



# THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

### **Myxomatous Degeneration of the Canine Mitral Valve: From Gross Changes to Molecular Events**

**Citation for published version:**

Markby, G, Summers, K, MacRae, V, Del-Pozo, J & Corcoran, B 2017, 'Myxomatous Degeneration of the Canine Mitral Valve: From Gross Changes to Molecular Events' *Journal of Comparative Pathology*, vol 156, no. 4, pp. 371-383. DOI: 10.1016/j.jcpa.2017.01.009

**Digital Object Identifier (DOI):**

[10.1016/j.jcpa.2017.01.009](https://doi.org/10.1016/j.jcpa.2017.01.009)

**Link:**

[Link to publication record in Edinburgh Research Explorer](#)

**Document Version:**

Peer reviewed version

**Published In:**

*Journal of Comparative Pathology*

**General rights**

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

**Take down policy**

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



**REVIEW ARTICLE**

**SPONTANEOUSLY ARISING DISEASE**

**Short Title: Canine Mitral Valve Myxomatous Degeneration**

**Myxomatous Degeneration of the Canine Mitral Valve: from Gross Changes to  
Molecular Events**

**G. Markby\*, K. M. Summers\*, V. E. MacRae\*, J. Del-Pozo† and B. M. Corcoran†**

*\*Roslin Institute and †Royal (Dick) School of Veterinary Studies, the University of Edinburgh,  
Easterbush Veterinary Centre, Roslin, Scotland, UK.*

Correspondence to: B. M. Corcoran (e-mail: [Brendan.Corcoran@ed.ac.uk](mailto:Brendan.Corcoran@ed.ac.uk)).

## Summary

Myxomatous mitral valve disease (MMVD) is the single most common acquired heart disease of the dog, but is also of emerging importance in human medicine, with some features of the disease shared between both species. There has been increased understanding of this disease in recent years, with most research aiming to elucidate the cellular and molecular events of disease pathogenesis. For gross and histological changes, much of our understanding is based on historical studies and there has been no comprehensive re-appraisal of the pathology of MMVD. This paper reviews the gross, histological, ultrastructural, cellular and molecular changes in canine MMVD.

*Keywords:* myxomatous mitral valve disease; dog; pathology; gene expression

## Introduction

Myxomatous mitral valve disease (MMVD) is the most common acquired cardiac disease of the dog and is a major cause of morbidity and mortality in this species (Darke, 1987; Beardow and Buchanan, 1993; Hamlin *et al.*, 1996; Haggstrom *et al.*, 2004). Much is known about the diagnosis and treatment of MMVD, but only recently has attention focussed on better understanding of the pathobiology of this disease. Recent research builds on original pathological studies from more than 30 years ago, and with available molecular tools an exciting era of research in canine MMVD is now underway. While there are similarities with human MMVD, there are also clear differences in morphological, genetic and protein expression parameters, and extrapolating between species must be done with caution (Hulin *et al.*, 2013; Roberts *et al.*, 2014; Thalji *et al.*, 2015). For example, in human MMVD a large component of the pathology is fibrosis, while in canine MMVD, accumulation of a

myxomatous substance within the core of the valve is the main change (McDonald *et al.*, 2002b; Roberts *et al.*, 2014)

### **Valve Structure**

The mitral valve consists of two leaflets, anterior (septal) and posterior (mitral) and is connected to the heart by the annulus fibrosus and to the papillary muscles by the chordae tendineae (Fig. 1). The chordae attach either to the valve edge (first order) or to the ventricular surface close to the valve edge (second order). The latter insertions give a rough and irregular surface to the underside of the leaflets, while the atrial surface is smooth. There can be marked variation in chordae/papillary muscle organization, and such variation is regarded as normal. Overall the normal leaflet has a partially translucent appearance and is roughly divided into proximal, mid and distal zones, the latter also known as the 'free edge'.

Microscopically, each leaflet has an atrial and ventricular surface and a single layer of endothelial cells, continuous with that lining the atrium and ventricle, covers each surface (Fig. 2). In the mid-zone there are three identifiable layers, the atrialis, spongiosa and fibrosa, each containing varying amounts of collagen I, III, IV and VI, laminin, fibronectin and heparan sulphate (Aupperle *et al.*, 2009a). The thin atrialis is also rich in elastin with a mixed amount of scattered collagen fibres and valvular interstitial cells (VICs). The spongiosa consists of loose collagen fibres, scattered collagen bundles and small numbers of thin elastin fibres embedded in glycosaminoglycan (GAG)-rich ground substance with scattered VICs. Immediately beneath the spongiosa is the dense, well-organized fibrosa layer with well-defined collagen bundles and scattered VICs. The fibrosa is continuous with the annulus fibrosus and branches off to form the core of the chordae tendineae. The thin subendothelial layer on the ventricular side has collagen fibres and occasional elastin, and is

suggested to be an additional fourth layer (ventricularis) similar to that seen in the aortic valve (Buchanan, 1977; Anderson and Wilcox, 1996; Fox, 2012). Towards the distal zone (free edge) the distinction between spongiosa and fibrosa, at least in some dogs, can become indistinct and the valve at this level appears to almost entirely consist of spongiosa covered by endothelium (Han *et al.*, 2013b). Cardiac muscle extends from the atrial myocardium for a variable length along the valve leaflets, and while this lessens with age, likely has important functional implications in valve mechanics (Culshaw *et al.*, 2010; Fox, 2012).

### **Gross Pathology**

Gross changes to the leaflets in MMVD are often described according to the Whitney classification (Fig. 3), and are graded 0 to 4 with 0 being normal and 4 the most advanced form of the disease (Pensinger, 1965; Pomerance and Whitney, 1970; Whitney, 1974; Han *et al.*, 2010). These grades have a close association with advancing age and the intensity of cardiac murmur (Han *et al.*, 2010). Typically, the disease starts with small numbers of discrete nodules developing on the free edge of the leaflet with intervening areas of translucent leaflet (grade 1), progressing to the development of larger nodules (grade 2), coalescence of these nodules (grade 3), involvement of the chordae, and finally, gross distortion and ballooning of the leaflets with marked chordal thickening and eventual chordal rupture (grade 4). The main difficulty with grading can be in distinguishing between normal and grade 1, and an alternative approach is to use measurement of free-edge thickness as an index of severity, but this has not been adopted widely (Disatian *et al.*, 2008, 2010).

Change in the thickness of the edge of the valve, associated with disease progression, has also been confirmed on histological samples, with the posterior leaflet being slightly thicker than the anterior (Han *et al.*, 2010). Additionally, there is lengthening of the valve with advancing disease as evidenced by alteration in the ratio of the anterior-to-posterior

leaflet length, but the anterior leaflet is always longer than the posterior leaflet, irrespective of disease severity (Han *et al.*, 2010). Chordal changes mirror those seen with the valve proper with swelling, thickening and eventual rupture, but chordal changes have not been investigated in detail (Fox, 2012).

### **Microscopical Changes**

On histopathology the most striking changes are expansion of the spongiosa and loss or destruction of the fibrosa (Fenoglio and Tuan duc, 1972; Pomerance and Whitney, 1970; Whitney, 1974; Buchanan, 1977; Kogure, 1980; Hadian *et al.*, 2007; Han *et al.*, 2010) (Fig. 4). The myxomatous changes involve expansion of the loose connective tissue of the spongiosa and an increase in amorphous metachromatically-stained mucoid material (myxomatous degeneration) (Pensinger, 1965; Pomerance and Whitney, 1970; Buchanan, 1977; Han *et al.*, 2010). Amyloid deposition has been reported in a small number of dogs with MMVD (Schneider *et al.*, 1971). The remnants of the fibrosa can be appreciated even in advanced disease, but this layer is markedly disorganized (Han *et al.*, 2013b). Previously, these changes had been described as fibro-elastic proliferation, but this does not appear to be the case and it is connective tissue disorganization that is the main change (Pensinger, 1965; Whitney, 1967; Buchanan, 1977; Aupperle *et al.*, 2009c; Hadian *et al.*, 2009). Overall, depending on the grade of disease, there are changes in laminin, elastin and collagens IV and VI in the subendothelium, and elastin, fibronectin and collagens I and II deeper in the leaflet (Aupperle *et al.*, 2009c). In the myxomatous areas there is a measurable reduction in connective tissue density and increase in GAG content and this reduction equates with disease severity (Han *et al.*, 2010). An accumulation of fat cells adjacent to the fibrosa and mainly towards the valve base has been reported, but may in part be age related (Pomerance and Whitney, 1970).

Qualitative and quantitative differences in total collagen/connective tissue content have been established (Corcoran *et al.*, 2004; Hadian *et al.*, 2007, 2010; Han *et al.*, 2013a). There is a reduction in collagen content in lesion areas and these areas tend to be patchy and only coalesce into more contiguous areas as the disease progresses (Hadian *et al.*, 2007). Changes are typically close to the valve edge and difficult to identify towards the mid and basal zone, but image analysis of cell macerated (i.e. valves decellularized with sodium hydroxide) scanning electron microscopy images has identified mild changes in the mid-zone (Han *et al.*, 2013b). Overall, there is distinct alteration in collagen bundle orientation and fibril alignment in diseased areas (Black *et al.*, 2005; Hadian *et al.*, 2007; Han *et al.*, 2013b). There is a reduction in total tissue content (i.e. collagenous and non-collagenous) in myxomatous areas and increased expression of GAGs (Hadian *et al.*, 2007; Han *et al.*, 2010). Nevertheless, at least in mild to moderate disease, there is a 20% decrease in collagen fibril numbers, but only a 10% decrease in total collagen content (Hadian *et al.*, 2010).

Coupled with these change in collagen there is an increase in immature reducible covalent cross-linkage bonds in collagen in affected areas and a reduction in irreducible mature cross-linkages (Hadian *et al.*, 2010). This implies that there is failure to produce mature fibrillar collagen to replace normal collagen turnover, and improper collagen assembly which can be appreciated at the ultrastructural level (Corcoran *et al.*, 2004; Black *et al.*, 2005; Han *et al.*, 2013b). The production of irreducible (mature) cross-linkages requires lysyl-oxidase, which is known to become depleted in old age, and therefore, changes are also seen as part of the normal ageing process (Eyre *et al.*, 1988; McDonald *et al.*, 2002a, b).

However, these changes are seen in young dogs and the increased deposition of proteoglycans, which is known to occur with MMVD, may interfere with cross-linkages in collagen. The likely involvement of activated myofibroblasts (VICs) in this process, in both

the human and canine forms of the disease, is of major interest (Rabkin *et al.*, 2001; Black *et al.*, 2005; Disatian *et al.*, 2008; Han *et al.*, 2008). Similar changes are noted in the chordae and include significant reduction in collagen, increase in GAG content and altered GAG composition (Buchanan, 1977; Baker *et al.*, 1988). Fat cell accumulation has been reported in the chordae of affected dogs, and is an interesting observation as a change in fat content is also reported for the valve base (Ernst *et al.*, 1973b; Buchanan, 1977; Culshaw *et al.*, 2010). All of these changes are likely to contribute to the mechanical instability of the entire mitral valve complex, and have catastrophic consequences if the chordae rupture.

### **Cellular Changes**

#### *Interstitial and Stromal Cells*

Cellular changes are noted in diseased canine and human valves (Ernst *et al.*, 1973b; Soini *et al.*, 2001; Rabkin-Aikawa *et al.*, 2004; Disatian *et al.*, 2008; Han *et al.*, 2008, 2013a) (Fig. 5a). There is an overall decrease in cell numbers in overtly myxomatous zones in the centre of the leaflet with a significant increase in cell numbers in the peripheral subendothelial zone and close to areas of endothelial cell loss (Han *et al.*, 2010, 2013a, b). Cell shape does not change with advancing disease and the interstitial cells maintain a spindle shape (Han *et al.*, 2010). More importantly, there is phenotypic alteration of the valvular interstitial cells from a presumed quiescent vimentin-positive fibroblast-like cell to a more activated  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)-positive cell type (i.e. activated myofibroblasts) (Black *et al.*, 2005; Disatian *et al.*, 2008; Han *et al.*, 2008). The  $\alpha$ -SMA-positive cells increase in number and are located primarily close to the endothelium, with a partial preference for the atrialis layer (Disatian *et al.*, 2008; Han *et al.*, 2008, 2013a). Increased expression of Ki-67 suggests there is cellular proliferation, but what contribution cell migration might make to this is unknown (Lu *et al.*, 2016). The contribution from the endothelium via endothelial-to-mesenchymal



transition (EMT) should also be considered, and EMT and recapitulation developmental pathways have been partially identified in MMVD (Lu *et al.*, 2015a, 2016). Throughout the diseased valve stroma many cells express embryonic smooth muscle myosin (Smemb), another putative marker of myofibroblast activation, while some co-express  $\alpha$ -SMA and Smemb suggestive of heightened differentiation capacity (Disatian *et al.*, 2008; Lu *et al.*, 2016).

VICs can be found in all layers of the valve and may be divided into five identifiable phenotypes: (1) embryonic progenitor endothelial/mesenchymal cells, (2) quiescent VICs (qVICs), (3) activated VICs (aVICs), (4) progenitor VICs (pVICs), and (5) osteoblastic VICs (obVICs) (Liu *et al.*, 2007). During the earliest stage of valve development, endothelial cells invade the endocardial cushion and are transformed into embryonic progenitor endothelial/mesenchymal cells, and the cushion undergoes matrix remodelling (Armstrong and Bischoff, 2004). Transforming growth factor (TGF)- $\beta$ , bone morphogenetic proteins, Notch signalling, vascular endothelial growth factor and hyaluronic acid contribute to the regulation of this EMT (Choy *et al.*, 1990; Armstrong and Bischoff, 2004; Nosedá *et al.*, 2004; Lu *et al.*, 2015b). Once the valve is formed, qVICs predominate and are thought to maintain valve structure and function and inhibit angiogenesis in the leaflets (i.e. normal life-long valve remodelling). The qVICs are then presumed to become activated to aVICs in response to disease and injury, and then regulate the pathobiological responses of the valve (Liu and Gotlieb, 2007). Small numbers of desmin-positive VICs with chondroblast morphology (obVICs) can be seen in some diseased valves, but calcification is not a clearly identified feature of mitral valve disease in the dog (Han *et al.*, 2008). However,  $\alpha$ -SMA-positive cells can be located in the proximal and mid zones of normal and mildly-affected valves, but only begin to appear in large number in the distal zone as disease develops (Lu, personal communication). In diseased valves, a re-emergence of EMT has been identified,

with changes in expression of a range of genes associated with valve development and EMT. This is best exemplified by emerging co-expression of hyaluronic acid synthase (HAS)-2 and  $\alpha$ -SMA in endothelial cells, and in the stroma, reduced *CDH5* gene expression and increased expression of HAS-2 with stromal deposition of hyaluronic acid (Lu *et al.*, 2015a). Similar differentiation capacity has been identified in ovine mitral and porcine aortic valve endothelial cells (Bischoff and Aikawa, 2011; Wylie-Sears *et al.*, 2011; Mahler *et al.*, 2013). Furthermore, in diseased valves a population of von Willebrand factor (vWF)-positive cells can be identified in the stroma, which might be endothelial in origin (Lu *et al.*, 2016).

Irrespective of their origin or lineage, aVICs are found to lie in areas of loose collagen bundles and can be seen to exude fibrillar collagen on electron microscopy, with many localized away from the central myxomatous areas and close to the valve edge (Ernst *et al.*, 1973a, 1974a, b; Black *et al.*, 2005). This change in cell phenotype and localization coincides with disease progression with demonstrable temporal and spatial alteration in valve cellularity (Disatian *et al.*, 2008; Han *et al.*, 2008; Lu *et al.*, 2016). However, the increased numbers of Smemb-positive cells in the spongiosa, and to a lesser extent the fibrosa, suggests distribution of activated myofibroblasts throughout the entire depth of the valve (Disatian *et al.*, 2008). The relative contributions of these two activated myofibroblast subpopulations to the overall disease process are unknown. What is interesting is that the Smemb-positive cells show much greater expression of 5-hydroxytryptamine (HT) compared with  $\alpha$ -SMA-positive cells, considering the putative role of 5-HT/TGF- $\beta$  signalling in disease pathogenesis (Disatian *et al.*, 2008). In the more advanced stages of the disease, interstitial cells can appear more like smooth muscle, form syncytia and anchor to collagen bundles, and this has been suggested to compensate for the reduction in valve tensile strength (Ernst *et al.*, 1974b; Black *et al.*, 2005). Coupled with this regionalization of stromal cells, there is a reduction in cell number in overtly myxomatous areas compared with normal spongiosa, but no further

change in cell number as the disease progresses (Ernst *et al.*, 1973a; Han *et al.*, 2008). However, in both human and canine mitral valve disease, there is an increase in total cell number through the entire depth of the valve (Rabkin *et al.*, 2001; Rabkin-Aikawa *et al.*, 2004; Disatian *et al.*, 2008). A confounding factor in interpreting changes in cell number is the increased thickness of diseased valves compared with normal, and when cell numbers are indexed to area there is a decrease in cell density (Han *et al.*, 2010). It is clear that there is congregation of cells towards the periphery, close to the overlying endothelium and in areas of endothelial loss (Disatian *et al.*, 2008; Han *et al.*, 2008, 2013a). Experimental ovine valve injury models show the same effect (Lester *et al.*, 1992, 1993; Black *et al.*, 2005; Han *et al.*, 2010).

The exact identity of all of the cells present in myxomatous areas is not known as some are both vimentin and  $\alpha$ -SMA negative. However, in diseased valves the majority of the cells throughout the valve are Smemb positive and this, coupled with  $\alpha$ -SMA expression in some cells and their spindle shape, indicates that they mostly have an interstitial phenotype (Disatian *et al.*, 2008; Han *et al.*, 2008). In human MMVD, some cells are CD34 and CD117 positive, suggesting a circulating fibrocyte, mast cell and/or stem cell lineage, but further work is needed to determine their precise phenotypes and to see if the same populations occur in dogs (Barth *et al.*, 2004, 2005). In dogs with advanced disease there is evidence of a marginal increase in the number of mast cells in affected valves (Han *et al.*, 2008). However, in dogs with severe MMVD there is no evidence of involvement of the resident macrophage population in disease pathogenesis, or recruitment of inflammatory cells, as evidenced by the lack of expression of CD68, CD11c, CD45 and MAC387, despite increased expression of gene transcripts encoding for several inflammatory cytokines and adhesion molecules (Disatian *et al.*, 2008; Han *et al.*, 2008; Disatian and Orton, 2009; Lu *et al.*, 2016). Electron

microscopy has also failed to identify inflammatory cells in the stroma of diseased canine valves (Ernst *et al.*, 1974b; Black *et al.*, 2005).

### *Valvular Endothelial Cells*

Changes to the endothelium are clearly seen in MMVD. Scanning electron microscopy demonstrates areas of endothelial loss, cellular pleomorphism and increased numbers of surface micro-appendages (Ernst *et al.*, 1973a, 1974b; Corcoran *et al.*, 2004) (Fig. 5b). These changes are suggestive of basement membrane abnormalities, endothelial cell death and reactive changes in the endothelium in response to insult. Similar changes can be identified on transmission electron microscopy, with interstitial cell accumulation in the immediate subendothelium and disruption of the basement membrane (Black *et al.*, 2005; Han *et al.*, 2013a). Endothelial damage has also been detected using expression of the endothelial cell markers CD31 and vWF, with clear reduction of expression of these markers in canine MMVD, and further reduction as disease progresses (Disatian *et al.*, 2008; Lu *et al.*, 2016).

The transition of VECs as part of EMT is also an important change (Lu *et al.*, 2015a). It has been hypothesized for many years that repeated damage to the leaflet edge is likely to play a role in the aetiopathogenesis of canine MMVD and the localization of lesions to the valve edge with demonstrable changes to the endothelium would support that assertion. Functional changes in canine valve endothelium have been identified, with increased nitric oxide (NO) synthase activity (i.e. expression of NADH, NADPH reductase and diaphorase), increased endothelin (ET) receptor expression (associated with severity), subendothelial collagen degeneration and deposition of mucopolysaccharides (Ernst *et al.*, 1973b; Mow and Pedersen, 1999; Olsen *et al.*, 2003). This suggests that NO and ET might contribute to the development of MMVD, as both have an effect on extracellular matrix production (Olsen *et al.*, 2003). The migration and localization of activated myofibroblasts towards the

endothelium in MMVD may be a part of this process and would fit into the endothelial injury model of disease pathogenesis (Black *et al.*, 2005; Disatian *et al.*, 2008; Han *et al.*, 2008).

### **Molecular and Biochemical Changes**

Activation of VICs has been shown to be associated with increased extracellular matrix (ECM) secretion (Durbin and Gotlieb, 2002). Several lines of evidence have shown that phenotypic alteration in VICs is crucial to the development of myxomatous degeneration and probably accounts for the derangement of the valvular ECM seen in both canine MMVD and human mitral valve disease (Rabkin *et al.*, 2001; Rabkin-Aikawa *et al.*, 2004; Black *et al.*, 2005; Disatian *et al.*, 2008; Han *et al.*, 2008). The ECM derangement may in part be due to altered expression of matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), the ‘A disintegrin and metalloproteinase with thrombospondin motifs’ (ADAMTS) family of metalloproteases and cathepsins (Soini *et al.*, 2001; Rabkin-Aikawa *et al.*, 2004; Aupperle *et al.*, 2009a, 2010; Lu *et al.*, 2015c). In the dog, with advancing valvular disease, there is decreased expression of the gelatinase MMP-2, an increase in cell surface-bound MMP-14 and an increase in TIMP-2 and TIMP-3 expression, illustrating a change in the balance of catabolic enzymes and their inhibitors that are important in normal valve remodelling (Aupperle *et al.*, 2009b). Changes in gene expression for the MMPs and TIMPs in both human and canine MMVD do not always match that seen on immunohistochemistry, but a 4.5-fold increase in TIMP-1 has been reported (Oyama and Chittur, 2006; Lu *et al.*, 2015c; Greenhouse *et al.*, 2016). MMP-14 is important in the degradation of collagens I, II and III and its presence in the valves of dogs with MMVD, but not in human diseased valves, probably reflects a dog-specific alteration in VIC phenotype (Soini *et al.*, 2001; Aupperle *et al.*, 2009b). Mice with cardiac-specific transgenic expression of active MMP-2 have been

shown to develop marked mitral valve thickening and prolapse with a major loss of collagen bundles and a large accumulation of acidic GAGs, similar to the pathological changes observed in canine MMVD (Aupperle *et al.*, 2009b; Mahimkar *et al.*, 2008). MMP substrates can also include basement membrane components, and basement membrane loss is seen on transmission electron microscopy of diseased dog valves (Matrisian, 1990; Black *et al.*, 2005; Han *et al.*, 2013a).

Gene and protein expression changes implicated in basement membrane disarray have been reported, including alterations in expression of genes encoding *NIDI*, *LAMI*, *LAMA2*, *CTSS* *COL6A3*, and the proteins laminin, collagen IV and fibronectin (Aupperle *et al.*, 2009a; Lu *et al.*, 2015c). The cathepsin-encoding genes *CTSK* and *CTSS* are significantly upregulated in diseased valves and in human MMVD aVICs (Rabkin *et al.*, 2001; Lu *et al.*, 2015c). Expression of these genes is induced by a range of inflammatory cytokines, including interleukins and tumour necrosis factor (TNF)- $\alpha$ , and the substrates of these molecules include elastin, collagens, fibronectin, laminin and nidrogen. While tissue protein expression of the ADAMTS peptidases has not been reported, there are interesting gene changes found in both human and canine MMVD (Lu *et al.*, 2015c; Thalji *et al.*, 2015). There is significant down-regulation of *ADAMTS2*, *ADAMTS9*, *ADAMTS19* and *ADAMTSL4* in diseased canine valves (Lu *et al.*, 2015c). Additionally, a strain of mice with knockout of the gene encoding the proteoglycan cleaving protein ADAMTS5 has been shown to develop a MMVD phenotype due to a lack of proteoglycan clearance. Considering their function in pro-collagen maturation, deposition of the small leucine rich proteoglycan (SLRP) versican and regulation of fibrillin-1 and elastin production (Marfan syndrome) respectively, these changes in *ADAMTS* gene expression fit in with the known valve pathology. Of all of the catabolic enzymes that might be contributing to the pathology of MMVD, it is study of

molecules of the ADAMS family and the cathepsins, but not necessarily the MMPs, which might be most informative (Lu *et al.*, 2015c; Greenhouse *et al.*, 2016).

Alteration in proteoglycan (PG) and GAG expression is also a cardinal feature of mitral valve disease, but has been reported to a limited extent in the dog (Ernst *et al.*, 1973b; Han *et al.*, 2010). Increased PG content in canine valves has been extrapolated indirectly by using cell maceration scanning electron microscopy (Han *et al.*, 2013b). Reduction in fibrillar connective tissue components in human myxomatous mitral valve disease coincides with increased expression of GAGs (Cole *et al.*, 1984; Lis *et al.*, 1987; Whittaker *et al.*, 1987; Tamura *et al.*, 1995; Grande-Allen *et al.*, 2003). However, the relative proportions of the different GAGs also changes with age in normal human subjects, and this is a confounding factor when considering disease-associated changes in a disease that is age-dependent (Murata, 1981; McDonald *et al.*, 2002b). PGs and GAGs are necessary for the proper assembly of the ECM, interact with other important structural proteins such as fibronectin and laminin and have a role in elastin fibrogenesis (Fornieri *et al.*, 1987). Hyaluronic acid is the most abundant GAG in mitral valves, but other proteoglycans, particularly the SLRPs, are likely to have an equally important role in maintaining a healthy ECM (Radermecker *et al.*, 2003). SLRP gene transcripts have the highest signal intensity of all transcripts in normal and diseased canine valves and there is significantly decreased expression of *CHAD* and *KERA* (encoding keratocan) in the canine valve (Lu *et al.*, 2015c). Keratocan is of particular interest as it is important in collagen assembly. Decorin and biglycan are increased in the diseased canine valvular proteome and biglycan, decorin and versican are increased in human MMVD valve tissue (Gupta *et al.*, 2008; Lacerda *et al.*, 2009; Radermecker *et al.*, 2003). Decorin plays a critical role in collagen fibrillogenesis, while biglycan is found in both collagen- and elastin-rich regions and also influences elastic fibre formation (Reinboth *et al.*,

2002; Gupta *et al.*, 2008). The accumulation of GAGs in MMVD is a major contributor to valve thickening and contributes to the mechanical failure of the valve (Tamura *et al.*, 1995).

Elastin is particularly interesting as it has greater tensile strength than collagen and may contribute disproportionately to valve mechanical integrity and strength (Tamura *et al.*, 1995). An increase in elastic fibres has been reported in human and canine myxomatous mitral valves, but the fibres are thinner than normal, arranged into more distinct bundles, and the bundles are more widely separated than in normal leaflets (Tamura *et al.*, 1995).

The signalling pathways that drive all of these structural changes are complex and only understood partially. Global valve tissue transcriptomic profiling has identified a wide range of gene changes associated with MMVD in man and dogs (Oyama and Chittur, 2006; Hulin *et al.*, 2012; Sainger *et al.*, 2012; Lu *et al.*, 2015c; Thalji *et al.*, 2015; Greenhouse *et al.*, 2016). In human MMVD, further major gene changes include the expression of genes involved in control of oxidative stress (an endothelium function) and downregulation of the endothelin receptor type A (ET-A) gene *EDNRA* (Hulin *et al.*, 2012). In the dog there is upregulation of the gene encoding the 5-HT<sub>2B</sub> receptor, but this is not seen in human MMVD. In contrast, there is greater evidence of activation of TGF- $\beta$  signaling pathways in human MMVD that will drive fibrosis (Hulin *et al.*, 2012; Sainger *et al.*, 2012; Thalji *et al.*, 2015; Greenhouse *et al.*, 2016). Changes in TGF- $\beta$  protein and gene expression have been identified in canine MMVD using immunohistochemistry and reverse transcriptase polymerase chain reaction for TGF- $\beta$ <sub>1</sub>, -2 and -3 and TGF- $\beta$ <sub>2</sub>, but transcriptomic profiling has not found such changes (Oyama and Chittur, 2006; Aupperle *et al.*, 2008; Obayashi *et al.*, 2011; Moesgaard *et al.*, 2014; Lu *et al.*, 2015c). Furthermore, upregulation of members of the transcription factor family NFATc is found in human MMVD, but is downregulated in the canine transcriptome (Lu *et al.*, 2015c; Greenhouse *et al.*, 2016). NFATc has an important function in the production of ECM. These canine/human differences are not



unexpected considering that fibrosis is not a cardinal feature of canine MMVD (Buchanan, 1977; Roberts *et al.*, 2014).

Up-regulation of the 5-HT<sub>2B</sub> receptor in the dog is an interesting finding as it is reminiscent of the pathological change in the valve that occurs with use of the appetite suppressant phentermine-fenfluramine and various anti-Parkinsonian drugs (Connolly *et al.*, 2009). The 5-HT/TGF- $\beta$  signalling pathway is important in ECM production, including collagen and fibronectin, but 5HT has not been shown to transform VICs into  $\alpha$ -SMA-positive cells, a cardinal feature of MMVD (Connolly *et al.*, 2009; Oyama and Levy, 2010). The increased protein expression of the 5-HT<sub>2B</sub> receptor, the rate limiting enzyme in serotonin synthesis tryptophan hydroxylase-1 (primarily in  $\alpha$ -SMA-positive cells), phosphorylated ERK-1 and TGF $\beta$ -1 receptors in canine MMVD would support a possible role for enhanced serotonin signalling in MMVD (Arndt *et al.*, 2009; Disatian and Orton, 2009). However, the downregulation of proteins important for tryptophan hydroxylase function may mean that this potential mechanism of disease pathogenesis might not be activated, and the exact role of 5HT in the pathogenesis of MMVD is unclear (Lacerda *et al.*, 2009).

In the dog, cluster analysis of all gene changes suggests that the most affected biologically relevant functions are inflammatory response, cellular movement, cardiovascular development, extracellular matrix organization and epithelial-to-mesenchymal transition, as well as the endothelial function pathways, caveolar-mediated endocytosis signalling, endothelin-1 signalling and epithelial adherens junctions remodelling (Oyama and Chittur, 2006; Lu *et al.*, 2015c). Caveolar-mediated endocytosis regulates endothelial cell growth, endocytosis and cell migration and reduces TGF- $\beta$ -induced fibroblast activation (Del Galdo *et al.*, 2008; Sowa, 2012). In canine and human MMVD there is increased expression of genes encoding inflammatory molecules such as vascular cell adhesion molecule (VCAM)-1,

intercellular adhesion molecule (ICAM)-1, selectin E (SELE), Toll-like receptor (TLR)-4, TLR-8, interleukin (IL)-18 and IL-6, which can trigger ECM changes and EMT (Oyama and Chittur, 2006; Hulin *et al.*, 2012; Sainger *et al.*, 2012; Mahler *et al.*, 2013; Lu *et al.*, 2015c). Activation of low density lipoprotein receptor (LDLR) has been reported in valves affected by MMVD, where this molecule is predicted to upregulate expression of a variety of inflammatory mediators (Olsen *et al.*, 1999; Lu *et al.*, 2015c). An experimental mechanical endothelial shear stress model has been shown to generate reactive oxygen species that act as intracellular secondary messengers and result in elevation of inflammatory cytokines, particularly ICAM-1 (Chiu *et al.*, 1997). The increase in expression of genes encoding IL-6, IL-1 and IL-10, as well as the adhesion molecules VCAM-1 and ICAM-1, may represent a response to the endothelial damage associated with MMVD (Corcoran *et al.*, 2004; Han *et al.*, 2013a). Upregulation of genes associated with oxidative stress has been identified in canine and human MMVD and the increase in inflammation-associated cytokines might be in response to reactive oxygen species (ROS) generated by endothelial shear stress (Hulin *et al.*, 2012; Sainger *et al.*, 2012; Lu *et al.*, 2015c). All of these gene functions are not unexpected considering what is known about valve pathology. Groups of gene changes, which on first examination might not appear to have related functions, clearly contribute to valve pathology by affecting VEC function, transition and loss, VIC activation, proliferation and migration, catabolism of the ECM and aberrant remodelling of the same, without inducing a fibrotic response in the dog. A similar array of changes, but with subtle differences, illustrate the role of fibrosis in human MMVD and the contrast with the canine disease.

### **Conclusion**

There have been major advances in understanding of the pathobiology of canine MMVD in the last 10 years and research activities are ongoing and expanding at various centres. In

addition, there is a large amount of comparative data emerging from studies of human MMVD and associated diseases, but caution must be exercised in extrapolating new findings between man and the dog. The availability of dog-specific molecular tools has allowed the linking of pathological events to effects at the levels of gene and protein expression. However, since the pathological data are more than 30 years old, there is also a need to re-appraise the morphological changes seen with MMVD at the gross, histological and ultrastructural levels. An observation of the same problems in human MMVD research has recently been reported showing that fundamental aspects of valve pathology, particularly the fibrotic response, have been over-looked (Roberts *et al.*, 2014).

### **Acknowledgments**

The authors work has been funded by a variety of sources including the UK Kennel Club, the Cavalier Collection Scheme, the American Kennel Club, the UK Dogs Trust, the Roslin Institute, the University of Edinburgh and the Chinese State Scholarship Scheme.

Production of this review article did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. G. Markby is funded by the UK Dogs Trust and K. M. Summers and V. MacRae are funded by the UK Biotechnology and Biological Sciences Research Council (BBSRC BB/J004316/1).

### **References**

- Anderson RH, Wilcox BR (1996) The anatomy of the mitral valve. In: *Mitral Valve Disease*, FC Wells, LM Shapiro, Eds., Butterworth-Heinemann, Oxford UK pp. 4-27.
- Armstrong EJ, Bischoff J (2004) Heart valve development: endothelial cell signaling and differentiation. *Circulation Research*, **95**, 459-470.

Arndt JW, Reynolds CA, Singletary GE, Connolly JM, Levy RJ *et al.* (2009) Serum serotonin concentrations in dogs with degenerative mitral valve disease. *Journal of Veterinary Internal Medicine*, **23**, 1208-1213.

Aupperle H, Marz I, Thielebein J, Dinges G, Schoon HA (2010) Histomorphological findings and expression of matrix metalloproteinases and their tissue specific inhibitors (TIMPs) in normal tricuspid valves and in chronic tricuspid valvular disease in dogs. *Veterinary Journal*, **183**, 176-183.

Aupperle H, Marz I, Thielebein J, Kiefer B, Kappe A *et al.* (2009a) Immunohistochemical characterization of the extracellular matrix in normal mitral valves and in chronic valve disease (endocardiosis) in dogs. *Research in Veterinary Science*, **87**, 277-283.

Aupperle H, Marz I, Thielebein J, Schoon HA (2008) Expression of transforming growth factor- $\beta$ 1, - $\beta$ 2 and - $\beta$ 3 in normal and diseased canine mitral valves. *Journal of Comparative Pathology*, **139**, 97-107.

Aupperle H, Thielebein J, Kiefer B, Marz I, Dinges G *et al.* (2009b) An immunohistochemical study of the role of matrix metalloproteinases and their tissue inhibitors in chronic mitral valvular disease (valvular endocardiosis) in dogs. *Veterinary Journal*, **180**, 88-94.

Aupperle H, Thielebein J, Kiefer B, Marz I, Dinges G *et al.* (2009c) Expression of genes encoding matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) in

normal and diseased canine mitral valves. *Journal of Comparative Pathology*, **140**, 271-277.

Baker PB, Bansal G, Boudoulas H, Kolibash AJ, Kilman J *et al.* (1988) Floppy mitral valve chordae tendineae: histopathologic alterations. *Human Pathology*, **19**, 507-512.

Barth PJ, Koster H, Moosdorf R (2005) CD34<sup>+</sup> fibrocytes in normal mitral valves and myxomatous mitral valve degeneration. *Pathology Research and Practice*, **201**, 301-304.

Barth PJ, Schenck zu Schweinsberg T, Ramaswamy A, Moll R (2004) CD34<sup>+</sup> fibrocytes,  $\alpha$ -smooth muscle antigen-positive myofibroblasts, and CD117 expression in the stroma of invasive squamous cell carcinomas of the oral cavity, pharynx, and larynx. *Virchow's Archive*, **444**, 231-234.

Beardow AW, Buchanan JW (1993) Chronic mitral valve disease in cavalier King Charles spaniels: 95 cases (1987–1991). *Journal of the American Veterinary Medical Association*, **203**, 1023-1029.

Bischoff J, Aikawa E (2011) Progenitor cells confer plasticity to cardiac valve endothelium. *Journal of Cardiovascular Translational Research*, **4**, 710-719.

Black A, French AT, Dukes-McEwan J, Corcoran BM (2005) Ultrastructural morphologic evaluation of the phenotype of valvular interstitial cells in dogs with myxomatous

degeneration of the mitral valve. *American Journal of Veterinary Research*, **66**, 1408-1414.

Buchanan JW (1977) Chronic valvular disease (endocardiosis) in dogs. *Advances in Veterinary Science and Comparative Medicine*, **21**, 75-106.

Chiu JJ, Wung BS, Shyy JY, Hsieh HJ, Wang DL (1997) Reactive oxygen species are involved in shear stress-induced intercellular adhesion molecule-1 expression in endothelial cells. *Arteriosclerosis, Thrombosis and Vascular Biology*, **17**, 3570-3577.

Choy M, Armstrong MT, Armstrong PB (1990) Regulation of proliferation of embryonic heart mesenchyme: role of transforming growth factor- $\beta$ 1 and the interstitial matrix. *Developmental Biology*, **141**, 421-425.

Cole WG, Chan D, Hickey AJ, Wilcken DE (1984) Collagen composition of normal and myxomatous human mitral heart valves. *Biochemical Journal*, **219**, 451-460.

Connolly JM, Bakay MA, Fulmer JT, Gorman RC, Gorman JH 3<sup>rd</sup> *et al.* (2009) Fenfluramine disrupts the mitral valve interstitial cell response to serotonin. *American Journal of Pathology*, **175**, 988-997.

Corcoran BM, Black A, Anderson H, McEwan JD, French A *et al.* (2004) Identification of surface morphologic changes in the mitral valve leaflets and chordae tendineae of dogs with myxomatous degeneration. *American Journal of Veterinary Research*, **65**: 198-206

- Culshaw GJ, French AT, Han RI, Black A, Pearson GT *et al.* (2010) Evaluation of innervation of the mitral valves and the effects of myxomatous degeneration in dogs. *American Journal of Veterinary Research*, **71**, 194-202.
- Darke PG (1987) Valvular incompetence in cavalier King Charles spaniels. *Veterinary Record*, **120**, 365-366.
- Del Galdo F, Lisanti MP, Jimenez SA (2008) Caveolin-1, transforming growth factor- $\beta$  receptor internalization, and the pathogenesis of systemic sclerosis. *Current Opinion in Rheumatology*, **20**, 713-719.
- Disatian S, Ehrhart EJ 3<sup>rd</sup>, Zimmerman S, Orton EC (2008) Interstitial cells from dogs with naturally occurring myxomatous mitral valve disease undergo phenotype transformation. *Journal of Heart Valve Disease*, **17**, 402-411.
- Disatian S, Lacerda C, Orton EC (2010) Tryptophan hydroxylase 1 expression is increased in phenotype-altered canine and human degenerative myxomatous mitral valves. *Journal of Heart Valve Disease*, **19**, 71-78.
- Disatian S, Orton EC (2009) Autocrine serotonin and transforming growth factor- $\beta$ 1 signaling mediates spontaneous myxomatous mitral valve disease. *Journal of Heart Valve Disease*, **18**, 44-51.

- Durbin AD, Gotlieb AI (2002) Advances towards understanding heart valve response to injury. *Cardiovascular Pathology*, **11**, 69-77.
- Ernst E, Drommer W, Schneider P, Trautwein G (1973a) Electron microscopy studies on the normal structure of atrioventricular valves of the dog. *Anatomischer Anzeiger*, **134**, 309-326.
- Ernst E, Schneider P, Trautwein G (1973b) Etiology and pathogenesis of the endocardiosis and endocarditis in dogs. II. Anatomicopathological findings. *Deutsche Tierärztliche Wochenschrift*, **80**, 322-328.
- Ernst E, Schneider P, Trautwein G (1974a) Electron microscopic studies on amyloidosis of the atrioventricular valves of the dog (author's translation). *Beiträge zur Pathologie*, **152**, 361-382.
- Ernst E, Schneider P, Trautwein G (1974b) Endocardiosis of canine atrioventricular valves. IV. Electron microscopy studies. *Zentralblatt für Veterinärmedizin A*, **21**, 400-416.
- Eyre DR, Dickson IR, Van Ness K (1988) Collagen cross-linking in human bone and articular cartilage. Age-related changes in the content of mature hydroxypyridinium residues. *Biochemical Journal*, **252**, 495-500.
- Fenoglio J Jr, Tuan duc P (1972) Ruptured chordae tendineae. An electron microscopic study. *Human Pathology*, **3**, 415-420.



- Fornieri C, Baccarani-Contri M, Quaglino D Jr, Pasquali-Ronchetti I (1987) Lysyl oxidase activity and elastin/glycosaminoglycan interactions in growing chick and rat aortas. *Journal of Cell Biology*, **105**, 1463-1469.
- Fox PR (2012) Pathology of myxomatous mitral valve disease in the dog. *Journal of Veterinary Cardiology*, **14**, 103-126.
- Grande-Allen KJ, Griffin BP, Ratliff NB, Cosgrove DM, Vesely I (2003) Glycosaminoglycan profiles of myxomatous mitral leaflets and chordae parallel the severity of mechanical alterations. *Journal of the American College of Cardiology*, **42**, 271-277.
- Greenhouse DG, Murphy A, Mignatti P, Zavadil J, Galloway AC *et al.* (2016) Mitral valve prolapse is associated with altered extracellular matrix gene expression patterns. *Gene*, **586**, 56-61.
- Gupta V, Werdenberg JA, Mendez JS, Jane Grande-Allen K (2008) Influence of strain on proteoglycan synthesis by valvular interstitial cells in three-dimensional culture. *Acta Biomaterialia*, **4**, 88-96.
- Hadian M, Corcoran BM, Bradshaw JP (2010) Molecular changes in fibrillar collagen in myxomatous mitral valve disease. *Cardiovascular Pathology*, **19**, e141-148.

- Hadian M, Corcoran BM, Han RI, Grossmann JG, Bradshaw JP (2007) Collagen organization in canine myxomatous mitral valve disease: an x-ray diffraction study. *Biophysics Journal*, **93**, 2472-2476.
- Haggstrom J, Duelund Pedersen H, Kwart C (2004) New insights into degenerative mitral valve disease in dogs. *Veterinary Clinics of North America: Small Animal Practice*, **34**, 1209-1226.
- Hamlin RL, Benitz AM, Ericsson GF, Cifelli S, Daurio CP (1996) Effects of enalapril on exercise tolerance and longevity in dogs with heart failure produced by iatrogenic mitral regurgitation. *Journal of Veterinary Internal Medicine*, **10**, 85-87.
- Han RI, Black A, Culshaw G, French AT, Corcoran BM (2010) Structural and cellular changes in canine myxomatous mitral valve disease: an image analysis study. *Journal of Heart Valve Disease*, **19**, 60-70.
- Han RI, Black A, Culshaw GJ, French AT, Else RW *et al.* (2008) Distribution of myofibroblasts, smooth muscle-like cells, macrophages, and mast cells in mitral valve leaflets of dogs with myxomatous mitral valve disease. *American Journal of Veterinary Research*, **69**, 763-769.
- Han RI, Clark CH, Black A, French A, Culshaw GJ *et al.* (2013a) Morphological changes to endothelial and interstitial cells and to the extracellular matrix in canine myxomatous mitral valve disease (endocardiosis). *Veterinary Journal*, **197**, 388-394.

- Han RI, Impoco G, Culshaw G, French AT, Black A *et al.* (2013b) Cell maceration scanning electron microscopy and computer-derived porosity measurements in assessment of connective tissue microstructure changes in the canine myxomatous mitral valve. *Veterinary Journal*, **197**, 502-505.
- Hulin A, Deroanne C, Lambert C, Defraigne JO, Nusgens B *et al.* (2013) Emerging pathogenic mechanisms in human myxomatous mitral valve: lessons from past and novel data. *Cardiovascular Pathology*, **22**, 245-250.
- Hulin A, Deroanne CF, Lambert CA, Dumont B, Castronovo V *et al.* (2012) Metallothionein-dependent up-regulation of TGF- $\beta$ 2 participates in the remodelling of the myxomatous mitral valve. *Cardiovascular Research*, **93**, 480-489.
- Kogure K (1980) Pathology of chronic mitral valvular disease in the dog. *Nippon Juigaku Zasshi*, **42**, 323-335.
- Lacerda CMR, Disatian S, Orton EC (2009) Differential protein expression between normal, early-stage, and late-stage myxomatous mitral valves from dogs *Proteomics Clinical Applications*, **3**, 1422-1429.
- Lester WM, Damji AA, Gedeon I, Tanaka M (1993) Interstitial cells from the atrial and ventricular sides of the bovine mitral valve respond differently to denuding endocardial injury. *In Vitro Cellular and Developmental Biology*, **29A**, 41-50.

- Lester WM, Damji AA, Tanaka M, Gedeon I (1992) Bovine mitral valve organ culture: role of interstitial cells in repair of valvular injury. *Journal of Molecular and Cellular Cardiology*, **24**, 43-53.
- Lis Y, Burleigh MC, Parker DJ, Child AH, Hogg J *et al.* (1987) Biochemical characterization of individual normal, floppy and rheumatic human mitral valves. *Biochemical Journal*, **244**, 597-603.
- Liu AC, Gotlieb AI (2007) Characterization of cell motility in single heart valve interstitial cells in vitro. *Histology and Histopathology*, **22**, 873-882.
- Liu AC, Joag VR, Gotlieb AI (2007) The emerging role of valve interstitial cell phenotypes in regulating heart valve pathobiology. *American Journal of Pathology*, **171**, 1407-1418.
- Lu CC, Liu MM, Clinton M, Culshaw G, Argyle DJ *et al.* (2015a) Developmental pathways and endothelial-to-mesenchymal transition in canine myxomatous mitral valve disease. *Veterinary Journal*, **204**, 23-31
- Lu CC, Liu MM, Clinton M, Culshaw G, Argyle DJ *et al.* (2015b) Developmental pathways and endothelial to mesenchymal transition in canine myxomatous mitral valve disease. *Veterinary Journal*, **206**, 377-384.

Lu CC, Liu MM, Culshaw G, Clinton M, Argyle DJ *et al.* (2015c) Gene network and canonical pathway analysis in canine myxomatous mitral valve disease: a microarray study. *Veterinary Journal*, **204**, 23-31.

Lu CC, Liu MM, Culshaw G, French A, Corcoran B (2016) Comparison of cellular changes in cavalier King Charles spaniel and mixed breed dogs with myxomatous mitral valve disease. *Journal of Veterinary Cardiology*, **18**, 100-109.

Mahimkar R, Nguyen A, Mann M, Yeh CC, Zhu BQ *et al.* (2008) Cardiac transgenic matrix metalloproteinase-2 expression induces myxomatous valve degeneration: a potential model of mitral valve prolapse disease. *Cardiovascular Pathology*, **18**, 253-261.

Mahler GJ, Farrar EJ, Butcher JT (2013) Inflammatory cytokines promote mesenchymal transformation in embryonic and adult valve endothelial cells. *Arteriosclerosis Thrombosis and Vascular Biology*, **33**, 121-130.

Matrisian LM (1990) Metalloproteinases and their inhibitors in matrix remodeling. *Trends in Genetics*, **6**, 121-125.

McDonald PC, Wilson JE, Gao M, McNeill S, Spinelli JJ *et al.* (2002a) Quantitative analysis of human heart valves: does anorexigen exposure produce a distinctive morphological lesion? *Cardiovascular Pathology*, **11**, 251-262.

- McDonald PC, Wilson JE, McNeill S, Gao M, Spinelli JJ *et al.* (2002b) The challenge of defining normality for human mitral and aortic valves: geometrical and compositional analysis. *Cardiovascular Pathology*, **11**, 193-209.
- Moesgaard SG, Aupperle H, Rajamaki MM, Falk T, Rasmussen CE *et al.* (2014) Matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and transforming growth factor- $\beta$  in advanced canine myxomatous mitral valve disease. *Research in Veterinary Science*, **97**, 560-567.
- Mow T, Pedersen HD (1999) Increased endothelin-receptor density in myxomatous canine mitral valve leaflets. *Journal of Cardiovascular Pharmacology*, **34**, 254-260.
- Murata K (1981) Acidic glycosaminoglycans in human heart valves. *Journal of Molecular and Cellular Cardiology*, **13**, 281-292.
- Noseda M, McLean G, Niessen K, Chang L, Pollet I *et al.* (2004) Notch activation results in phenotypic and functional changes consistent with endothelial-to-mesenchymal transformation. *Circulation Research*, **94**, 910-917.
- Obayashi K, Miyagawa-Tomita S, Matsumoto H, Koyama H, Nakanishi T *et al.* (2011) Effects of transforming growth factor- $\beta$ 3 and matrix metalloproteinase-3 on the pathogenesis of chronic mitral valvular disease in dogs. *American Journal of Veterinary Research*, **72**, 194-202.

- Olsen LH, Fredholm M, Pedersen HD (1999) Epidemiology and inheritance of mitral valve prolapse in Dachshunds. *Journal of Veterinary Internal Medicine*, **13**, 448-456.
- Olsen LH, Mortensen K, Martinussen T, Larsson LI, Baandrup U *et al.* (2003) Increased NADPH-diaphorase activity in canine myxomatous mitral valve leaflets. *Journal of Comparative Pathology*, **129**, 120-130.
- Oyama MA, Chittur SV (2006) Genomic expression patterns of mitral valve tissues from dogs with degenerative mitral valve disease. *American Journal of Veterinary Research*, **67**, 1307-1318.
- Oyama MA, Levy RJ (2010) Insights into serotonin signaling mechanisms associated with canine degenerative mitral valve disease. *Journal of Veterinary Internal Medicine*, **24**, 27-36.
- Pensinger RR (1965) Comparative aspects of mitral valve disease in dogs. *Annals of the New York Academy of Science*, **118**, 525-534.
- Pomerance A, Whitney JC (1970) Heart valve changes common to man and dog: a comparative study. *Cardiovascular Research*, **4**, 61-66.
- Rabkin-Aikawa E, Farber M, Aikawa M, Schoen FJ (2004) Dynamic and reversible changes of interstitial cell phenotype during remodeling of cardiac valves. *Journal of Heart Valve Disease*, **13**, 841-847.

- Rabkin E, Aikawa M, Stone JR, Fukumoto Y, Libby P *et al.* (2001) Activated interstitial myofibroblasts express catabolic enzymes and mediate matrix remodeling in myxomatous heart valves. *Circulation*, **104**, 2525-2532.
- Radermecker MA, Limet R, Lapiere CM, Nusgens B (2003) Increased mRNA expression of decorin in the prolapsing posterior leaflet of the mitral valve. *Interactive Cardiovascular and Thoracic Surgery*, **2**, 389-394.
- Reinboth B, Hanssen E, Cleary EG, Gibson MA (2002) Molecular interactions of biglycan and decorin with elastic fiber components: biglycan forms a ternary complex with tropoelastin and microfibril-associated glycoprotein 1. *Journal of Biological Chemistry*, **277**, 3950-3957.
- Roberts WC, Vowels TJ, Ko JM, Hebler RF Jr (2014) Gross and histological features of excised portions of posterior mitral leaflet in patients having operative repair of mitral valve prolapse and comments on the concept of missing (ruptured) chordae tendineae. *Journal of the American College of Cardiology*, **63**, 1667-1674.
- Sainger R, Grau JB, Branchetti E, Poggio P, Seefried WF *et al.* (2012) Human myxomatous mitral valve prolapse: role of bone morphogenetic protein 4 in valvular interstitial cell activation. *Journal of Cellular Physiology*, **227**, 2595-2604.
- Schneider P, Ernst E, Trautwein G (1971) Amyloidosis of heart valves in dog. *Veterinary Pathology*, **8**, 130-145.



- Soini Y, Satta J, Maatta M, Autio-Harmainen H (2001) Expression of MMP2, MMP9, MT1-MMP, TIMP1, and TIMP2 mRNA in valvular lesions of the heart. *Journal of Pathology*, **194**, 225-231.
- Sowa G (2012) Caveolae, caveolins, cavins, and endothelial cell function: new insights. *Frontiers in Physiology*, **2**, 120.
- Tamura K, Fukuda Y, Ishizaki M, Masuda Y, Yamanaka N *et al.* (1995) Abnormalities in elastic fibers and other connective-tissue components of floppy mitral valve. *American Heart Journal*, **129**, 1149-1158.
- Thalji NM, Hagler MA, Zhang H, Casacang-Verzosa G, Nair AA *et al.* (2015) Non-biased molecular screening identifies novel molecular regulators of fibrogenic and proliferative signaling in myxomatous mitral valve disease. *Circulatory and Cardiovascular Genetics*, **8**, 516-528.
- Whitney JC (1967) Cardiovascular pathology. *Journal of Small Animal Practice*, **8**, 459-465.
- Whitney JC (1974) Observations on the effect of age on the severity of heart valve lesions in the dog. *Journal of Small Animal Practice*, **15**, 511-522.
- Whittaker P, Boughner DR, Perkins DG, Canham PB (1987) Quantitative structural analysis of collagen in chordae tendineae and its relation to floppy mitral valves and proteoglycan infiltration. *British Heart Journal*, **57**, 264-269.

Wylie-Sears J, Aikawa E, Levine RA, Yang JH, Bischoff J (2011) Mitral valve endothelial cells with osteogenic differentiation potential. *Arteriosclerosis, Thrombosis and Vascular Biology*, **31**, 598-607.

*Received, November 14<sup>th</sup>, 2016*

*Accepted,*

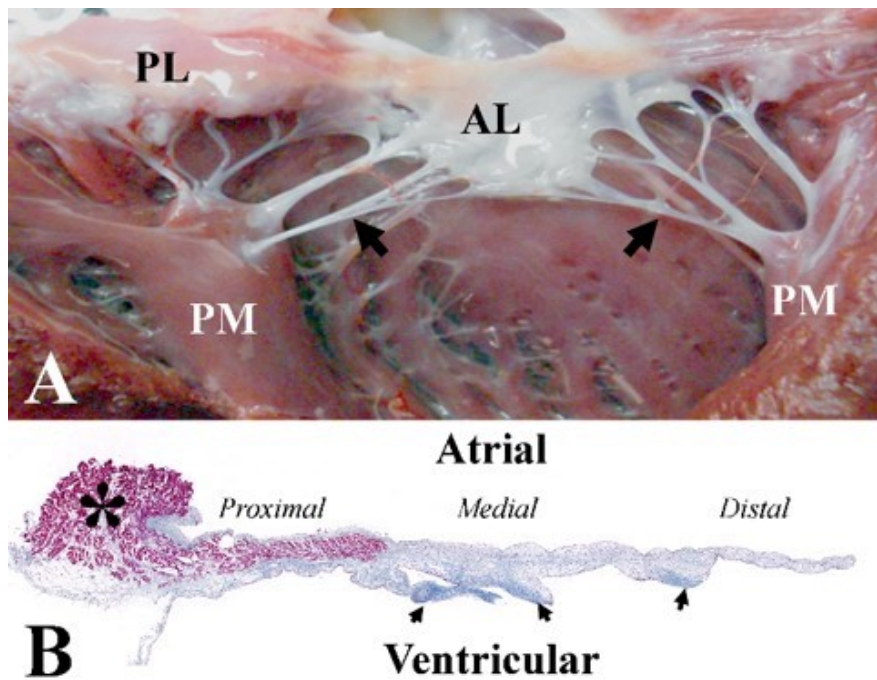


Fig. 1. Healthy canine mitral valve. (A) Gross view of the valve after dissection. Note the presence of an anterior leaflet (AL), posterior leaflet (PL) and chordae tendinae (arrows) connecting the leaflets to the ventricular papillary muscles (PM). (B) Subgross view of the anterior leaflet of the mitral valve, highlighting its two surfaces (atrial and ventricular), the proximal/medial/distal areas, the presence of myocardium extending into the base of the valve (\*), and the attachment of the chordae tendinae. Note that all of the chordae included are of second order. Masson's trichrome. Bar, 1 mm.

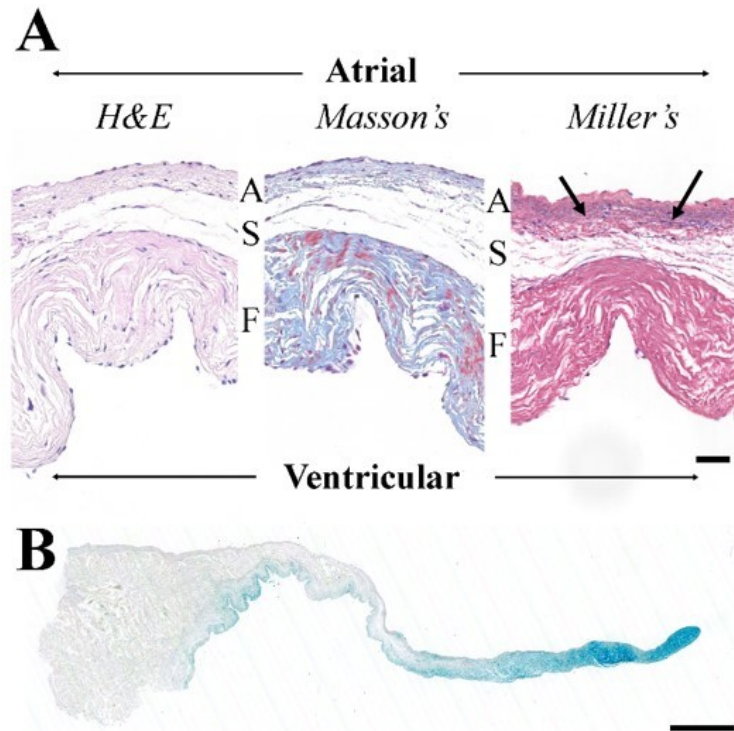


Fig. 2. Healthy canine mitral valve. (A) Images of the same valve at the mid-level displaying the histological features of the atrialis (A), spongiosa (S) and fibrosa (F), with three different histochemical stains. Note the endothelial lining on both the atrial and ventricular aspects of the leaflet, elastin accumulation in the atrialis (arrows), loose connective tissue of the spongiosa and dense collagen of the fibrosa. Haematoxylin and eosin, Masson's trichrome stain and Miller's elastin stain. Bar, 50  $\mu$ m. (B) A subgross view of the same valve stained with Alcian blue highlights the presence of mucopolysaccharide within the leaflet. Bar, 1 mm.

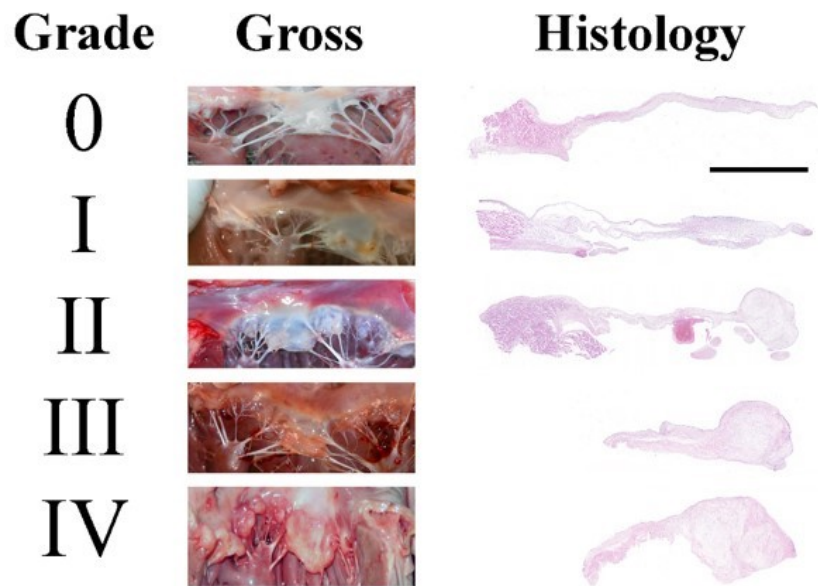


Fig. 3. Whitney grading scheme for myxomatous valvular degeneration. Both the gross and histological presentations are illustrated. Note the location of myxomatous nodules at the tip of the mitral leaflets. There is increase in nodule size with grade, which results in marked distortion of the leaflet tip at higher grades. In grade IV, there is evident thickening of chordae tendinae. HE. Bar, 2 mm.

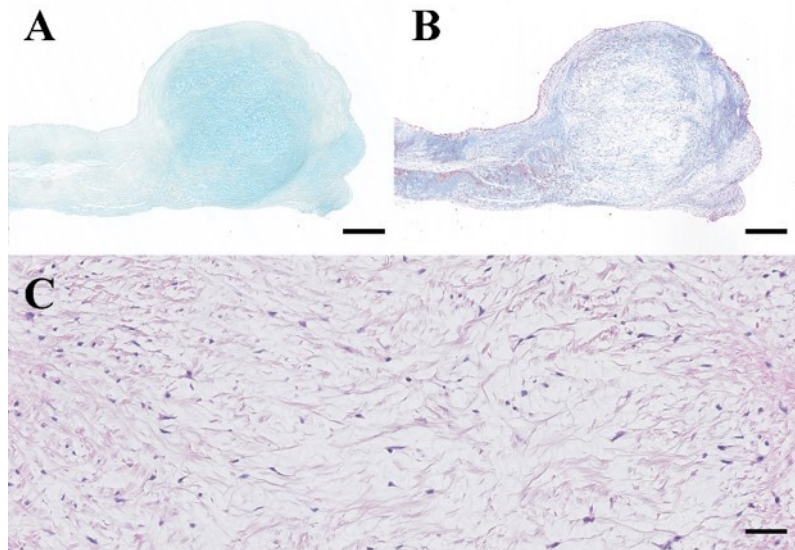
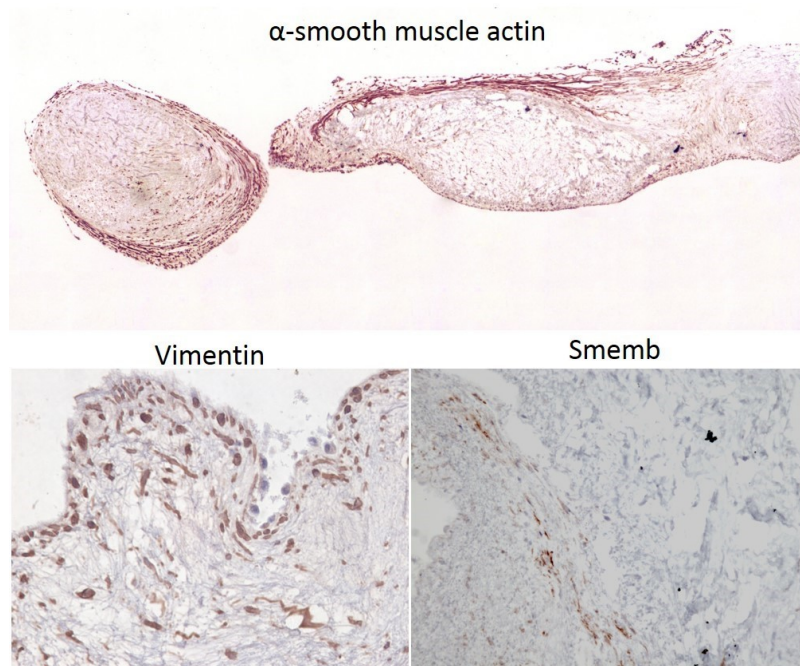


Fig. 4. Histological changes during myxomatous valvular degeneration. The atrialis aspect of the valves is at the top of the picture. (A) There is accumulation of alcian blue-positive myxomatous substance within the spongiosa and fibrosa layers. Bar, 200  $\mu\text{m}$ . (B) This results in distortion and reduction in density of the fibrosa layer, appreciable with Masson's trichrome stain. Bar, 200  $\mu\text{m}$ . (C) With HE staining, the centre of the lesion is characterized by accumulation of unstained material between haphazardly arranged bundles of eosinophilic material and scattered spindle-shaped interstitial cells. Bar, 100  $\mu\text{m}$ .



A



B

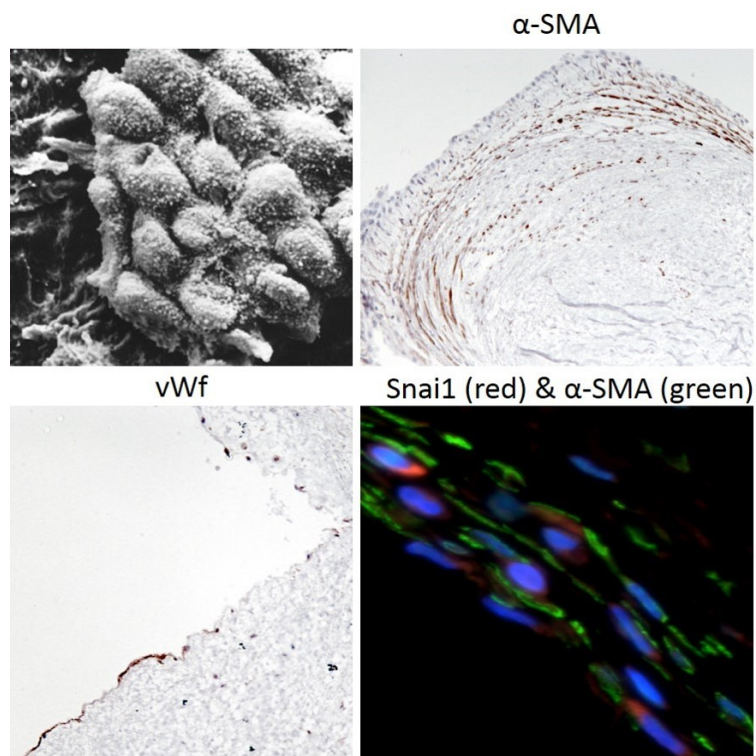


Fig. 5. (A) Representative images of immunohistochemical labelling showing the distribution of cells positive for  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA), embryonic smooth muscle myosin (Smemb) and vimentin in mitral valve myxomatous degeneration. Cells expressing

$\alpha$ -SMA are predominantly close to the valve edge surrounding the central myxoid core (cross section [left] and longitudinal section [right]; Bar, 1mm). Smemb has similar distribution, but with fewer labelled cells (Bar, 50 $\mu$ m) and vimentin labelling is throughout the valve leaflet, but again with increased labelling at the periphery, where there are increased cell numbers (Bar, 25 $\mu$ m). (B) Representative photomicrographs of mitral valve myxomatous degeneration showing: endothelial loss and increased endothelial surface micro-appendages, with exposure of the valve collagen (upper left, scanning electron microscopy; Bar, 10 $\mu$ m), accumulation of  $\alpha$ -SMA-positive cells (activated myofibroblasts) close to intact endothelium overlying a relatively acellular central myxoid core (upper right, immunohistochemistry [IHC]; Bar, 50 $\mu$ m), partial endothelial expression of von Willebrand factor (vWF) (lower left, IHC; Bar, 50 $\mu$ m), and endothelial and interstitial expression of zinc-finger protein Snail (red) with  $\alpha$ -SMA also shown (green) (lower right, dual fluorescence immunolabelling; Bar, 25 $\mu$ m).