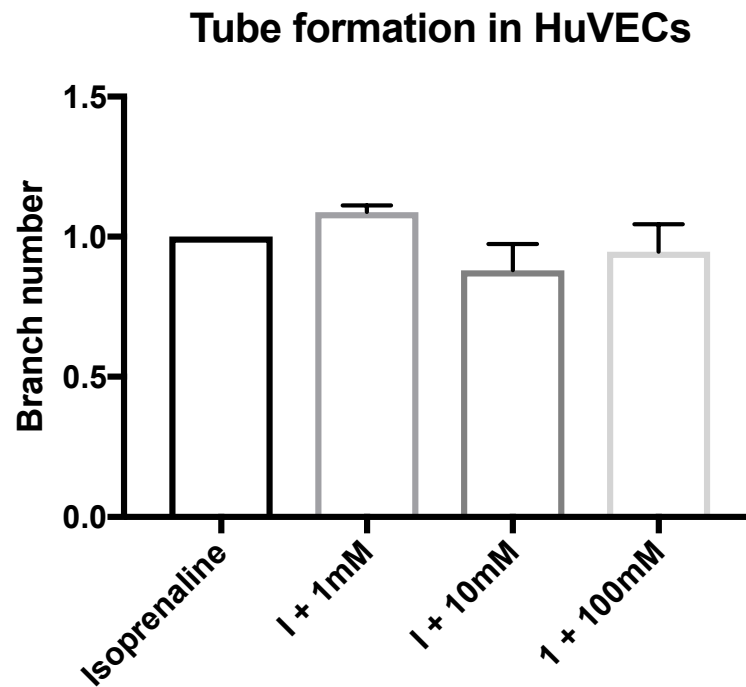
Figure 7. 1: Incremental metoprolol treatment of HuVECs (n=4) while performing a tubule formation assay.