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Isometric myography is a feasible method to identify and quantify endothelial dysfunction in dogs with myxomatous mitral valve disease

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OBJECTIVE
To assess the feasibility of isometric myography in pet dogs with myxomatous mitral valve disease (MMVD) to determine its use in quantifying endothelial dysfunction.

ANIMALS
9 dogs euthanized for medical reasons.

METHODS
Femoral, renal, and mesenteric arteries were collected postmortem and stored in physiological saline solution at 4 °C for myography. Mitral valves were scored for myxomatous degeneration (grades 1 to 4). Sections of arteries were mounted in wells, immersed in physiological saline solution perfused with 95% O_2 and 5% CO_2 at 37 °C, and stretched to an internal circumference (IC) that generated the maximal difference between active and passive wall tension (IC_1). Normalization factors were calculated by dividing the IC_1 by the IC at which the passive wall tension was 100 mm Hg (IC_{100}). Vasoconstriction to phenylephrine and vasodilation to acetylcholine (endothelial dependent) and sodium nitroprusside (endothelial independent) were assessed by cumulative dose-response curves.

RESULTS
Median MMVD grade was 3. Mean values of normalization factors were 1.00 ± 0.14 (renal, n = 15), 1.00 ± 0.10 (femoral, 8), and 1.05 ± 0.12 (mesenteric, 6). Responses to phenylephrine were similar between dogs (P = .14). Reduced responses to acetylcholine compared with sodium nitroprusside were identified in 15 arteries, suggesting endothelial dysfunction.

CLINICAL RELEVANCE
Isometric myography of arteries from pet dogs is feasible and can identify loss of endothelial-dependent relaxation in dogs with MMVD postmortem. Its use in further research can lead to a better understanding of the pathophysiology mechanisms of this disease.

Keywords: endothelial dysfunction, canine, cardiology, myxomatous mitral valve disease, myography

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease in dogs and is similar to mitral valve prolapse observed in people.[1] Progression of many cardiovascular diseases, including mitral valve prolapse, but also noncardiovascular ones, is associated with progressive dysfunction within the vasculature.[2,3]

Vascular dysfunction is defined as an imbalance between vasoconstriction and vasorelaxation within the vascular tree.[4] When this imbalance is due to the inability of the endothelium to release vasoactive factors, it is referred to as endothelial dysfunction.[5] To date, few studies[6–9] in veterinary medicine have assessed the relationship between MMVD and endothelial dysfunction, and none has used the "reference standard" technique of myography, which directly quantifies the mechanical components of vascular function.[10]

Isometric myography (often referred to as wire myography) is an ex vivo technique in which a section of blood vessel is mounted on wires or pins

and immersed in a physiological salt solution (PSS). It is then "normalized" by stretching to an internal circumference (IC) and tension that optimize measurable constrictive and relaxation responses to vasoactive substances.[11] A multitude of studies[12–14] have used this technique to study vascular function in different laboratory animal species and assess their suitability as experimental models of human disease. However, to the best of our knowledge, myography has not yet been used to study vascular function in pet dogs with known cardiac disease. Normalization permits the comparison of responses between vessels and subjects.[11] The IC is first stretched to a wall tension of 100 mm Hg (13.3 KPa), denoted IC_{100}. The IC is then adjusted so that the active force production of the vessel is maximal, denoted IC_1. The ratio between the IC_1 and the IC_{100} is called the normalization factor. Routinely, and to save time, a blanket normalization factor of 0.9 is applied.[11] However, normalization factors can vary according to the type of artery as well as species,[15] and if the normalization factor varies from 0.9 in pet dogs, it could prevent the generation of accurate and repeatable datasets.

The aims of this study were to establish the feasibility of isometric myography in pet dogs, to identify the normalization factors of different arteries, and to identify and quantify endothelial dysfunction. To achieve these aims, we utilized a recently validated mathematical model that simplifies the calculation of normalization factors.[16]

Methods

Dog selection

This prospective study was conducted at the Hospital for Small Animals of The University of Edinburgh and The Roslin Institute. Client-owned dogs euthanized for welfare or medical reasons and donated by the owners for research were enrolled. Ethical approval was obtained from the local Veterinary Ethical Review Committee (reference No. 120.19). No exclusion criteria were applied.

All dogs were euthanized with an intravenous injection of highly concentrated pentobarbitone solution and moved quickly (< 10 minutes) to a dissection room so that arteries could be collected as quickly as possible. After collection, all arteries were immediately placed in cold PSS **(Supplementary Table S1)** that had been previously gassed with a mixture of 95% O_2 and 5% CO_2 and kept at 4°C to reduce bacterial growth and decomposition.

Femoral arteries were collected first because they are superficial and easy to access. An incision was made medially over the level of the mid femur. Muscles were carefully dissected to expose the artery, which was then isolated from the accompanying vein and nerve. A section approximately 2 cm long was collected. Renal arteries were collected after each kidney was exposed by a lateral incision through the body wall just below the lumbar muscles. Blood vessels were cut as close as possible to the renal hilus taking care not to stretch them. The artery was then isolated from the vein, and a section was removed. Both left and right femoral and renal arteries were collected.

The lateral abdominal incision was extended, and the jejunum was exposed. Two sections of the mesenteric artery and vein were removed from 2 different sites of the mesentery. Only samples without macroscopic lesions were selected. A second-order mesenteric artery (following the first bifurcation in the mesentery) was then isolated from the vein with the aid of a dissection microscope immediately before performing myography.[1] Portions of all arteries were also fixed in 10% formalin, stained with hematoxylin and eosin and Masson trichrome **(Figure 1)**, and examined histologically to confirm they were indeed arteries.

Once all the arteries had been collected, the thoracic cavity was opened from the left side. The heart was incised, and the mitral valve was macroscopically examined. Myxomatous lesions were graded (1 to 4).[17] The whole dissection process took no longer than 1 hour from euthanasia to completion.

Isometric myography

Isometric myography was performed on day 1 and day 3 **(Supplementary Table S2)** after collection using a multiwire myograph system (Multi Wire Myograph System Model 610 M; Danish Myo Technology) connected to a multichannel data acquisition system (Powerlab. ADInstruments Ltd) that used dedicated software (LabChart Software; ADInstruments Ltd). A ring section 2 mm in length

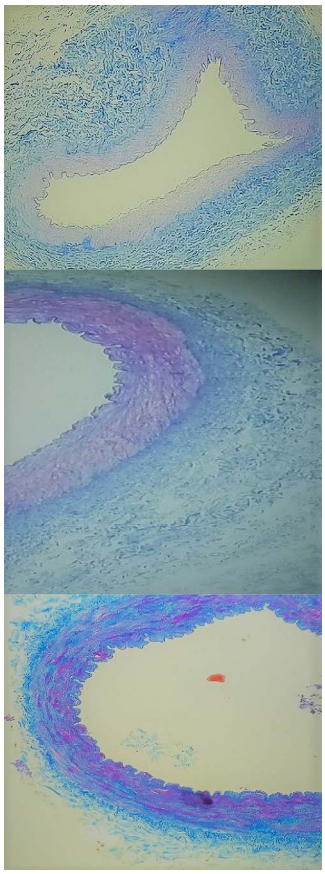

Figure 1—Histology of a renal artery (top), femoral artery (middle), and mesenteric artery (bottom). Masson trichrome staining, all the images are magnified at X20; muscle layer is red, and collagen is blue.

was cut from each artery and cleaned from its perivascular tissue under a dissecting microscope. Two sections of the same artery were prepared, 1 for normalization and 1 for pharmacological assessment. Both the right and left femoral and renal arteries and both of the mesenteric arteries that were collected were tested. Every artery was placed in a well filled with 6 mL PSS at a controlled temperature of 37 °C and constantly perfused with a gas mixture of 95% O_2 and 5% CO_2. Renal and femoral arteries were mounted on pins in a fixed-mount myography bath, while mesenteric arteries were mounted in a wire myography bath. Every artery was normalized using the method described by Hugelshofer et al.[16] Briefly, arteries were stretched with a micrometer to induce a passive response, and then the baths were filled with high-potassium PSS (KPSS) to induce an active response. The reading on the micrometer was used to calculate the IC of the arteries, 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 these 2 curves was plotted as a third curve whose apex was the IC at which IC_1 was generated. The IC_{100} was determined as the intersection of the passive tension curve with the linear isobar for 13.3 KPa (100 mm Hg). The normalization factor was identified as the ratio between IC_1 and IC_{100}. To avoid changes in vascular function secondary to overstretching of the arterial wall during normalization,[11] a different section of the same artery was used for the response test but stretched to the IC_1 that had just been determined.

PSS washes were performed after every treatment in the response test. First, tissue viability was assessed in triplicate by inducing vasoconstriction with KPSS (Supplementary Table S1). Sections that did not show any constriction with KPSS were excluded from the rest of the study. After washout, vasoconstriction was quantified by adding increasing concentrations of phenylephrine (PE) (Sigma; 1 nM to 3 μM) to the bath. Once the maximum concentration was achieved, PE was washed out to allow the arteries to relax. Vasoconstriction by PE was expressed as a percentage of the corresponding vessel's maximum response to KPSS.

The dose of PE that achieved half of the maximal response was used to induce a second vasoconstriction. Once the constrictive response achieved a plateau, increasing concentrations of acetylcholine (ACh) and sodium nitroprusside (SNP) (both Sigma; 1 nM to 3 μM) were cumulatively added to the bath. The response was expressed as a percentage of the preconstriction induced by PE.

Statistical analysis

Curve fitting for normalization factors and all analyses were performed using commercially available statistical software. The distribution of data was assessed with a Shapiro-Wilk test.

Data are shown as means ± SD if normally distributed; otherwise, they are shown as median (interquartile range).

A mixed effect ANOVA was used to compare the effect of different arterial sites and of different dogs on the normalization factor and the interaction between them. Coefficients of variance between normalization factors obtained from the same artery site were also calculated.

Responses to PE, ACh, and SNP were fitted with nonlinear regression curves, both against –log of the molar drug concentration.

Dose responses between groups were compared by comparing their logarithmic half-maximal effective/inhibitory concentrations ($logEC/IC_{50}$). For vasoconstriction with PE, the $logEC_{50}$ values from each artery site from each dog were compared using a Kruskal-Wallis test. For relaxation, the $logIC_{50}$ values of ACh for all arteries were compared between dogs with mild MMVD (grades 1 to 2) and dogs with moderate-severe MMVD (grades 3 to 4) and between arteries assessed on day 1 after collection and day 3 after collection, using mixed-effect ANOVA. The same comparisons were made for the response to SNP.

Results

Arteries were collected from 9 dogs in total. Characteristics of individual dogs are summarized (gender, age, MMVD pathology grade, day of test, and reason for euthanasia; Supplementary Table S2). All vessels examined by myography were confirmed as arteries on histology (Figure 1).

Of the arteries collected, 9 pairs of renal, 6 pairs of femoral, and 5 pairs of mesenteric arteries were viable. Two pairs of femoral arteries did not show any response to KPSS, and 1 pair of femoral arteries was excluded because although it responded to KPSS and PE, it was unable to hold a constriction plateau. Four pairs of mesenteric arteries did not show any response to KPSS.

Normalization factors were 1.00 ± 0.14 for renal arteries, 1.00 ± 0.10 for femoral arteries, and 1.05 ± 0.12 for mesenteric arteries. Individual values for every dog are summarized **(Figure 2)**. Overall, there was no effect of artery site (P = .32) or between dogs (P = .37) on the normalization factor. There was no interaction between the dogs and arterial site (P = .62). The data spread varied between arterial sites, with a coefficient of variation of 14% for renal arteries, 10% for femoral arteries, and 11% for mesenteric arteries.

The $logEC_{50}$ values for PE were 5.38 (4.91 to 5.95) –log(M) (renal artery), 5.40 (4.93 to 5.71) –log(M) (femoral artery), and 5.33 ± 0.41 –log(M) (mesenteric artery). Differences between the artery sites were not significant (P = .84; **Figure 3**). An impaired response to ACh (endothelial-dependent relaxation) was observed in dogs with moderate-severe MMVD compared to dogs with mild MMVD **(Figure 4)**. Out of the 3 dogs with mild MMVD, there was a reduced response to ACh in both renal arteries of dog 1 and both mesenteric arteries of dog 2. In moderate-severe MMVD, a reduced response to ACh was observed in all the artery sites examined. It was not possible to identify a $logIC_{50}$ for ACh in vessels that had impaired endothelial relaxation

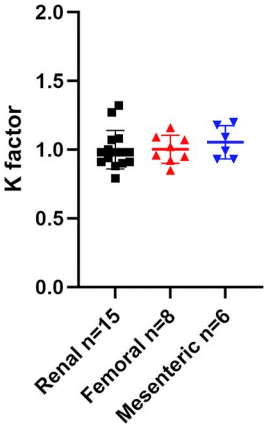

Figure 2—Scatter plots of normalization factors identified for femoral, renal, and mesenteric arteries. Each dog contributed 1 value with the exception of dogs 1, 2, and 3, who contributed 3 values for the renal artery and 2 values for femoral and mesenteric arteries (dog 2 only). Normalization factors are calculated as the ratio between the internal circumference (IC) at which there is maximum active contraction (IC_1) and the IC at which the passive contraction achieves a wall tension of 100 mm Hg (IC_{100}). K factor = Normalization factor.

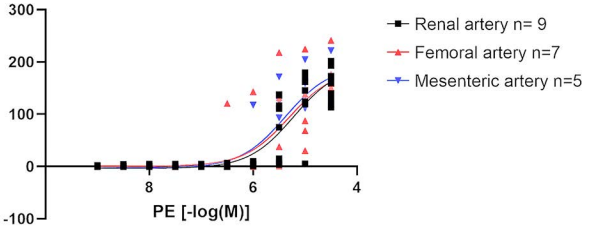

Figure 3—Nonlinear regression curve for renal (black line, square), femoral (red line, triangle), and mesenteric (blue line, reversed triangle) arteries showing the response to a cumulative dose of phenylephrine (PE) against the percentage of the maximum response to high potassium physiological salt solution (KPSS). Each data point represents 1 dog.

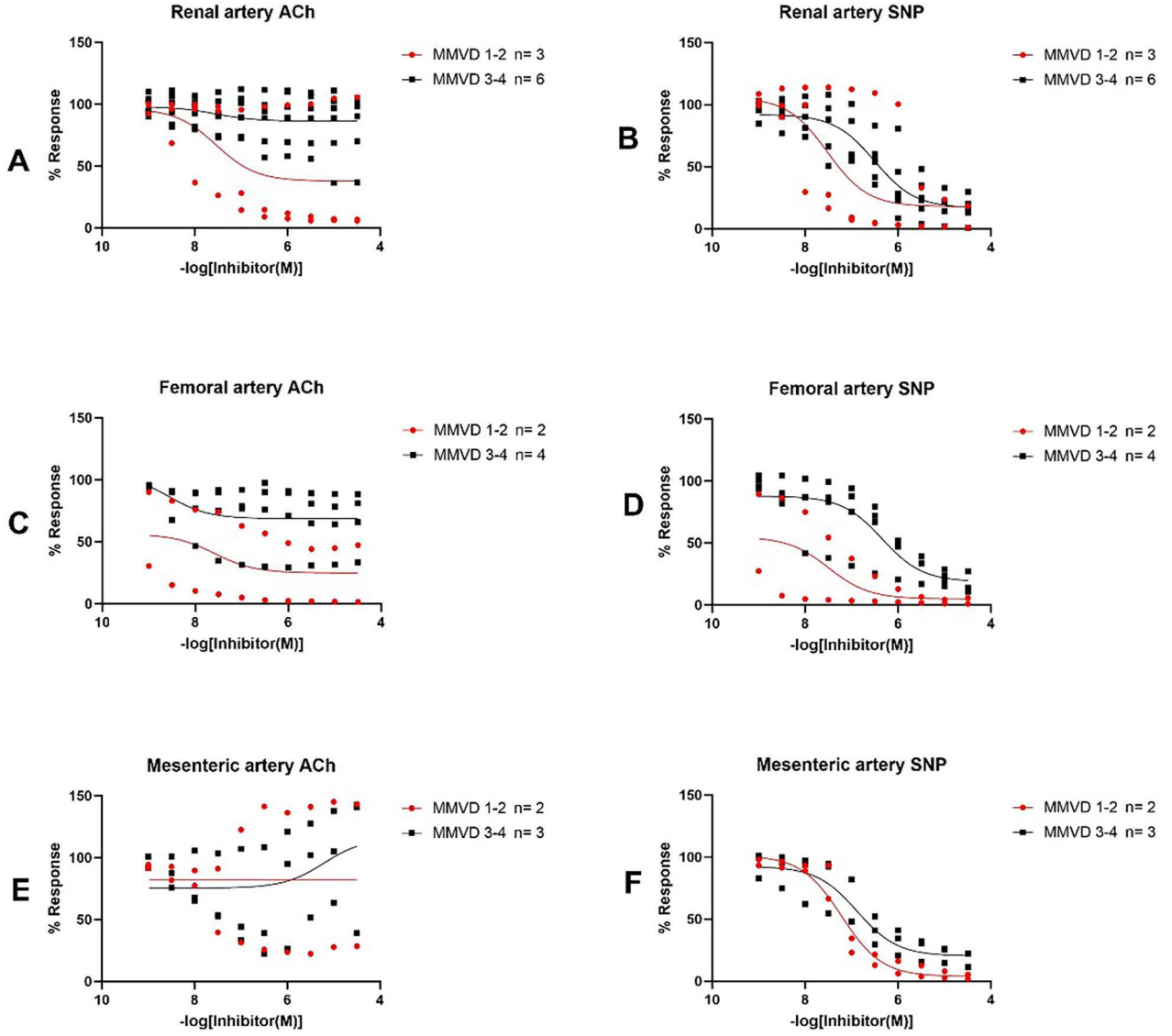

Figure 4—Nonlinear regression curves for renal (A and B), femoral (C and D), and mesenteric (E and F) arteries showing responses to acetylcholine (ACh) and sodium nitroprusside (SNP) in dogs with mild myxomatous mitral valve disease (MMVD; grades 1 to 2) and moderate-severe MMVD (grades 3 to 4). Each data point represents a value for a single dog.

because there was frequently no relaxation at all. In mild MMVD dogs, a $logIC_{50}$ was calculated from a total of 6 pairs of arteries: 3 pairs all obtained from dog 9 and tested on day 1 ($logIC_{50}$ = 6.621 ± 0.913), and 3 pairs obtained from dog 1 (femoral) and dog 2 (mesenteric and renal) and tested on day 3 ($logIC_{50}$ = 9.321 ± 1.068). In moderate-severe MMVD dogs, a $logIC_{50}$ was calculated from a total of 5 pairs of arteries: 1 pair (femoral) from dog 8 and tested on day 1 ($logIC_{50}$ 7.501), and 4 pairs obtained from dog 3 (femoral, mesenteric, and renal) and dog 7 (renal) and tested on day 3 ($logIC_{50}$ = 4.343 ± 2.168). $LogIC_{50}$ values were similar between days (*P* = .99) and MMVD groups (*P* = .12) with no interaction between them (*P* = .08; **Figure 5**). For the response to SNP, day 1 $logIC_{50}$ values were 7.408 ± 0.158 (mild MMVD) and 6.776 ± 0.522 (moderate-severe MMVD). Day 3 $logIC_{50}$ values were 7.54 6 ± 1.646 (mild MMVD) and 6.182 ± 0.553 (moderate-severe MMVD). Again, there was no difference in $logIC_{50}$ values between days (*P* = .58) or MMVD groups (*P* = .055) and no interaction (*P* = .40; **Figure 6**).

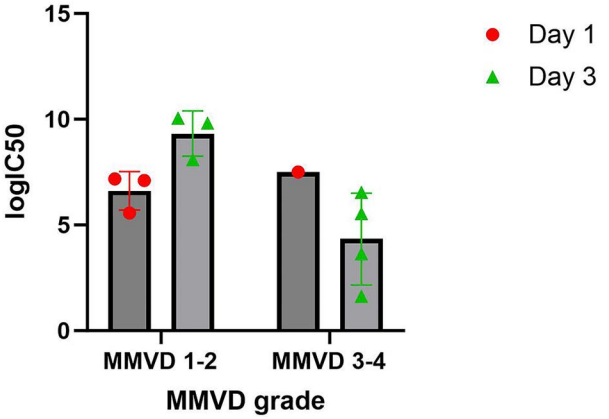

Figure 5—Scatter plots showing the $logIC_{50}$ values for ACh between dogs with MMVD mild (grades 1 to 2) and moderate-severe (grades 3 to 4) and between day 1 and day 3 from the day of collection. Each dot represents a value for a single dog.

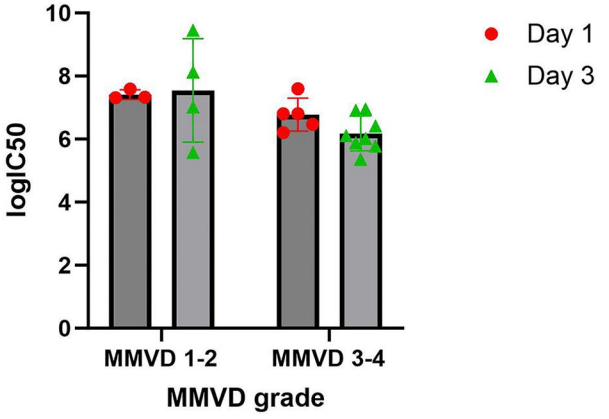

Figure 6—Scatter plots showing the $logIC_{50}$ values for SNP between dogs with MMVD mild (grades 1 to 2) and moderate-severe (grades 3 to 4) and between day 1 and day 3 from the day of collection. Each dot represents a value for a single dog.

Discussion

This is the first study to use isometric myography to assess vascular function in different arteries in pet dogs. We have demonstrated that the viability of arteries collected from specific sites postmortem can be maintained and that constriction and both endothelial-dependent and -independent relaxation can be measured. Importantly, we have used the normalization process to determine the optimal tension required to study vascular function and identify differences between dogs and between artery sites. We conclude that isometric myography is feasible to study vascular function in pet dogs but that use of a fixed normalization ratio does not permit accurate comparison between samples.

Previously, isometric myography has been applied to arteries from experimental dogs used as models of human disease or for physiology studies.[18,19] Extending its use to pet dogs creates logistical difficulties in terms of rapid collection of arteries after euthanasia. Although some arteries that were collected were not viable, we have shown that the technique we used for rapid collection, within 1 hour of euthanasia, maintained viability in the majority of renal, femoral, and mesenteric arteries we examined. Furthermore, viability was maintained for at least 3 days in PSS at 4 °C, and this time period did not appear to significantly affect endothelial-dependent and nonendothelial-dependent relaxation. This means that vascular function can still be performed on arteries collected from 1 geographical site and then transported to another site for myography, increasing the accessibility of multiple centers to this technique.

From the arteries collected, the renal arteries were viable most often, although the femoral and mesenteric arteries were also frequently viable. We speculate that this may reflect ease of collection, despite the more peripheral location of femoral arteries, since renal arteries are easy to identify and can be removed rapidly. This is advantageous, since cardiac and renal functions are closely linked, making the renal artery and its function an attractive experimental target when studying the systemic effects of cardiac diseases such as MMVD. Our finding raises the possibility of future research exploiting the more robust nature of the renal artery with myography to study important cardiorenal vascular interactions.

Having confirmed arterial viability, renal, femoral, and mesenteric arteries were set at tensions that optimize their vasoactive responses (normalization). Normalization factors were calculated to determine whether these could be applied to future studies as an experimental shortcut. Isometric myography is most frequently performed in rodent species, and it is standard practice to use a normalization factor of 0.9 to set the resting tension of the artery being studied. This factor was obtained from work originally conducted on rat mesenteric arteries.[20] Slezák et al[15] have shown that this value is not even appropriate for the rat femoral artery, so it was unsurprising that our study has shown that a normalization

factor of 1.0 rather than 0.9 would be more appropriate for pet dogs. However, although our value of 1.0 is a mean value obtained from each of the 3 arterial sites, the degree of variation between dogs for the same arterial site, especially the renal artery, would suggest that a blanket normalization factor would not be appropriate and runs the risk of introducing experimental error. Instead, we suggest that optimal tensions should be determined for every individual artery before starting a myography study of canine arteries. Since the mathematical method developed by Hugelshofer et al[16] that we used was rapid and simple, we do not believe the additional time required is prohibitive to the application of myography in pet dogs.

The main application for isometric myography is to study vascular function, in particular, endothelial function.[11] Therefore, a key focus of our study was to assess endothelial-dependent and independent vasodilatory functions. Both utilize the nitric oxide pathway but, in endothelial-dependent vasodilation, nitric oxide is released by the endothelium, whereas in endothelial-independent vasodilation, nitric oxide is donated by application of exogenous SNP.[21,22] Our data suggest that endothelial-dependent vasodilation was impaired in arteries from all the sites examined. When identified, the function of sections from the same artery or the contralateral artery was tested. In all cases, endothelial dysfunction was replicated. Furthermore, on histological examination, the endothelial layer appeared structurally intact after arterial collection (Figure 1). Combined, these findings suggest isometric myography can identify vascular dysfunction in pet dogs. Impairment to endothelial-dependent relaxation was observed more frequently in dogs with moderate-severe MMVD (Figure 4), but the study numbers were low and the significance of this could not be determined. However, our work is in agreement with in vivo studies that provide evidence for progressive endothelial dysfunction with worsening canine MMVD.[6,7] Additional studies with larger numbers of dogs subject to ex vivo assessment of vascular function should allow us to identify the underpinning mechanism. The feasibility of myography, using the technique we have described, should facilitate these studies. Since myography assesses the integrity of functional pathways, such studies could identify biomarkers for in vivo assessment of vascular function as well as potential therapeutic targets for endothelial dysfunction in MMVD and other diseases with cardiovascular impact.

It is important to highlight the limitations of this study, in addition to the small sample size. We believe that some arteries were unviable because of the collection technique. Since this is the first study to use myography in pet dogs, our initial approach was similar to the one employed in laboratory rodents. However, arterial collection from rodents is more rapid because of their small size, and multiple sites can be accessed quickly. In our study, significant care was required to dissect down to the arteries of interest, identify them, and then remove them without damage. This delay may have led to the loss of viability. All renal arteries were suitable for the entire experiment, suggesting that if only 1 type of artery is available for collection, the renal artery should be prioritized. An additional important delay in obtaining vessels was related to the euthanasia process. However, we took great care when studying our design not to compromise the welfare and experience of the patient and owner, and this delay must be accepted as integral to future myographic studies in pet dogs. Finally, this study does not include a control group of samples from young dogs. This reflects the postmortem nature of myography and means that the role of age rather than MMVD cannot be discounted in the endothelial dysfunction we identified. For this reason, as well as because of the small numbers, we do not attempt to define the relationship between vascular dysfunction and MMVD but rather advocate a future study to address this question, using the technique we describe.

In conclusion, isometric myography is feasible in pet dogs when arteries are collected within 1 hour of euthanasia. Once collected and stored in PSS at 4 °C, the samples can be analyzed up to 3 days later. Optimal tensions for individual arteries should be determined without the use of a fixed normalization factor. Endothelial dysfunction can be identified and quantified using isometric myography, although further studies are required to establish the relationship between endothelial function and MMVD.

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Disclosures

The authors have nothing to disclose. No AI-assisted technologies were used in the generation of this manuscript.

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Supplementary Materials

Supplementary materials are posted online at the journal website: avmajournals.avma.org.