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Vascular function in dogs with myxomatous mitral valve disease

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Declaration

I hereby declare that all work carried out during this MScR was performed by myself, unless otherwise stated, under the supervision of Dr Geoff Culshaw and Dr Natalie Jones. This thesis has not previously been submitted for any other degree of qualification.

Signed: Date: 30/08/2022

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Abstract

Myxomatous mitral valve disease (MMVD) is the commonest heart disease in dogs. Vascular dysfunction contributes to human heart disease but has not been assessed in pet dogs by myography. We hypothesised that vascular dysfunction contributes to progression of MMVD and can be quantified with myography.

Isomeric myography was performed on femoral, mesenteric, renal, and pulmonary arteries from pet dogs euthanised for welfare reasons. Testicular arteries were collected from dogs undergoing routine castration as a control group. Histopathology analysis was made for confirmation of arterial tissue. MMVD was graded 1-4 (Whitney classification). Arteries were stretched to an internal circumference (IC_1) that generated maximal tension (mmHg) to high potassium concentrations (normalisation). The ratio of $IC_{1:}IC_{100mmHg}$ (normalisation factor) was calculated for every artery. Vasoconstriction to phenylephrine, and vasodilation to acetylcholine (endothelial dependent) and sodium nitroprusside (endothelial independent) were assessed by cumulative response curves. Concentrations of agents that gave 50% maximal response (EC_{50}/IC_{50}) were calculated. Response to high potassium physiological salt solution (KPSS) was calculated as variation in percentage between minimum and maximum response and results for tissue from the same dog but on different days were compared.

Histopathology confirmed all the samples processed were arteries. Normalisation factors were determined for renal, femoral and mesenteric artery. Impaired endothelial dependent vasorelaxation was identified in all dogs with MMVD graded 3 and 4 and in some arteries of dogs with MMVD graded 1 and 2. Pulmonary and testicular arteries failed to show response on myograph. No significant difference was identified in KPSS response in the same dog over seven days.

Isomeric myography is feasible in pet dogs and can be used to investigate vascular dysfunction. Impaired endothelial-dependent relaxation was

identified more frequently in dogs with higher MMVD grade. Further investigation is required to establish the relationship between MMVD and vascular dysfunction.

Lay Summary

Myxomatous mitral valve disease (MMVD) is the commonest heart disease in dogs and can lead to congestive heart failure and death. The blood is pumped by the heart to the body through a series of tubes called blood vessels. The flow is regulated by several mechanisms, one of this is the ability of blood vessels to contract and relax. This ability is called vascular function and is controlled by different signalling proteins. Vascular dysfunction is when the between contraction and relaxation is abnormal. In people vascular dysfunction is well known to play a role in heart diseases. Isometric myography is the gold standard technique after death to study vascular function but has never been used in pet dogs. In this study we used isometric myography in pet dogs euthanised for welfare reasons to study vascular function and to look at whether it might be linked to MMVD. The results showed that vascular dysfunction was present in dogs with more advanced MMVD compared to dogs with mild MMVD. We conclude that isometric myography can be performed on tissue from pet dogs and can be applied to lots of conditions that might affect vascular function. More work needs to be done to determine whether vascular dysfunction is clinically relevant to MMVD.

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List of abbreviation

ACE: angiotensin converting enzyme

ACEi: angiotensins converting enzyme inhibitor

ACh Acetylcholine

ADMA: asymmetric dimethyl arginase

ANG II Angiotensin II

ANOVA Analysis of variance

ATE Arterial thromboembolism

cAMP Cyclic adenosine monophosphate

ECE Endothelin converting enzyme

EDHF Endothelium derived hyperpolarisation factors

ET Endothelin

KPSS High potassium physiological salt solution

logEC_{50} logarithm half maximal effective concentration

logIC50 logarithm half maximal inhibitory concentration

MMVD Myxomatous mitral valve disease

NO Nitric oxide

NOS Nitric oxide synthase

PE Phenylephrine

PSS Physiological salt solution

ROS Reactive Oxygen Species

SD Standard deviation

SNP Sodium nitroprusside

TxA_2 Thromboxane A_2

VEGF Vascular endothelial growth factor

VSMC Vascular smooth muscle cells

Up4A Uridine adenosine tetraphosphate

Contents

Chapter 1 Introduction

Myxomatous mitral valve disease (MMVD) is the commonest heart disease in dogs[1]. Although new treatments have improved the outcome for many affected dogs in recent years, the prognosis for individual dogs and response to treatment is highly variable. This reason for this remains unclear. In people, the role of endothelial dysfunction in cardiovascular disease is well established[2]. Research over the last decades has linked vascular function with outcome, raising the possibility that the same might apply to dogs. Although there are studies that have looked at vascular and endothelial dysfunction in dogs, there are no studies in which isometric myography, the gold standard *ex vivo* technique to study vascular function, has been used in arteries collected from pet dogs.

1.1 History about vascular research

The study of the cardiovascular system originates in ancient times. In the 2nd century CE, Galen (126 – c. 210 CE) was the leading medical authority in the Roman Empire and his model was considered valid in the following centuries[3]. He believed that the liver was the source of all the veins, and the site where food digested by the stomach (chyle) was transformed into blood; the heart was the source of innate heat (particularly the left ventricle), and the function of the lungs was to cool it down via the pneuma (originating from the air inspired into the lungs) that accompanied the blood in the arteries. To explain the presence of blood in the arteries, Galen thought that there were pores in the interventricular septum of the heart that allowed the blood to pass from the right ventricle to the left[3]. In 1628 William Harvey (1578 – 1657) published *"Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus"* (An Anatomical exercise on the Motion of the Heart and Blood in Animals) describing a model in which the blood circulates through the body, moving out through the arteries and back through the veins, with the movement made possible by the mechanical activity of the pumping heart[3]. Harvey could not demonstrate the connection between arteries and veins, but in 1661 Marcello Malpighi (1628 – 1694) discovered the presence of capillaries under the microscope[4]. In 1865 Wilhelm His (1831 – 1904) introduced the term endothelium, triggering subsequent research on its role and dysfunction in cardiovascular

disease[5]. Following Harvey's discovery, in 1840 Friedrich Henle (1809 – 1885) suggested that the muscle fibres in the walls of blood vessels were controlled by nerve fibres, which was experimentally confirmed a decade later by Claude Bernard (1813 – 1878) and by Charles-Édouard Brown-Sèquard (1817 – 1894)[6] opening the way to study vasoconstriction and vasodilation as the components of vascular function. In the last century more discoveries have been made, using new methods to study the cardiovascular system, and, in particular, vascular function and its impairment. The historical progression of discovery made over centuries can teach us the importance of looking in different directions even when physiology and pathophysiology mechanisms seem to be already fully understood. Likewise, in veterinary medicine the study of cardiovascular diseases has almost exclusively focused on the heart. Only in recent years researchers have started to look to the vascular compartment for answers.

1.2 Overview of the vascular function

1.2.1 The vascular system

The vascular system is a complete circuit of blood, which is distributed through the body via a system of arteries, capillaries and veins[9]. The aim of this system is to maintain the homeostasis of the body. This role is achieved by transporting nutrient molecules to the tissues, transporting away waste products, carrying chemical information molecules, and distributing heat energy in the body[9]. It is divided into the pulmonary circulation, which contributes to gas exchange in the lungs, and the systemic circulation, which provides nutrients to the organs[9]. A typical blood vessel can be divided into three layers (figure 1): the innermost *tunica intima* (formed mainly by endothelial cells), the *tunica media (*elastic fibre and smooth muscle cells) and the outermost *tunica adventitia* (connective tissue)[10]. Arteries can be divided into conducting arteries (e.g., aorta), which contain a large amount of elastin that allows the vessel to expand and recoil to accommodate pulsatile ventricular output; muscular and elastic conduit arteries (e.g., femoral artery) that conduct the blood to specific regions of the body, and arterioles or resistance arteries that are characterised by a *tunica media* of predominantly smooth muscle cells highly innervated by sympathetic nervous fibres. Arterioles regulate blood flow via

constriction or dilation of their diameter[11]. Downstream of arterioles, the capillaries form the microcirculation, where the diffusion and transfer of gases, nutrients and metabolites occur between the vascular system and the tissues[10]. Many capillaries arise from each arteriole, in this way the combined capillary cross-sectional area is greater than for each arteriole, but the flow velocity is less, aiding exchange[11]. From the capillaries the blood passes through the venules and then veins, which function as capacitance vessels, returning to the heart[11]. The cardiovascular system, therefore, guarantees delivery of nutrients to the tissues and removal of waste products.

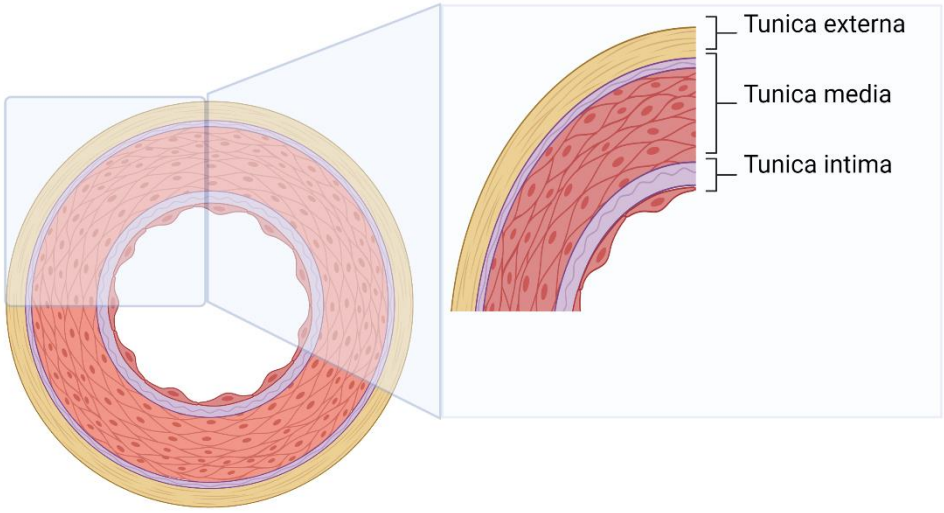


Figure 1. Section of a muscular artery in which is possible to visualise all the layers, from the innermost: *tunica intima* with endothelial cells*, media* with smooth muscle cells*,* and *adventitia (externa)* with perivascular tissue. Image created with BioRender.com.

1.2.2 Vascular function and blood pressure

Blood pressure is partly regulated through vascular function: vasoconstriction increases it, and vasodilation reduces it[11]. In human medicine, several conditions such as diabetes mellitus, obesity, dyslipidaemia, and aging can induce arterial stiffness, impairing vascular function and contributing to increased blood pressure

and heightened cardiovascular risk[11]. Therefore, measuring blood pressure represents an important and easy-to-measure biomarker, although not the only one, that monitors vascular function and risk of cardiovascular disease. In veterinary medicine, there is lack of studies on vascular function and its contribution to disease morbidity and mortality, including its role in systemic hypertension. Indeed, the consequences of disruption to blood pressure regulation in are poorly understood in veterinary species, although a role for hypertension in target organ damage, especially of the heart, kidneys, retinas, and brain, is at least recognised [12]. One of the reasons for this lack of information could be the difficulty in reliably measuring blood pressure in companion animals[12]. There is therefore an unmet need to determine the significance of vascular dysfunction in veterinary medicine to provide a basis for subsequently measuring it more effectively *in vivo.*

1.2.3 Role of the nervous system in vascular function

The central nervous system controls the vascular function via inducing vasoconstriction through the sympathetic system (release of catecholamine) and vasorelaxation through the parasympathetic system (release of acetylcholine (ACh). The sympathetic nervous system acts on the vasculature via releasing noradrenaline which binds to α_1-adrenergic receptors present on the vessels[13]. As consequence of this, phospholipase C is activated, which leads to production of diacylglycerol and inositol triphosphate, ending up in the release of calcium (Ca^{++}) that causes contraction of the vascular smooth muscle cells (VSMC)[13]. Phenylephrine (PE) is an α_1-adrenergic receptor agonist; therefore, it acts like noradrenaline on these receptors to cause vasoconstriction[14]. The parasympathetic system plays a relatively minor but significant role in control of vascular tone[13], however, the administration of ACh induces vasorelaxation by stimulating the endothelium to produce nitric oxide (NO) and endothelial-derived relaxation factors[15], described in more detail in the next session. Where there is endothelial dysfunction, ACh can have a paradoxical effect and induce vasoconstriction at high doses[16]. Sodium nitroprusside (SNP) is an endothelium-independent vasorelaxation factor that acts as a NO donor, so it induces vasorelaxation even if the endothelium is damaged and cannot produce NO by itself[17].

1.3 The endothelium

The endothelium coats the inner surface of entire vascular system, providing an interface between circulating blood and the wall of the blood vessels[18]. In arteries and veins, the endothelial cells appear to be more continuous and thicker compared to those present in the capillaries, which can be fenestrated, sinusoidal and thinner[19]. Moreover, endothelial cells have a different response to stimuli in different vascular beds[20]. Although it was originally considered to be only a barrier, now it is clear that the endothelium plays an important role in vascular function[21]. Endothelial cells control vascular function by responding to various hormones, neurotransmitters and vasoactive factors[21]. They also, themselves, release vasoactive factors with vasodilatory or vasoconstrictive activity[21].

1.3.1 Endothelial vasodilator factors

Nitric Oxide (NO) is a freely diffusible free radical that is permeable to cell membranes[22]. It is synthesised by endothelial cells from L-arginine in a two-step oxyreduction reaction led by the enzyme NO synthase (NOS) in the presence of NADPH, tetrahydrobiopterin (BH_4), guanosine monophosphate cyclic (cGMP) and the Ca^{2+}/ Calmodulin protein[23]. First, there is the hydroxylation of L-arginine to N-hydroxy-L-arginine; then the oxidative deamination, with L-citrulline and NO as final products[23]. NO exerts a vasodilatory effect via activation of guanylyl cyclase and accumulation of cGMP[22]. It has other downstream effects. NO is an antioxidant, inhibitor of leukocyte adhesion, protector against toxicity and peroxidation, regulator of cell adhesion and vascular permeability, neurotransmitter, bronchodilator, and inhibitor of platelet activation[24]. NO has also deleterious effects in association with excessive production and reaction with superoxide radicals; these effects include inhibition of enzymatic function, induction of DNA damage, induction of lipid peroxidation, and increased susceptibility to radiation, alkylating agents and toxic metals[24].

Prostacyclin is a prostaglandin produced from membrane-bound phospholipid by a series of enzymes including phospholipase A_2, cyclooxygenase and prostacyclin synthase. Its vasodilatory effect is via activation of adenylate cyclase that leads to cyclic adenosine monophosphate (cAMP) formation[25].

Endothelium Derived Hyperpolarising Factors (EDHF) are not fully characterised but cause vasodilation even when the actions of prostacyclin and NO are inhibited[26]. Their action is inhibited by calcium activated potassium channels (K_{Ca}) inhibitors or by excessive extracellular potassium, suggesting they cause vasodilation via hyperpolarisation of the endothelial cells and, subsequently, VSMC[27]. Currently, the most likely candidates as EDHF are arachidonic acid metabolites[28], hydrogen peroxide ($H2O_2$)[29], potassium ions[30] and gap junction transmitters between VSMC and endothelial cells[31]. More recently, another factor, hydrogen sulphide (H_2S), has emeged[32]; low concentrations of this gas can regulate vascular tone, although the exact mechanisms have not yet been elucidated[27]. H_2S may be more important in resistance arteries, inducing hyperpolarisation of the plasma membranes, endothelial cells and VSMC[33].

1.3.2 Endothelial vasoconstrictor factors

Endothelin (ET-1) is a protein released by the endothelium in a biologically inactive form (pro-ET-1) and converted into an active form by endothelin converting enzyme (ECE)[34]. It is a potent vasoconstrictor, the effect is mediated by two receptors, ET_A ET_B, in the VSMC[35], although the effect is buffered by ET_B receptor-mediated NO release from endothelial cells. Stimuli of ET-1 release include thrombin, adrenaline, angiotensin II, bradykinin, hypoxia, high- and low-density lipoproteins, insulin, ischemia, shear stress, and growth factors[35]. In people, high levels of circulating ET-1 were observed in patients with severe cardiovascular disease[35]. Particularly, pulmonary arteries seem to be highly sensitive to ET-1, and inhibitors of ET_A and ET_B receptors are used as treatment of pulmonary arterial hypertension[36].

Angiotensin (Ang II) is a hormone that reduces sodium and water loss, maintaining the volume of extracellular liquid and arterial pressure[37]. All the constituents of Ang II, which include angiotensinogen, renin, angiotensin converting enzyme (ACE), have been found in different vascular layers, and the tissue formation of Ang II is independent of circulating angiotensinogen and renin, although the importance of this is under debate[38]. On the vasculature, it induces vasoconstriction, decreases blood flow, and increases vascular resistance[38]. Pro-inflammatory, pro-oxidative, proliferative, and pro-fibrotic properties of Ang II have also been observed[38]. It also

stimulates the release of aldosterone as part of the renin – angiotensin – aldosterone system, a well-known mechanism associated with heart failure[38]. The vasoconstriction effect is explained by increased cytosolic Ca^{2+} that induces contraction of the VSMC[39, 40].

Reactive Oxygen Species (ROS) are highly reactive molecules characterised by the presence of oxygen with one or more unpaired electrons in the outer orbital[40]. It was observed that these molecules have vasoconstrictive effects[41]. This action is explained by two mechanisms, one in which ROS reduce the capacity of the endothelium to promote relaxation via NO, and a second one in which ROS interact with signalling pathways involved in the proliferative responses of VSMC[42, 43].

Thromboxane A_2 (TxA_2) is an eicosanoid formed from the metabolism of arachidonic acid or other lipid substrates[21]. It binds the thromboxane-prostanoid receptors (TP) present on the VSMC causing vasoconstriction[44]. This effect seems to be greater in hypertensive animal models than in physiological conditions, possibly due to an increased levels in the presence of hypertension[45].

Uridine adenosine tetraphosphate (Up4A) is a dinucleotide containing purine and pyrimidine that has vasoconstrictive properties, although the mechanisms are unknown[46]. Up4A has also been shown to induce vascular calcification, activation of inflammatory mediators, proliferation and migration of VSMC, and generation of ROS[47, 48].

1.3.3 Endothelial dysfunction and cardiovascular diseases

Endothelial dysfunction is characterised by an imbalanced vasomodulatory function, leading particularly to decreased vasodilator activity and increased vasoconstrictive activity[49]. There is evidence that endothelial dysfunction plays a central role in several conditions in humans[50]. A classification of endothelial dysfunction has been proposed by Evora et al.[51] and is summarised in Table 1.

Endothelial dysfunction classification	a	b
I Etiological classification	*Primary or "genotypic" endothelial dysfunction*: shown in children with homozygous Homocystinuria, and in normotensive patients with a family history of essential arterial hypertension	*Secondary or "phenotypic" endothelial dysfunction*: present in all cardiovascular diseases (atherosclerosis, coronary artery diseases, arterial hypertension, diabetes, and others)
II Functional classification	*"Vasotonic" endothelial dysfunction*: present in cardiovascular diseases, implying risk of vasospasm and thrombosis	*"Vasoplegic" endothelial dysfunction*: present in distributive shock status (sepsis, anaphylactic shock, anaphylactoid reactions, and vasoplegy related to extracorporeal circulation) due to the action of cytokines that stimulate increased pathological release of endothelial relaxing factors, especially NO
III Progressive or prognostic classification	*"Reversible" endothelial dysfunction*: most probable occurrence in the initial stages of "vasoplegic" dysfunctions. "Vasotonic" dysfunctions associated with cardiovascular diseases are hardly completely reverted	*"Irreversible" endothelial dysfunction*: present in advanced states of cardiovascular diseases and sepsis

Table 1. Classification for endothelial dysfunction proposed by Evora et al.[51].

1.3.4 Mechanisms of endothelial dysfunction

Patients with heart failure are known to have activation of the renin-angiotensin-aldosterone system and sympathetic nervous system, increased production of inflammatory cytokines, all leading to a reduction of NO bioavailability and consequently increasing oxidative stress[52]. Ang II causes a BH_4 deficiency in the endothelium via downregulation of dihydrofolate reductase, an enzyme involved in BH_4 bioavailability[53]. As a consequence of the lack of this cofactor, endothelial NOS transfers electrons from NADPH to O_2 instead of L-arginine, forming superoxide[54]. Superoxide reacts with NO, forming peroxynitrite anion, a molecule with cytotoxic potential that can alter mitochondrial function, DNA structure, and induce apoptosis of endothelial cells[55]. Although apoptosis is a defensive mechanism of a cell to spare neighbour cells, in chronic insults this process may be sustained with a greater loss of endothelial cells, compromising endothelial function[56]. Endothelial dysfunction is induced by heart failure and cardiovascular disease; however, it can also contribute to these conditions at central and peripheral levels[57]. As consequence of endothelial dysfunction, the arteries become stiff and less compliant, which can lead to left ventricular end-systolic stress, dilation and failure[57]. In patients with heart failure, ET-1 levels are increased, inducing hypertrophy of VSMC and vascular remodelling[57]. ET-1 can also induce hypertrophy of the heart by enhancing expression of foetal genes in cardiomyocytes[57].

1.3.5 Role of endothelial dysfunction in cardiovascular diseases

In some conditions, endothelial dysfunction seems even to be an aetiologic factor. In a study of biomarkers of oxidative stress, endothelial function was assessed in patients with mitral valve prolapse before and after surgical treatment, there was a reduction in markers of oxidative stress, whereas markers of endothelial dysfunction remained high six months after mitral valve repair, suggesting a primary role for endothelial dysfunction in the pathogenesis of the disease[58]. Few studies have investigated the association between endothelial dysfunction and the canine equivalent of mitral valve prolapse, MMVD. In one of these, flow-mediated vasodilation was measured in cavalier King Charles spaniels with MMVD at different stages, showing that it decreased as progressed. Although this study had

limitations such as the small number of dogs and repeatability of the ultrasonic method of assessment, it does raise the possibility of a role for endothelial dysfunction similar to that observed in people[59]. Another study compared reactive hyperaemia in healthy dogs with dogs in congestive heart failure due to MMVD or dilated cardiomyopathy and identified a reduction in vascular function when congestive heart failure developed, although the level of dysfunction was similar in MMVD and dilated cardiomyopathy[60]. Endothelial dysfunction may also be important in pulmonary arterial hypertension, where increased levels of ET-1 and oxidative stress increase the reactivity of the VSMC in the pulmonary arteries[61]; moreover, levels of ET-1 and NO are considered prognostic factors in patients with pulmonary arterial hypertension[61]. A similar role is supposed to occur also in dogs with pulmonary arterial hypertension secondary to MMVD, although no studies in dogs are available to my knowledge[62].

1.4 Vascular aging

Aging has several effects on both micro- and macrovasculature[63]. Several cellular and molecular mechanisms are described below:

- oxidative and nitrate stress: as described before, oxidative stress is associate with endothelial dysfunction through reduced NO availability. This can promote a proatherogenic vascular phenotype for aging[64].
- chronic low-grade sterile inflammation provoked by continuous antigenic load and stress, recently this inflammatory state has been named “inflamm-aging” to highlight the association between aging and inflammation. The overall effect is impaired vascular function[65].
- mitochondrial dysfunction: with age there is a reduced efficacy of the respiratory chain that increases the production of mitochondrial ROS, associated with age-related vascular dysfunction[66].
- cellular senescence is a process in which cells, including vascular and endothelial cells, undergo phenotypical changes in response to endogenous and exogenous stressors[67]; in particular endothelial senescence is implicated in the pathophysiology of congestive heart failure[68].

The main results of these mechanisms are an increased production of vasoconstrictor mediators and increased arterial stiffness, all factors that contribute to vascular dysfunction[69].

1.5 Clinical parameters that assess endothelial dysfunction

Due to its clinical importance, non-invasive diagnostic methods and biomarkers of endothelial dysfunction have been studied in patients with cardiovascular disease. Flow-mediated vasodilation can be measured non-invasively with ultrasonography. It measures the degree of vasodilation due to reactive hyperaemia of an artery (brachial artery in people) after ischaemia induced by compression with a blood pressure cuff or a torniquet[69]. Although the measurement seems to be mainly correlated with NO-dependent vasodilation, other pathways can affect the result[70]. As previously discussed, this technique has been studied in veterinary medicine, although with some limitations[59, 60]. Other non-invasive techniques include plethysmography of the forearm circulation, finger plethysmography and laser Doppler flowmetry[70, 71]. Biomarkers assess NO availability such as ROS and asymmetric dimethylarginine (ADMA), cell adhesion, inflammation and coagulation[72]. ADMA is a competitive inhibitor of NO production from NOS in plasma and its levels are correlated with NOS activity[73]. A recent study[74] in dogs with MMVD has also correlated levels of ADMA with different classes of MMVD, although a previous study showed that differences in ADMA levels in dogs can be affected by factors such as breed, sex and age[75].

1.6 Therapeutic targets for endothelial dysfunction

Endothelial dysfunction is a therapeutic target in people with cardiovascular diseases. Blockade of the renin-angiotensin-aldosterone mechanism is one the most explored therapeutic options, and in people, several studies have shown a beneficial effect from ACE inhibitors (ACEi) linked with restoring endothelium-dependent vasodilation[76]. Spironolactone, a mineralcorticoid receptor antagonist, also seems to have a beneficial effect by enhancing endothelial progenitor cells recruitment[77]. In dogs, a recent multicentre study showed that treatment with an ACEi and spironolactone did not delay the onset of congestive heart failure in dogs

with pre-clinical MMVD, although it did have a beneficial effect on cardiac remodelling that might be explained by improved endothelial function[78]. Another drug that seems to restore endothelial function is atorvastatin, a member of the statins group, that reduces cholesterol levels but also has pleiotropic and antioxidant effects, increases NO bioavailability, reduces of blood thrombogenicity, and inhibits proinflammatory responses[79]. Atorvastatin has been tested safely in healthy dogs and dogs with congestive heart failure, although further studies are needed to assess efficacy in congestive heart failure[80]. Carvedilol, a third generation β-blocker, improves endothelial-dependent vasodilation in people with dilated cardiomyopathy[81]. Cardiac resynchronisation therapy also has beneficial effects on endothelial dysfunction in patients with dilated cardiomyopathy[82]. Pimobendan is an inodilator with phosphodiesterase 3 inhibitory and calcium sensitising properties[83]. It has showed to delay the onset of congestive heart failure in MMVD[84], although, to my knowledge, no studies have been conducted in the prospective of endothelial function.

1.7 Myxomatous mitral valve disease

This is the commonest acquired heart disease in dogs and a common cause of left-sided congestive heart failure[1]. It is characterised by a myxomatous degeneration[85], which leads to failure of the valve to close tightly and therefore, regurgitation of into the left atrium during every systolic cycle, at the expense of output into the aorta (figure 2).

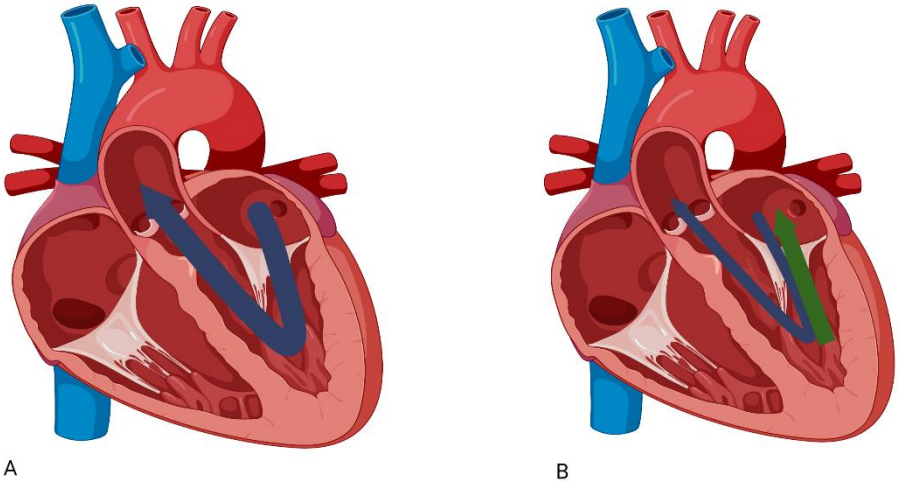


Figure 2. Cross section of the heart in which the pulmonary artery has been removed to allow visualisation of left ventricular outflow tract. A. The blue arrow indicates the blood flowing from the left atrium into the left ventricle and then into the aorta. B. The blue arrow is smaller to indicate less amount of blood is pumped into the aorta (blue arrow) because part of the total amount is pumped backward into the left atrium (green arrow). Images created with BioRender.com.

Gross pathological alterations are described according to the Whitney classification[86] (figure 3):

- Grade I: Few small discrete nodules in the area of contact associated with areas of diffuse opacity in the proximal portion of the valve.
- Grade II: Larger nodules are evident in the area of contact that tend to coalesce with their neighbours. Areas of diffuse opacity may be present.
- Grade III: Large nodules may be seen but many have coalesced into irregular, plaque-like deformities. These lesions extend to involve the proximal portions of the chordae tendineae.
- Grade IV: There is gross distortion and 'ballooning' of the valve cusp, the chordae tendineae are thickened proximally.

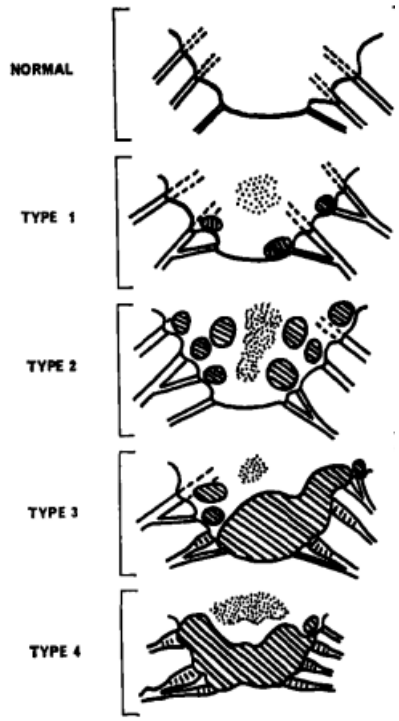


Fig 3. Schematic representation of the Whitney classification. Modified from Pomerance and Whitney, 1970.

Grades I and II are not associated with mitral valve insufficiency, which is instead present for grades III and IV lesions[86]. Valvular endothelial changes have also been described, particularly loss of endothelial cells and cellular pleomorphism, indicative of a reactive response by the endothelium and endothelial cells death[87]. Although there is evidence of valvular endothelial damage, to my knowledge there have examined endothelial function directly, using methods such as myography.

1.8 Isometric myography

A myograph is a device used to measure the force produced by a muscle during contraction under the influence of agonists and antagonists such as those described above[88]. VSMC contraction is most frequently studied by pressure and isometric myography (figure 4), both are *ex vivo* methods[88].

Fig 4. Multiwire Myograph System 610 M (DMT) at The Roslin Institute, with four wells which can be equipped with pin or jaws.

Pressure myography more reliably mimics physiological conditions and allows direct evaluation of the relationship between intraluminal pressure and VSMC contractility[88]. Isometric myography allows better access to the vascular endothelium and is the method of choice to discern the contribution of different mechanisms to vascular reactivity[88]. The myograph consists of a number of wells (figure 5) with jaws on which to mount wires or pins. One of the jaws or pins is connected to a micrometre (figure 6) that allows the operator to increase or decrease the distance between the two jaws or the two pins, thus stretching the vessel after mounting.

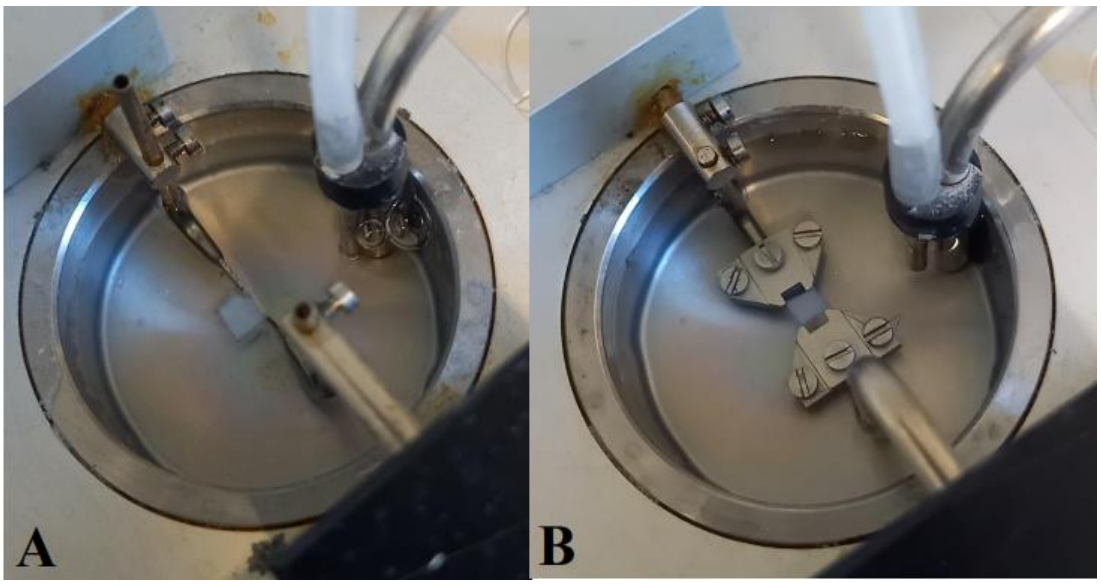


Fig 5. Wells with arteries mounted on pins (A) and wires (B). It is possible to notice the bubble coming from the more internal tube and connected with a gas cylinder to maintain the gas infusion into the medium (physiologic salt solution or high potassium concentrated physiological salt solution). The tube behind is connected to a vacuum system which allows to remove the medium by opening a valve. The arteries present a pale colour which is typical of the tissue after the collection and preservation.

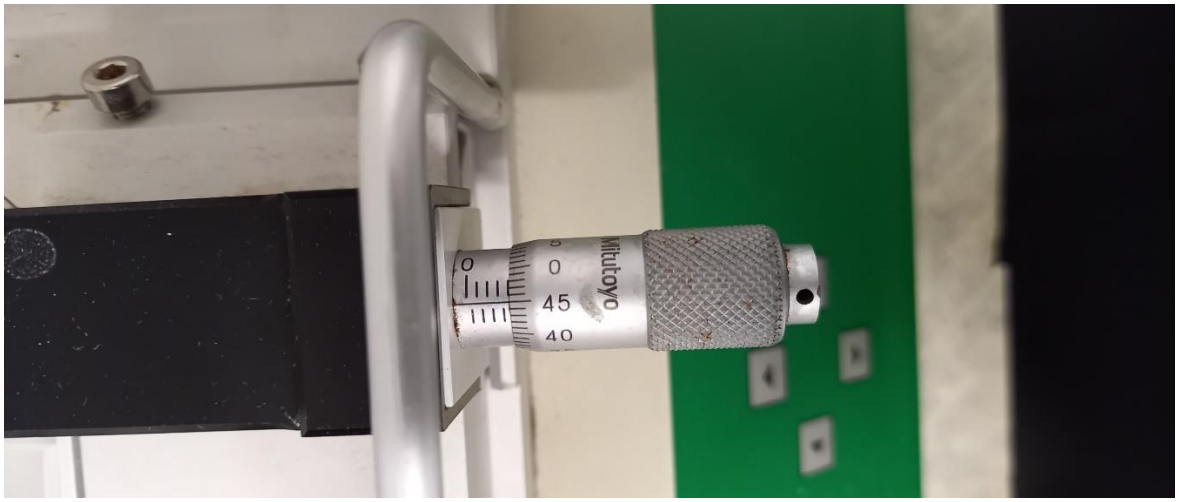


Fig 6. Detail of a micrometre. By turning it anti clockwise it allows to increase the distance between the pins or jaws, stretching the wall of the mounted artery. The sleeve has a total length of 25 mm divided into fifty equal parts, while the thimble is divided into fifty equal parts, a complete turn of the thimble equals 0.5 mm on the sleeve. The reading in the image is 3.5 mm on the sleeve to which are added 0.45 mm from the thimble, equalling 3.95 mm (3950 µm).

The reading on the micrometre allows measurement of the internal circumference (IC) of the vessel through the following formula[89]:

$$IC = r(2 + 2\pi) + 2gap$$

Where r is the radius of the pin or of the wire and gap is the distance between the two pins or wires from the micrometre reading at which they are almost touching.

1.8.1 Overview of the technique for myography

Arteries are dissected from samples taken *post-mortem* or from biopsies. The artery needs to be immersed quickly in a cold physiological salt solution (PSS) and then, with the aid of a dissection microscope, the vessel must be cleaned of all surrounding perivascular tissue, being careful not to traumatise it. Then a ring section is cut, and the vessel is mounted on two wires or pins (figure 5), according to its size, while in a bath filled with PSS kept at 37° C and regularly gassed with a mixture of 95% O_2 and 5% carbon dioxide (CO2). At this point it is important to perform normalisation.

Normalisation is a procedure that mimics physiologic conditions by stretching the vessel to the IC that optimises vasoconstriction of the vessel. This permits appropriate comparison between vessels and between subjects. To perform normalisation, the IC stretched to a wall tension of 100 mmHg (13.3 KPa) and is defined as IC_{100} (figure 7). The normalisation factor is the ratio between the IC at which the active forces production of the vessel is maximal, defined as IC_1, and IC_{100}. Routinely, the normalisation factor is assumed to be 0.9 which is considered valid for the rat mesenteric artery[89], however normalisation factors can vary according to the type of artery and species. For example, one study has shown that for the rat femoral artery, the normalisation factor used should be 1.1 rather than 0.9[90]. Recently, Hugelshofer and colleagues[91] have established a mathematical method to calculate the normalisation factor, via administration of a high potassium concentrated physiological salt solution (KPSS) after the vessel has been stretched (figure 8), in this way both passive and active forces can be measured in relation to a certain IC, and then used to calculate both IC_1 and IC_{100}, obtaining the normalisation factor from their ratio. Once the vessel is normalised it is possible to

continue with the experiment via administration of vasoactive substances such as PE, ACh and SNP. The responses can be measured and used to assess the endothelial function of the vessel.

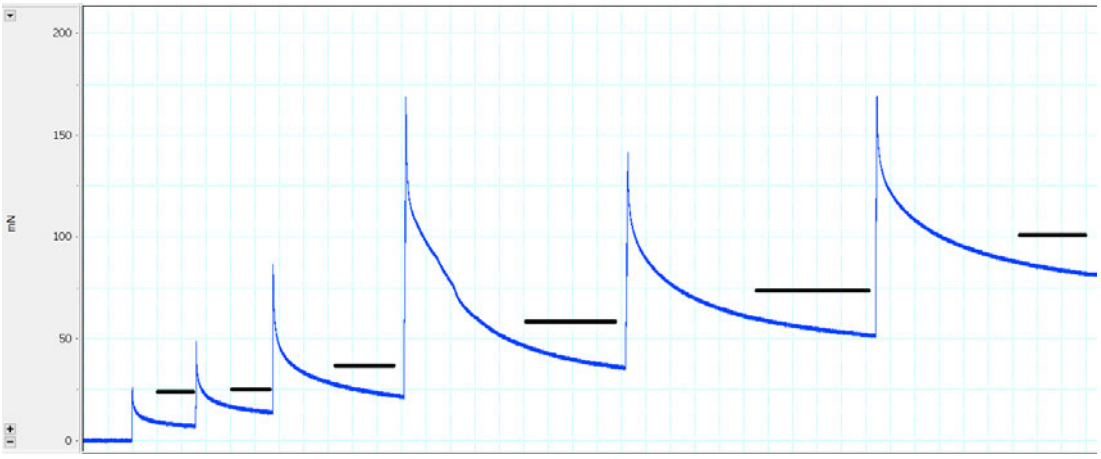


Figure 7. Normalisation procedure with known normalisation factor. The black lines indicate where to read the passive forces generated by stretching the vessel.

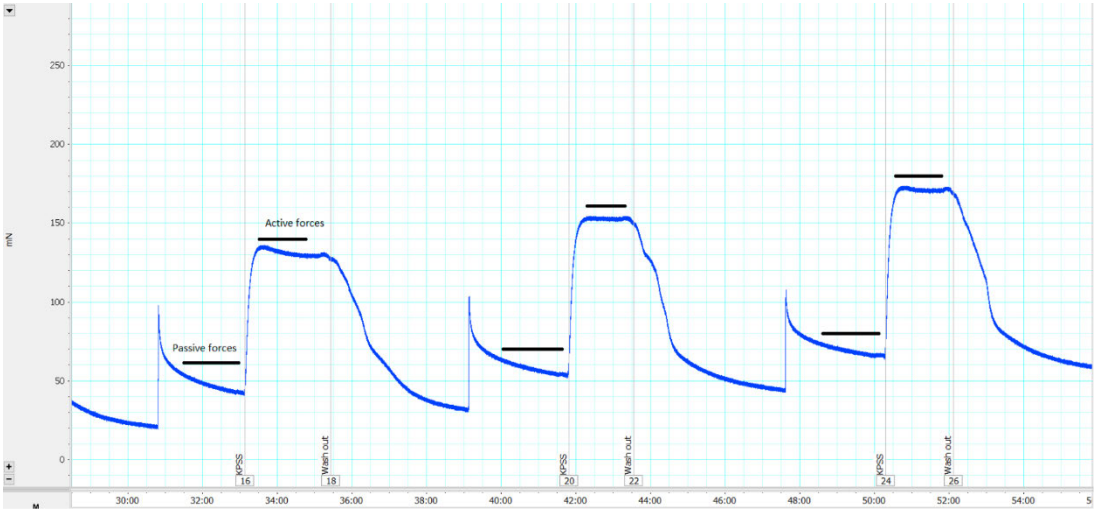


Figure 8. Procedure to identify the normalisation factor; after the vessel is stretched the well is filled with KPSS to induce constriction, active forces can therefore be measured as passive forces. Before the following stretch, KPSS is washed out with PSS to induce relaxation.

1.8.2 Applications of isometric myography

A large number of studies have used this technique to assess VSMC function, endothelial function and response to different drugs or vasoactive substances in experimental models or human arteries[92-94]. Applications in veterinary medicine are limited but isometric myography has been used to investigate the role of endothelial dysfunction in horses with laminitis[95] and the effect of racing on mechanical properties of pulmonary microvasculature in horses[96]. A study was conducted on mesenteric arteries of experimental dogs to induce pharmacologically endothelial hyperpolarisation, to identify a possible treatment for hypertension[97].

1.9 Hypothesis and aims

In conclusion, the hypothesis I have investigated this year is that MMVD and age can affect vascular function. I aimed to investigate this by:

- Determining the feasibility of applying isometric myography to different types of pet dog arteries.
- Measuring vascular function in different arteries from different sites.
- Comparing the vascular function in dogs of different ages and different grades of MMVD.

Chapter 2 Materials and methods

2.1 Population

Only pet dogs euthanised for welfare reasons were used in this study. Informed consent was obtained from owners for body donation prior to euthanasia, and the option of individual cremation after sample collection was available. Testicular arteries were collected as an age control from dogs undergoing routine gonadectomy, again with owner's informed consent to use them for vascular research. As part of protocol optimisation, bovine and sheep arteries were collected from animals slaughtered for welfare reasons and available for teaching purposes. Ethical approval was obtained by the R(D)SVS Veterinary Ethical Review Committee, reference number 120.19.

2.2 Optimising methods

Before starting work on canine arteries, I collected samples from bovines and sheep to optimise collection methods and familiarise myself with the myograph.

Samples were collected soon after the animal's death. Due to their use for teaching, only femoral arteries were collected. Once the femoral artery was isolated from the surrounding tissue, a sample of a few centimetres in length was collected and placed in ice-cold PSS that had been prepared on the same day. Ingredients used to make PSS and their concentrations are listed in table 2. The day after collection, arteries were prepared and mounted and always had a good contractile response to high potassium physiological salt solution (KPSS, table 2), indicative of their vitality and that they were feasible for myograph studies.

Normalisation factors were calculated using the method described by Hugelshofer and colleagues[86]. With practice, the time to determine the normalisation factor decreased from an entire working day to two hours.

2.3 Sample collection from dogs

Sections of femoral, mesenteric, renal, and pulmonary arteries were collected from dogs euthanised for welfare reasons at the Hospital for Small Animal of the Royal (Dick) School of Veterinary Studies and donated by the owners for vascular research. All dogs were euthanised via intravenous administration of high concentrated pentobarbitone (Pentoject 20% Injection, Animalcare limited). The collection was performed within one hour from the time of death. Both right and left femoral and renal arteries were collected. For the mesenteric arteries, samples were collected from two different random sites of mesentery. Different techniques were used to collect pulmonary arteries suitable for myography. These included
1) collecting branches exterior to lung parenchyma

2) using wires to isolate more distal branches and collecting the sample distal to the wire to avoid possible injury to the endothelium

3) isolating terminal arteries within pulmonary tissue.

Testicular arteries were isolated from testicles excised after gonadectomy performed by one of the veterinary surgeons of the Dick Vet General Practice.

Soon after collection, all samples were placed in ice-cold PSS gassed with a mixture of 95% O_2 and 5% CO_2. Samples were stored in a room at a controlled temperature of 4° C. For every dog, the mitral valve was macroscopically examined for MMVD and classified according to Whitney classification[86]. Reason for euthanasia and other signalment (i.e., sex, neutered status, age) were collected retrospectively from case records.

	PSS	KPSS
NaCl	119.0 mM	0 mM
KCl	3.7 mM	125 mM
$CaCl_2$	2.5 mM	2.5 mM
$MgSO_4$	1.2 mM	1.2 mM
$NaHCO_3$	25.0 mM	25.0 mM
KH_2PO_4	1.2 mM	1.2 mM
EDTA	27.0 µM	27.0 µM
D-Glucose	5.5 mM	5.5 mM

Table 2. Ingredients and concentrations expressed as millimolar (mM) for physiological salt solution (PSS) and high potassium physiological salt solution (KPSS).

2.4 Isometric myography

Samples were processed for isometric myography using a multi-wire myograph system (Multi Wire Myograph System Model 610 M, DMT) on the days following collection. Wells were filled with 6 mL of PSS constantly perfused with 95% O_2 and 5% CO_2, the wells were heated to keep the temperature at 37° C. Before every experiment, the myograph was calibrated setting force output for each channel according to the type of vessel mounted in the associated well (femoral and mesenteric artery up to 300 mN for normalisation and up to 200 mN for response test, for mesenteric artery up to 250 mN for normalisation and up to 150 mN for response test, for pulmonary up to 20 mN). Vessels were carefully cleaned from perivascular fat tissue and 2mm ring sections were cut under a dissecting microscope. Femoral and renal arteries were mounted on fixed pins, mesenteric arteries were mounted on 40 µm wires, pulmonary arteries were mounted on wire or pin according to the size of the artery collected. Once mounted, the vessels were left to acclimatise for 30 minutes.

2.4.1 Establishing normalisation factor

For every artery of every dog, normalisation factors were established prior to running the response experiment. Vessels were stretched with the micrometres to induce a passive response, then the wells were filled with KPSS (table 2) to induce an active response. Reading on the micrometres were used to calculate the internal circumference (IC) of the vessels, and for each IC both passive and active forces were recorded. Passive and active wall tensions were fitted to exponential and linear regression curves respectively, the difference between active and passive wall tensions was obtained, and the apex of the curve was calculated to identify $IC_{1.}$ IC_{100} was determined as the intersection of the passive tension curve with the linear isobar for 13.3 KPa (100 mmHg) as described by Hugelshofer and colleagues[91]. The normalisation factor was identified as the ratio between IC_1 and IC_{100}.

2.4.2 Response test

Once the normalisation factor was identified, a different 2 mm ring section of the same vessel was used for the response test. The decision to use a different section was made due to the fact that the process used to identify the normalisation factor necessitated overstretching the vessel, which could have potentially led to injury of the wall and possibly affected the results of the response test. Vessels were normalised according to normalisation factor previously identified. After normalisation and between each part of the response test, the vessels were left to rest for 30 minutes.

Wells were filled with KPSS three times to assess the vitality of the vessels and to normalise the absolute contractive forces generated by PE. Vessels that did not show any response to KPSS were considered not vital and therefore excluded from the rest of the experiment. Once the KPSS was washed out, increasing concentrations of PE (Sigma 1 nM – 3 µM) were then added to PSS. The concentration of PE that gave 50% of the maximum response was then used to preconstrict the vessels prior to adding increasing concentration of vasodilators (ACh, Sigma 1 nM – 3 µM; SNP, Sigma 1 nM – 3 µM). Vessels that were unable to maintain constriction after stimulation with PE were excluded from this part of the study.

2.5 Histopathology and immunohistochemistry

Segments of each vessels used were fixed in 10% formalin and sent to the Pathology Department of the R(D)SVS for histopathology and immunohistochemistry to confirm their identity and to look for possible microscopic alterations that could be associated with different responses on the myograph. Histopathology slides were stained with haematoxylin and eosin (H&E) and Masson's trichrome. Immunohistochemistry slides were stained with antibodies against α-1B adrenergic receptor (Novus Biological) and against muscarinic acetylcholine receptors (Novus Biological), as control dog adrenal gland and dog brain were used for adrenergic and muscarinic receptors respectively. All the slides were reviewed by me.

2.6 Statistical analysis

Data were obtained using LabChart Software (AD Instruments). Curve fitting for normalisation factors and all statistical analysis were performed using GraphPad Prism 9. Normalisation factors were compared between each type of artery and between dogs with mixed effect analysis of variance (ANOVA) to analyse the variation between different types of arteries and within the same arteries for different dogs.

The response to KPSS was expressed as a percentage of variation between the first and the third administration of KPSS which was calculated dividing the maximum response to KPSS by the minimum response and multiplying the result by 100; the percentage of variation was used to compare the vessels of the same dog at two different times to determine whether time after collection affected the response of the vessels. This comparison was made with a paired t-test or Wilcoxon test if the values were not normally distributed (Shapiro-Wilk test).

Response to PE normalised to KPSS and response to ACh and SNP were fitted in non-linear regression curves as routinely described[88]. Contractility data are presented as a percentage of the maximum KPSS response, and dilatory data as a percentage of the vessel's preconstriction with PE, both against –log of the molar drug concentration.

All data are shown as mean±SD. Dose responses between groups were compared by comparing logEC/IC_{50} with mixed effect ANOVA. For the constriction test, the types of arteries of each dog were compared to measure if there was any difference comparing logEC_{50}. For the relaxation tests, the curves of ACh and SNP were compared according to MMVD grade (1 and 2 vs 3 and 4) to determine whether dogs with more advanced MMVD were more likely to have impaired endothelial-dependent relaxation.

Differences were considered statistically significant when P value was <0.05.

Chapter 3 Results

3.1 Optimising responses

For the part first on farm animals, arteries were collected from five sheep. Samples from the first two sheep were used only to define the technique of isolating the femoral artery, cleaning, dissecting and mounting. Samples from the last three sheep were also processed on the myograph.

Arteries were successfully collected, isolated, mounted and interrogated on the myograph. The responses showed that arteries in these animals can have a strength of contraction much higher than laboratory rodents, as a strength >200mN was obtained with KPSS. Therefore, it was important to calibrate the myograph each time before the experiment to set a higher value of force output according to the type of vessel. These vessels also maintained vitality for up to 10 days if preserved in PSS and kept in a controlled cold room at 4°C.

Two dogs were also used in this preliminary part of the study. The samples were collected in both cases the morning after the dogs had been euthanised and placed in a cold room (time between death and collection was about 12 hours). In both cases, the vessels failed to show any response to KPSS making them not suitable for the myograph and confirming the need to remove arteries immediately after death.

3.2 Population for experiment

Samples were collected from a total number of 11 dogs, of which nine dogs were euthanised for welfare reasons and two dogs underwent routine gonadectomy. Data of the euthanised group are summarised in table 3. Both dogs that underwent gonadectomy were six months old Labrador retrievers and clinically healthy.

Dog	Age	Sex	Breed	MMVD Grade	Cause of euthanasia
1	8 y	MN	Boxer	2	Pituitary tumour
2	16 y	FN	Greyhound	1	Paraparesis
3	15 y	FN	Bulldog	3	unknown (difficult to walk)
4	13 y	FN	Whippet	4	Congestive Heart Failure
5	13 y	FN	Cocker Spaniel	3	Neurological signs
6	13 y	F	Cocker Spaniel	3	Pyometra
7	17 y	F	Cross breed	3	Vestibular signs
8	12 y	F	Cocker Spaniel	3	Vestibular signs
9	14 y	FN	Labrador	2	Pituitary macroadenoma + nasal mass

Table 3. Clinical details of the dogs' population. Y: years old; MN: male neutered; F: female; FN: female neutered

3.3 Histopathology and immunohistochemistry

All the samples examined were confirmed to be arteries (figure 9). There was small atherosclerotic plaque in the femoral artery of dog number 2 (figure 10), and severe fibrosis of the *media* layer in dog number 6 (figure 11). A mild degree of fibrosis was observed also in other arteries, and no other significant alterations were identified on the histopathology analysis. Immunohistochemistry staining failed with both anti-adrenergic and anti-cholinergic receptors antibodies; only mild staining was apparent in the control tissue.

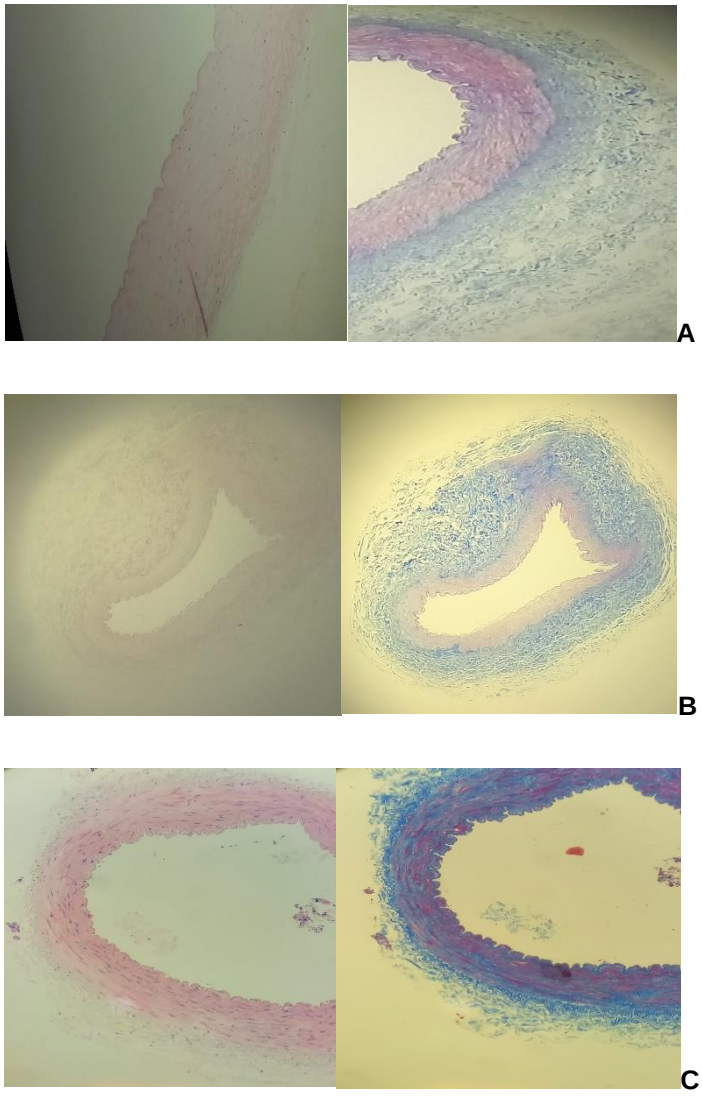


Fig 9. Histology of a femoral artery (A), renal artery (B) and mesenteric artery (C). On the right staining with haematoxylin and eosin (H&E), on the left Masson's trichrome, all the images are magnified at 20x. H&E stains: pink extracellular material and blue/purple nuclei. Masson's trichrome stains: red muscle, blue collagen.

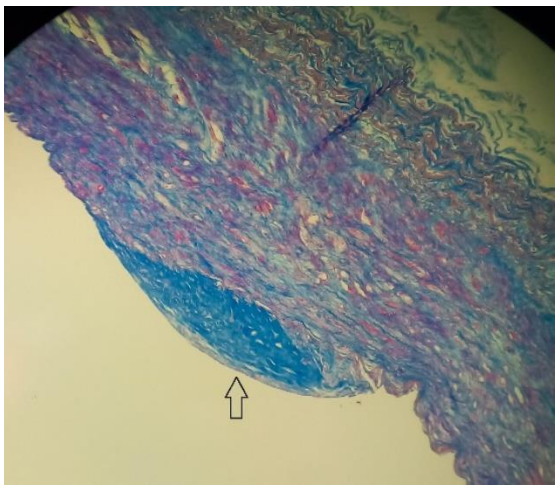


Fig 10. Section of femoral artery from dog number 2, showing the presence of an atherosclerotic plaque in the lumen adhered to the wall (arrow), stained in blue due to the presence of collagen in the plaque. Masson's trichrome, 20x.

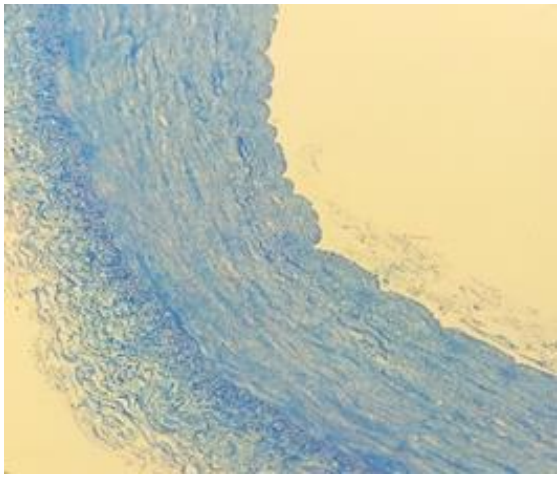


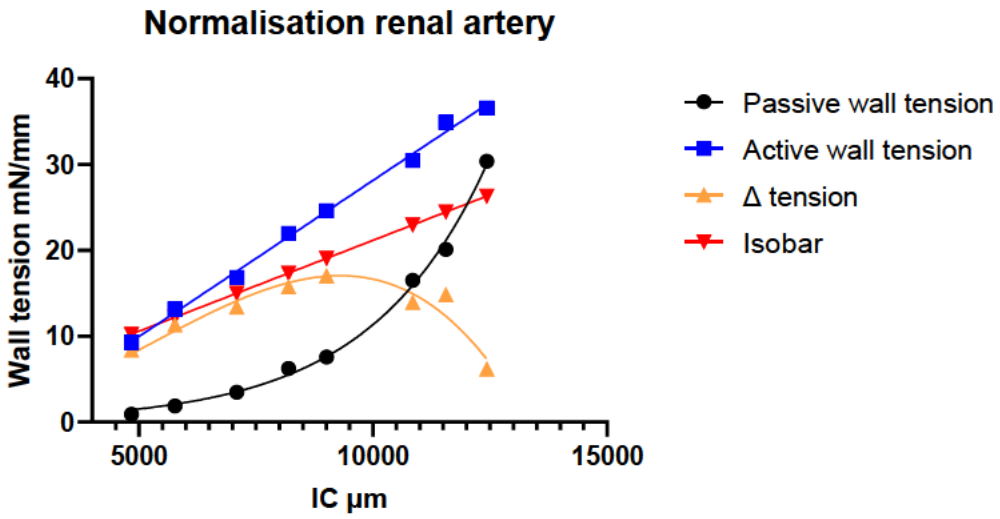
Fig 11. Femoral artery of dog number 6. It is possible to notice the lack of red staining, which indicates lack of muscular tissue substituted with fibrotic tissue (stained blue). Masson's trichrome, 20x.

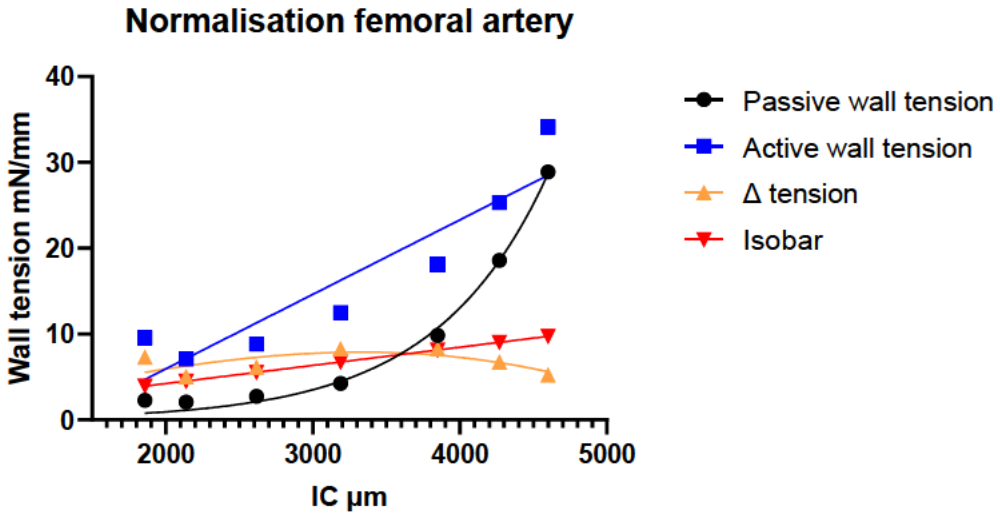
3.4 Isometric myography

All euthanised dogs had at least one type of artery that was suitable for all the experiments. All the renal arteries collected were suitable, femoral arteries from dogs number 4 and 5 had no responses to KPSS, the femoral artery from dog number 2 was suitable for normalisation and the constriction response but was unable to maintain constriction afterwards and was therefore excluded from the relaxation experiment. Mesenteric arteries from dogs 1, 4, 5 and 6 did not show a response to KPSS. None of the pulmonary arteries nor testicular arteries were suitable for any experiment as they did not show any response to KPSS.

3.4.1 Determining normalisation factors

Normalisation factors were determined for femoral (1.00±0.10), renal (1.00±0.14; figure 12) and mesenteric (1.05±0.12) arteries. The results with individual values are summarised in figure 13. Values were not different between different arteries (P=0.32) or between the same artery of different dogs (P=0.37), despite the wide data spread, especially for the renal artery which had a coefficient of variation of 14%. Coefficient of variation for femoral artery was 10% and for mesenteric artery was 11%.


Normalisation renal artery
Wall tension mN/mm
0
10
20
30
40
5000
10000
15000
IC µm
Passive wall tension
Active wall tension
Δ tension
Isobar


Normalisation femoral artery
Wall tension mN/mm
0
10
20
30
40
2000
3000
4000
5000
IC µm
Passive wall tension
Active wall tension
Δ tension
Isobar

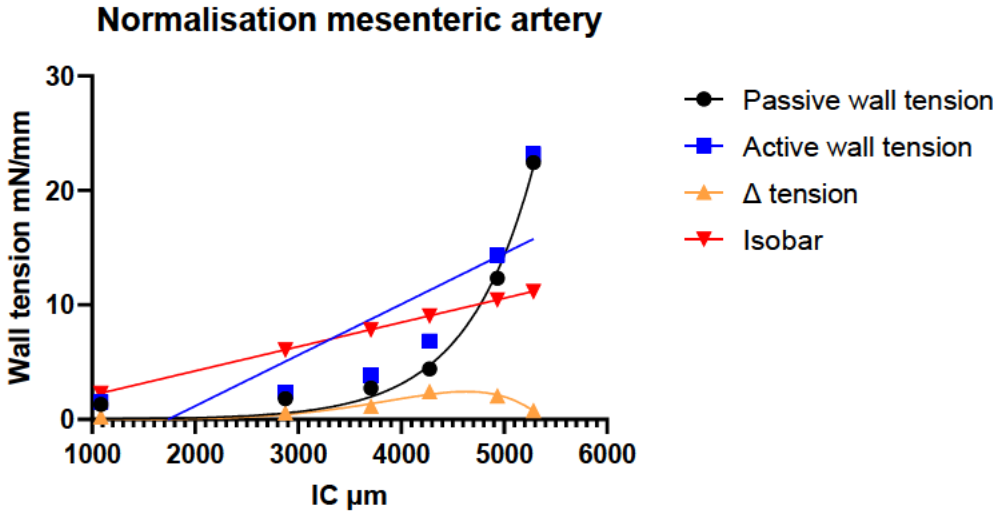

Normalisation mesenteric artery
Wall tension mN/mm
0
10
20
30
1000
2000
3000
4000
5000
6000
IC µm
Passive wall tension
Active wall tension
Δ tension
Isobar

Fig 12. Examples of curves fitting for determining normalisation factor for renal, femoral and mesenteric artery. Black circles are value of passive wall tension with the black line representing the non-linear regression curve fitted, blue squares are the value of active wall tension and blue line is the linear regression curve fitted, Δ tension values are made from the difference between active and passive wall tension and the orange line is the non-linear regression curve fitted, last the red triangles are values mathematically calculated to build the linear regression curve of the isobar set at 13.3 KPa. IC_{100} is determined as the intercept of the isobar with the passive wall tension curve, IC_1 is the vertex of the Δ tension curve, obtained from the difference between active wall tension curve and passive wall tension curve.

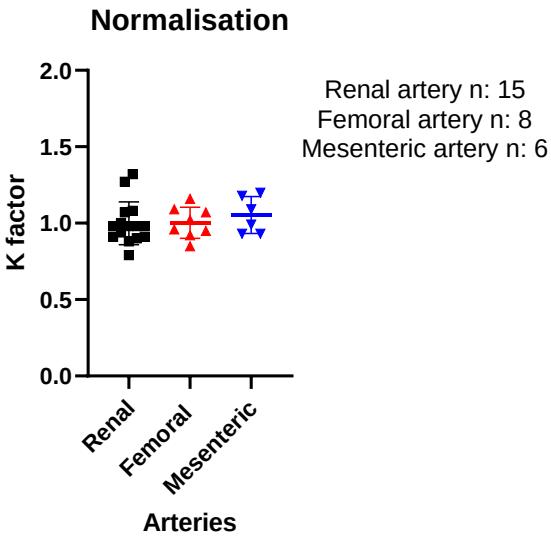


Fig 13. Scatter plots for normalisation factors identified for femoral, renal and mesenteric arteries, each dog contributed with one value except dog 1, 2, and 3 who contributed with three values for renal artery and two values for femoral and mesenteric artery (only dog number 2). K factor: normalisation factors, n: number of dogs.

3.4.2 Variation of KPSS response

Due to the unpredictability of the samples' availability (whenever dogs were being euthanised) it was not possible to standardise an equal time between one analysis and the other. Therefore, this analysis was made considering time 0 (T_0) as the time most close to the time of death (up to four days later) and time 1 (T_1) any days after the first analysis (up to seven days from the time of death). For the renal artery, it was possible to compare dogs numbers 3, 5, 6, 8, and 9, while for femoral and mesenteric arteries, it was possible to compare dogs number 3, 8 and 9 (figure 14). The values expressed as percentage variation at T_0 and at T_1 are summarised in table 4. No significant difference was found for any of the arteries, although for the renal artery was almost significant (P=0.055).

	%KPSS variation		
	T_0	T_1	P
Renal artery	134.27%±26.06	111.8%±36.73	0.055
Femoral artery	190.20%±62.99	165.4%±45.33	0.14
Mesenteric artery	169.2%±75.12	148.3%±63.37	0.10

Table 4. Summary of percentage of variation of KPSS response at time 0 (T_0) and time 1 (T_1) with P values from the comparison of each artery, data are expressed and mean±SD.

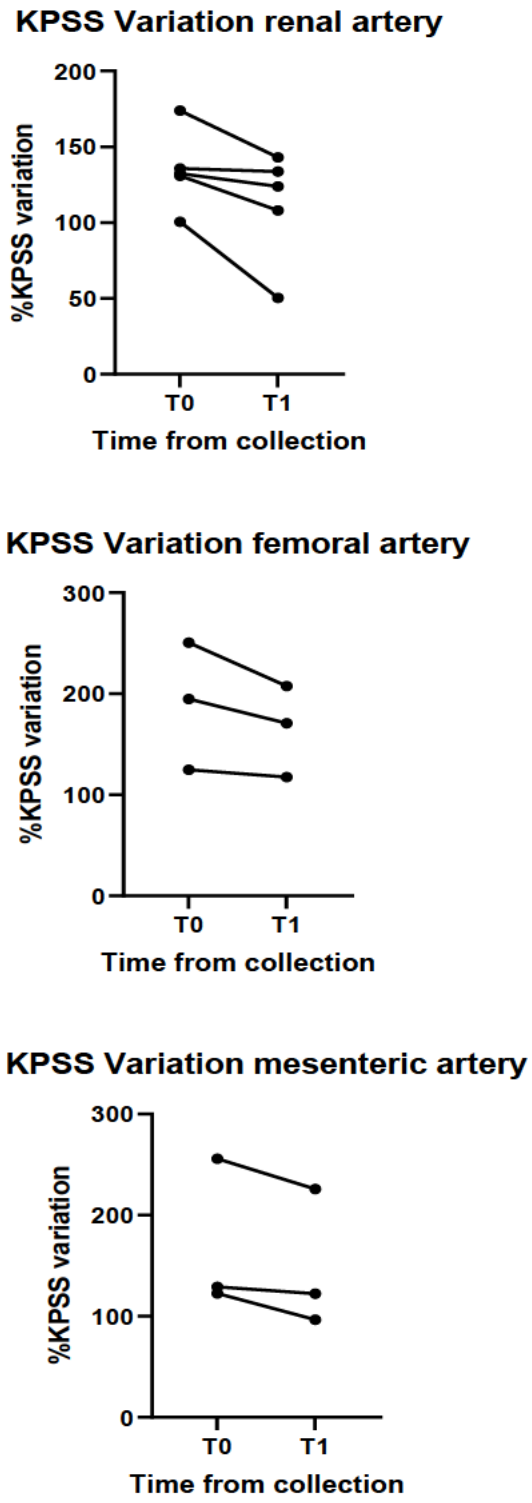


Fig 14. Estimation plot comparing the variation between time 0 (T_0) and time 1 (T_1) for renal, femoral, and mesenteric artery. There was difference between the two times periods for any artery.

3.4.3 Constriction and relaxation tests

The $logEC_{50}$ for the renal artery was 4.78±1.56, for the femoral artery it was 4.50±2.00, and for the mesenteric artery it was 5.33±0.41 (figure 15). Differences between the types of arteries were not significant (P=0.49).

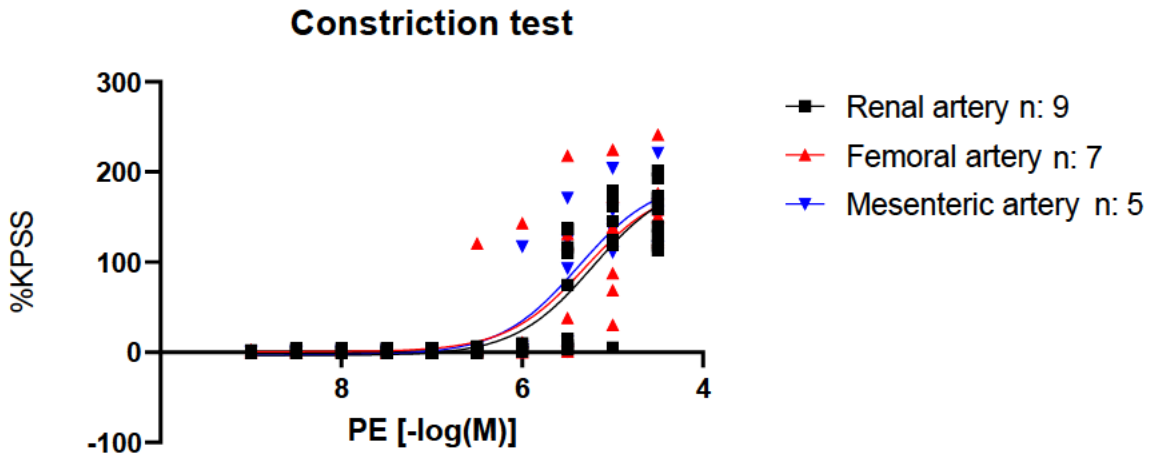


Fig 15. Nonlinear regression curve for cumulative response dose to phenylephrine as percentage of the maximum response to KPSS. Each plot represents one dog, n: number of dogs.

Impairment of endothelial-dependent relaxation was observed in dogs with both MMVD stages 1 and 2, and MMVD stages 3 and 4 (figure 16), compared to endothelial-independent relaxation induced by SNP. In the dogs with advanced MMVD stages 3 and 4, this was present in all the artery types examined, although not in every individual artery. In MMVD stages 1 and 2, it was observed in one artery in two of the three dogs (renal artery but not femoral artery for dog number 1, and mesenteric artery but not renal artery for dog number 2). Table 4 summarises mean and SD of $logIC_{50}$ for ACh and SNP based on type of artery and MMVD grade group. It was not possible to identify a $logIC_{50}$ for ACh for the vessels that showed an impaired response as in none of them the response to ACh achieved 50% of the preconstriction levels achieved when administered PE. Therefore, it was not possible to perform a statistical analysis to compare the $logIC_{50}$ ACh response between dogs with different grades of MMVD. The $logIC_{50}$ for SNP did not differ between mild MMVD and moderate to severe MMVD between any of the arteries.

	LogIC$_{50}$ MMVD 1-2		LogIC$_{50}$ MMVD 3-4		P value
	logIC$_{50}$ ACh	logIC$_{50}$ SNP	logIC$_{50}$ ACh	logIC$_{50}$ SNP	SNP
Femoral artery	7.99±1.23	10.18±4.10	N/A	6.63±1.09	0.36
Renal artery	*7.75±0.82	7.23±1.43	N/A	6.55±0.62	0.51
Mesenteric artery	*7.87	7.22±0.20	N/A	6.98±0.41	0.46

Table 5. Summary of logIC$_{50}$ for ACh and SNP for dogs with MMVD grade 1-2 and grade 3-4, values expressed and mean±SD expressed for mesenteric artery in the group MMVD grade 1 and 2. Statistical comparison is made between SNP in dogs of group MMVD graded 1 and 2 and MMVD graded 3 and 4. *Missing one value as was not possible to identify a logIC$_{50}$; N/A: not applicable; ACh: acetylcholine; SNP: sodium nitroprusside; MMVD: myxomatous mitral valve disease.

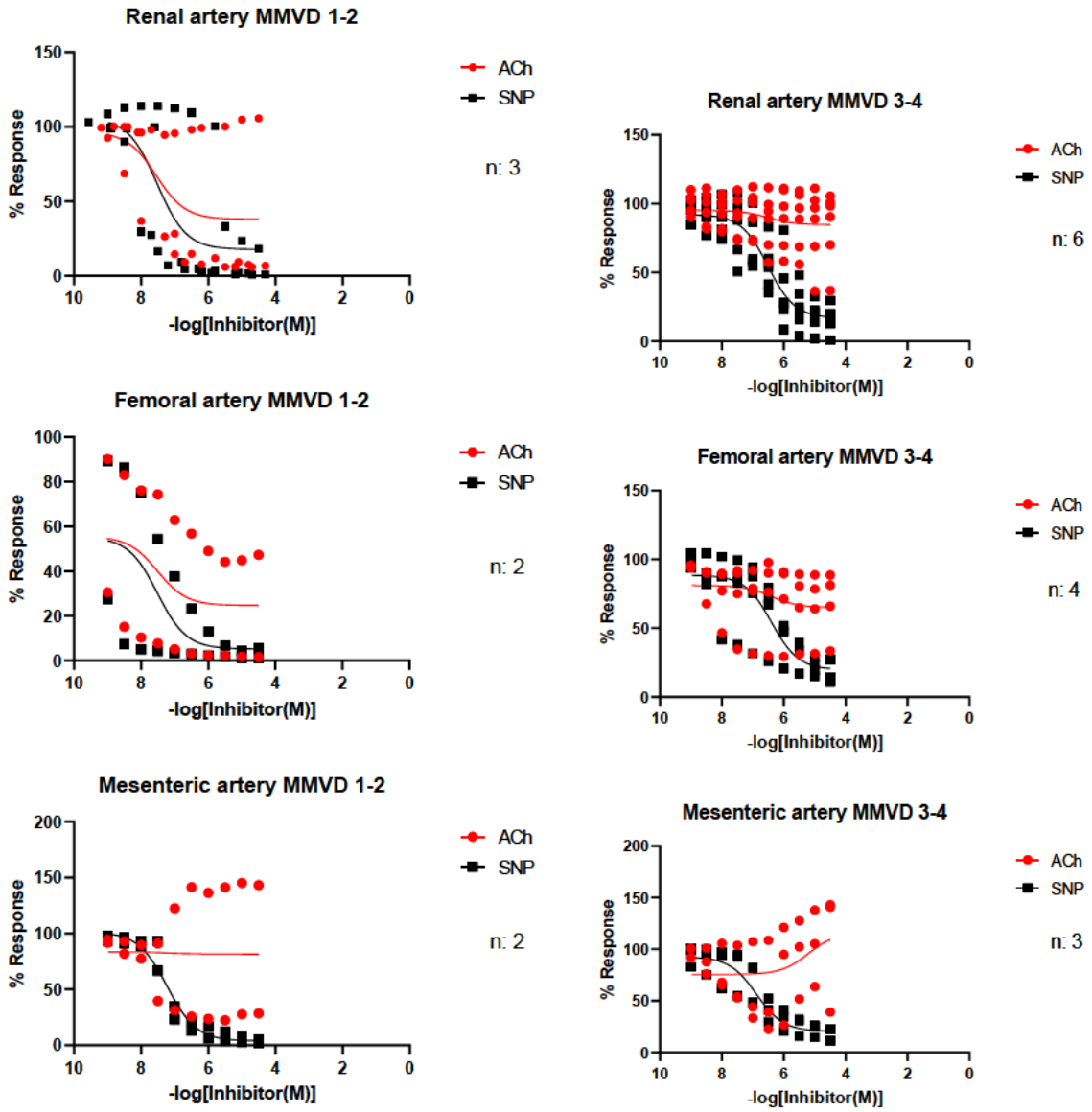


Fig 16. Nonlinear regression curves for renal, femoral, and mesenteric arteries showing responses for acetylcholine (ACh) and sodium nitroprusside (SNP) in the groups of mild myxomatous mitral valve disease (MMVD grade 1-2) and in the group of moderate to severe MMVD (3-4). Each data point represents a value for a single dog. N: number of dogs

Chapter 4 Discussion

This study represents the first time that myography has been performed on arteries collected from pet dogs. Although there were previous studies in which isometric myography was used to analyse canine arteries, these were collected from experimental dogs and normalisation studies were not performed. This study demonstrates the variability of normalisation factors between different arterial sites and between different dogs. It has also identified impaired ACh-mediated vasodilation. I conclude that normalisation performed *de novo before* every myographic response study, is required to identify endothelial dysfunction in pet dogs.

The preliminary part of the study had method optimisation as an objective. From this, it was determined that the vessels, once collected and placed in cold PSS, can remain viable for up to ten days. This represented an important result for the next step, as it meant that samples did not have to be fully processed on the same day of collection. The importance of this discovery is in the fact that one of the intrinsic difficulties of clinical research is the impossibility to predict when a sample will be available. In some cases, I had to collect samples from dogs euthanised late in the evening, making it difficult to stay to run an entire experiment on the same day. There was also an occasion when two dogs (dogs numbers 4 and 5) were donated on the same day. If long term viability was not sustained, further application of myography to study vascular function in MMVD and other diseases might not be feasible.

The first two dogs that I collected samples from were euthanised the previous evening and stored in a refrigerated room at 4°C. The failure to obtain viable arteries form these dogs confirmed that samples must be collected and placed in PSS immediately after euthanasia. They can be stored at 4°C for up to ten days, although in this study I usually run all the samples within seven days of collection. Furthermore, not only do vessels remain viable, but the low percentage of variation in response to KPSS would suggest reliable and repeatable results can be obtained across a range of days, although the almost significant difference in the renal artery could suggest that experiments need to be run within the first days from the

collection rather than waiting an entire week. This reveals that isometric myography is more feasible, and applicable to clinical research of naturally occurring disease in companion animals than previously believed.

The histology confirmation that all the samples were arteries without exception proved that the collection technique is sound and can be performed by anyone with a basic anatomic knowledge, again increasing the feasibility of myography for future studies.

It had been hoped that immunohistochemistry would link the functional and pharmacological aspects of myography. However, the failure to establish strong, repeatable staining on positive control samples within the time constraint of one year meant that this aspect of the study was incomplete. There are no commercially available anti-canine α-1 and ACh receptor antibodies. Instead, I used antibodies raised against other species that were predicted to work on canine tissue, based on similar or identical RNA transcripts of the receptor epitopes. As well as being linked to use of an inappropriate antibody, the only mild staining on the control tissue could be a result of poor optimisation of technique or the use of 10% formalin as the fixative, which tends to increase cross-linking, minimising epitope exposure[98]. This is despite the datasheets for the antibodies used stating that 10% formalin was the fixative of choice.

It is interesting that in dog number 6, there was severe fibrosis of the arterial wall on Masson's trichrome staining. Vascular fibrosis is commonly reported in old people, and it is one of the mechanisms that leads to arterial stiffness and systemic arterial hypertension[99]. This dog was euthanised for pyometra, an infection of the uterus that happens secondary to endometrial hyperplasia after the oestrus, and in severe cases can lead to sepsis[100]. This dog was not suspected to be septic before being euthanised and no abdominal effusion was noticed during the collection of samples. Possible effects of pyometra on the vascular function have not been investigated to the best of my knowledge. This dog was also 13 years old, so it is possible that the fibrosis could be age related and independent from her cause of death. Unfortunately, her blood pressure status is unknown. Other dogs from this study had some mild degree of vascular fibrosis but the clinical impact of this was

unknown. Since the fibrosis involves the *media* rather than the endothelium, a severe alteration would expect to impair also the endothelium-independent vasorelaxation and, interestingly, although the $logIC_{50}$ was not statistically different from the other dogs, the maximum response to SNP expressed as percentage of inhibition of the preconstriction given by the PE was 29% for the renal artery and 27% for femoral artery, while for all the other arteries the maximum response to SNP was below 20% with some cases even below 5%. As this was only one dog, a statistical analysis was not feasible, but it does raise the possibility of a link between vascular fibrosis and vascular dysfunction in pet dogs.

Normalisation factors were determined for canine femoral, renal, and mesenteric arteries. In the studies in which dogs were used, the normalisation factor used is not stated. It is possible that a normalisation factor of 0.9 was employed as this is the standard used for rodents. The value of 0.9 is based on a study in which only rat mesenteric arteries were examined. Overall, this study suggests a value of 1.0 would be more appropriate. However, although statistical analysis showed no significant difference in normalisation factors between dogs and between arteries, the data spread was wide, particularly for the renal artery as showed by the coefficient of variation, and the possibility of a Type II error should be considered. The wide range of values is not surprising, but it is important. There is no evidence that laboratory rodents of different species or strains have identical normalisation factors for different arteries, and, where this has been investigated, rat femoral arteries have a value different to the 0.9 normalisation factor obtained from mesenteric arteries[90]. A population of pet dogs of different breeds may be far more heterogeneous than a population of experimental rodents. Therefore, this study would support calculation of normalisation factors for individual arteries and individual dogs before proceeding to response studies. Furthermore, it raises that the possibility of inappropriate normalisation factors being used in rodent studies. This should be clarified experimentally.

My study also suggests that isometric myography can identify and quantify endothelial dysfunction in pet dogs' arteries. Due to the small population and the distribution of age and grade of MMVD, it was not possible to assess properly the

relationship between impaired endothelial-dependent relaxation and grade of MMVD or age. An impaired response to ACh was observed more frequently in older dogs with more advanced MMVD but this may reflect the larger number of dogs in this group and the larger number of viable arteries. The possibility of endothelial injury associated with sample collection or preparation should also be considered. However, impaired endothelial-dependent relaxation was observed in multiple sections from the same or corresponding arteries in dogs. For example, where an impaired response to ACh was observed in one renal or femoral artery, it was also observed in the contra-lateral renal artery, which is more suggesting of genuine endothelial dysfunction. Furthermore, if tissue collection had damaged the vessel sufficiently to injure the internal endothelial layer, injury to the artery media, manifesting as an impaired response to SNP, might also be expected. This was not identified.

If the endothelial-dependent relaxation I recorded is genuine, then it is possible it could be related to factors other than MMVD or aging. This was not possible to elucidate further because of small numbers and an absence of clinical information. In some dogs, endothelial-dependent relaxation was not equally impaired in all arteries, this could be explained by to conditions that could have affected certain arteries more. For example, it is possible that dysfunction of the renal artery reflected subclinical chronic kidney disease. It is interesting to note that the dog number 2 that was euthanised for paraparesis, had an adequate response to KPSS and PE in the femoral artery, but was unable to hold the constriction for longer than two minutes, making it not unsuitable for testing the response to ACh and SNP. One might speculate that this lack of ability to maintain the vasoconstriction in the femoral artery might be linked to the aetiology of its paraparesis. This dog was a greyhound, a breed overrepresented for aortic thromboembolism (ATE)[101]. Furthermore, this same dog had an atherosclerotic plaque in the femoral artery. Since greyhounds are a popular breed and ATE and the protein losing nephropathy that predispose to it are not rare, this condition is an example to which myography may provide mechanistic information.

One disappointment of my study was that the pulmonary arteries from pet dogs did not remain vital for isometric myography, when collected by conventional means. Another difficulty was locating a pulmonary artery of a suitable diameter. The main pulmonary arteries were too large to be mounted, even on pins. Pulmonary arteries isolated from within the parenchyma lung tissue were suitable for mounting but showed no response to KPSS. The lack of response could be due to injury caused by excessive tissue manipulation during collection because there are difficult to locate. This also prolonged the collection time, further compounded by the fact that they were always the last arteries to be collected from any cadaver. Pulmonary arteries from horses have been shown to be suitable for isometric myography, so it should be possible to improve collection and viability in dogs. Future studies could prioritise pulmonary artery collection over other arteries to minimise the contribution of time. This is important to address because pulmonary vascular function may play a role in the morbidity and mortality of MMVD, suggested by the frequent development of pulmonary hypertension without significantly high left atrial pressures[102].

Testicular arteries also did not show any response to KPSS. It is possible that these arteries were also subjected to excessive manipulation during gonadectomy, possibly from surgical clamping. I was not present during the surgery to oversee collection, and it may be that there was a delay between removal and being placed in ice-cold PSS. Since testicular arteries represent an easy resource of arteries from both young and healthy dogs (and potentially older dogs with testicular neoplasia or prostatic disease), it would also be advisable that the collection method is optimised in the future.

All the other artery types (femoral, mesenteric, renal) were suitable for myography. The renal artery was the only one shown to be vital in all dogs. While this reflect deficiencies in collection technique at other sites, it does suggest that if only one artery can be studies in future studies, or if a high likelihood of vitality is required for a study, then the renal artery would be the artery of choice.

This study had several limitations. The number of dogs used was small and only around half the number of dogs that had been predicted. Several factors have

contributed to this low number. One is intrinsic to clinical research, as the availability of samples is entirely dependent on donors, and the willingness of dog owners to donate their much loved pet to research after euthanasia. Euthanasia is always very emotionally intense, and it can be difficult for the veterinarian to broach the subject of donation with the owner. To aid this process, I offered the possibility of individual cremation, and all cadavers were sutured closed after sample collection. Another important factor has been the pandemic of Covid-19, since the new O-variant was spreading through the UK during the first half of my study. This has limited access to the laboratory and the hospital, created clinical staff shortages, reduced patient numbers coming into the clinic and made it less easy for busy clinicians to spend time with owners going through the donation process.

In many cases, all the clinical details of dogs may not have been known or apparent. Dogs scheduled for euthanasia have often not undergone extensive investigations, and the nature of their disease of organ health may not have been fully known. The decision to euthanise is often based on age, severity of non-specific signs and financial constraints, so comprehensive diagnoses are frequently not made.

Although these limitations do not allow me to achieve all the aims of the study, it is still relevant that the results in viable tissue seemed to be repeatable and this should encourage further studies.

Chapter 5 Conclusions

Isometric myography is feasible in pet dogs' arteries and can identify and quantify endothelial dysfunction, therefore it can be used to investigate the effect of MMVD and other cardiovascular and non-cardiovascular diseases on vascular function.

Rapid collection of femoral, renal and mesenteric arteries and transfer into cold buffer soon after death gives a high rate of viability.

Collection of pulmonary and testicular arteries may be more problematic and additional studies are required to optimise the technique.

Normalisation factors can vary between different artery types in the same dog and the same artery types in different dogs. Therefore, normalisation should be performed in every artery at the beginning of every individual experiment.

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