University of Salford MANCHESTER

Master's by Research Thesis

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Characterisation of cardiac cellular and vascular function in Ischemic Heart Disease.

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Abstract

Ischemic heart disease (IHD) is the most common cause of death across the globe, of which global mortality rates are projected to continue rising into the future. Therapies used to treat the most chronic cases of IHD are focused on the revascularisation of the ischemic region and the subsequent restoration of blood flow. As such, the most prevalent therapeutic option is the coronary artery bypass graft (CABG). In these procedures, the internal mammary artery (IMA) is deemed to be the "gold standard" vascular model for use in grafting, offering a better patency (over 90%) than the next most frequently used model, the saphenous vein (SV). Prior to bypass grafting, endogenous adipose deposits (perivascular adipose tissue or PVAT) surrounding the arteries are removed. PVAT acts as an endocrine organ and has vast and complex interactions with its surrounding tissues, aiding in the maintained health, reactivity and function of its underlying vasculature. Subsequently, PVAT and its functions are directly and often negatively impacted in cardiovascular disease, sometimes driving and worsening disease states. PVAT exerts its effects via the production and secretion of adipocyte-derived cytokines (adipokines), of which there are numerous, eliciting a myriad of functions. One such mechanism of influence is the enzyme eNOS, an important protein responsible for generating nitric oxide (NO), which plays an important role in mediating healthy PVAT and vascular function, though it also becomes dysregulated in obesity.

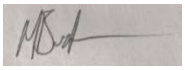
With these considerations, the aim of this study is to identify the potential for PVATs usage in revascularisation procedures, in an effort to improve successful patient outcomes post-surgery. This will be performed by investigating PVATs role on vascular contractility in the conductance vessels used in bypass grafting, and secondarily by investigating the role and importance of eNOS in bypass graft vessels. In collaboration with the Blackpool Victoria Hospital, human IMA and SV samples were provided for human tissue trials, which we supported with data from murine aortic control and eNOS knockout ($eNOS^{-/-}$) models, by assessing the contractile and distensile properties via myography based assays.

In control murine models it was observed that PVAT presence elicited a pro-contractile effect when subjected to noradrenaline (NA) dose responses (P = 0.0058, n = 10) compared to PVAT-removed controls under the same test conditions, showing PVAT's potential to effect vasoconstriction in response to a pro-contractile factor. Further investigation into the mechanisms regarding NA-induced contractility and PVAT's perceived contractile augmentation effect led to us to observe that these contractile events are not influenced by beta-3 adrenergic receptor (β3ADR) interactions (and subsequent NO release) (+PVAT models: P = 0.9064; -PVAT models: P = 0.9064, n = 8), nor are they affected by the pro-contractile adipokine chemerin (P = 0.0564, n = 9), suggesting this phenomenon is removed of both these mechanisms, though it was observed that in PVAT-removed models subjected to a chemerin blocker that contractility increased (P = 0.0137, n = 9). The same NA dose response assays were also performed on $eNOS^{-/-}$ models, in which the same pro-contractile effect of PVAT was observed (P = 0.0295, n = 10), which further suggests no NO involvement in the perceived augmentation effect. Early, proof-of-concept myography testing of human models was able to be performed and optimised, based around the same NA dose response assays as used within our murine models, from which future studies can be based and rapidly scaled from.

The results of this study gave new insights into the vascular reactivity to vasoconstrictors in murine models, allowing us to extrapolate information to human models. PVAT's perceived pro-contractile effects challenge our previous understanding regarding its functionality as a vascular endocrine organ and how it could affect vascular function and revascularisation, leading to new questions and exciting avenues of research.

Declaration

I declare that, with the exception of any statements to the contrary, the contents of this thesis are my own work, that the data presented herein has been obtained by my own experimentation and that no part of the report has been copied from previous reports/dissertations, books, manuscripts, research papers or the internet.

Signed  Print name Matthew Bushdyhan

Date 18/01/2021

Acknowledgements

I would like to begin by thanking the University of Salford, for which I have spent the last five years studying with in my undergraduate degree, placement year and this Master's by Research degree. During this time, I have had the absolute pleasure of learning and growing both academically and as a person, as well as being introduced to some of the most inspiration and motivational people I have had the amazing fortune to work with and learn from.

I would like to particularly thank my incredible supervisors Sarah Withers and David Greenwood, without whom I would never have been able to take this step in my academic life. Without the support, feedback, and excellent company I would not have been able to complete this project as I have. Thank you both for all the fantastic opportunities and experiences you have afforded me, I wouldn't be here without you.

Next, I would like to thank the University of Manchester, the BSF and particularly Anthony Heagerty and Sophie Saxton. I was welcomed into a new environment with open arms and respect and I cannot put into words how much your support helped me throughout the year, be it via meetings or idle musings and long chats in the lab. I look forward to and I am eager to continue working with you both.

A thank you next to my family, particularly my parents who have always encouraged me and supported me through all my life's endeavours, be it academic or otherwise. I stand where I am today only because of the person you made me. And to my mother I thank you for your constant inquisitiveness and enthusiasm for my work, I know so much is like a foreign language to you but your eagerness to hear what I have learned has always meant more than I can put into words.

To my wonderful friends, who have lifted my spirits through a tumultuous year and have always been at hand to give me words of encouragement when I needed them and to bounce ideas off when I have been stuck, you have all made this year infinitely easier and I can't thank you enough.

Finally, a huge thank you to Nidal Bittar and everyone in his team at NHS Blackpool Victoria, without whom this project would not have been possible.

Abbreviations

α-NETA	2-(α-naphthoyl) ethyltrimethylammonium iodide
ACE	Angiotensin converting enzyme
Ang-II	Angiotensin II
β3ADR	Beta-3 adrenergic receptor
BAT	Brown adipose tissue
CABG	Coronary artery bypass graft
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanoside monophosphate
CMKLR1	Chemokine-like receptor-1
CPB	Cardiopulmonary bypass (pump)
CVD	Cardiovascular disease
EC	Endothelial cell
ECM	Extracellular matrix
eNOS	Endothelial nitric oxide synthase
eNOS$^{-/-}$	Endothelial nitric oxide synthase knockout (B6.129P2-Nos3tm1Unc/J)
ET - 1-3	Endothelin 1-3
IHD	Ischemic heart disease
IMA	Internal mammary artery
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NA	Noradrenaline
NADPH	Nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
PDRF	PVAT-derived relaxing factors
PGI2	Prostaglandin I2
PVAT	Perivascular adipose tissue
RAS	Renin-angiotensin system
SV	Saphenous vein
TNFα	Tumour necrosis factor
TXA2	Thromboxane A2
VSMC	Vascular smooth muscle cells
WAT	White adipose tissue

1. Introduction

1.1 Cardiovascular disease and its influence on public health

Any disease of the heart and its circulatory system is termed a cardiovascular disease (CVD), which covers a wide array of conditions including congenital heart disease, ischemic heart disease, cardiopulmonary arrest, angina and myocardial infarction (Mendis et al., 2011). CVD is the second highest cause of mortality in the United Kingdom, second only to cancer, causing more than a quarter of all deaths (27%) and is responsible for more than 170,000 deaths per year (ONS, ISD, NISRA. 2018). Statistics show that CVD is also the predominant cause of death worldwide, with coronary heart disease alone killing 9.43 million globally in 2016 (WHO. 2018).

Collectively, cases of CVD are predicted to rise due to the increase in behavioural risk factors such as smoking, sedentary lifestyles, diet-related health complications and hypercholesterolemia. However, while cases of CVD are rising in the UK, the number of deaths (mortality) are reducing, meaning despite higher survival rates there is a growing population encumbered with the burden (morbidity) of CVD (Nichols et al., 2012). Studies across Europe have shown that despite the declining mortality of CVD in more affluent countries the prevalence of both diabetes and obesity provides one of the biggest obstacles in reducing the burden of CVD and its morbidity, with lower income countries suffering most in both terms of morbidity and mortality, especially as populations age more (Bhatnagar et al., 2016; Timmis et al., 2019). The economic strain of an aging population and the associated rise in CVD morbidity is one of the biggest challenges the National Health Service in the UK must contend with, spending £7 billion to combat CVD in 2019 (Leal et al., 2006; Nichols et al., 2012; Kearney, 2019).

1.2 Vasculature structure and tone in health and disease

The human body maintains itself by transporting cells, nutrients, oxygen, hormones and carbon dioxide via an organ system known as the vascular, circulatory or cardiovascular system. The transport of these elements lets the body maintain homeostasis; the stable living condition our bodies operate within. Homeostasis is a delicate equilibrium the body must maintain, with the cardiovascular system interacting with many other bodily systems in tandem to meet this goal (Informed Health 2019). Thus, the importance of a cohesive and cooperative cardiovascular system and its structure in health cannot be understated. The disruption of any of the numerous elements that allows vasculature to function and transport blood and its contents correctly can quickly lead to illness on a potentially life-threatening scale (Mendis et al, 2011).

1.2.1 Vascular Smooth Muscle

During development, the body created a closed transport system with which to facilitate the movement and delivery of biological elements to their desired locations and organs, to maintain homeostasis. During the development of this transport system, the body underwent a critical mechanical adaptation via the addition of a smooth muscle layer within the vascular walls. This adaptation allows for the contraction and expansion of the blood vessels, giving them the ability to alter their luminal diameter. Macrocirculation, the term given to describe the circulation of blood to and from the body's various organs, allows the hearts pulsatile blood flow to be directed as needed to end organs. In microcirculation, the vascular contractility afforded by the smooth muscle allows for vasomotion; the contraction of vasculature independent of the heart's pulsations, which permits organs to maintain blood pressure levels specific to their needs (Hilenski and Griendling, 2013). This

maintenance of blood pressure and blood flow is due to smooth muscle arteries ability to readily change their diameter, thus creating resistance when and where needed.

To accomplish the contractile and distensile properties required, the majority of the vascular cell wall is made up of vascular smooth muscle cells (VSMCs). VSMCs are comprised of a complex arrangement of myofibers (actin and myosin), organelles, dense bodies, dense plaques, ion channels and surface receptors which allow for the facilitation of cellular contraction and relaxation (Metz, Patterson and Wilson, 2011).

Contraction events (Figure 1.1) begin with an increase in intracellular Ca^{2+}, which occurs either through influx via voltage-gated ion channels or from the release of internally stored Ca^{2+} (such as that stored within the sarcoplasmic reticulum). The predominant means of extracellular entry occurs via L-type calcium channels, after which the now intracellular Ca^{2+} binds to calmodulin, an intermediate messenger protein. Calmodulin proceeds to activate the enzyme myosin light chain kinase (MLCK) which in the presence of ATP begins to phosphorylate subunits of the myosin filaments head, termed myosin light chains (MLC). The phosphorylation of the MLCs causes the cross-bridging of myosin filament heads with the neighbouring actin filaments, generating force and inducing contraction within the cell (Hashimoto et al. 1990). Inversely, relaxation begins upon the reduction of intracellular Ca^{2+}, and thus a decrease in the phosphorylation of MLC. Other distensile measures also occur via the inhibition of MLCK or through MLC dephosphorylation with the use of regulatory enzymes (Webb, 2003).

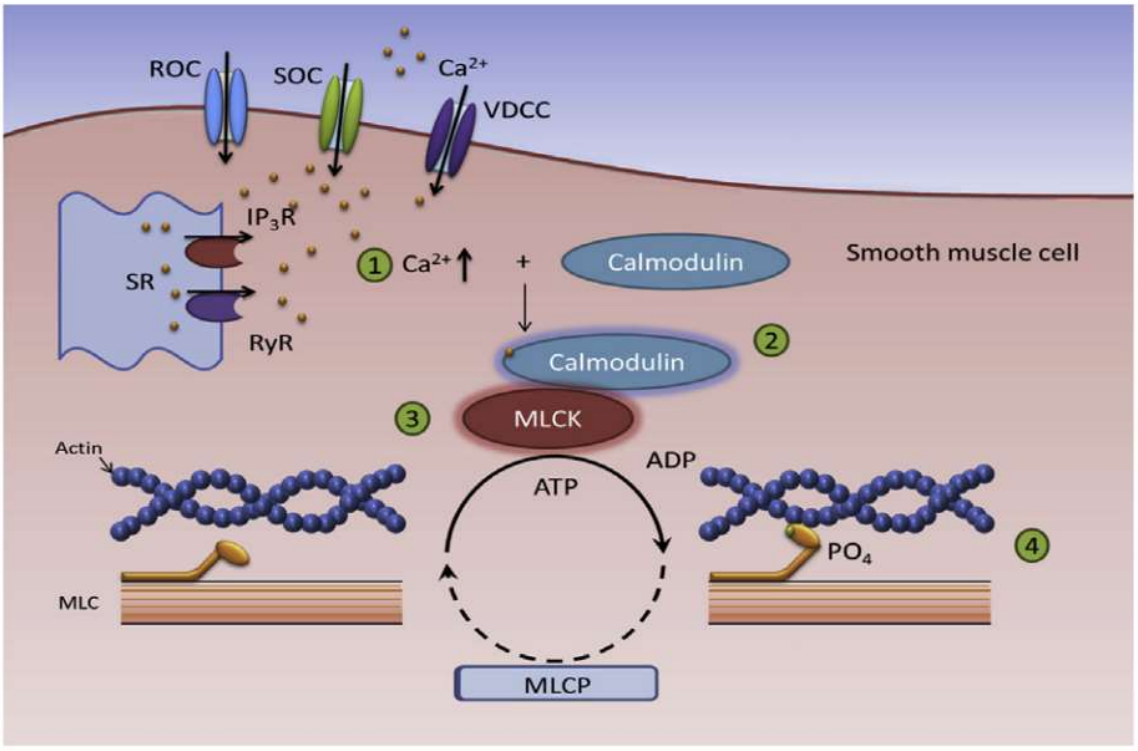


Figure 1.1 Elevated intracellular calcium in vascular smooth muscle cells instigates contraction
(1) Intracellular calcium levels increase by means of internal stores (sarcoplasmic reticulum, SR) or through influx via calcium channels in the cellular plasma membrane. (2) Intracellular calcium binds to calmodulin, which in turn activates myosin light chain kinase (MLCK). (3) Now activated MLCK phosphorylates the myosin light chain (MLC). (4) Myosin heads form cross bridges with actin filaments, resulting in contraction events. Zhao, Vanhoutte and Leung, 2015.

The VSMCs themselves are oriented in a ring around the vascular lumen, forming numerous layers in the medial section of the vessel wall, in a section termed the *tunica media,* which in turn is located between the *tunica intima* (the interior layer, containing the endothelium) and the *tunica adventitia* (the external, elastic membrane layer) (Figure 1.2). The *tunica media* is then separated from these respective sections by the *lamina elastica interna* and the *lamina elastica externa.* The number of cell layers is dependent on the type and size of vessel, with large resistance vessels having as many as 60 layers while medium layers can have up to 40 (Bacakova et al., 2018).

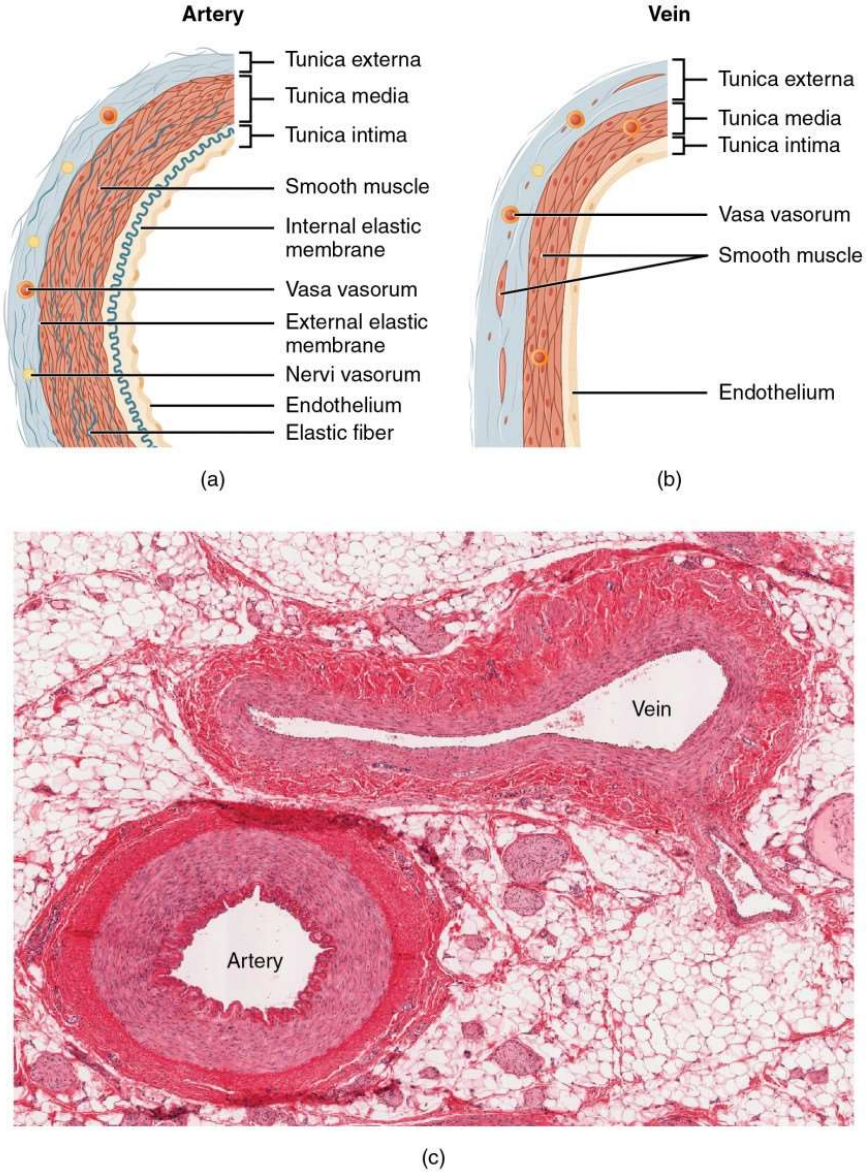


Figure 1.2 Arterial and venous transverse structures

Arteries (A) and veins (B) exhibit the same generalised features, but with significant differences in layer and wall thickness. This being due to the differences in blood pressure that flows through them, with arterial blood pressure being much higher. (C) A micrograph showing the difference in relative thickness between an artery and vein. LM × 160. Image adapted from the University of Michigan Medical School, 2012.

The VSMCs are then arranged in a highly organised manner within layers, combined by a dense architecture of cytoskeletal proteins which allow for the maintenance of contractility and vascular tone. They further synthesise, organise and secrete extracellular matrix (ECM) components with elastic resilience and recoil properties to aid them with distensibility (Figueroa et al., 2003). The muscle cells can also adapt the expression of contractile proteins and the levels of ECM component synthesis based on extrinsic and intrinsic stimuli, such as in development, injury or disease (Shinohara et al., 2012). This ability to modulate its elastic phenotype is what distinguishes vascular smooth muscle from other differentiated muscle cells but is also a major component in vascular disease (Hilenski and Griendling, 2013).

VSMCs are the key component in vascular tone modulation and their activation can be controlled via a series of stimuli, be it mechanical, electrical or hormonal (Brozovich et al., 2016).

1.2.2 Vascular Endothelium

The innermost surface of the vascular assembly, the endothelium, is a structure that lines the interior lumen of every artery, capillary and vein in the body; creating a haemocompatible surface layer by which blood passes and can interact where necessary. This establishes the endothelium as a major controlling factor that actively regulates the flow of fluids and substances from the blood stream into and out of tissues (Cahill and Redmond, 2016).

The endothelium itself is comprised of a single layer of squamous endothelial cells (ECs) anchored to a sub-surface layer called the *basal lamina,* which together form the *tunica intima*. The ECs are polarised, with their luminal side exposed to the blood and its constituent elements while their basolateral sides are separated from any other surrounding tissue by the *basal lamina*, which is synthesised and secreted by the ECs themselves. The shape and size of the individual vascular ECs varies based on location, though typically they are thin and elongated, roughly 30-50 µm in length, 10-30 µm in width and 0.1-10 µm in thickness (Gimbrone, 1987). The cells orientate themselves parallel to the bloodflow, minimising the pressure (shear) stress from the passing blood and thus lowering the potential for damage (Reidy and Langille, 1980; Levesque and Nerem, 1985) (Figure 1.3). Each cell is linked together and to the layer's subsurface extracellular matrix via membrane-cytoskeletal proteins, which are found grouped in bundles termed focal adhesion plaques. The primary protein in EC adhesion is vinculin, which causes cell surface integrin adhesion molecules to link to neighbouring cells actin cytoskeleton (Lee et al., 1999).

ECs have a number of functions which are specific to their location in the body and their location in the vascular tree, therefore they have shown to exhibit remarkable phenotypic heterogeneity across the entirety of the vascular bed (Aird, 2007). Despite this variability, all ECs are actively involved in the binding and extravasation of solutes, fluid, hormones, and macromolecules (Mehta and Malik, 2006) as well as platelets and blood cells. They also have a key role in suppressing the cells of the intermediate vascular layers (such as VSMCs in the tunica media) to avoid overgrowth, which if left unrestricted would cause disruption of the normal vascular layers and thus disturb normal vascular functions (Krüger et al., 2013). Under healthy conditions, ECs also inhibit platelet activation and inhibit thrombus formation to prevent unneeded clot formations (Alheid, Reichwehr and Förstermann, 1989).

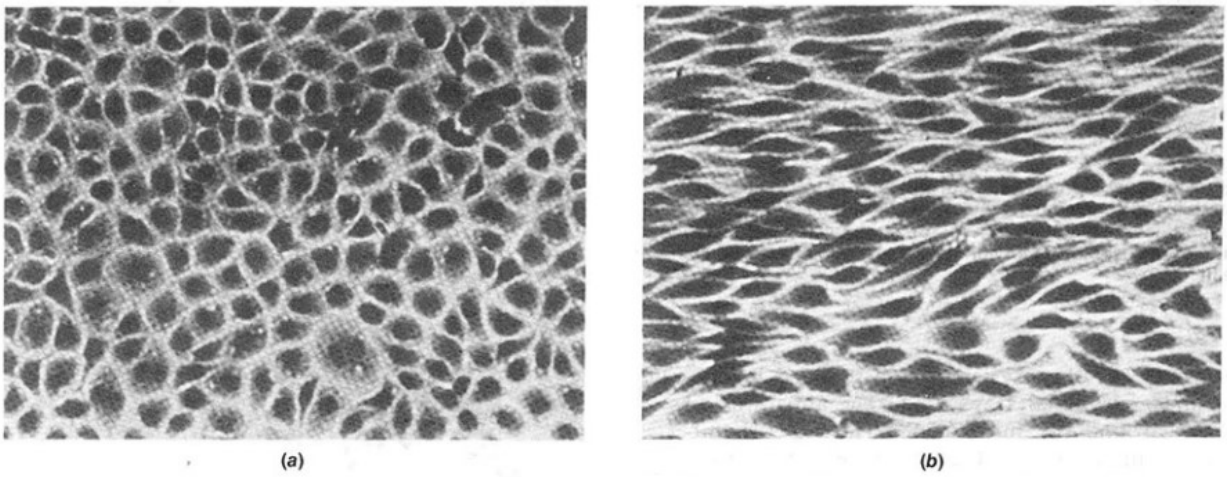


Figure 1.3 Under shear stress bovine aortic endothelial cells change orientation
Cultured bovine aortic endothelial cells imaged under control conditions (A) and under shear stress conditions (B) (85 dynes/cm, L-R) after 24 hours. Image adapted from Levesque and Nerem, 1985.

1.2.3 Modulation of Vascular Tone

The cells of the endothelium are also active regulators of vascular dilation and constriction via the synthesis and secretion of vasodilators and vasoconstrictors, such as prostacyclins, prostaglandins (Weksler, Marcus and Jaffe, 1977) and nitric oxide (NO) (Tousoulis et al., 2012). Upon production and secretion, these molecules pass through the *basal lamina* of the *tunica intima* and into the *tunica media* where they are absorbed by the VSMCs, causing relaxation or expansion of the vascular walls.

1.2.3.1 Modulation via Vasodilation

Nitric oxide (NO), originally termed the endothelium derived relaxing factor (EDRF), is a widely circulated free radical gas and a key component of vasodilation in the body. Produced endogenously by vascular endothelial cells, it is derived from L-arginine, oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) in a reaction catalysed by the intracellular enzyme; endothelial nitric oxide synthase (eNOS) (Stryer, 1995). Once produced, NO freely diffuses across the EC membrane and into the VSMCs, where it causes an increase in cyclic guanoside monophosphate (cGMP) via the activation of the enzyme guanylate cyclase (Figure 1.3). The rising levels of cGMP cause the VSMCs to extrude Ca^{2+}, leading to muscular relaxation and thus dilation of the vascular wall (Ghalayini, 2004). NO has been proven to play a significant role in the maintenance of vascular tone, accomplished via the introduction of an eNOS inhibitor (such as NG-Methyl-L-arginine acetate) which lead to an increase in latent blood pressure (Ahmad et al., 2018). Because of this importance, deficiencies in NO production from ECs can lead to hypertension and disease states such as atherosclerosis (Tibballs, 1993).

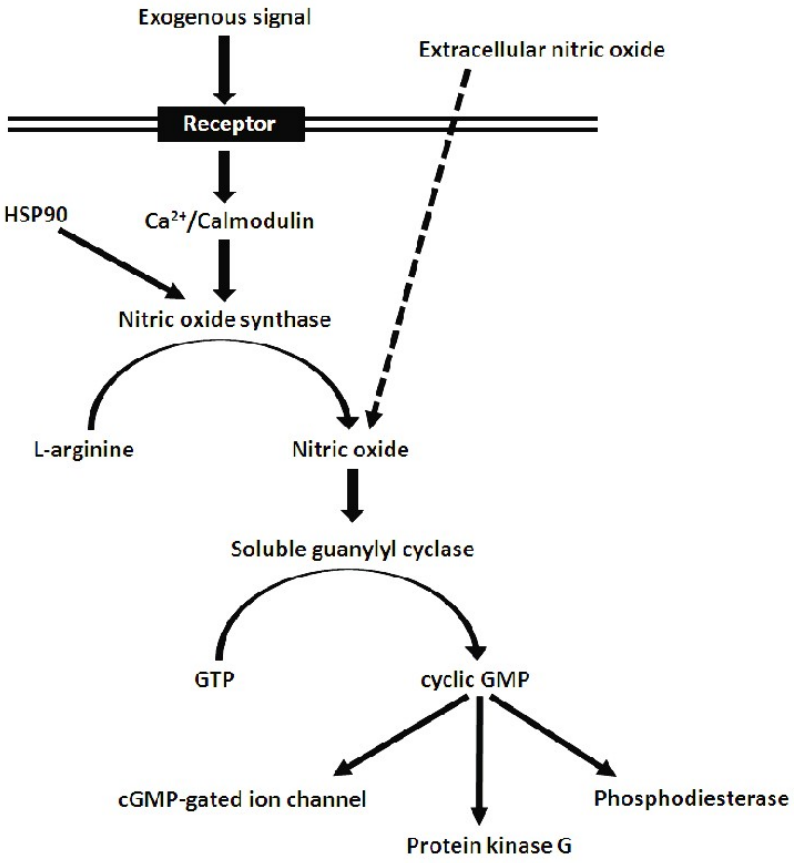


Figure 1.4 The nitric oxide-cyclic GMP signalling pathway
In brief, NO will freely diffuse across the cell membrane, catalysed from eNOS, interacting with the intracellular soluble guanylate cyclase enzyme to cause an increase in cyclic guanoside monophosphate (cGMP) from guanosine triphosphate. cGMP is shown to act as a secondary messenger, activating intracellular protein kinases and leading to downstream vasodilatory effects such as smooth muscle relaxation, platelet inhibition and gene expression modulation. Image adapted from Zhang et al., 2012.

Despite this dependence, NO is not the only endothelium derived vasodilator. Also produced by ECs is the eicosanoid signalling molecule prostaglandin I2 (PGI2), also referred to as prostacyclin. This lipid molecule is primarily involved in platelet activation inhibition, stopping the formation of blood clots, but also acts as a potent smooth relaxant. PGI2 stimulates an increase in cAMP and protein kinase A via G protein-coupling, causing a decrease in Ca^{2+} and the inhibition of Rho kinase, leading to VSMC relaxation (Majed and Khalil, 2012). PGI2 also counterbalances the effects of the molecule thromboxane A2 (TXA2) a potent vasoconstrictor and platelet aggregator. When these molecules fall out of balance vascular disorders such as pulmonary arterial hypertension (PAH) may arise, reinforcing PGI2's importance in vasodilation (Miller, 2020).

While vasodilation is predominantly controlled via NO and PGI2, it has been demonstrated that dilation still occurs when the activity and expression of both these molecules are downregulated, indicating the presence of other vasorelaxant mechanisms (Wang et al., 2003). In the event of the concurrent NO and PGI2 loss, it has been demonstrated that endothelium-derived hyperpolarization factor (EDHF), a metabolite synthesised within ECs, causes dilation via hyperpolarisation of the VSMCs through the AMP and PKA signalling pathways (Arendshorst and Bello-Reuss, 2010).

1.2.3.2 Modulation via Vasoconstriction

The endothelium also produces and secretes multiple vasoconstrictive factors, including endothelin 1 (ET-1), angiotensin II (Ang-II/A-II), prostaglandin H2 (PGH2) and the previously mentioned thromboxane A2 (TXA2) (Schiffrin, 2001).

Endothelins are a family of potent vasoconstricting peptides which consist of three related members; endothelin 1 (ET-1), endothelin 2 (ET-2) and endothelin 3 (ET-3) which act as endogenous modulators of voltage-gated ion channels, allowing the influx of Ca^{2+} into the VSMCs (Yanagisawa et al., 1988). Of the three peptides, ET-1 is the most vasoactive and exerts its physiological effect via interactions with G-protein coupled receptors (ET_A and ET_B). Endothelins themselves are not readily stored in the ECs but synthesised as a response to various signalling mechanisms (interleukins, tumour necrosis factors), other active factors (Ang-II, thrombins) or due to physical signals (hypoxia, sheer stress) (Haque, Welch and Loizidou, 2013). Once synthesised as a pro-endothelin, it is released from the ECs basal layer and converted to endothelin by extracellular, membrane bound endothelin coverting enzymes (Krüger et al., 2019).

One of the most essential mechanisms of vascular constriction in the body is the renin-angiotensin system (RAS), responsible for regulating blood pressure and vascular resistance. When blood pressure is sensed to be too low via baroreceptors, the RAS begins by releasing the enzyme renin, which converts the protein angiotensinogen into angiotensin I (Ang-I), which then interacts with an angiotensin converting enzyme (ACE), culminating in the development of the peptide Ang-II (Paul, Poyan Mehr and Kreutz, 2006). Once Ang II has been produced, it directly binds to Ang-II type 1 (AT_1) receptors of VSMC surfaces, causing the firing of action potentials, an influx of Ca^{2+} and thus vascular constriction (McKinley and Oldfield, 2009).

As previously stated (section 1.2.3.1), thromboxane A2 (TXA2) is also found within the endothelium and is another potent vascular constrictor as well as a platelet aggregator, used in the formation of clots and, as before, maintains a delicately balanced homeostasis with the vasodilator PGI2 (Ding and Murray, 2005). In the event of vascular damage, PGI2 release rates decline, allowing for an excess of TXA2 to arise, causing a decrease in blood flow and a rise in platelet aggregation to support the damaged tissue (Engelking, 2015). TXA2 itself is produced via a metabolic reaction between thromboxane-A synthase (TBXAS1) and the prostanoid precursor PGH2, after which its primary mechanism of action in vasoconstriction is via TXA2 receptors (TP). TPs are G-protein coupled receptors and found widely expressed on both VSMCs and ECs, which upon activation promote multiple downstream signalling pathways which cause VSMC constriction (Nakahata, 2008). The TXA2-TP axis has also been shown to promote sustained contraction effects in endothelin-induced vasoconstriction events (Reynolds and Mok, 1990).

Vasoconstrictive modulation can also be influenced by hormonal factors derived from other bodily organs, such as noradrenaline (NA). Produced in the adrenal medulla of the adrenal gland, NA is secreted as a response to the sympathetic nervous system and binds to VSMCs via α_1-adrenergic receptors, whereupon constriction occurs (Graham et al., 1996).

1.2.4 Perivascular Adipose Tissue

Surrounding the majority of the body's blood vessels is a layer of adipose tissue, termed perivascular adipose tissue (PVAT) (Figure 1.5). Until recently, PVAT was classically thought of as a simple, supporting connective tissue layer with some functions in thermogenesis and lipid deposition, and because of this assumption it is mostly removed in vascular surgical procedures and studies (Szasz, Bomfim and Webb, 2013).

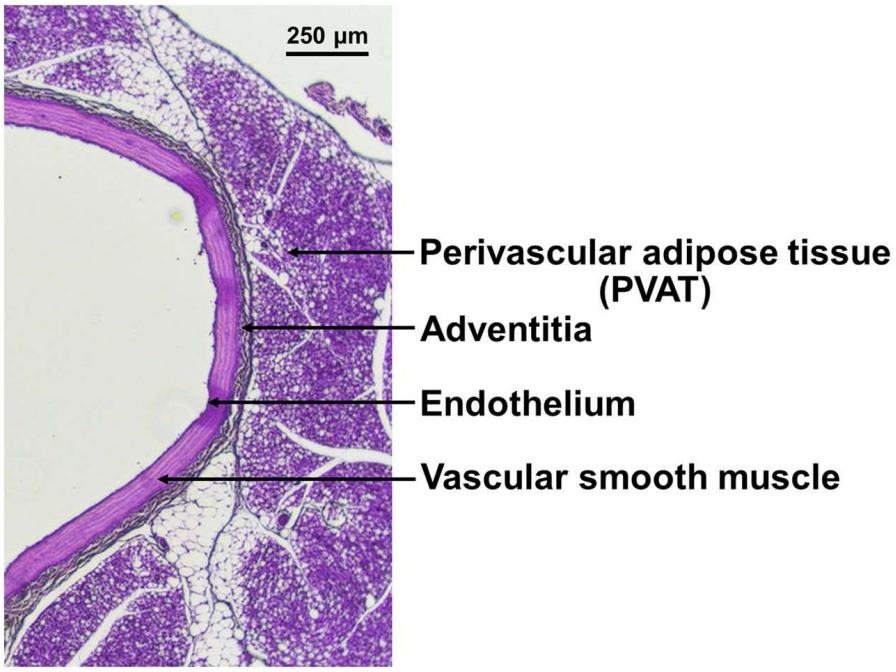


Figure 1.5 Aortic ring structure and surrounding endogenous PVAT

An aortic ring dissected from a rodent model and processed with haematoxylin-eosin staining. Marked are the vascular layers, including the endothelium, VSM and adventitia with the PVAT surrounding it. Scale bar 250 µm. Adapted from Liu et al., 2020.

Studies since have highlighted PVATs regulatory role in vascular constriction, demonstrated first in a 1991 study where rat aortic contractility was shown to be significantly less influenced by NA (less contractile) in the presence of PVAT than without (Soltis and Cassis, 1991). Since then, many studies have shown PVATs effectiveness in regulating vascular tone in multiple species and across a the vascular tree (Verlohren et al., 2004; Ketonen et al., 2010) and with its vasorelaxant effects being further exhibited via demonstration of the release of anti-contractile factors within the PVAT itself (Löhn et al., 2002).

1.2.4.1 Types and definitions of classic adipose

Adipose tissue, composed mostly of adipocytes, but also containing fibroblasts, vascular endothelial cells and a collection of immune cells (Birbrair et al., 2013), has the typical biological role of storing energy in the form of lipid molecules as well as being a cushioning and insulating tissue layer for the body's varying and numerous organs, protecting against mechanical shock (Zwick et al., 2018). In more recent years, adipose tissue's role as an endocrine organ has been further recognised, as the production and secretion of a myriad number of hormones has been discovered. Some such hormones, termed adipokines, include; adiponectin, tumour necrosis factor (TNFα), leptin, resistin and oestrogen (Kershaw and Flier, 2004). With adipose tissue no longer being considered solely as an

inert storage tissue, its role and relationship in inflammatory diseases is being further explored and expanded upon (Coelho, Oliveira and Fernandes, 2013). Adipose tissue itself is divided into two classical types; white adipose tissue (WAT) and brown adipose tissue (BAT), of which both have differing biological roles.

BAT is an important type of adipose tissue in the body, featured more prominently in infants and but found in adults in the para-aortic, paravertebral, supraclavicular, mediastinal suprarenal areas (Nedergaard, Bengtsson and Cannon, 2007). BAT is responsible for non-shivering thermogenesis across most mammalian clades and thus is highly metabolically active in its thermoregulatory role (Fox, 2011). BAT contains a unique protein called uncoupling protein-1 (also known as thermogenin) which uncouples protons created in the process of ATP synthesis, which causes energy to be released as heat (Cannon and Nedergaard, 2004). While both types of adipose tissue are rich in microvasculature, BAT is the more heavily vascularised of the two, containing many more capillaries and parenchymal nerve fibres, giving it it's "brown" colour (Cinti, 2005).

WAT is the most abundantly found type of adipose tissue in human adults and has multiple differing biological functions but is predominantly used as a form of energy storage, accomplished by the white fat cells which each house a large, singular lipid droplet of triglycerides (Dawkins and Stevens, 1966). These triglycerides can be broken down into fatty acids via lipolysis, which are then used by muscle and cardiac tissue as a source of biological fuel (Pollard et al., 2017). Lipolysis itself is induced via the catecholamines noradrenaline and adrenaline, which activate β-adrenoceptors (1,2 and 3), activating lipase, an enzyme responsible for triglyceride breakdown via hydrolysis (Straznicky, Nestel and Esler, 2009). WAT is also responsible for secreting signalling factors that regulate appetite and energy homeostasis (White and Copps, 2016) as well as playing a role in thermal insulation along with BAT, supporting the maintenance of bodily temperatures. Appetite regulation occurs via the production and secretion of leptin, a hormone which inhibits hunger and in turn encourages the usage of stored fat within adipocytes as energy. Acting predominantly on the hypothalamus to regulate hunger, leptin also interacts with other hormones such as insulin, insulin-like growth factor and glucagon amongst others, which indirectly mediates the effects of metabolism focused processes and further promotes stored energy expenditure (Margetic et al., 2002).

Despite the distinct differences between the two adipose types, a key feature of adipose tissue is its plasticity and adaptability (Sethi and Vidal-Puig, 2007). Both tissue types have the capacity to change their size, phenotype and function based on varying environmental and metabolic conditions; even going so far as to change phenotypes in what is termed the "browning process", in which WAT can exhibit BAT-like characteristics (Petruzzelli et al., 2014). Within WAT, this change has been demonstrated by large increases of brown-like adipocyte cells (termed beige adipocytes) in white adipose deposits, which causes a rise in the adipose tissue's thermogenic capacities and capabilities (Ro et al., 2019). The presence and growth of these beige adipocytes is often found in response to chronic adrenergic stimuli such as cold temperature exposure or exercise, leading to p38 MAPK/ERK pathway activation and cell differentiation; showcasing adipose tissue's plasticity and dynamism (Bartelt and Heeren, 2013). BAT can also transform back into WAT, and occurs most commonly during aging, as humans develop from infants into adults and the larger stores of BAT become less metabolically active (Yoneshiro et al., 2011), but also has been shown to occur in adults in states of obesity (Shimizu and Walsh, 2015).

1.2.4.2 Characteristics of PVAT

PVAT is similar to other visceral adipose tissue depots in that it provides mechanical protection for vascular beds. In addition to this role, its unique secretory profile of vasoactive molecules make it vital in vascular homeostasis. Although the specific composition of PVAT varies based on its anatomical location, and can change in response to multiple different factors, it largely remains a dense matrix of constituent components, such as; nerve bundles, collagen bundles, fibroblasts, mesenchymal stem cells and immune system cells, sharing similarities with both brown and white adipose (Szasz, Bomfim and Webb, 2013).

PVAT has no physical barrier between it and the outer vascular *tunica adventitia*, thus the PVAT has been shown to have a paracrine effect on vascular tone (Saxton et al., 2019) by secreting molecules that can be moved into direct contact with the vascular wall to exert their effects (Rajsheker et al., 2010), of which there are both anti-contractile and pro-contractile properties as well as inflammatory responses in the event of vascular injury (Okamoto et al., 2001).

As an endocrine organ, PVAT has the ability to synthesise, store and secrete a wide array of differing bodily messengers. For example, it has been shown that subcutaneous and visceral adipose depots secrete adiponectin, leptin and resistin in much larger quantities than in aortic PVAT (Szasz, Bomfim and Webb, 2013). Also, human coronary PVAT has been shown to exhibit a morphology similar to that of WAT, in which adipocytes are small and heterogenous in shape and size, while undergoing decreased levels of differentiation (Chatterjee et al., 2013), whereas the PVAT surrounding the upper thoracic aorta has been shown to exhibit qualities more similar to that of BAT (Omar et al., 2014).

1.2.4.2.1 Adipokines and their function in PVAT

PVAT produces, secretes and interacts with a large variety of cell signalling factors which belong to a family of proteins termed cytokines, of which those secreted from the adipose tissue itself are further termed adipokines. Adipokines are responsible for the modulation of many, varied physiological functions within the body such as glucose and lipid metabolism, thermogenesis, neuroendocrine function, inflammatory and anti-inflammatory responses (Carbone, Mach and Montecucco, 2015). Most importantly to this study, adipokines are responsible for the induction and modulation of both anti-contractile and pro-contractile effects within vasculature.

Some of the members of the adipokine family found to be present within PVAT include adiponectin, resistin, chemerin, visfatin, leptin, lipcalin-2 and tumour necrosis factor alpha (TNFα) to name a few (Lynch et al., 2013; Park et al., 2013; Soehnlein et al., 2013; Li et al., 2014; Wang et al., 2016). Each of these adipokines serve different physiological roles, working in tandem to maintain a constant homeostatic balance.

Adiponectin and Leptin: As before, some adipokines are anti-contractile, such as; adiponectin via nitric oxide (NO) production enhancement and leptin via endothelium interactions (Gálvez-Prieto et al., 2012), though they also serve other roles. Specifically, adiponectin has been found to increase glucose uptake and storage in adipocytes, control energy metabolism and reduces levels of TNFα (Vasseur et al., 2003) while leptin manages adipose storage and has also been shown to promote angiogenesis and, contradictorily, promotes contraction of vascular beds via sympathetic stimulation of the hypothalamus (Elias et al., 1998). All of which is to say that PVAT-derived adipokines and their interactions within the adipose tissue and with the underlying vascular are wide ranging and variable.

Chemerin: The chemoattractant protein chemerin is a ligand for the G protein-linked chemokine-like receptor 1 (CMKLR1 or ChemR23) (Bozaoglu et al., 2007). Both chemerin and CMKLR1 itself are

predominantly expressed in adipocytes and stromal-vascular cells and are both implicated in the process of inflammation, adipogenesis and adipocyte lipid metabolism activation (Goralski et al., 2007). More recently, studies have highlighted chemerin as an endogenous vasoconstrictor, acting upon CMKLR1 expressed within the *tunica media* and the endothelium. One such study showed CMKLR1 to express profound contraction events when subjected to the agonist chemerin-9 and inversely showed largely inhibited levels of contraction when subjected to the antagonist CCX832, demonstrating chemerin and CMKLR1's role in vascular tone (Watts et al., 2013).

Resistin: A peptide hormone derived from adipose tissue, which is typically linked with obesity, type II diabetes and predominantly inflammation, though also plays a role in the regulation of vascular tone (Kusminski et al., 2007; Lazar, 2007). Resistin has been shown to augment the release of the potent vasoconstrictive peptide endothelin-1 from the endothelium via endothelial cell activation (Verma et al., 2003) and in *in vitro* porcine models has been shown to increase superoxide anion (O_2^-) production (Kougias et al., 2005) which interacts with endothelium derived NO to diminish its anti-contractile effects; further bolstering vasoconstriction (Tesfamariam and Cohen, 1992). Resistin has shown to be a selective antagonist of vasorelaxation though, having no effect on Ach-induced relaxation but has been shown to impair bradykinin-induced relaxation (Dick et al., 2006).

1.2.4.2.2 The anti-contractile properties of PVAT

As previously stated (sections 1.2.4.1-1.2.4.2), PVAT has come to be known as an important paracrine and endocrine organ integral to vascular health and function, displaying the ability to synthesise and secrete a wide array of active molecules used to affect its mechanically supported vasculature. As discussed earlier (section 1.2.3.1), the endothelium is a key component of vascular dilation in which it synthesises and releases factors which drive "endothelium-dependent vasodilation" (Laurindo et al., 2018). PVAT has been shown to also directly cause and influence VSMC relaxation and thus vasodilation by itself, with its own suite of previously mentioned (section 1.2.4.2.1) endogenous adipokines, causing non-endothelium dependent relaxation (Lynch et al., 2013; Park et al., 2013; Soehnlein et al., 2013; Li et al., 2014; Wang et al., 2016). Despite these two different avenues of vascular relaxation standing on their own, PVAT has also demonstrated the ability to affect and direct endothelium-derived responses to further amplify and co-opt vasodilatory action. Evidence of this cooperation with the endothelium has been shown in studies highlighting the impairment of vasodilation in cases of hypertension (Gálvez-Prieto et al., 2012) and metabolic syndrome (Marchesi et al., 2009).

The physical presence of PVAT also moderates the overall response of vasoconstrictive agonists (such as NA and Ang-II) in both arteries and veins in what is termed the "sponging effect", by acting as a storage and processing site and thus diminishing the concentration and effects of active vasoconstrictors (Löhn et al., 2002; Gao et al., 2005; Greenstein et al., 2009; Saxton et al., 2018).

As there are numerous factors which have been shown to promote anti-contractile effects, they as a group have been termed PVAT-derived relaxing factors (PDRF), though different members of this family have been shown in greater or lesser quantities based on the PVATs anatomical location and the subjects state of health (Löhn et al., 2002). There are multiple factors that have been classed as PDRFs, including angiotensin 1-7, adiponectin and palmitic acid methyl ester (Brown et al. 2014). Also, prostacyclin, a typical endothelial cell derived vasodilator is thought to belong to the PDRF family via studies showing it to be readily found within PVAT, wherein it was shown to block induced vessel constriction, and further proved when a prostacyclin agonist blocked the vasorelaxant effect when PVAT was present (Chang et al., 2012).

It is important to note that in health both vasodilatory and vasoconstrictive agents work together in a form of homeostatic balance, where the action of one will cause the release of another, which is an important mechanism regarding the release of the above-mentioned vasodilator adiponectin which itself is stimulated to be secreted via the activation of beta-3 adrenoceptors by NA, which itself is a vasoconstrictor (Saxton et al., 2018).

1.2.4.2.2.1 Nitric oxide as a vasodilator within PVAT

PDRFs as a whole coordinate vasorelaxation via many different mechanisms, some of which previously discussed and again linked or co-opted with other tissue derived relaxation mechanisms.

One such mechanism is the aforementioned NO. Just as it has been shown to be produced in endothelial cells (section 1.2.3.1), PVAT has been shown to produce NO via present eNOS and was shown to be one of the earliest and most well documented vasorelaxant elements within PVAT (Dashwood et al. 2007). The presence and interaction of NO has been well demonstrated via assays in which PVAT's anti-contractile effect was diminished when using several NO and K^+ antagonistic measures. NO dependence was highlighted via the removal of the endothelium, the inhibition of NO synthase and the scavenging of NO while K^+ dependant relaxation was shown via the inclusion of high extracellular K^+ levels and the blockade of calcium dependent K^+ channels, all of which again diminished PVATs typical anti-contractile effect (Gao et al., 2009; Gao et al., 2000; Greenstein et al., 2009).

To further prove the idea of PVAT's dependence on NO as a vasorelaxant, a study was aimed at inhibiting protein kinase G, a kinase activated by cGMP downstream of NO (Rapoport et al. 1983), which showed that PVAT did not exert its typical anti-contractile effects upon successful protein kinase G blockage (Withers et al., 2013).

1.2.4.2.3 Pro-contractile properties of PVAT

As with the documented vasodilatory effects, PVAT has also been shown to exhibit vasocontractile effects on its underlying vasculature (such as across the aortic tree, mesenteric arteries and coronary arteries), in moderation in states of health and in excess during states of disease (Ramirez et al., 2017). In health, PVAT accomplishes this via several different mechanisms, some of which are shared with and featured in surrounding tissues such as those in the endothelium as discussed previously (section 1.2.3.2).

One such mechanism shared with the endothelium is again the renin-angiotensin system (RAS), of which PVAT contains each necessary component (with the exception of the enzyme renin itself, which is secreted from the kidneys) (Hermenegildo et al., 2005), including Ang-II receptors type 1a (AT_{1a}) and 1b (AT_{1b}) (Gálvez-Prieto et al., 2008). The inclusion of RAS within PVAT has been proven over multiple different studies, one such confirmation being via the electrically stimulated contraction of vascular rings which was dependent on the presence of intact PVAT deposits, which mediated its effects via detected Ang-II (Lu et al., 2010).

The potent contractile agonist NA is also found stored in the adipose cells of PVAT through uptake via NET and OCT3 plasma membrane transporters, whereupon in the cytosol it is taken up through the vesicular monoamine transporter to be stored in vesicles for later use as needed (Ahmad et al., 2019). Anecdotally, this NA storage system can also be an anti-contractile function as discussed previously (section 1.2.4.2.1), as its storage effect can diminish NA-induced contractions due to uptake, again

known as the “sponging effect” (Saxton et al., 2018, Zimlichman et al., 2018). Further, there is also evidence of a transferable pro-contractile factor in swine models that is released from coronary PVAT deposits, which is secreted in response to high concentrations of potassium and prostaglandin F2α (Owen et al., 2013) and can be blocked via beta-3 antagonism (Saxton et al., 2018).

1.2.4.2.4 The role of PVAT in inflammation

While having a key role in vascular modulation, PVAT also has an essential and extensive role in inflammation, which when exacerbated can lead to disease states such as in atherosclerosis (Ross, 1993). As previously mentioned, PVAT exerts different characteristics based on its anatomical location, with some depots showing more pro-inflammatory features than others. One such example of this is in the PVAT surrounding the coronary artery, which has been shown to exhibit a heightened pro-inflammatory state when compared to that of other local PVAT depots (Omar et al., 2014).

The pro-inflammatory phenotype is made possible by the production of factors which promote VSMC migration and immune cell recruitment. Chemotactic factors causes the migration of monocytes, T lymphocytes and granulocytes that amplify inflammatory events, which in heightened disease states can promote the development of atherosclerotic lesions, wherein large accumulations of lipids, cells and ECM components cause a thickening of the intima, deforming the arterial wall and restricting blood flow (Ozen et al., 2015). Within PVAT itself, under normal conditions, immune cells are also found in abundance, with six primary cell types being identified: macrophages, mast cells, neutrophils, T-cells (CD4 and CD8), B-cells and natural killer (NK) cells (Kumar et al., 2020).

In states of health, the inflammatory capabilities of PVAT is regulated by anti-inflammatory adipokines such as adiponectin, which modulates the production and effects of pro-inflammatory cytokines such as TNFα and interleukin-6 (IL-6) (Ouchi and Walsh, 2007). Adiponectin has been shown to be downregulated in conditions of excess adiposity, such as in obesity, where TNFα suppresses its production at a transcriptional level (Maeda et al., 2001). This dysregulation then can give rise to heightened pro-inflammatory effects, contributing to further vascular damage or the progression of inflammation-mediated disease.

1.2.4.2.5 PVAT in obesity

In obesity, PVAT has been shown to become dysfunctional regardless of anatomical position or phenotype via several means, including; hypertrophy of its adipocytes, dysfunction of its secretory profile and the elevation of pro-inflammatory conditions (Xia and Li, 2016).

Obesity induces the whitening of BAT and BAT-like PVAT, reducing its thermogenic capacity and causing lipid accumulation and mitochondrial dysfunction, leading to the onset of atherosclerosis (atherogenesis) (Hung et al., 2014). The hypertrophic expansion of both WAT and BAT also results in elevated basal rates of lipolysis, leading to enhanced levels of fatty acid release and the secretion of pro-inflammatory factors which contribute to inflammation and the development of insulin resistance (Frayn, 2000; Cao, 2014), a driving component of type-2 diabetes.

Also, instead of its usual homeostatic balance of vascular tone effecting adipokines, obesity affected PVAT releases elevated levels of pro-inflammatory factors and cytokines directly upon the vascular wall, leading to immune cell recruitment, heightened adipokine dysregulation and further exacerbating atherogenesis, all while adipocyte hypertrophy contributes to cellular hypoxia (Fernández-Alfonso et al., 2013). Atherogenesis and atherosclerosis are further discussed below.

1.3 Ischemic Heart Disease

Ischemia is a condition which occurs when there is an inadequate blood supply to a tissue or organ, typically due to a blockage in the blood vessel that would normally enervate that area, leading to a shortage of blood and oxygen. In the condition ischemic heart disease (IHD), also termed coronary heart disease or coronary artery disease, circulation is lost or restricted via a blockage or narrowing in the coronary arteries which enervates the heart itself (Ross, 1975). This narrowing can be caused by blood vessel constriction or blood clotting, but it most commonly due to the build-up of plaque in the arterial walls, termed atherosclerosis. When the coronary artery is sufficiently blocked, the hearts myocardiocytes will begin to die and the heart will lose its ability to contract, thus fail to perfuse blood throughout the body. This event is called a myocardial infarction (MI) or more commonly as a heart attack and can lead to death (*Institute of Medicine US*, 2010).

The survival rate of IHD and subsequently MI has increased due to the development of better treatment options but there is a wide portion of secondary comorbidities that coincide with the decreased performance of the heart (Bozkurt et al., 2016). One such problem is heightened blood pressure within the pulmonary circulation, termed pulmonary hypertension, in which increased pressure within the left ventricle and atrium of the heart causes an increased workload for the right ventricle, damaging the right side of the heart and leading to further serious health problems. Hypertension is just one secondary morbidity of many that can arise from the presence of IHD of which their incidences are all increasing.

IHD is a disease which progresses over the course of decades, with most people afflicted by early IHD (where vascular lumen narrowing is 50% or below) do not experience any symptoms. As the disease progresses symptoms can begin to show, typically during events of high oxygen demand such as with exercise or in emotional stress.

1.3.1 The pathophysiology of ischemic heart disease

Atherosclerosis is the leading cause of IHD, in which a plaque consisting of adipose, cholesterol, blood products and calcium build up and narrow the vascular lumen leading to decreased blood flow. The process of plaque development, atherogenesis, is a slow process, taking a period of many years and consisting of multiple complex cellular events.

1.3.1.1 The mechanisms of atherosclerosis

Early onset atherogenesis begins with the adherence of monocytes to the endothelium via the blood, wherein they migrate to the *tunica intima* and differentiate into macrophages and cause the formation of “fatty streaks” (Schwartz et al., 1993).

This process is driven by elevated levels of blood glucose or low-density lipoproteins (LDLs), which invade the endothelium and become oxidised via endothelium-derived free radicals, causing vascular wall damage. This initial event causes the instigation of an inflammatory response, in which signalling factors induce immune cell recruitment, such as via vascular cell adhesion molecule-1 (VCAM-1) which attracts and allows for the adhesion of monocytes to the vascular wall. Once within the *tunica intima,* monocytes begin to differentiate into macrophages, which is induced by macrophage colony stimulating factor (M-CSF), whereupon they will proliferate locally and begin to ingest the oxidised LDL causing them to turn into large foam cells, which are now rich in lipid content (Robbins et al.,

2013). This presence and propagation of foam cells forms a “fatty streak”, the beginning stage of an atherosclerotic lesion. Also, in response to the vascular wall damage, cytokines are released by the damaged endothelial cells, causing the proliferation and migration of VSMCs into the *tunica intima* from the *tunica media*. This then leads to the VSMC ingestion of lipids, the production of ECM elements such as collagen and ultimately the formation of a fibrous cap over the fatty streak, thickening the now-forming lesion (Figure 1.6) (Basatemur et al., 2019).

As time passes, the capped fatty streak becomes an atheromatous plaque which interferes with normal cellular processes, such as with calcium deposition. Normally, extracellular calcium deposits derived from dead cells are removed from the local area as needed, but the atheroma disrupts this process, causing the accumulation and crystallisation of calcium and the further thickening of the plaque (Miller, 2013). The atheroma also releases enzymes which cause the artery to enlarge over time, which can actually cause the vasculature to compensate for the growing plaque without losing luminal diameter, but can also lead to the formation of an aneurism if the enlargement grows beyond the thickness of the atheroma (Glagov et al., 1987).

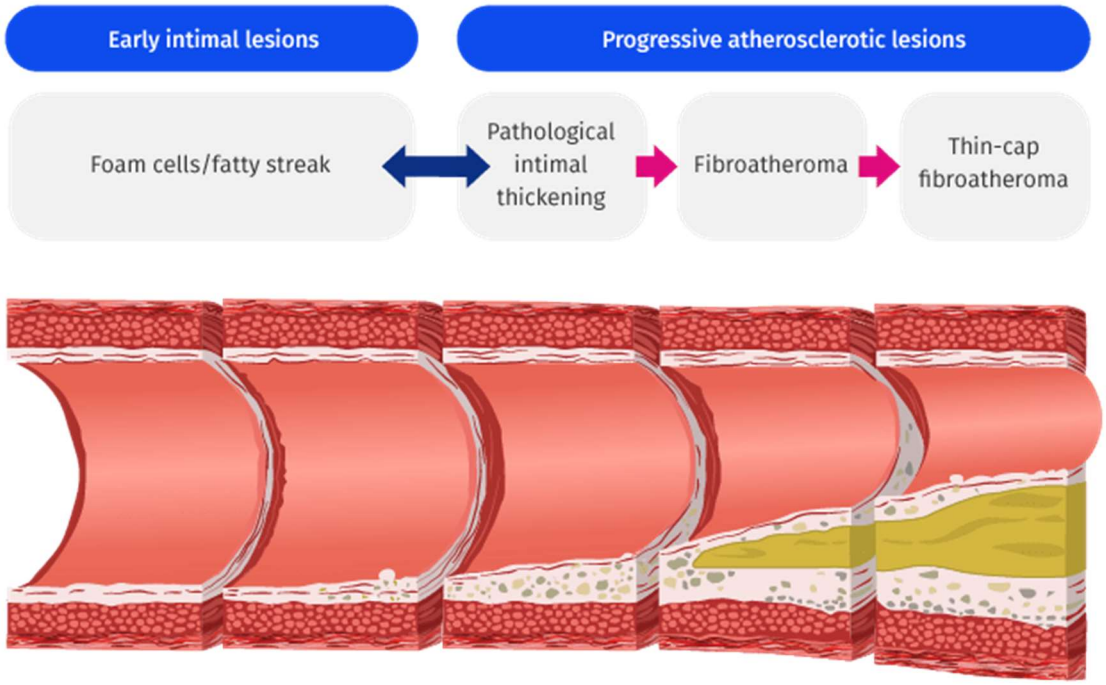


Figure 1.6 The developmental stages of atherosclerotic lesions
The invasion of immune cells and production of foam cells leads to the formation of a fatty streak between the endothelium and VSMCs. Propagation of the leads to the development of atherosclerotic lesions and the formation of fibrous atheroma caps. Image adapted from Vascular Adviser, 2020.

1.3.1.2 Vascular blockage and rupture

If the atherosclerotic state of the vessel progresses sufficiently, the interior lumen of the artery can become significantly reduced causing high levels of blood pressure in the narrowed area, in what is termed stenosis. If an atheroma grows this significantly it has to possibility to ulcerate and rupture, penetrating the vascular endothelium which leads to the acute formation of a blood clot. Coupled with the narrowed lumen, the formation of an enlarging clot has the possibility to completely obstruct blood flow, leading to ischemia of the surrounding tissue and a myocardial infarction in the heart (Frostegård, 2013).

If the clot is non-fatal and left untreated it will heal the offending wound, covering the ulcerated plaque and depositing more fibrous material which will compound the closure of the lumen and cause further stenosis. Heightened blood pressure and the further invasion of the atheroma can lead to the formation of an aneurism, leading to the rupture of the artery and the onset of internal bleeding (Frostegård, 2013).

1.3.2 Ischemic heart disease treatment

There are a multitude of preventative and interventive treatments available to combat IHD, with a large focus on lifestyle changes to lower blood pressure and cholesterol. Many pharmacologic treatments are also available to aid in the prevention of arterial rupture or blockage and to lower blood pressure, with prescriptions varying based on the severity of the atherosclerosis.

Commonly used are medicines which thin the blood and prevent clotting, for example; rivaroxaban, clopidogrel, ticagrelor and low-dose daily aspirin. Also, as high cholesterol is implicit in plaque build-up, statins such as simvastatin, atorvastatin and rosuvastatin are used to block cholesterol formation. Beta-blockers, including; bisoprolol, metoprolol and atenolol are also common treatments, used to attenuate the presence of hormones such as adrenaline which raise blood pressure and heart rate. Angiotensin-converting enzyme (ACE) inhibitors and Ang-II receptor blockers are also a popular form of treatment, blocking the activity of vasoconstrictive Ang-II, with examples of treatment including ramipril and lisinopril. Finally, pharmaceuticals such as nitrates and calcium channel blockers are also used to instigate vasodilation, further aiding in blood pressure reduction (NHS UK, 2020).

Nonpharmacologic treatments are used in the case of severe atherosclerotic build-up and typically consist of either open chest coronary artery bypass graft (CABG) surgery, or the more minimally invasive percutaneous coronary intervention (PCI, angioplasty or stenting).

1.3.3 The coronary artery bypass graft

CABG is defined as the surgical process of grafting a section of vasculature from the aorta to the coronary artery to bypass a blockage within the latter and to improve the supply of blood to the heart. The first concept of CABG was proposed in 1910 by Alexis Carrel (Shumacker, 1992) but was not brought into practice until 1950 in Montreal, Canada where Vineburg and Buller were successfully able to use the internal mammary artery (IMA) to treat cardiac ischemia and angina by implanting it into the myocardium (Shrager, 1994). Following, the process of CABG improved and increased with the advent of new surgical techniques until 1964 where Kolesov performed the first successful IMA-coronary artery anastomosis, developing the standard for modern CABG (Olearchyk, 1988). In more recent years, advancements in robotics has given rise to more minimally invasive techniques (Falk et al., 2000; Prasad et al., 2001) leading to an array of options under which CABG can be conducted.

In the UK, the rate at which CABG is being performed has been steadily dropping, from as many as 16,786 procedures in 2014 to 14,527 in 2017 and is continuing to fall on an annual basis, reportedly due to a decrease in elective procedures, with treatment instead being focused on more preventative and interventive methods. Despite this, the number of annual urgent CABG procedures remain roughly the same, from 6885 in 2014 to 6895 in 2017, with little deviation in the intervening years (National Adult Cardiac Surgery Audit UK, 2019).

1.3.3.1 Traditional coronary artery bypass grafting

Traditional CABG involves the use of a cardiopulmonary bypass (CPB) pump, which keeps blood oxygen rich and moving through the body throughout surgery while the heart is stopped. During surgery the thoracic cavity is opened via a sternotomy, in which a surgical incision is made through the chest bone to open the ribcage, exposing the heart. Medicines are then used to induce cardioplegia; the intentional and temporary stoppage of cardiac activity and the CBP pump is activated to take over blood flow regulation and oxygenation (UCSF, 2020).

Arteries or veins are harvested from the body and prepared for use as grafts by stripping them of their endogenous PVAT. Both arteries and veins can be used in surgical procedures where multiple bypasses are needed. Arterial grafts are much less likely to become blocked over time when compared to veins, of which the left-IMA is the most commonly used, harvested from within the thoracic cavity, though the radial artery of the arm is also used frequently. Venous grafts typically use a section of the saphenous vein (SV), harvested from the inner side of the leg, though as above are more prone to developing plaque and becoming blocked over time. This higher failure rate is offset by SV's inherent expendable nature within the body, as deeper vessels are still able to maintain blood flow after its removal from the leg, which coupled with its extensive length (60cm~) allows for easy access and an abundance of usable vascular tissue to harvest (Loesch and Dashwood, 2018). After the graft is successfully applied, blood flow is restored to the heart, after which the heart will resume beating on its own and the CPB pump is thereafter disconnected. Finally, excess fluid will be drained from the chest cavity, the chest bone closed and affixed via the insertion of wires and the chest incision will be stitched or stapled back together (UCSF, 2020).

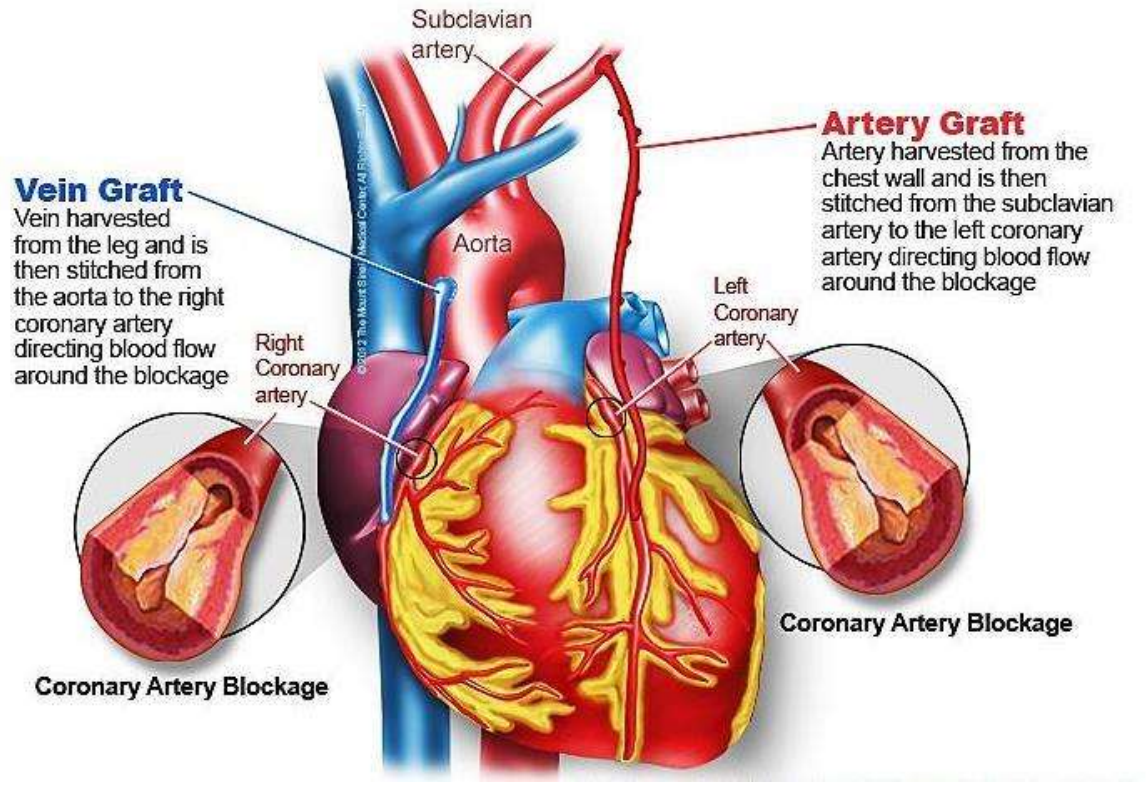


Figure 1.7 Coronary artery bypass grafting

Vein and artery bypass grafts are attached to the heart to circumvent atherosclerotic blockages which would reduce bloodflow to the heart tissue. Image adapted from Icahn School of Medicine at Mount Sinai, 2012.

1.3.3.2 Non-traditional coronary artery bypass grafting

As technology has advanced, CABG has seen many technical modifications to decrease the morbidity and increase the successful outcome of the operation. Typically, this has come in the form of using less invasive methods or by eliminating the used of the CPB pump.

Off-bypass coronary surgery, without the use of the CPB pump is performed on a still beating heart though with a reduction of cardiac motion via different pharmacologic and mechanical aids and devices. Pharmacological aids include the use of β-blockers and Ca^{2+} channel blockers to slow the hearts while mechanical stabilizing devices hold the target vessel. This technique still involves the use of a sternotomy, so it is equally as invasive as traditional CABG, but its main benefit is the avoidance of a CPB pump, from which it has been reported that there are many adverse health effects associated with its use in traditional CABG. Effects of CPB use include microembolic showering (the formation of blood clots in the brain), whole-body inflammatory responses and multiple organ dysfunction (de Jaegere, 2002) and the proportion of patients who recovered with no adverse complications during traditional CABG was found to only be 64.3% (Duhaylongsod, 2000).

Minimally invasive direct CABG (MID-CAB) forgoes the use of a sternotomy and a CPB bypass and is performed by via a small left anterior thoracotomy, in which an incision is made between the ribs in the fourth intercostal space to expose the heart. Due to the limited exposure of the heart, this surgical procedure is only performed when there are one or two coronary bypass targets, limiting its potential patient group (Eagle et al., 1999). It has been reported that event-free survival is improved following this surgical process, with excellent long-term outcomes including 10-year actuarial survival of 84.8%, 10-year freedom from repeat vascularisation at 89.9% and freedom from MI being 96.7% (Farid et al., 2018).

Finally, port-access cardiac surgery (PACS) is a closed-chest technique in which cardioplegia is induced and a CPB pump is used to regulate blood-flow, much like traditional CABG. Unlike traditional CABG, there is no sternotomy needed, using surgical techniques akin to MID-CAB. Also, vascular access for the CPB is via the femoral artery and vein, in which a triple-lumen catheter with a distal inflatable balloon is used to block the aorta and decompress the left ventricle of the heart. This allows for the decompressed heart to be operated on via small ports incised in the thoracic wall with the use of camera assisted robotics. As with MID-CAB, the minimally invasive nature of this procedure alleviated post-surgery bodily stress and improved patient discharge and recovery time, though at the cost of increased surgical complexity and operating theatre time (Reichenspurner et al., 1998; Chaney et al., 2000).

1.3.4 Perivascular adipose tissue in coronary artery bypass grafting

It is now clear that PVAT is an important endocrine and paracrine organ with supportive functions relevant to vascular health and tone. As previously discussed, PVAT becomes dysfunctional in cases of obesity; contributing to the onset of atherosclerosis. Instances of IHD strongly correlate with obesity, therefore PVAT in obese patients impacts the propagation of the disease state leading to concerns about PVAT's helpfulness in post-CABG recovery.

In the majority of CABG procedures, the IMA is considered the "gold standard" of vascular tissue to be used as a bypass, offering a superior patency of 90% in uncomplicated patient cohorts when compared to that of the SV (van der Meer et al., 1994). It is typically harvested with its PVAT left intact, though as stated above, adipose changes in obesity render IMA PVAT use limited. Also, the use of IMA

grafts in obese patients is limited by an increased risk of mediastinitis, a life-threatening complication after sternotomy (Milano et al., 1995).

With these considerations in place regarding the IMA, its PVAT and obesity, surgeons prefer to opt for the use of the SV in obese patients. The SV is typically harvested with its endogenous PVAT removed as outlined in its original harvesting technique, in which it is stated that "care must be taken to dissect only the vein, avoiding as much as possible the adventitia that surrounds it" (Favaloro, 1969). The network of micro-vessels surrounding vasculature, termed the *vasa vasorum*, which enervates the PVAT is damaged in this removal process, potentially impacting graft performance, especially as it is shown to run much more deeply in veins when compared to arteries, possibly alluding to the SVs higher failure rate in revascularisation when compared to the IMA and radial artery (Loesch and Dashwood, 2018).

An atraumatic "no-touch" (NT) method of harvesting the SV has been introduced, but the typical "conventional" (CT) method (Figure 1.8) is still employed by the majority of cardiac surgeons (Souza, 1996), despite NT SV grafts showing improved vascular functions than CT SV grafts (Samano et al., 2015).

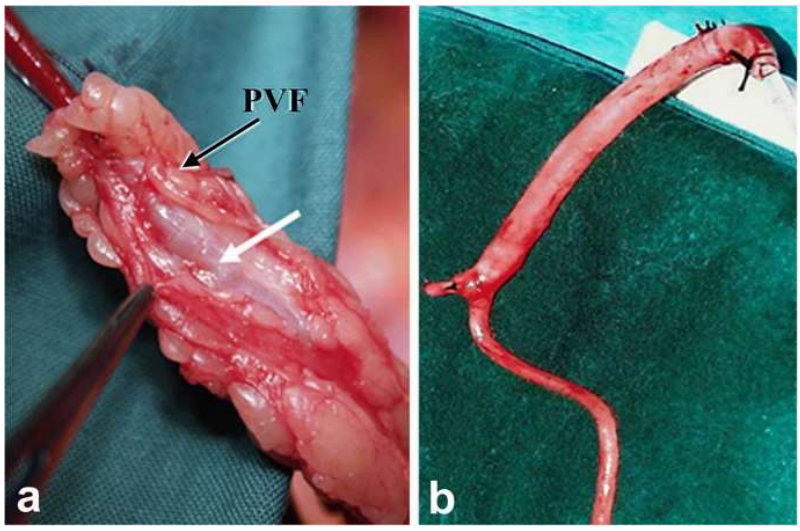


Figure 1.8 Saphenous veins harvested by no touch and conventional techniques
(a) Saphenous vein (SV) harvested via no touch (NT) methodology.The SV is indicated via the white arrow, and the surrounding PVAT can be identified by the black arrow (PVF) (b) SV harvested via conventional (CT) methodology, in which all PVAT has been removed. Image adapted from Loesch and Dashwood, 2018.

Additionally, it was suggested that the preservation of the SV's endogenous PVAT increases its patency due to the observed preservation of vascular eNOS and NO sources, highlighting NO's beneficial role in vascular distension and health post-graft and further promoting PVATs health benefits in CABG (Dashwood et al., 2007). This was then proven and demonstrated to be true when harvesting SV samples using the above discussed NT method displayed an increased maintenance and presence of eNOS and NO as well as eNOS activity. Use of the NT method and preservation of PVAT also displayed a significant drop in distension-induced endothelial damage, further highlighting perceived benefits in post-graft recovery in CABG (Dashwood et al., 2009).

With the rising obesity epidemic across the globe, it is likely that surgeons will be presented with increasing amounts of complicated patient cohorts, displaying obesity-induced disease states and therefor vascular disturbances. This will have deleterious effects on graft success and lead to more complex choices regarding tissue type in CABG.

1.4 Hypothesis and aims

The importance of PVAT in vascular health and tone and its key function as an endocrine organ has been made increasingly clear, with a mounting body of evidence to support this. As stated, when undergoing CABG to alleviate the effects of IHD, it is common practice for PVAT to be removed from some elements of the harvested graft tissue whether by tradition or in the face of obesity-induced disease states. Therefore, the hypothesis of this study is that the maintained inclusion of PVAT will improve cardiac revascularisation, patient recovery time and long-term survival rates in IHD patients undergoing CABG. The broader goal of this study is to investigate the role of PVAT on vascular contractility of vessels used for bypass grafting and the contribution of eNOS to the contractility of conductance vessels.

Using human and animal models, in sequence this present study aims to:

1. Record the quantitative effects of PVAT in mouse aortic contractility.
2. Discern the importance of eNOS in the contractile function of mouse aortae.
3. Ascertain the presence and importance of specific adipokines in vascular contractile events.
4. Extrapolate these data to human models with the goal of optimising experiments before utilising limited human tissue samples.
5. Conduct proof of concept experiments using human tissue samples and record preliminary data to support further investigation of PVATs effects in CABG patients.

In collaboration with Dr Nidal Bittar and the cardiac surgery group at Blackpool Victoria Hospital, we will receive blood, cardiac and vascular tissue samples from IHD patients, harvested at the time of surgery. The vascular samples received will be portions of the IMA and SV, left ideally with its endogenous PVAT intact. Human IMA and SV samples from obese and non-obese patients are to have their vascular functions assessed, both with (+) and without (-) PVAT, using different vasodilators and vasoconstrictors. This is to determine the efficacy of PVATs modulatory effects and to elucidate potential effector adipokines in IHD.

Also, investigations will be conducted into the influence of endogenous PVAT on the aortic rings of control and eNOS knockout mice, as the mouse aorta is surrounded by BAT much like human IMA. eNOS knockout mice will used be to observe the role of eNOS in conductance vessels and discern if its previously documented and discussed importance in SV samples (Dashwood et al., 2007; Dashwood et al., 2009, section 1.3.4) is mirrored in mouse aortae samples, to which we can relate back to human IMA models.

With these elements, the study will characterise vascular cellular function in IHD, identify IHD-associated tissue biomarkers which can be correlated to vascular dysfunction, with the ultimate aim of optimising surgical outcome by revascularisation following this and future studies.

2. Materials and Methods

2.1 Animal Models

All experiments conducted with animal models were performed in accordance with the Animals (Scientific Procedures) Act 1986. Mouse colonies were kept at The University of Manchester's Biological Services Unit under a 12-hour light and dark cycle, whilst provided with food and water as needed (project licence P3A97F3D1, Dr Elizabeth Cartwright).

2.1.1 Control and eNOS knockout mouse models

The $eNOS^{-/-}$ mice (strain B6.129P2-Nos3tm1Unc/J) were kindly provided by Dr Elizabeth Cottrell (University of Manchester) and were generated in-house at the Biological Services Unit. Control mice, C57BL/6J, were acquired from Charles River Laboratories at 18 weeks of age for immediate use.

2.2 Human Tissue

The collection, use and disposal of human tissue was ethically approved and performed in accordance with the standards of the International Conference on Harmonization, Good Clinical Practice Guideline, Research Ethics Committee and applicable government, Trust and Research Office policies, regulations and guidelines. The University of Salford approved this project (STR1920-07) and it also received IRAS approval (REC reference: 18/LO/2219).

2.2.1 Inclusion Criteria for Human Studies

All patients included into this study had previously diagnosed IHD and were scheduled for routine coronary revascularisation surgery, on pump or off pump.

Prior to surgery, patients were recruited to the study during their CABG preoperative assessment by the Blackpool Victoria Hospital research team, whereupon they gave written and informed consent for their participation in the study. BMI was recorded, and two research groups were identified: BMI < 25 and BMI > 30.

All participants were to be over the age of 18 and English speaking or were otherwise subject to study exclusion.

2.3 Tissue collection

2.3.1 Mouse models

At the required time of testing, mice were sacrificed by subjection to rising levels of CO_2 to cause asphyxiation, after which an incision was made along the midline of the abdomen to expose the entirety of the ventral cavity. The ribcage was then dissected away to expose the thoracic cavity, the aorta, vena cava and the heart. The heart was completely removed with the thoracic aorta and vena cava intact down to diaphragm.The aorta was then excised from below the aortic arch to the diaphragm and placed in chilled physiological salt solution (PSS) and left on ice before use.

2.3.2 Human models

A 5mL sample of blood was taken prior to surgery at NHS Blackpool Victoria Hospital at the point of anaesthesia and stored under refrigeration within an EDTA tube prior to collection. During surgery, vascular tissue was harvested as is typical in CABG procedures (IMA and SV). Upon successful completion of the bypass graft, excess vascular tissue and cardiomyocytes removed in the grafting procedure, that would otherwise be discarded, were collected and stored in MACS® Tissue Storage Solution (Miltenyi Biotec, UK) within a single sample tube. The tissue and blood samples were then stored within a UN3373 bag alongside an absorbent pad, then further placed within a polystyrene lined UN3373 box and kept refrigerated with frozen gel packs.

All collected samples were then to be transported to the University of Salford via a courier service, to be delivered within 1-1.5 hours of initial storage.

2.4 Assessment of vascular reactivity via myography

2.4.1 Mouse models

2.4.1.1 Preparation of mouse model thoracic aorta

As described above in section 2.3.1, the aortae were removed from the mice and pinned onto a dissection plate, submerged in cold PSS. The aorta was then cut into four 2mm rings, with two sections being stripped of their endogenous PVAT and the following two sections having their endogenous PVAT left intact, this allowing for the functional study of vascular tone in the presence of and without PVAT.

2.4.1.2 Mounting and normalisation of mouse aortic rings

Aortic rings were transferred into pre-heated, temperature controlled (37°C) myograph baths, as part of an automated multi-wire myograph (Danish Myo Technology, Denmark). The baths were filled with 6ml of PSS and gassed with 5% CO_2 balanced air, whereupon the aortic rings were individually mounted onto two 200µm pins, passing through the vascular lumen (Figure 2.1). The rings were left with no tension applied for 30 minutes in an equilibration period. Upon completion of equilibration, the aortic rings were exposed to an incrementally increasing tension, up to a stable 5nM, over 30 minutes, which was then treated as the relaxed baseline. This method has been outlined and adapted from previous studies (Judkins et al., 2006; Xu et al., 2012).

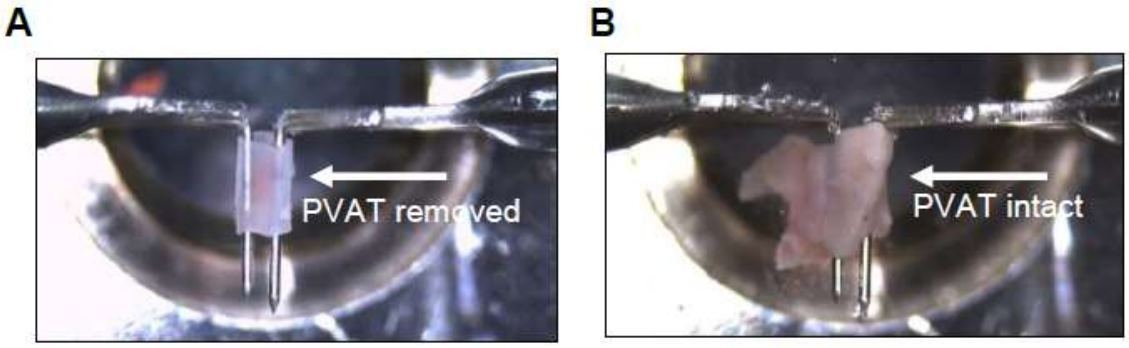


Figure 2.1 Mounted vasculature in myograph baths

Vascular tissue mounted onto myograph pins, with PVAT removed (A) and with the PVAT left intact (B). Image adapted from and courtesy of Rachel Walker, University of Manchester 2016

2.4.1.3 Assessing aortic ring tissue integrity and viability

The condition and contractile efficacy of the mounted aortic rings was initially tested after equilibration and tensile stabilisation with the addition of high potassium physiological salt solution (KPSS, a standard PSS solution with sodium chloride replaced with a 100 mM of potassium chloride). The PSS within the baths was drained and 6ml of KPSS was added. Contraction was observed until the maximal contraction of the tissue plateaued, after which the KPSS was drained and three washes of PSS were introduced to remove contractile stimuli, allowing the aortic rings to dilate back to their relaxed baseline tension (Figure 2.2).

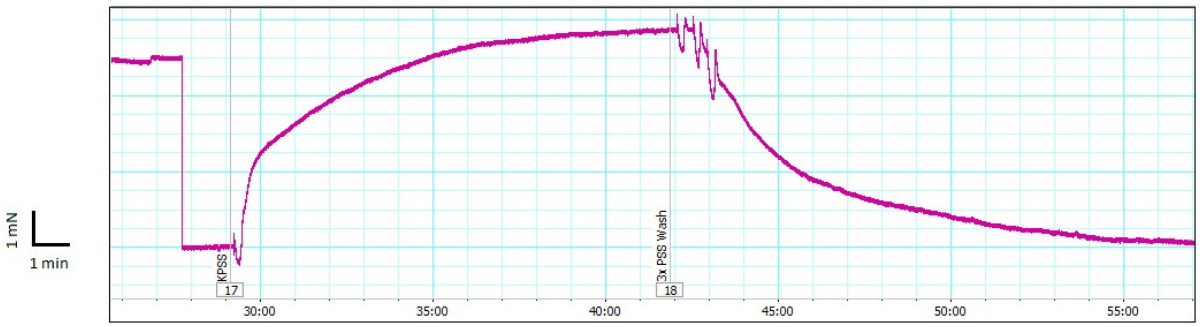


Figure 2.2 Mouse aortic ring constriction test via the addition of 100mM KPSS and PSS washes
Y axis displays change in tension (mN), X axis displays time (minutes). Scale bar displays 1mN (Y) and 1min (X). Points of KPSS addition (29min) and PSS washes (42min) are marked via the gray lines and tagged text.

2.4.1.4 Cumulative dose responses to noradrenaline and acetylcholine

After successful assessment of the aortic rings contractile function, cumulative concentration-response curves were created to the vasoconstrictor NA ($1x10^{-6}$ - $3x10^{-2}$ mol/L) (Figure 2.3) and vasodilator Ach ($1x10^{-4}$ - $3x10^{-2}$ mol/L) (Figure 2.4).

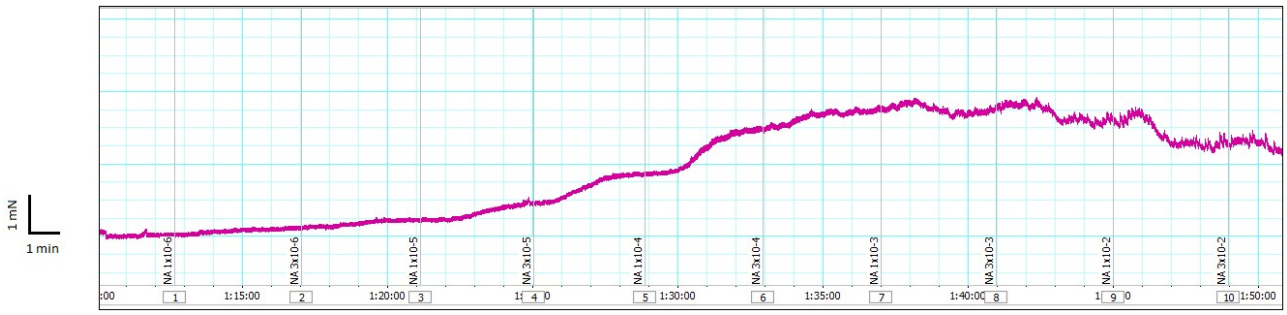


Figure 2.3 Mouse aortic ring constriction via NA concentration-response ($1x10^{-6}$ - $3x10^{-2}$ mol/L)
Aortic ring exposed to cumulative concentrations of NA, with each successive concentration timestamped by the tagged grey lines. Y axis displays change in tension (mN), X axis displays time (minutes). Scale bar displays 1mN (Y) and 1min (X).

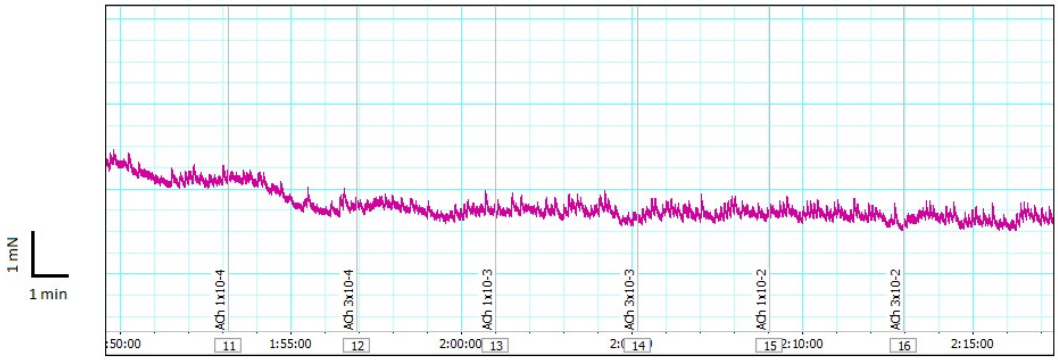


Figure 2.4 Mouse aortic ring constriction via Ach concentration-response ($1x10^{-6}$ - $3x10^{-2}$ mol/L)

Aortic ring exposed to cumulative concentrations of Ach, post-NA concentration-response, with each successive concentration timestamped by the tagged grey lines. Y axis displays change in tension (mN), X axis displays time (minutes). Scale bar displays 1mN (Y) and 1min (X).

2.4.1.5 Cumulative concentration-responses to NA with a β-3 adrenoceptor antagonist

Aortic rings were collected, prepared and mounted as outlined above (2.4.1.1 and 2.4.1.2), with the inclusion of a β-3 adrenoceptor (β3ADR) antagonist (SR59230A). Following the KPSS and PSS washes, SR59230A was added at a concentration of 1μM (1μmol/L) (as outlined in previous studies (Saxon et al., 2018)) to one bath containing a -PVAT aortic ring and to another bath with a +PVAT aortic ring and left to incubate for 30 minutes. A standard NA concentration-response as outlined above (2.4.1.4) followed.

2.4.1.6 Cumulative concentration-responses to NA with a chemerin receptor antagonist

Aortic rings were prepared and mounted as above (2.4.1.1 and 2.4.1.2), but with the inclusion of CMKLR1 antagonist 2-(α-naphthoyl) ethyltrimethylammonium iodide (α-NETA). Following the KPSS and PSS washes, α-NETA was added at a concentration of 10μM (10μmol/l) (as outlined and adapted from previous studies (Graham et al., 2014)) to one bath containing a -PVAT aortic ring and to another bath with a +PVAT aortic ring and left to incubate for 30 minutes. A standard NA concentration-response as outlined above (2.4.1.4) followed.

2.4.2 Human models

2.4.2.1 Preparation of internal mammary artery and saphenous vein

Upon receiving the human tissue from Blackpool Victoria Hospital via courier, the various human tissues were separated out and stored in MACS® Tissue Storage Solution to preserve tissue integrity and viability during transport and before use in testing. Vasculature was removed from the MACS solution and pinned to a dissection plate, submerged in cold PSS (Figure 2.5). IMA and SV samples were then cut into 4-5mm rings, both with either PVAT removed (-PVAT) or with PVAT left intact (+PVAT), in numbers applicable to the testing scenario, for example: four IMA samples, two with +PVAT and two with -PVAT , or; two IMA samples and two SV samples, with one +PVAT and one with -PVAT for both respectively. Care was taken during PVAT removal to not invade or damage the fibrous connective tissue of the adventitia.

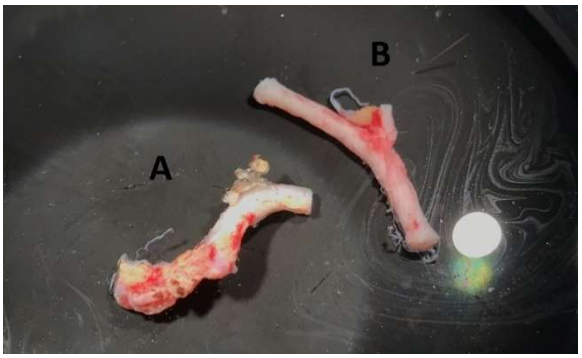


Figure 2.5 Human tissue samples received from Blackpool Victoria Hospital

Human internal mammary artery (A) and saphenous vein (B) samples as received from NHS Blackpool Victoria Hospital, submerged in cold PSS, prior to dissection and mounting on myographs.

2.4.2.2 Mounting and normalisation of internal mammary artery and saphenous vein

IMA/SV rings were transferred into pre-heated, temperature controlled (37^{o}C) myograph baths, filled with 6ml of PSS and gassed with 5% CO_2 balanced air, whereupon the IMA/SV rings were individually mounted onto two 200µm pins therein. The rings were left with no tension applied for 30 minutes in an equilibration period, upon completion of which, the were to be exposed to an incrementally increasing tension, up to a stable 40nM, over 60 minutes, which was then to be treated as the relaxed baseline. This method has been outlined and adapted from previous studies (Gao et al., 2003).

2.4.2.3 Assessing internal mammary artery and saphenous vein integrity and viability

The condition and contractile efficacy of the IMA/SV rings was tested after equilibration and tensile stabilisation, with the use of KPSS as outlined above (2.4.1.3). The PSS within the baths was drained and KPSS (6ml) was added in its place. Contraction was observed until the maximal contraction of the tissue plateaued, after which the KPSS was drained and three washes of PSS were introduced to remove contractile stimuli, allowing the aortic rings to dilate back to their relaxed baseline tension. Using this, the condition and health of the vasculature was observed and its viability for further study confirmed.

2.4.2.4 Cumulative Concentration-Response to NA

After successful assessment of the human vascular tissue's health and viability, cumulative concentration-response curves were created to NA ($1x10^{-6}$ - $3x10^{-2}$ mol/L).

2.5 Data analysis and statistics

All myograph data acquired in the assessment of vascular contractility was recorded using LabChart and exported using LabChart Reader (V7; AD Instruments, Oxford, UK). Measurements were based on the maximum stable contractions when presented with agonists and were presented as changes in tension (in mN). Datapoints were exported to Microsoft Excel and the change in tension was converted to change as a percentage from the baseline value, whereupon values for repeats were compiled and the mean determined to the n value of the data set.

Compiled values were exported to GraphPad Prism (V7; GraphPad Software, USA), whereupon they were graphed and analysed via two-way ANOVA followed by Bonferroni post-hoc tests to assess variance in contractility between the presence and absence of PVAT. Probability (P) values < 0.05 were considered to be significant.

3. Results

3.1 PVAT elicits a pro-contractile effect on mouse aortic rings

To firstly determine and confirm PVATs pro-contractile effect on the mouse aortea, contractility tests of aortic models both with and without PVAT were conducted using Noradrenaline (NA).

Aortic rings of control mice with intact PVAT (+PVAT) displayed significantly increased levels of contractility (P = 0.0058, n = 10) when compared to aortic rings with their endogenous PVAT removed (-PVAT), after being subjected to a NA concentration-response curve (Figure 3.1).

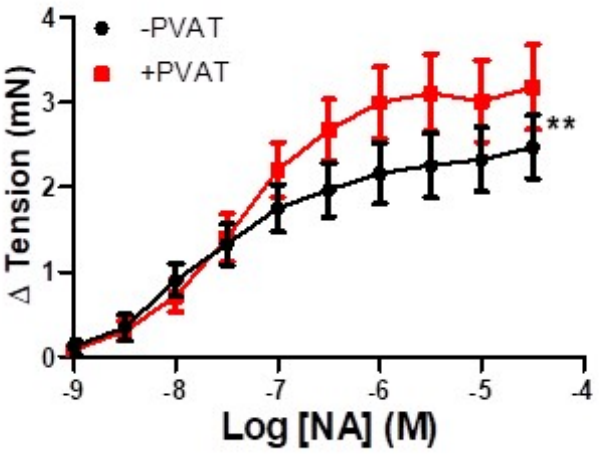


Figure 3.1 Removal of aortic PVAT attenuates vasoconstriction in C57BL/6J mice
*Contractions to NA ($1x10^{-6}$ - $3x10^{-2}$ mol/L) were significantly (P = 0.0058) diminished in -PVAT aortic rings when compared to PVAT-intact aortic rings of control mice. Data expressed as mean ± S.E.M., n = 10, ** P < 0.01, two-way ANOVA followed by Bonferroni post-hoc test.*

3.2 β-3 adrenoceptors do not influence vascular contractility in the aorta, or the PVAT pro-contractile effect

Following confirmation that PVAT stimulates a pro-contractile response, testing began to determine NAs point of interaction, of which the β-3 adrenoceptor (β3ADR) is one. A β3ADR antagonist (+SR59230A) was utilised to block any NA-β3ADR interactions, wherein contractility was recorded and compared.

In control mice, -PVAT aortic rings did not show any statistically significant difference (P = 0.0659, n = 8) in vasoconstriction when subjected to a β3ADR antagonist (+SR59230A), in comparison to antagonist-negative (-SR59230A) controls, when subjected to a NA concentration response ($1x10^{-6}$ - $3x10^{-2}$ mol/L) (Figure 3.2A).

Also, there was no statistically significant difference (P = 0.9064, n = 8) in vasoconstriction between β3ADR antagonist-positive +PVAT aortic rings and β3ADR antagonist-negative +PVAT aortic rings, upon subjection to a NA concentration-response (Figure 3.2B).

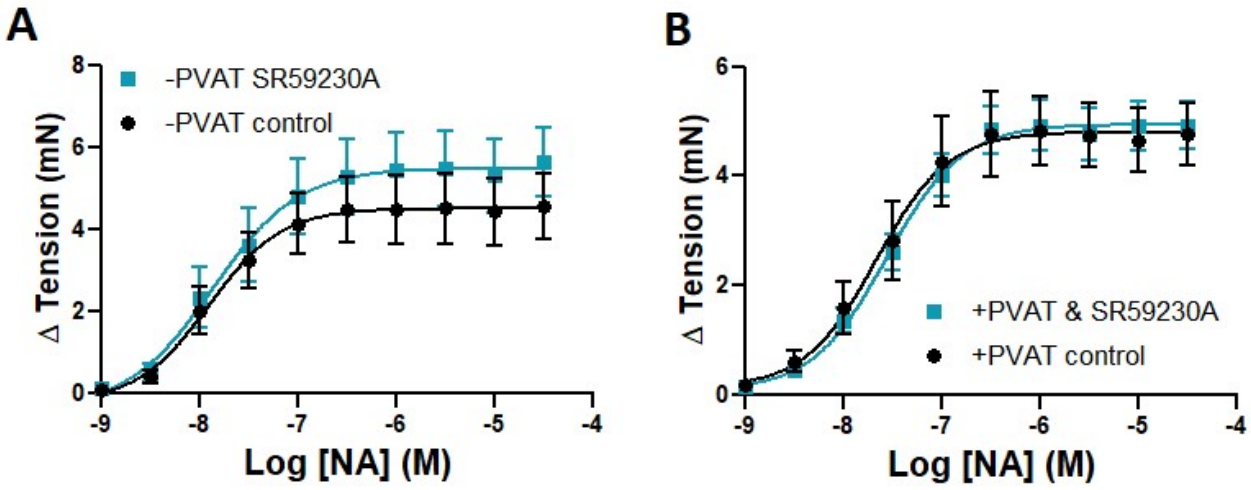


Figure 3.2 The inclusion of a β-3 adrenoceptor antagonist has no effect on contractility in C57BL/6J mice aortae

*A) In -PVAT aortic rings, contractions to NA concentration-response showed no statistical significance (P = 0.0659), both with and without the inclusion of β3ADR antagonist SR59230A in control mice. B) In PVAT-intact aortic rings, contractions to NA concentration-response showed no statistical significance (P = 0.9064), both with and without the inclusion of β3ADR antagonist SR59230A in control mice. Data is expressed as mean ± S.E.M., n = 8, * P < 0.05, two-way ANOVA followed by Bonferroni post-hoc test.*

3.3 Inhibition of CMKLR1 increases the contractile response to NA in the absence of PVAT, although baseline tension is increased by chemerin inhibition in both the presence and absence of PVAT

To further test possible points of NA influence and interactions with other compounds in contractility events, the study moved to test the effect of vasocontractile adipokines, of which Chemerin was selected. As Chemerin is a ligand to the CMKLR1 receptor, the use of a CMKLR1 antagonist was employed (α-NETA) to determine if there would be any significant change in contractility, in turn displaying Chemerin contribution or lack thereof in said contractile effects.

The -PVAT aortic rings of control mice showed a statistically significant difference (P = 0.0137, n = 9) in vasoconstriction, displaying greater constriction rates with the presence of a CMKLR1 antagonist (α-NETA), in comparison to -PVAT controls, when subjected to standard NA concentration-response (Figure 3.3A).

However, there was no statistically significant difference (P = 0.0564, n = 9) in vasoconstriction between control and +α-NETA, PVAT-intact control aortic rings, again when subjected to a NA concentration-response (Figure 3.3B).

When recording baseline tensions and averaging (n = 9), it was observed that constriction significantly increased in both PVAT-intact and PVAT-removed models which were incubated with α-NETA when compared to PVAT-intact and PVAT-removed models (Figure 3.3C).

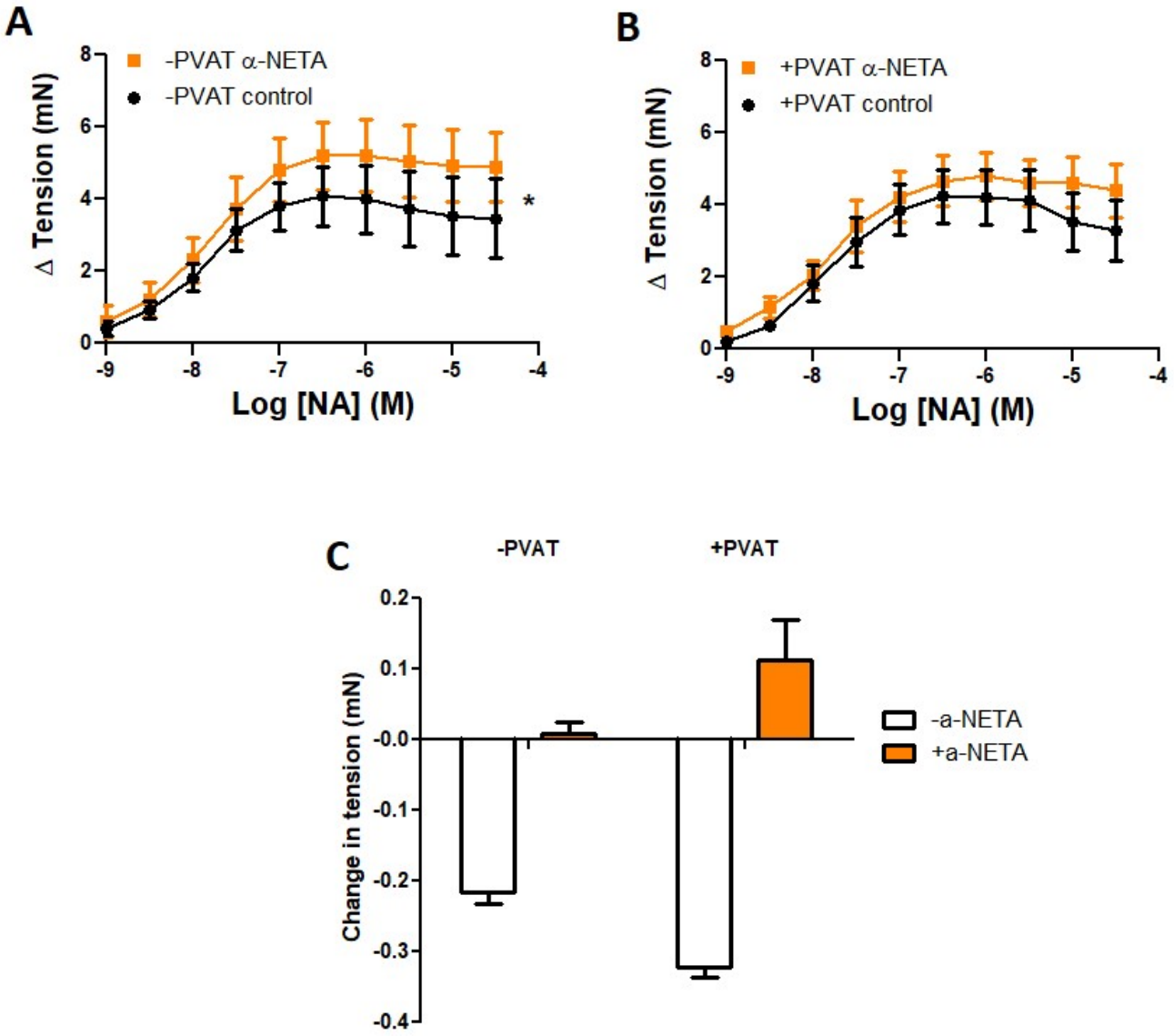


Figure 3.3 CMKLR1 antagonism increases NA-induced contractility in PVAT-removed C57BL/6J mouse aortae but does not in PVAT-intact aortae

A) In -PVAT aortic rings of control mice, contractions to a standard NA concentration-response ($1x10^{-6}$ - $3x10^{-2}$ mol/L) showed a statistically significant difference (P = 0.0137), with higher levels of contraction, in the presence of the CMKLR1 *antagonist α-NETA. B) In PVAT-intact aortic rings, contractions to NA dose response showed no statistical significance (P = 0.0564), both with and without the inclusion of* CMKLR1 *antagonist α-NETA. Data is expressed as mean ± S.E.M., n = 9, * P < 0.05, two-way ANOVA. C) The average resting baseline values of -PVAT and -PVAT mouse aortic samples show that baseline tension is increased in both when subjected to* CMKLR1 *antagonist α-NETA.*

3.4 Contractile properties of PVAT in eNOS$^{-/-}$ mice

To accomplish the aim of investigating the role of eNOS in the contractility of conductance vessels, the previous NA concentration response was performed on aortic tissue taken from eNOS knockout mice. The use of eNOS$^{-/-}$ models was to remove NO and its vasorelaxant functions from the tissue so that its efficacy at counterbalancing vascular constriction could be measured and quantified.

In eNOS$^{-/-}$ mice, aortic rings with intact PVAT (+PVAT) displayed significantly increased levels of contractility (P = 0.0295, n = 9) when compared to aortic rings with their endogenous PVAT removed (-PVAT), after being subjected to a NA concentration-response curve ($1x10^{-6}$ - $3x10^{-2}$ mol/L) (Figure 3.4).

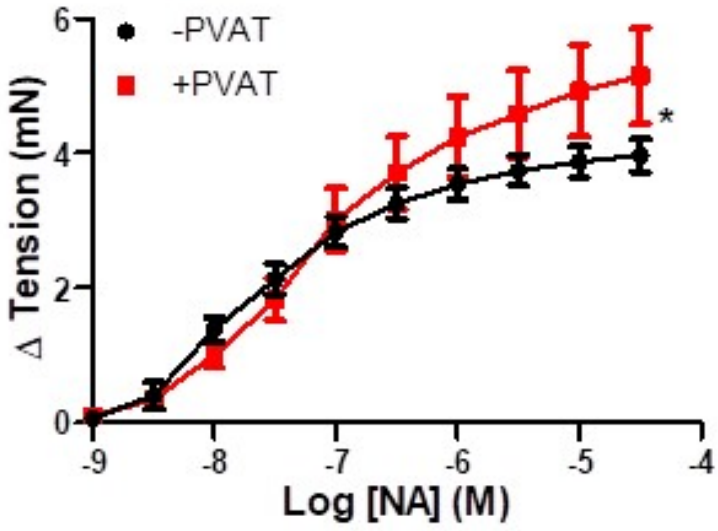


Figure 3.4 PVAT exerts a pro-contractile effect on the aortae from eNOS$^{-/-}$ mice
*Contractions to NA concentration-responses ($1x10^{-6}$ - $3x10^{-2}$ mol/L) were significantly (P = 0.0295) diminished in -PVAT aortic rings when compared to PVAT intact aortic rings of eNOS$^{-/-}$mice. Data expressed as mean ± S.E.M., n = 9, * P < 0.05, two-way ANOVA followed by Bonferroni post-hoc test.*

3.5 eNOS$^{-/-}$ mice exhibit increased aortic contractility compared to C57BL/6j mice

To draw comparisons between aortic mouse models with and without functioning eNOS the previous dataset of control models with intact and removed PVAT (section 3.1) was compared and contrasted to the dataset of eNOS$^{-/-}$ models with intact and removed PVAT (section 3.4).

When comparing control and eNOS$^{-/-}$ mice, there is a significant degree of variation in contractility between the two, with both the presence (+PVAT) and absence (-PVAT) of adipose tissue, when subjected to a NA concentration-response ($1x10^{-6}$ - $3x10^{-2}$ mol/L). In -PVAT models (Figure 3.5A) (P < 0.0001, n = 9) we observed greater levels of constriction in the -PVAT eNOS$^{-/-}$ subjects compared to those in the -PVAT controls. The same is reflected in +PVAT models (Figure 3.5B) (P < 0.0001), wherein +PVAT eNOS$^{-/-}$ models constricted at much higher tensions compared to +PVAT controls.

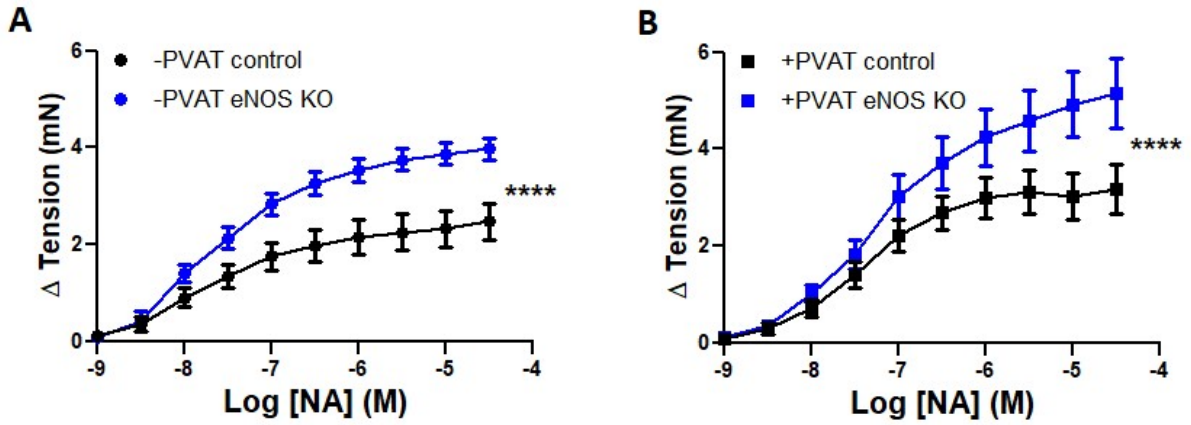


Figure 3.5 The inhibition of eNOS enhances the contractile effects of NA in PVAT-removed and PVAT-intact mouse aortae

*A) In the -PVAT aortic rings of control (C57BL/6J) and eNOS$^{-/-}$ mice, there is a significant difference in contractility (P < 0.0001), with eNOS$^{-/-}$ displaying higher levels of contractility overall B) In the PVAT-intact aortic rings of control and eNOS$^{-/-}$ mice, there is again a significant difference in contractility (P < 0.0001), with eNOS$^{-/-}$ once more displaying higher levels of contractility. Data expressed as mean ± S.E.M., n = 9, **** P < 0.0001, two-way ANOVA.*

3.6 Responses to KPSS were greater in the presence of PVAT

To normalise and test the viability of the murine models prior to concentration-response assays using other compounds, both control and eNOS$^{-/-}$ models were mounted to a myograph and subjected to a high K^+ stimulus (KPSS) to observe and confirm contractile efficacy and PVATs effect on this.

The constriction response of aortic rings to 100mM KPSS was greater in both C57BL/6J and eNOS$^{-/-}$ mouse models when PVAT was left intact. Though with the eNOS$^{-/-}$ models, the overall contractile effect was lower when compared to control models, in both -PVAT and +PVAT groups (Figure 3.6).

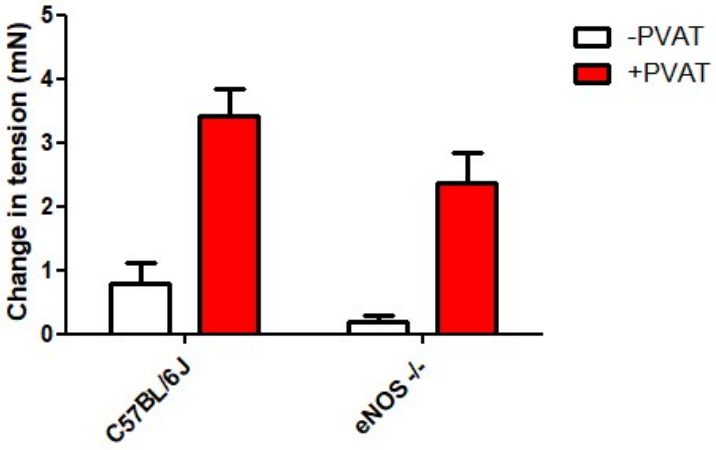


Figure 3.6 KPSS induced constriction is stronger in PVAT-intact aortae of both C57BL/6J and eNOS$^{-/-}$ models

Contractions to 100mM KPSS were enhanced in the presence of PVAT, in both models, though overall contraction was diminished in eNOS$^{-/-}$ aortic rings. Data is expressed as mean ± S.E.M., n = 9.

3.7 Human internal mammary artery myography proof of concept and optimisation

By obtaining human tissue samples via Blackpool Victoria Hospital as previously outlined (section 2.3.2) we were able to begin preliminary testing and optimisation of our myography assays on IMA samples (section 2.4.2), based on protocols previously used on murine models and as detailed in other studies (Gao et al., 2003).

Through iterative testing, optimisation of the dissection, myography and concentration-response processes was achieved, culminating in a final proof-of-concept dataset which displays the viability of human tissue samples when subjected to our adapted myography protocol (Figure 3.7). The sample BVF008 was the final human IMA sample able to be procured and tested during the timespan of this study (see; section 5.1) and as such, any further optimisation or subsequent data was unable to be obtained beyond this.

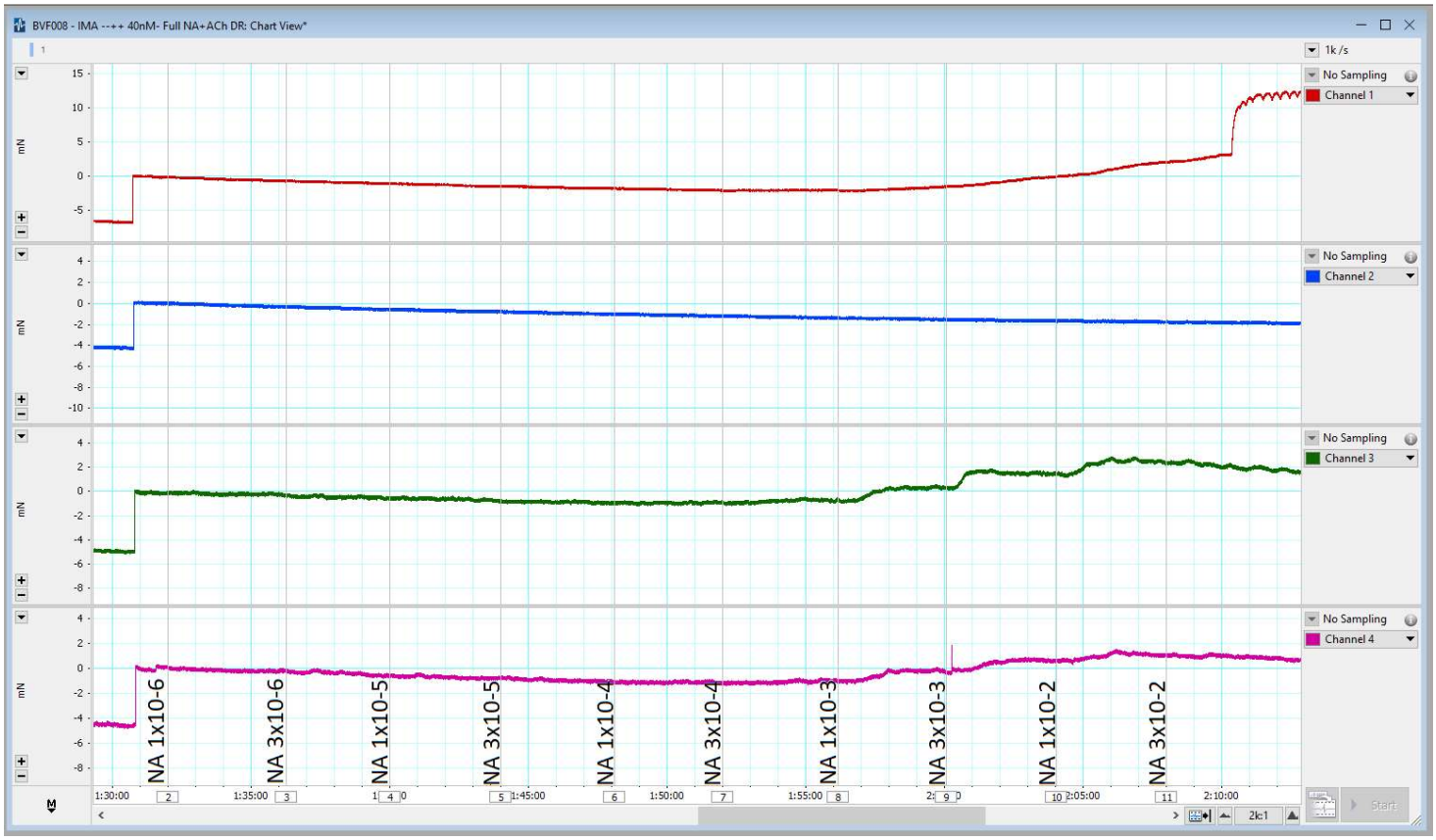


Figure 3.7 Human IMA constriction testing of sample BVF008 via NA concentration-response
Displayed is a myography trace of four (4) human internal mammary artery (IMA) rings. Channels 1 and 2 feature IMA samples with their PVAT removed (-PVAT), while channels 3 and 4 feature IMA samples with their PVAT left intact (+PVAT). All samples were exposed to cumulative concentrations of NA, with each successive concentration timestamped by the tagged grey lines. Y axis displays change in tension (mN), X axis displays time (minutes). Scale bar displays 1mN (Y) and 1min (X). Of note, the tissue sample featured in channel 2 (-PVAT) was deemed unviable as no reaction was observed to NA.

4. Discussion

4.1 Overview

The importance of PVAT in vascular health cannot be overstated, with an ever-increasing body of evidence to show its regulatory and supportive effects. With key functions in modulating vascular contractility, distensibility and recovery, it is becoming clear that PVAT should be considered a fundamental component in bodily revascularisation and procedures which focus on revascularisation, such as CABG.

With the rise of the global obesity epidemic, heart and vascular disease is increasing in tandem. Within these obesity derived disease states, PVAT's core functionality becomes maladjusted, propagating diseases such as atherosclerosis and causing further deleterious effects on the health of the neighbouring vasculature. The present study aimed to investigate and discern the usefulness of PVAT in aiding revascularisation across a variety of health states, in an effort to understand in what situations PVAT can be used to increase the rate of successful revascularisation and at what point it actively antagonises it.

The key findings of this study were:

- NA induced contractile events were greater in mouse aortic models with intact PVAT, than those with their PVAT removed.
- β3ADR and NA interactions do not exhibit any significant effect on contractility within mouse aortae both in the presence of and without PVAT.
- Chemerin, a pro-contractile adipokine, does not significantly augment contractility when applied in conjunction with NA in murine aortic models.
- The removal of eNOS and the subsequent loss of vasorelaxant NO production causes increased contractility in all models subjected to NA.
- The novel early optimisation of human IMA model contractility testing show promise when subjected to modified versions of myographic testing as outlined and adapted from murine models.

4.2 Use of mouse models mice for this study

The maintenance of vascular health falls upon a highly complex series of bodily mechanisms, which become dysregulated under multiple disease conditions and with age. Together with the challenges of procuring and utilising human tissue samples, this can make elucidating the true effects of PVAT difficult. Therefore, the use of the C57BL/6J mouse is a valid model with which we can draw comparisons to human models. In this study, while pursuing the aim of modelling PVATs efficacy in human tissue regarding contractility, it was decided that there would be a supportive study conducted, wherein mouse models were used as an analogue, in which we could draw comparisons to the human models.

Within the mouse studies there were several key aims; to quantitively measure the effects of PVAT in mouse aortic contractility, to ascertain the importance of eNOS in the contractile function of mouse aortae, to elucidate the presence and importance of specific adipokines in vascular contractile events and to extrapolate these data to human models in order to optimise experiments before utilising limited human tissue samples

The mouse is the animal model of choice when studying human heart disease, and has been for the last 15 years, as 99% of human genes have direct orthologs within the murine genome (Guenet, 2005).

Also due to their high breeding rate and relatively short lifespan, models can be bred rapidly and have the natural history of their disease states observed and recorded at an accelerated pace when compared to the self-same disease states in humans (Recchia and Lionetti, 2007). Also, due to their high breeding rate, genetically modified mice can quickly be selected for and bred up across different generations, allowing for the rapid procurement of data to be used as "proof-of-principle", which can then be translated up to larger animal models and eventually humans (Milani-Nejad and Janssen, 2014).

Despite this, the translational value of murine-derived data must be interpreted with a degree of caution, as the heart model found in mice is far removed from that of the human heart, both in contractile function and capability, this being mainly due to their small size and short-lived lifespan. Although mouse models recapitulate some phenotypic characteristics of a self-same disease found in humans, they will not mirror all aspects, thus we must be mindful of these discrepancies when drawing direct comparisons (Milani-Nejad and Janssen, 2014).

Regarding eNOS$^{-/-}$ mouse models, the use of mice which are homozygous for the endothelial nitric oxide synthase knockout allele has been a staple in the study of hypertension, cardiovascular dysfunction and wound healing. As eNOS plays a key role in blood pressure regulation and vascular tone, its absence leads to naturally hypertensive vasculature, making it an ideal model for contractile studies (Huang, 2000). Under natural conditions, eNOS derived NO also supresses VSMC proliferation during vascular injury. As VSMC proliferation and intimal invasion is a key component of atherosclerotic lesion formation, eNOS$^{-/-}$ models allow for the study of atherosclerotic disease progression (Moroi et al., 1998), which in relation to this study allows us to observe the contractile function of vasculature in a disease state similar to that of human patients undergoing CABG.

Two considerations that must be observed regarding the use of mouse models, when drawing comparisons to human tissue studies, are the relative ages and levels of obesity of the mice used in testing. In this study, mice were used at the age of 18+ weeks (4.5 months), which with an average lifespan of approximately 30 months identifies them as mature, sexually active adults (Fox et al., 2007) and all mice were fed with non-special diets, ergo with no induced obesity. Mice have also been documented to begin developing age-associated pathologies by the age of 12 months, which include hallmarks of vascular aging that lead to vascular dysfunction (Georgeon-Chartier et al., 2012; Rammos et al., 2014). As the human model component of this study includes variations in age and BMI, with tissue samples taken from patients typically in some form of disease state, these variables must be considered, and caution used when drawing direct conclusions and comparisons between murine and human models.

4.3 PVATs augments the contractile response to KPSS in mouse aortae

When testing the viability of the murine vascular models prior to concentration-response assays with other compounds, the myograph mounted vascular rings were firstly subjected to a high K^+ stimulus (KPSS) (as outlined in section 2.4.1.3) to observe their contractile function and confirm their continued efficacy. This confirmation occurs via K^+ induced vascular contraction which functions via VSMC depolarisation and the rapid ingress of Ca^{2+} into the intracellular space through ion channels (Hashimoto et al. 1990).

As above, KPSS response assays are typically confirmatory in nature, but within this study it was observed that the presence of PVAT heavily augmented the contractile effect elicited by KPSS in both control and eNOS$^{-/-}$ models (Fig 3.6), which is contradictory to some studies using murine aortic rings with similar KPSS response assays, in which there is no significant difference in contractility between

PVAT-intact and -PVAT aortic rings exposed to PVAT (Greenstein et al. 2009; Lynch et al., 2013; Meyer et al., 2013; Withers et al., 2014).

Upon searching for comparative or similar results regarding PVATs seemingly pro-contractile response to KPSS, it was found that this augmentation effect had also been observed in the coronary arteries of swine previously, and was linked to $Ca_v1.2$ calcium channels, whereupon inhibition of said channels abolished the PVAT-enhanced, KPSS-induced contractile events (Owen et al., 2013; Villacorta and Chang, 2015). Despite the difference in animal model, if there were parallels to be drawn, this could suggest that the aortic PVAT of both the control and $eNOS^{-/-}$ mice used in this study was phenotypically similar to coronary PVAT. With coronary PVAT being composed of brown adipose tissue (BAT) in health (Aldiss et al., 2017) and thoracic periaortic tissue being morphologically similar to BAT (Fitzgibbons et al., 2011), this assumption becomes further validated, especially when we consider that brown adipose cells have been shown to possess high levels of voltage-dependant K^+ channels (Lucero and Pappone, 1989). Furthermore, coronary arteries are significantly different to aortic vessels morphologically, with aortic models being substantially bigger and designed to bear much higher-pressure tolerances, exhibiting greater contractile stability than that of the smaller coronary models and this distinction between the two could disqualify the above comparative assessment, despite the interesting parallels.

4.4 PVAT augments the contractile response to NA in mouse aortae

NA is a catecholamine which is a core component of the mammalian bodies "fight or flight" response, in which its general function is to instigate brain and bodily mobilisation. Regarding vascular function, NA is a highly potent mediator of blood pressure via vasoconstriction, acting via direct interaction with α_1-adrenergic receptors on VSMC cell surfaces to initiate contractile events (Graham et al., 1996). In this study, NA was chosen to both confirm and quantify its vasoconstrictive properties in our mouse models and to determine what effect the presence or absence of PVAT would have on vascular contraction under these conditions.

The aortic rings of control mice subjected to a NA concentration-response showed significantly diminished contractility in -PVAT samples when compared to +PVAT samples, inferring that PVAT augments NA-induced contractions. This is counter to other literature, in which it has been documented that there is typically no difference in contractile events between +PVAT and -PVAT samples when subjected to NA, and in some instances the removal of PVAT can enhance arterial contractions, this being due to NA uptake, metabolism and storage within the PVAT when it is present and a loss of PVAT-derived relaxing factors when it is not (Soltis and Cassis, 1991; Ayala-Lopez et al., 2014; Bussey et al., 2018).

Inversely, there has been evidence to show that PVAT can promote arterial contraction in response to exogenous agonists, though this typically occurs in disease states, including obesity and hypertension (Gao et al. 2006; Lee et al. 2009; Owen et al. 2013). Phenotypic modifications to the PVAT in the mouse models used in this study (such as beiging, or changes in adipokine production and release) could cause them to behave like PVAT in the above disease states, but without further testing this hypothesis is currently unclear.

To further postulate the observed contractile augmentation of NA in PVAT, it is possible to look towards PVAT's own innate stores of catecholamines. Evidence to support the storage of functional catecholamines within PVAT has been shown via the induction of tyramine to PVAT-intact rat aortic tissue, in which NA release was detected and recorded (Ayala-Lopez et al., 2014). Thus, one cause for

the observed pro-contractile effects of PVAT in response to NA could be due to the release of these stored NA reserves, though this assumption is potentially tenuous as there has been no currently observed mechanism of release for PVAT-stored NA in response to exogenous NA. Further studies could confirm if PVAT-stored NA is released upon introduction to exogenous NA via the use of an enzyme-linked immunosorbent assay (ELISA). Measured concentrations of NA could be introduced to a PVAT sample in which quantitative levels of NA could be recorded via the ELISA. Heightened NA levels than the administered dose would suggest NA does in fact modulate PVAT-stored NA release.

Though following this, a compelling query for this pro-contractile effect lies in NA's potential to release other pro-contractile elements from within the PVAT. Based on the NA response and with this route of questioning in mind, the decision was made to selectively test for other specific vasoconstrictive mechanisms and elements in following assays to discern the reasoning behind these results.

4.5 Interactions between β3 adrenoceptors and NA do not attenuate or augment contractility in mouse aortae both with and without PVAT

In an effort to ascertain NA's point of interaction and any potential interactions involved in its augmentation effect, one of its known targets within PVAT was chosen for study; the β-3 adrenoceptor (β3ADR). Typically, NA binds to β3ADR causing the activation of G proteins, specifically the Gs alpha subunit (Gαs), which leads to the instigation of the Gαs-signalling pathway and the activation of adenylyl cyclase. Subsequently, this causes an increase in cyclic adenosine monophosphate (cAMP), finally leading to the release of vasorelaxant NO (Bussey et al., 2018).

In relation to the results generated, it was expected that NA would cause the release of PVAT-derived NO, offsetting or diminishing the contractile effect, which based on our results did not occur. This prompted the use of the β3ADR antagonist SR59230A to observe how blocking this receptor and subsequent mechanisms would affect contractility in the same NA dose response assays of our control mouse aortic samples.

Interestingly, the results showed that the blockade of β3ADR receptors in PVAT showed no change in contractility when compared to control PVAT. This demonstrated that the observed contractile events were uninfluenced by NA-β3ADR interactions. This suggests that β3ADR interactions did not cause the release of NO in enough quantity to affect contractility. Another possibility is NA is modulating the effects of NO release in some other capacity whether β3ADR is blocked or not, possibly by activation of other pro-contractile elements. Or there were diminished or minimal levels of free NO within the PVAT itself before testing began.

In the same assay, performed on PVAT-removed aortic tissue, there was again no significant difference in levels of contractility between control models and those subjected to the β3ADR antagonist. While not wholly relevant to the topic of PVAT, this at least suggests that the NA-induced contractions are not influenced in the vascular tissue by β3ADR interactions either.

It should be worth noting that in using a β3ADR antagonist, β3ADR was not just blocked within PVAT but also in the surrounding tissue itself. These data suggest that β3ADRs do not play an active role in controlling contractility within the mouse aorta, or that β3ADRs are simply not present. Currently, there is no research documenting the presence of β3ADRs in the aortae of murine models, but in relation to the wider topic of study in this project it has been shown that β3ADRs are present in the human IMA (Rozec et al., 2005), to which our murine aorta has been presently used as an analogue. This should be taken into consideration if future studies see the use of β3ADR antagonists in human IMA models.

In progressing with further testing involving β3ADR receptors and their blockade, a continuation of this line of testing could be to confirm and quantify NO levels within the tissue samples, potentially via high performance liquid chromatography (HPLC) determination or fluorometric determination assay as is typically used (Bryan and Grisham, 2007).

4.6 Chemerin does not augment the vasoconstrictive response to NA in mouse aortic models

Furthering efforts to identify other pre-contractile or pro-contractile factors that could augment NA-induced contractions in PVAT, the study turned to look at vasocontractile adipokines. Of the selection previously discussed (section 1.2.4.2.3), examination of the chemoattractant adipokine chemerin was chosen. As before, chemerin has been found in abundance within adipocytes and stromal-vascular cells (Bozaoglu et al., 2007; Goralski et al., 2007), whereup it interacts with its ligand CMKLR1, which has been shown to induce pronounced contraction events (Watts et al., 2013).

CMKLR1 was selected to be targeted and blocked, to observe any change in contraction within the mouse aortic models, under the influence of NA as before, again to elucidate chemerin's involvement or lack thereof. To accomplish this the compound α-NETA was selected for use, as it was found to be a small molecule CMKLR1 antagonist, acting by inhibiting chemerin-stimulated β-arrestin2 association to CMKLR1 (Graham et al., 2014).

If chemerin were to have a positive vasoconstrictive effect in our tests we would expect to see a decrease in contractility in the CMKLR1-inhibited models. Interestingly though, the use of α-NETA and subsequent blocking of CMKLR1 caused no significant difference in contractility in our PVAT-intact models, indicating that chemerin does not augment or influence NA-induced contractions in PVAT. This agrees with other literature which also observed that chemerin did not directly amplify NA-induced contractions (Darios et al., 2016).

Interestingly, PVAT-removed models showed a small degree of heightened contractility when subjected to α-NETA as opposed to controls, which may indicate a pro-contractile interaction between α-NETA and the vascular adventitia when undergoing NA-induced contractions. Of note are the large error bars and the low value of statistical difference in these tests, meaning results should be interpreted with caution until repeated assays can be performed, and this phenomenon further confirmed and quantified.

One further observed effect on the underlying vasculature is that the resting baseline tension of both -PVAT and +PVAT models incubated with α-NETA were significantly higher than the resting baseline tension of -PVAT and +PVAT control models, indicating that α-NETA itself could potentially have some vasoconstrictive properties or could antagonise another form of vasoconstrictive action, unrelated to NA-induced contraction, which is unknown to us at this time. Confirmation of this effect would require further research but would suggest α-NETA exhibits minor vasoconstrictive action, despite blocking the stronger vasoconstriction events which would be brought about via Chemerin/CMKLR1 interactions. Contrary to this, the α-NETA concentration used within this study has been documented as selectively only effecting CMKLR1 (Graham et al., 2014), suggesting hitherto unknown interactions between α-NETA and other pro-contractile receptors and pathways.

Based on these results, it would be pertinent to continue future studies under the same template; identify potential vasoconstrictive adipokines and adapt testing conditions to inhibit or block their effector receptors to elucidate further NA-related constrictor actions. Adipokines identified for further and future testing include those mentioned previously (section 1.2.4.2.3); resistin and resistin as

vasoconstrictors that aid in the augmentation of constriction when NA is present, and adiponectin and visfatin as vasorelaxants which may become inhibited by NA.

4.7 The loss of eNOS augments contractile events in both PVAT-intact and -removed mouse aortic models

To satisfy the second criteria of the study, wherein the aim to investigate the role of eNOS in the contractility of conductance vessels was presented, a repeat of the previous model of testing using eNOS knockout mice ($eNOS^{-/-}$) was performed. This was to remove the vasorelaxant ability of NO and subsequently assess its importance in counterbalancing vascular constriction. By performing the same NA concentration-response assay as previously with the control mouse studies, NO's presence and potency in conductance vessels both with and without PVAT was hoped to be elucidated.

In the results it was observed that the loss of eNOS caused greater contractility in both the PVAT-removed and PVAT-intact $eNOS^{-/-}$ models when compared to control mice under the same conditions, as was expected with the loss of NO, aligning with other previously documented results (Hodges et al., 2006; Atochin and Huang, 2010; Li, Youn and Cai, 2015) and confirming eNOS's vasorelaxant role as well as its presence and importance in aortic vessels.

Interestingly, when comparing contractility in our PVAT-intact $eNOS^{-/-}$ models to contractility in our PVAT-removed $eNOS^{-/-}$ models, we again observed that PVAT elicited a pro-contractile effect when subjected to NA, again affirming our previously observed results in control models wherein NA's efficacy is augmented by PVAT, regardless of NO interaction.

These results highlight the importance of NO and its role as a vasorelaxant, specifically in rodent conductance vessels. In disease states that withdraw or reduce NO it has been observed to lead to higher degrees of contractility, such as in hypertension (Li, Youn and Cai, 2015), which we can then potentially confer to human models in the same test scenarios. Also, our testing with $eNOS^{-/-}$ models further reinforced previously observed results that PVAT contains pro-contractile functionality and augments NA-induced contractile events.

An interesting counterpart to this line of testing would be to observe the effects of NO in excess, via a transgenic mouse model modified to over-express eNOS either globally or specifically within aortic PVAT, this to further determine and quantify NO in aortic vasorelaxation. One study showed that mouse models overexpressing eNOS in the vascular wall are hypotensive, displaying strictly attenuated vasoconstrictive responses due to elevated basal NO (Ohashi et al., 1998) while another demonstrated that mice overexpressing eNOS displayed a six-fold increase of eNOS within the aorta itself (Sansbury et al., 2012).

4.8 Human IMA and SV myography testing and optimisation

In receiving human tissue samples of the IMA and SV, the initiation of proof-of-concept myography assays proceeded. The assays were based on the previous mouse model myography protocols but scaled to account for the larger physical presence and stronger tensile properties of human tissues and adapted from previous human aortic myography protocols (Gao et al., 2003). Human tissue assays were unable to progress past optimisation-based staging (See: Study Limitations) but early models in which human IMA was subjected to a NA dose response (section 2.4.2.4) showed promising results.

5. Study limitations

5.1 Global COVID-19 pandemic

With the onset and development of the global coronavirus 2019 (COVID-19) pandemic and the ensuing country-wide lockdown in the United Kingdom taking effect in March 2020, sudden limitations in the ability to continue laboratory operations and testing were imposed, and as a result all documented data were recorded in the time period between October 2019 and March 2020, halting thereafter.

Regarding our collaboration with the Blackpool Victoria hospital, their ability to perform CABG surgical procedures and our subsequent acquisition of human tissue samples became untenable as all hospital work was directed to pandemic-focused areas, non-critical surgical procedures were delayed, and all clinical trials were suspended. As such, human tissue sampling was unable to progress past the stated optimisation staging.

5.2 Animal models and their clinical relevance to human models

As studies in human models are the most direct and valuable source of understanding regarding human physiology and, with regards to this study, the mechanisms underlying vascular activity there are significant limitation associated with both the acquisition and use of human tissue. Logistically human tissue is less abundant in its procurement, especially when related to the topic of study, as the rate of CABG surgical procedures directly limited the rate at which tissue was harvested, with patient recruitment and approval to the clinical trial further limiting the potential sample number. Regarding human samples generally, the influence of lifestyle and comorbidities can affect tissue phenotype and thus cause variable results. With these considerations in mind, the necessity of animal models becomes clear, as they can be procured in quantities to meet testing requirements at much more rapid rates due to high breeding cycles and relatively low cost. Environmental factors in animal models can also be controlled much more easily than that in human models, such as in variables like diet, health and age-range.

It should be noted that control mice do not spontaneously develop atherosclerosis or hypertensive disorders (Lakatta, 2002) and because of this are considered to functionally represent states of vascular health. Inversely, the physiology of mice is considerably different to that of humans, despite many similarities, and thus phenomena observed in mouse models may not be entirely reflected in human models. Additionally, the use of a genetic knockout model ($eNOS^{-/-}$) confers an irregular change that is not present in standard human or mouse models, and because of this there may be hitherto unforeseen changes in physiology which could potentiate a change in results which may not be observed or occur in control models.

All mice used in this study were male in order to bypass the continuous polyoestrous effects in female rodents which alter NOS enzyme levels (Caligioni, 2009; Menazza and Murphy, 2016), thus eliminating one variable.

With regards to human models in this study, tissue samples were obtained from patients qualifying for CABG surgical procedures and thusly their vasculature would be identified to be in a state of unhealth, be it via intimal thickening, hypertension or otherwise, which should be taken into consideration on analysis. Relating back to the variable differences between humans, gender also plays a role in vascular health and phenotype, thus there may be inherent difference between male and female samples even of the same age range and health. For example, it has been shown that women develop atherosclerotic conditions on average 10 years later than in men (Pérez-López et al., 2010).

With this considered, while no animal models will perfectly mirror human physiology and bodily conditions, mice offer a close analogue which can be easily and quickly tested, to inform and give an underlying knowledge regarding the mechanisms that underpin vascular health and disease.

5.3 Assessment of vascular function via myograph

Myography as a technique is invaluable in its ability to study isolated vessels and allows us to quantifiably measure the vascular mechanisms of vasorelaxation and vasoconstriction in controlled test environments, bypassing the many complications presented by working in vivo, including sympathetic nervous stimulation, activity and the resultant endogenous hormone release and action. Despite its benefits, myography presents its own complications when directly assessing data. For example, the substance concentration used in myography assays is often non-comparable to physiological conditions, as substances are usually introduced in levels considered to be in excess of natural levels. Additionally, the environment the vessels are subjected to within the myograph baths causes the luminal surface of the vascular tissue to be directly exposed to substances and surfaces it would be otherwise be closed off to naturally, and luminal tissue also becomes directly exposed to exogenous PVAT products, which would otherwise be buffered by passage through the adventitia in vivo (Gollasch, 2012).

Mechanistically, it is also possible to damage the vasculature either within initial dissection of vascular rings prior to mounting, or in the mounting process itself. If mechanical handling is poorly performed, damage can easily occur in the vascular endothelium and adventitia, either skewing or invalidating any observed results. Care must also be taken when considering tensile levels, especially during equilibration periods, so as not to over-stretch vascular rings and avoid damage and distress of the tissue.

6. Concluding remarks

These data have provided interesting insights into PVAT and its role on vascular health and reactivity, with novel interactions between aortic vasculature and NA in both control and genetically modified eNOS knockout ($eNOS^{-/-}$) mouse models, giving us new perceptions of how the complex systems underpinning vascular contractility and relaxation function. Also, groundwork regarding the optimisation of human tissue studies has allowed us to develop proof-of-concept protocols for the continuation and propagation of human vascular model trials, so that future testing may commence apace upon resumption.

In this study it was further reinforced that the role of PVAT in vascular function is not simply one of support, with our models repeatedly demonstrating that its presence actively augmented contractility in response to the catecholamine NA. With some literature displaying the inverse, this knowledge allows further questioning of the complex and delicate balance that exists within PVAT regarding active pro-contractile and pro-relaxant elements, and their interactions with one another. When investigating this balance, we were able to conclude that certain mechanistic pathways were not involved in this process, ruling out chemerin involvement and ADRB3 interactions as compensatory or inhibitory factors, respectively. It was observed that this pro-contractile involvement was also present in models lacking eNOS, demonstrating that this pro-contractile effect was not influenced by vasorelaxant NO.

When discussing these elements, and in relation to how they could potentially effect revascularisation efforts, we must consider the type of adipose tissue featured in these tests, namely BAT. With BAT having a different function and physiological makeup to that of WAT, it should be clear that interactions and outcomes observed in one adipose tissue type may not occur the same, if at all, in the other. With that considered, and when relating to the overarching purpose of understanding and improving revascularisation within CABG, it is important that we aim to further identify if these pro-contractile interactions occur in human tissue featuring the same or similar adipose deposits, such as in BAT and BAT-like deposits in the human IMA, and how this may affect the post-surgical recovery of CABG patients.

While the results of this study have answered some questions, it has also posed more regarding vascular contractility and how we might improve tissue revascularisation procedures, leaving us with the exciting potential for more continued and focused investigation.

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