The Left Ventricle in Dogs with Myxomatous Mitral Valve Disease

Remodeling and Overall Performance

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The Left Ventricle in Dogs with Myxomatous Mitral Valve Disease. Remodeling and Overall Performance.

Abstract

The concept of left ventricular (LV) remodeling in dogs with myxomatous mitral valve disease (MMVD) includes changes in the LV occurring in response to mitral regurgitation (MR). The general aim of this thesis was to study LV remodeling and function in dogs with different severities of naturally acquired MMVD using 1) digital signal analysis technique for murmur and heart sound assessment, 2) biomarker analyses of circulating cardiac troponin I (cTnI), C-reactive protein (CRP), and matrix metalloproteinases (MMPs), and 3) real-time three-dimensional (RT3D) echocardiography for assessment of changes in LV shape and volume.

Digital linear and nonlinear analyses (using seven different sound variables) of cardiac sounds showed that more severe MR produced a murmur of "harsher" quality, longer duration, and with more complexity in the signal. The energy of the first heart sound was not associated with MR severity (assessed by echocardiography), whereas the energy of the second heart sound decreased with increasing MR severity. Biomarker assessments showed circulating (cTnI) concentration to increase with increasing disease severity, whereas circulating C-reactive protein (CRP) concentration was not associated with disease severity. Neither MMP-2 nor -9 were associated with the MMVD severity groups (which was mainly based on volume overload status), however, MMP-9 activity decreased with worsening systolic function. The RT3D echocardiographic examinations showed prominent LV volume expansions only in dogs with more severe MMVD. The mid LV segment contributed the most to the global volume increase. The LV shape changed from elliptical to more globular in response to increasing volume overload, with the basal and apical segments contributing the most to the increase in sphericity.

In conclusion, the findings in the present thesis provide data that might contribute to the understanding of the complex pathophysiology of MMVD; thereby potentially impacting both clinical management and prediction of outcome for affected dogs in the future.

Keywords: Left ventricle (LV), remodeling, myxomatous mitral valve disease (MMVD), murmur, biomarker, real-time three-dimensional (RT3D) echocardiography.

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To My Family

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ljungvall I, Ahlstrom C, Höglund K, Hult P, Kvart C, Borgarelli M, Ask P, Häggström J. 2009. Use of signal analysis of heart sounds and murmurs to assess mitral valve regurgitation attributable to myxomatous mitral valve disease in dogs. *American Journal of Veterinary Research*, 70, 604-613.

II Ljungvall I, Höglund K, Tidholm A, Olsen LH, Borgarelli M, Venge P, Häggström J. 2010. Cardiac troponin I is associated with severity of myxomatous mitral valve disease, age, and C-reactive protein in dogs. *Journal of Veterinary Internal Medicine*, 24:153-159.

III Ljungvall I, Rajamäki MM, Crosara S, Olsen LH, Kvart C, Borgarelli M, Höglund K, Häggström J Matrix metalloproteinase-9 is associated with systolic function in dogs with myxomatous mitral valve disease. *American Journal of Veterinary Research*. In press.

IV Ljungvall I, Höglund K, Carnabuci C, Tidholm A, Häggström J. Assessment of global and regional left ventricular volume and shape using real-time-three-dimensional echocardiography in dogs with myxomatous mitral valve disease. Revised manuscript.

Abbreviations

AUC	Area under the curve
BNP	B-type natriuretic peptide
CHF	Congestive heart failure
CKCS	Cavalier King Charles spaniel
CRP	C-reactive protein
cTnI	Cardiac troponin I
ECG	Electrocardiogram
ECM	Extracellular matrix
EDV	End-diastolic volume
ESV	End-systolic volume
HR	Heart rate
LA	Left atrium
LA/Ao	Left atrial to aortic ratio
LV	Left ventricle
LVIDd	End-diastolic left ventricular internal dimension
LVIDs	End-systolic left ventricular internal dimension
$LVIDd_{inc}$	Percentage increase in end-diastolic left ventricular internal dimension
$LVIDs_{inc}$	Percentage increase in end-systolic left ventricular internal dimension
MMP	Matrix metalloproteinase
MMVD	Myxomatous mitral valve disease
MR	Mitral regurgitation
MVP	Mitral valve prolapse
PCG	Phonocardiogram
2D	Two-dimensional
3D	Three-dimensional
RT3D	Real-time three-dimensional
RA	Right atrium
RV	Right ventricle
ROC	Receiver operating characteristic curve

SAP	Systolic arterial pressure
S1	First heart sound
S2	Second heart sound

1 Introduction

1.1 General background

The concept of ventricular remodeling, which was coined in the 1980s (Pfeffer *et al.*, 1985), is applied when describing ventricular changes occurring in response to hemodynamic changes of various etiologies (Opie *et al.*, 2006). The ventricular remodeling processes include left ventricular (LV) dilation, changes in LV shape, and LV muscle mass hypertrophy (Cohn, 1995); which all might adversely affect cardiac performance.

In 1817, Delabere Blaine described abnormal heart beats to "afford a decided characteristic of the complaint", detectable as a thrill when placing the hand on the side of the chest of a dog; thus indicating the presence of a cardiac disease (Blaine, 1817). Pathological changes of the mitral valve apparatus in dogs, possibly caused by valve degeneration, was described in the literature as early as 1935 (Münich, 1935). The knowledge in veterinary cardiology has improved tremendously since then, and assessment of cardiac diseases can nowadays be performed using various diagnostic techniques. As a result, myxomatous mitral valve disease (MMVD) has been proven the most prevalent cardiac disease in dogs (Buchanan, 1977; Whitney, 1974; Das & Tashjihan, 1965; Detweiler & Patterson, 1965), and hence, the disease most commonly causing LV remodeling in dogs. The highest disease prevalence in dogs is seen in small to medium-sized breeds, such as Cavalier King Charles Spaniel (CKCS), Dachshunds, miniature Poodles, and Yorkshire Terriers (Egenvall *et al.*, 2006; Olsen *et al.*, 1999; Haggstrom *et al.*, 1992; Darke, 1987; Buchanan, 1977). The prevalence increases with age and is, at a given age, higher in males (Olsen *et al.*, 1999; Häggström *et al.*, 1992; Buchanan, 1977; Whitney, 1974; Das & Tashjihan, 1965; Detweiler

& Patterson, 1965). Affected dogs have no signs of valve abnormalities at birth, but develop MMVD later in life. The etiology of MMVD is currently not known, but the current leading scientific hypothesis is that a genetically determined dystrophic process, rather than succession of repeated trauma of the valve leaflets, initiates the valve degeneration (Olsen *et al.*, 1999; Swenson *et al.*, 1996). This hypothesis is strengthened by the knowledge that some breeds, such as the CKCS and Dachshund, are predisposed to an early onset of MMVD (Lewis *et al.*, 2011; Olsen *et al.*, 1999; Swenson *et al.*, 1996; Häggström *et al.*, 1992). The disease, which has been described to strongly resemble primary mitral valve prolapse (MVP) syndrome in people (Pedersen & Häggström, 2000), is characterized by progressive degeneration of the mitral valve (Kogure, 1980; Buchanan, 1977; Whitney, 1974). The valve degeneration leads to mitral valve leakage, referred to as mitral regurgitation (MR), and subsequently chronic volume overload with dilation of the left atrium (LA) and the LV. Progression of the disease varies between individuals, but affected dogs can usually compensate the MR for years. Eventually the heart might become incapable of meeting the increased work load imposed upon it and congestive heart failure (CHF) develops. An increased cardiac mortality before the age of 10 years has been shown in dog breeds affected by an early onset of MMVD (Egenvall *et al.*, 2006).

The mechanisms involved in the remodeling process of MMVD remain poorly characterized and understood; partly owing to the slow progression from early degenerative changes to development of CHF; which complicates studies of disease progression. Cardiac remodeling processes in experimental settings as well as in acute vascular diseases in other species have been more extensively documented (Lang *et al.*, 2006a; Stewart *et al.*, 2003; Dell'Italia *et al.*, 1995). However, these cardiovascular events differ fundamentally from naturally acquired chronic cardiac disease in origin and progression, which emphasizes the importance of investigating the LV remodeling process also in dogs with naturally acquired MMVD. Studying LV remodeling in dogs with different severities of MMVD using recently developed diagnostic techniques, such as sound analysis techniques, magnetic resonance imaging, real-time three-dimensional (RT3D) echocardiography, and high sensitivity biomarker assays, as well as more established techniques, might reveal new concepts of pathophysiological changes occurring during MMVD progression. Increased knowledge of these complex pathophysiological processes might, hopefully, be of value for improved clinical management of the individual dog affected by the disease.

2 The mitral valve apparatus

2.1 Normal anatomy and function

The mitral valve apparatus includes six anatomical elements; the posterior LA wall, the mitral orifice, the leaflets (the posterior and anterior), the chordae tendineae, the papillary muscles, and the LV wall; which all work in fine concert to maintain competence (Perloff & Roberts, 1972).

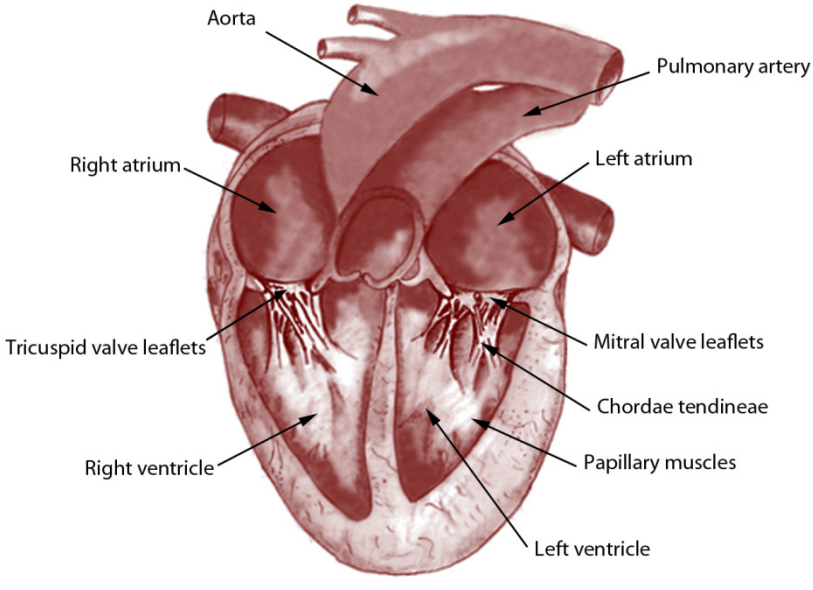

Figure 1. Normal anatomy of the canine heart.

In addition to serving as a hinge for the leaflets, the mitral orifice reduces the area required for the leaflets to bridge during ventricular systole (the ventricular contraction period) by decreasing its circumferential size (Ahmed *et al.*, 2009; Brolin, 1967; Davis & Kinmonth, 1963). A larger surface area of the leaflets compared to the annulus area, as described in people, works as a reserve for leaflet coaptation; hence preventing backflow of blood (Ahmed *et al.*, 2009; Perloff & Roberts, 1972). Each leaflet is supported by fibrous strands (chordae tendineae), which insert on papillary muscles arising from the apical and middle sections of the LV wall. When the LA pressure exceeds the LV pressure, the mitral valve opens and the LA empties into the LV. The mitral valve closes completely during LV systole to prevent retrograde flow. The mechanisms involved in closure of the mitral valve are not fully understood, and different events are probably involved in this process (Little, 1979). The LV filling pressure stretches the LV wall to its greatest geometric dimension (preload) during ventricular diastole. The increased intraventricular pressure during early systole forces the mitral valve to close by synergistic contraction of the LV walls; resulting in an appropriate application of vertical force to the chordae tendineae and hence, prevention of leaflet-eversion into the LA (Perloff & Roberts, 1972). Likely, significant reduction in the mitral orifice area contributes to closure of the mitral valve (Tsakiris *et al.*, 1971; Chiechi *et al.*, 1956). The mitral valve orifice is finally sealed when the free edges of the mitral valve leaflets firmly coapt.

2.2 Myxomatous mitral valve degeneration

Myxomatous mitral valve disease in dogs is characterized by progressive myxomatous degeneration of the mitral valve apparatus (Kogure, 1980; Buchanan, 1977; Whitney, 1974). Although the myxomatous degeneration most commonly affects the mitral valve; any of the four intracardiac valves can be affected. However, the pulmonary and aortic valves (the semilunar valves) rarely develop such degenerative changes (Buchanan, 1977). Histopathological findings include myxomatous degeneration (which refers to a characteristic pathological weakening and disturbance in the organization of the connective tissue) in which the spongiosa component is unusually prominent, and the collagen fibers are disorganized in the fibrosa layer (Hadian *et al.*, 2010; Hadian *et al.*, 2007; Black *et al.*, 2005). Proteolytic enzymes, such as the matrix metalloproteinases (MMPs), might be involved in the degenerative processes leading to atypical organization of

connective tissue components (Aupperle *et al.*, 2009b; Aupperle *et al.*, 2009c; Dreger *et al.*, 2002). An increased amount of mucopolysaccarides, and glycosaminoglycans are commonly seen within affected valves (Han *et al.*, 2010; Hadian *et al.*, 2007; Kogure, 1980). Characteristic findings of endothelial cells covering the valve surface include pleomorphism and damage to the cell-lining. The endothelial damage, which is most commonly evident near the edges of the valve leaflets, can cause regional loss of endothelial cells; hence exposing underlying basement membranes or subendothelial matrix (Corcoran *et al.*, 2004). Endothelial damage induces the release of vasoactive peptides, such as endothelin-1, which potentially is involved in transforming subendothelial valvular interstial cells (VICs) from a predominantly fibroblast phenotype into more active myofibroblast and smooth muscle cell phenotypes (Black *et al.*, 2005; Corcoran *et al.*, 2004; Mow & Pedersen, 1999). The transformation of VICs has also been suggested to be initiated by serotonin (5HT) (Oyama & Levy, 2010; Arndt *et al.*, 2009).

The mitral valve leaflets, which normally are thin, translucent and soft, become thickened and elongated with disease progression (Han *et al.*, 2010; Corcoran *et al.*, 2004; Kogure, 1980). The chordae tendineae also become affected by the myxomatous degeneration (Corcoran *et al.*, 2004; Beardow & Buchanan, 1993); resulting in elongation (Kogure, 1980), which together with distortion of the valve architecture, contributes to systolic atrial displacement of the mitral valve leaflets (i.e. mitral valve prolapse) (Pedersen *et al.*, 1996). The thickened and elongated chordae tendineae might rupture, which potentially worsens MR by causing leaflets to partially or completely prolapse (valve flail) into the LA (Beardow & Buchanan, 1993).

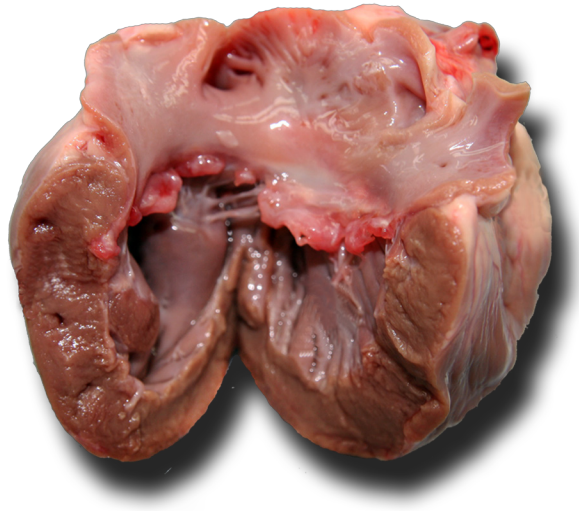

Figure 2. Post-mortem specimen of a dog with end-stage MMVD. The mitral valve leaflets appear irregularly thickened and contracted. There is also evidence of chordal engagement. Chordal rupture, particularly of lesser-order chordae, is a common finding, but not clearly apparent in this image. The left atrium and left ventricle are dilated and there is evidence of jet lesions on the atrial wall (which occur when the regurgitant jet of blood from the left ventricle strikes the atrial wall)

2.3 Mitral regurgitation

The degenerative changes of the mitral valve and the corresponding chordae tendineae lead to abnormal coaptation of the mitral valve leaflets during ventricular systole; why a percentage of the LV stroke volume is ejected backwards into the LA. The retrograde ejection of LV stroke volume starts already in early systole; leading to a short isovolumetric contraction period (defined as the interval between closing of the atrioventricular valves and opening of the semilunar valves) (Lord, 1974; Eckberg *et al.*, 1973). The extra pathway (into the LA) for stroke volume ejection reduces the LV afterload (the resistance to LV emptying). Most commonly, dogs with MMVD have a laterally directed MR jet, presumably because the anterior leaflet is longer and has a greater mobility than the posterior leaflet (as described in dogs and people) (Ahmed *et al.*, 2009; Borgarelli, 2004), and hence, is more likely to prolapse than the posterior leaflet. However, the spatial orientation of the jet is not constant, particularly not in mild cases of MR, probably due to changes in the shape and orientation of the mitral orifice area during LV contraction (Tsakiris *et al.*, 1971).

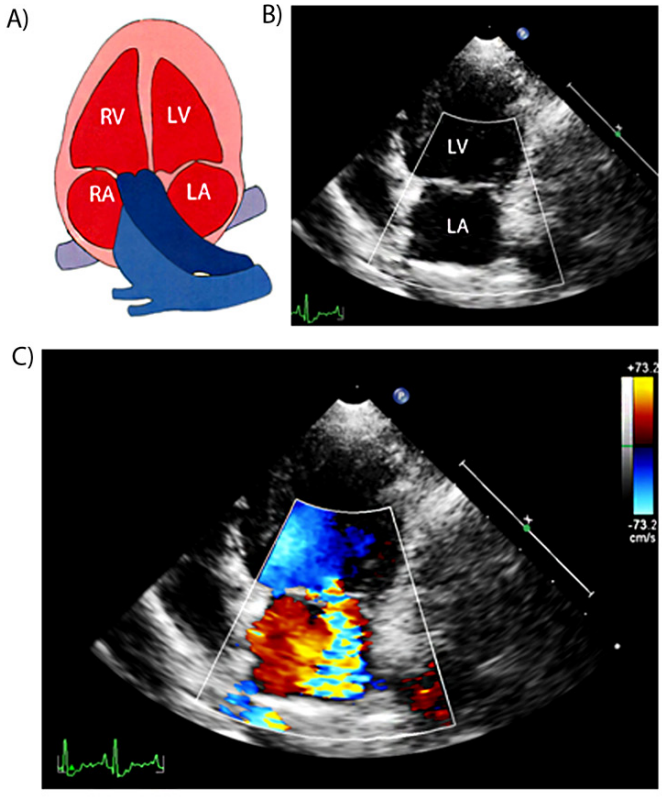

Figure 3. Left apical four-chamber views of the heart in a dog with mitral regurgitation caused by MMVD. A schematic drawing of the anatomy of the heart is shown in the same orientation as the echocardiograms (A). The echocardiograms show the identical image frame with the color mode off (B) and on (C). There is systolic displacement of the mitral valve leaflets (B) and valve leakage (C) as indicated by the turbulent flow (mosaic appearance on the color echocardiogram) in the left atrium. On the left ventricular side, blood is accelerating towards the regurgitant orifice, after which the flow becomes turbulent and is primarily directed laterally in the left atrium. The driving force for the jet is the pressure gradient between the left ventricle and atrium, which typically leads to flow velocities between 5.5 to 6 m/sec until late stages of the disease. Because the cross-sectional area changes from being comparably small at the regurgitant orifice to large on the atrial side, the flow becomes turbulent. In the left atrium, the kinetic energy of the jet is transformed into heat and vibrations, which are audible as a heart murmur on the thoracic wall. LA-left atrium, LV-left ventricle, RA-right atrium, RV–right ventricle.

The MR volume has been described to depend on the mitral valve orifice area, and the systolic pressure gradient between the LA and the LV (Mihalatos et al., 2007; Pierpont & Talley, 1982), of which the latter is influenced by the intra-atrial pressure, the LV function, as well as the systemic arterial blood pressure. The myxomatous degeneration of the mitral valve apparatus causes an abnormal leaflet-apposition, and hence primary MR, whereas left sided cardiac dilation exaggerates the abnormal valve apposition, leading to a secondary MR (Ahmed et al., 2009; Buchanan, 1977). Consequently, regurgitation begets regurgitation. Several mechanisms have been described involved in production of secondary MR: The LV dilation prevents the orifice from fully decreasing its circumferential size during ventricular systole (Buchanan, 1977; Brolin, 1967). In addition, the altered LV shape causes the laterally displaced papillary muscles to exert a more lateral, rather than vertical, force of exertion on the leaflets; hence further separating the mitral leaflets in systole (Hung et al., 2004; Lapu-Bula et al., 2002; Otsuji et al., 1997; Kono et al., 1992; Buchanan, 1977). Left atrial dilation might also aggravate the preexisting MR due to further leaflet displacement (Levy & Edwards, 1962). Expansion of the LA buffers the increasing MR volume, thereby allowing the intra-atrial pressure to remain comparably low, and blood can easily be ejected into the LA during ventricular systole, even in dogs with severe MMVD. More than 75% of the total LV stroke volume has been reported to be ejected into the LA during systole in dogs with severe MMVD (Kittleson & Brown, 2003). The severity of left sided cardiac dilation is linked to MR severity, suggesting that MR volume is the major determinant factor for the degree of left sided cardiac dilation (Eriksson et al., 2010; Kittleson & Brown, 2003).

3 The left ventricle

3.1 Myocardial histology

The LV myocardium in dogs has been shown to be composed of muscle layers with a characteristic three-dimensional (3D) organization, running radially across the LV wall from subendocardium to subepicardium (LeGrice *et al.*, 1995). Most of the myocardium is occupied by cardiac myocytes. Each myocyte is composed of bundles of myofibrils, which comprise longitudinally arranged microanatomical units termed sarcomeres. The sarcomeres, which represent the basic contractile units of the myocyte, are composed of thick and thin filaments; myosin and actin, respectively. Chemical and physical interactions between actin and myosin cause myocyte contraction; effected by sliding filaments along one another (Katz, 2001; Walker & Spinale, 1999).

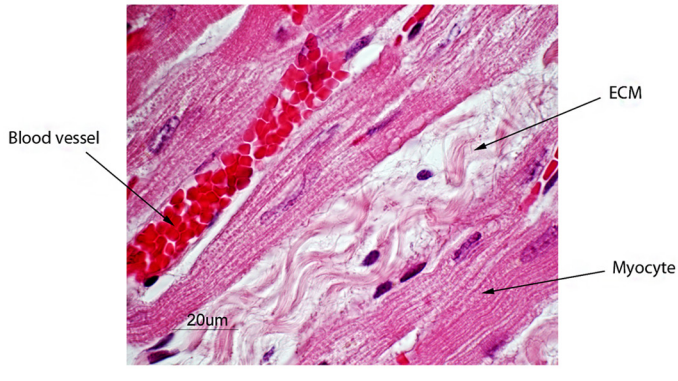

Figure 4. Histologic section of the myocardium showing myocytes, extracellular matrix (ECM) and a blood vessel. Haemotoxylin & eosin staining. (Courtesy of Fredrik Södersten)

Structural integrity and overall geometry of the heart is maintained by the extracellular matrix (ECM), which tethers individual myocytes together in proper alignment within the myocardium, hence working as a cardiac framework (Spinale, 2002; Weber *et al.*, 1988; Robinson *et al.*, 1983). This structural organization provides tensile strength to the myocardium; enabling transduction of contractile force generated by the myocytes in systole (Pelouch *et al.*, 1993; Weber, 1989; Robinson *et al.*, 1986). The cardiac fibroblast is the most abundant cell type within the ECM and most ECM components are produced exclusively by these cells (Pelouch *et al.*, 1993; Robinson *et al.*, 1983). The fibroblasts can also secrete enzymes that regulate ECM turnover; such as the MMPs, their tissue inhibitors (TIMPs), as well as other proteolytic enzymes (Hutchinson *et al.*, 2010). Collagen subtypes and fibronectin compose the majority of the ECM proteins responsible for maintaining structural integrity of the myocardium (Pelouch *et al.*, 1993).

3.2 Left ventricular changes in response to mitral regurgitation

Mitral regurgitation results in an increased total LV stroke volume as blood is ejected both forward into the aorta and retrograde into the LA. In order to accommodate the increase in preload, the LV undergoes various compensatory responses.

3.2.1 Changes in the myocardium

Changes in the composition and structure of both the ECM and the myocytes have been reported in dogs with MR, but the underlying cellular and molecular bases for these changes remain poorly understood. The ECM is a highly adaptive structure, which has been shown to play a fundamental role in myocardial adaptation to pathological stress; thereby facilitating remodeling (Weber *et al.*, 1992). The remodeling process of the ECM has been suggested to occur as a mechanically mediated response to stretch caused by volume overload and/or due to selective induction of proteolytic enzymes, such as the MMPs (Woessner, 1991). Normal collagen chains are fractured and replaced by poorly cross-linked collagen, resulting in loss of normal structural support and myocyte slippage (Zheng *et al.*, 2009; Dell'italia *et al.*, 1997; Kato *et al.*, 1995). The myocytes are exposed to an abnormal stress-and-strain pattern during the cardiac cycle, particularily during diastole owing to increased filling pressure (preload) (Spinale, 2002; Pelouch *et al.*, 1993; Grossman *et al.*, 1975). This triggers an unnatural growth response with myocyte hypertrophy and replication of sarcomeres in

series; so-called eccentric hypertrophy (Grossman *et al.*, 1975), thereby increasing myocyte length (Katz, 1990). Ventricular muscle mass is determined by the net difference between synthesis and degradation rate, and development of myocardial hypertrophy due to volume overload has been suggested to be caused by a decrease in degradation, rather than an increase in synthesis of contractile proteins (Carabello, 2002). Myocardial hypertrophy has also been suggested stiumulated by an increased neurohormonal activation, such as by increased formation of angiotensin II (AII) (Morgan & Baker, 1991; Schelling *et al.*, 1991). In dogs with naturally occurring MR caused by MMVD, circulating levels of AII appear comparably unchanged during progression from mild MMVD to overt CHF (Haggstrom *et al.*, 1997). However, the canine myocardium is most likely capable of forming AII locally in the heart in response to hemodynamic wall stress: Angiotensin converting enzyme (ACE), chymase and cathepsin D have all been reported capable of promoting tissue AII formation in the volume over-loaded canine heart (Stewart *et al.*, 2003; Barlucchi *et al.*, 2001; Dell'Italia *et al.*, 1995).

The structural changes in the cardiac ECM and myocytes allow for chronic dilatation without overstretching of the myocytes, thereby minimizing the effects on the myocardium from the increased volume overload (Komamura *et al.*, 1993; Anversa *et al.*, 1986). The resulting eccentric hypertrophy is characterized by chamber enlargement, but with maintained relative wall thickness; sufficient to normalize pressure in the volume overloaded LV and maintain an adequate forward stroke volume (Carabello, 2002). However, the LV mass-to volume ratio has been shown to decrease with progression of MR in dogs (both naturally acquired and experimentally induced MR) and in people (Borgarelli *et al.*, 2007; Carabello, 2000; Dell'Italia *et al.*, 1995; Katz, 1995; Urabe *et al.*, 1992; Grossman *et al.*, 1975), indicating insufficient degree of hypertrophy to accommodate severe volume overload.

The compensatory mechanism, such as LV hypertrophy, dilation, and enhanced activity of the neurohormonal system, are all initially considered beneficial in order to provide the hemodynamic support needed to maintain sufficient cardiac output despite MR. However, with progression of disease, these mechanisms themselves become factors leading to deterioration of the failing heart; such as by myocyte injury and accumulation of collagen fibers (i.e. myocardial fibrosis) (Opie, 2002; Cohn *et al.*, 2000; Sabbah *et al.*, 1995; Grossman *et al.*, 1975).

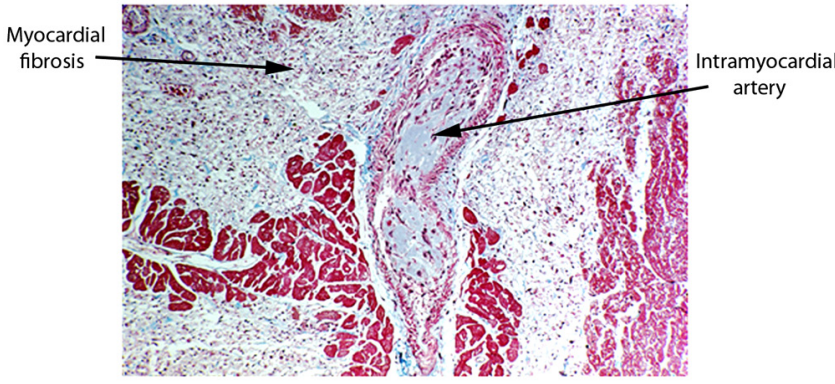

Figure 5. Histologic section of the myocardium in a dog. The center of the image shows an intramyocardial artery with arteriosclerotic changes of the vessel wall. The vessel is surrounded by demarcated myocardial fibrosis. Masson trichrome staining. (Courtesy of Lennart Jönsson).

3.2.2 Changes in left ventricular size, shape, and function.

Changes in the myocardium induced by chronic volume overload in MMVD dogs will, in turn, impact size, shape and mechanical function of the heart. The LV has been suggested to obtain a more globular rather than elliptical shape with increasing volume overload, as seen in human and canine cardiac diseases of varying etiologies (Di Donato *et al.*, 2006; Monaghan, 2006; Lord, 1974). However, sparse information is available regarding the transition into a more globular shape in dogs with naturally occurring MMVD. Interactions between myocardial structure and function exist, and the systolic function has been shown to decline in dogs with naturally acquired MMVD and in dogs with experimentally induced MR (Borgarelli *et al.*, 2007; Urabe *et al.*, 1992). Mechanisms underlying this deterioration are poorly understood, but various hypotheses have been suggested. Left ventricular apical rotation and twist, which have been proven of fundamental importance for cardiac performance, might be influenced by alterations in spherical geometry; thereby changing the normal pattern of contraction (van Dalen *et al.*, 2010; Sengupta *et al.*, 2008). Myocardial fibrosis, perhaps in combination with inadequate myocyte hypertrophy, might also contribute to the loss of force of transmission through the LV in MR dogs (Borgarelli *et al.*, 2007; Falk *et al.*, 2006; Urabe *et al.*, 1992; Carabello *et al.*, 1989; Detweiler *et al.*, 1968). Furthermore, an overall reduction in the volume fraction of myofibrils per myocyte unit volume, as seen in dogs with experimentally induced chronic MR and in human patients with chronic cardiac diseases, could potentially be a major

contributing factor to contractile dysfunction (Spinale *et al.*, 1993; Urabe *et al.*, 1992; Zimmer *et al.*, 1992). In addition, downregulation and desensitization of the β_1- adrenergic receptor (which is the primary adrenergic receptor in the normal heart) due to chronic overstimulation, as seen in human CHF patients (Bristow *et al.*, 1986; Bristow *et al.*, 1982) and in dogs with dilated cardiomyopathy (DCM) (Re *et al.*, 1999), could potentially also play a role in development of systolic dysfunction in dogs with advanced MMVD.

3.3 Assessment of left ventricular remodeling and function

Various diagnostic methods can be used when investigating LV remodeling and function in dogs with MMVD:

3.3.1 Heart sounds and murmurs

Heart murmurs

A systolic heart murmur is a prominent clinical finding in dogs with MMVD (Häggström *et al.*, 1995). The cross-sectional area of the mitral orifice is much smaller than the cross-sectional area of the LA, which causes turbulence on the atrial side in systole when blood is ejected backward from the LV up into the LA (*Figure 3*). In addition, changes in valvular motion and function due to myxomatous degeneration alterations, might contribute to the production of turbulence. The kinetic energy of the regurgitant jet is known to be dependent on the mass (m) of the regurgitant volume, and the velocity (v) of the jet, according to the formula $E=mv^2/2$. The kinetic energy is transformed into heat and vibrations in the LA cavity: The vibrations set particles in motion, which propagate as wave-sequences of alternating pressure toward the chest surface, where they are accessible for interpretation as a murmur.

Heart sounds

Heart murmur assessment is one of the major objectives when performing cardiac auscultation, but valuable information can also be obtained from assessment of heart sounds. The first heart sound (S1) is concurrent with the closure of the atrioventricular valves, whereas the second heart sound (S2) is concurrent with closure of the semilunar valves. Disagreement still exists regarding the origin of heart sounds and two main hypotheses have been presented (Durand & Pibarot, 1995): According to the valvular hypothesis, the heart sounds are caused by transient vibrations arising when the valves

come to a sudden halt at the end of coaptation. Alternatively, the cardiohemic hypothesis assumes the heart sounds to be created by vibrations in the whole cardiac structure. Likely, the origin of heart sounds is best described by a combination of these hypotheses (Durand & Pibarot, 1995). Regardless which of these mechanisms that best describes the origin of heart sounds; LV stroke volume and function are likely involved in the production of heart sounds, indicating a diagnostic potential of heart sound assessment when investigating LV remodeling and function in MMVD dogs.

Assessment of heart sounds and murmurs

Sounds can be described by their frequency (unit hertz; Hz), intensity (unit decibel; dB) and duration (unit ms). Frequency is a physical entity, which is perceived by the human senses in pitch (unit mel), while intensity is perceived in loudness (unit phon). However, neither frequency and pitch, nor intensity and loudness are linearly related to each other (Ahlstrom, 2008). Because a perceived increase in "murmur intensity" can be caused by either a change in frequency or a change in absolute intensity (or both),

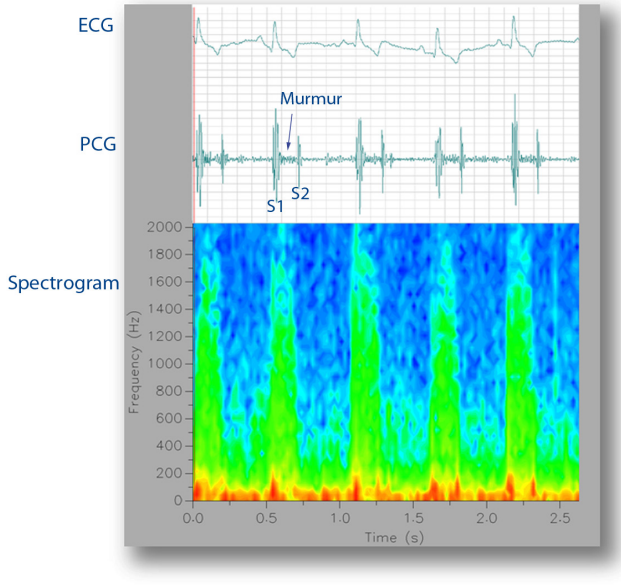

Figure 6. Phonocardiogram (PCG) from a dog with a moderately audible murmur caused by MMVD. The recording is displayed in two modes, which are timed with respect to each other: The upper mode shows synchronous electrocardiographic (ECG) and

phonocardiographic (PCG) traces; and the lower mode shows a time-frequency graph where different frequencies are displayed according to intensity, with high-intensity frequencies in red and low-intensity frequencies in blue. Note that the murmur is composed of sounds with frequencies up to approximately 1500 Hz. S1 - first heart sound and S2 –second heart sound.

it might be discussed if the term "murmur intensity" is adequate for describing murmur severity. "Murmur audibility" is possibly a more appropriate terminology.

Previous studies evaluating heart sounds and murmurs in dogs with MR have either been carried out by subjective assessment of auscultatory findings or by assessment on standard phonocardiographic (PCG) recordings (Häggström *et al.*, 1995; Gould *et al.*, 1968). Acoustic stethoscopes used for auscultation propagate sounds from a chest piece, which is either of diaphragm type (covered by a membrane) or of bell type (without a membrane), through a tubing system into two ear pieces. In the electronic stethoscope, which was introduced to avoid the resonances created in the tubing system, the bell and diaphragm are replaced by a broad-band acoustic sensor and an amplifier, and the tubing and the ear pieces are replaced by wires and head phones. These improvements lead to enhanced sound quality, potentially facilitating detection of low audibility murmurs (Höglund, 2007). Some information in the cardiac sound signal is inaccessible by standard auscultation due to physical limitations in the human auditory system (Selig, 1993): The human auditory system, which is adapted to speech, has an audible range of sounds between 20 Hz and 20 kilohertz (kHz); with sounds in the frequency range between 1000 and 5000 Hz being most easily perceived. This range is much higher compared to the range of most cardiac sounds; which often are band-limited to about 10-1000 Hz (Ahlstrom, 2008). The interpretation of auscultatory findings has, furthermore, been shown highly dependent on experience and a considerable inter-observer variation in the ability to detect and interpret hearts sounds and murmurs exists (Höglund *et al.*, 2004; Pedersen *et al.*, 1999; Rajakumar *et al.*, 1999; Kinney, 1988).

Phonocardiography (PCG), which is a quantitative graphic representation of cardiac sound waveforms, does not have these limitations, and the technique allows visual interpretation of cardiac sounds. Previous studies in dogs with MMVD have shown increasing severity of MR to be associated with certain characteristic features on the PCG recording: The murmur duration increases from early or late systolic to holosystolic, the murmur intensity (amplitude) increases, and there is a shift in the amplitude ratio between the first heart sound (S1) and the second heart sound (S2) (Häggström *et al.*, 1995). However, manual interpretations of PCG

recordings cannot reveal detailed information about the cardiac sounds. The more recent possibility to process the recorded PCG signals by use of signal analysis technique has the potential to provide an objective and more comprehensive characterisation of heart sounds and murmurs (Ahlstrom, 2008); thereby potentially increasing information about the hemodynamic alterations occurring in the heart during different severities of MMVD.

3.3.2 Circulating biomarkers

The cardiac remodeling process stimulates release of various circulating cardiac biomarkers, and the potential of using biomarkers for diagnosing and monitoring dogs with cardiac diseases has recently gained interest. Besides the obvious utility of biomarkers for optimizing clinical management of MMVD dogs, biomarkers can also provide information of potential value to increase our understanding of the complex cardiac remodeling process.

Cardic troponins

Cardiac troponins are myofibril proteins, which by regulating the calcium-mediated action between actin and myosin filaments in the myocytes are crucial for muscle contraction (Katz, 2001). The cardiac troponin complex is composed of three subunits (cardiac troponin C, cardiac troponin I, and cardiac troponin T), of which cardiac troponin I (cTnI) is the only one uniquely expressed in the myocardium (O'Brien *et al.*, 1997).

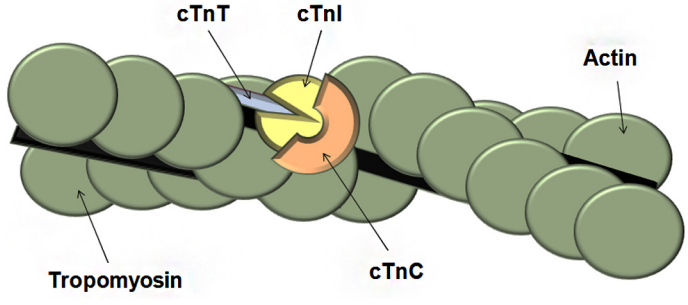

Figure 7. Schematic illustration of the cardiac troponin complex. Cardiac troponin C - cTnC, cardiac troponin I - cTnI, and cardiac troponin T – cTnT.

Most cTnI is structurally bound within the myocyte, and it is released into the circulation only after myocyte injury (O'Brien *et al.*, 2006). If the rate of cTnI release exceeds the rate of synthesis, the myocardium might become partially depleted of troponins; thereby potentially affecting the

contractile function (Van der Laarse, 2002). The release of cTnI from the myocardium has been shown to correlate with the amount of myocyte injury, as shown in experimental animal studies (O'Brien *et al.*, 2006; Feng *et al.*, 1998; Ricchiuti *et al.*, 1997; Smith *et al.*, 1997), and cTnI has for this reason become established as a clinical biomarker of cardiac injury. The value of cTnI as a cardiac biomarker in dogs is supported by studies demonstrating increased circulating cTnI concentrations in dogs with a variety of etiologies of cardiac injuries (Segev *et al.*, 2008; Linde *et al.*, 2006; Oyama & Sisson, 2004; Schober *et al.*, 2002; Schober *et al.*, 1999). Although cardiac troponin assays are most commonly used in the diagnosis of acute cardiovascular events, a growing interest exists in evaluating troponin concentrations in dogs with chronic cardiac diseases. Increased circulating troponin concentrations have previously been shown in dogs with MMVD (Spratt *et al.*, 2005; Oyama & Sisson, 2004), and improvements in the sensitivity of available cTnI assays might further increase the knowledge of the cardiac remodeling process in dogs.

C-reactive protein

Stimulation with inflammatory mediators causes a time-dependent increase in LV remodeling in experimentally induced heart failure models in animals (Bozkurt *et al.*, 1998), and changes in inflammatory pathways occurring locally in mitral valve leaflets have been described in dogs with naturally acquired heart diseases (de Laforcade *et al.*, 2003; Mow & Pedersen, 1999). However, it is unknown if inflammatory processes contribute to the progression of cardiac remodeling in MMVD in dogs. C-reactive protein (CRP), which is an acute-phase protein mainly produced in the liver, has been shown a valuable marker of systemic inflammatory activity in various diseases in dogs (Eckersall & Conner, 1988). C-reactive protein increases rapidly with the onset of tissue destruction or inflammatory stimuli (Eckersall & Conner, 1988). In human medicine, CRP concentration is reported to be related to severity of heart failure, and to be a strong predictor of adverse outcome in cases of acute cardiovascular diseases (Sakkinen *et al.*, 2002; Pye *et al.*, 1990). Less is known about CRP in chronic cardiac diseases and previous studies of CRP in dogs with MMVD have shown divergent results (Tarnow *et al.*, 2007; Rush *et al.*, 2006).

Matrix metalloproteinases

The matrix metalloproteinases, the MMPs, is a family of zinc-dependent proteolytic enzymes known to be responsible for degeneration and remodeling of extracellular components (Woessner, 1991). The net

proteolytic activities of MMPs are regulated by the tissue inhibitors of the MMPs, the TIMPs, which form irreversible complexes with the MMPs; hence blocking access to extracellular matrix substrates (Woessner, 1991). Various cell types within the myocardium, including myocytes and fibroblasts, can express and synthesize MMPs (Hutchinson *et al.*, 2010; Coker *et al.*, 1999; Ries & Petrides, 1995). The MMPs are secreted into the extracellular space in a latent form (pro MMP), which remains enzymatically silent until activation when an amino-terminal propeptide domain is removed; resulting in the ability to degradate extracellular matrix components.

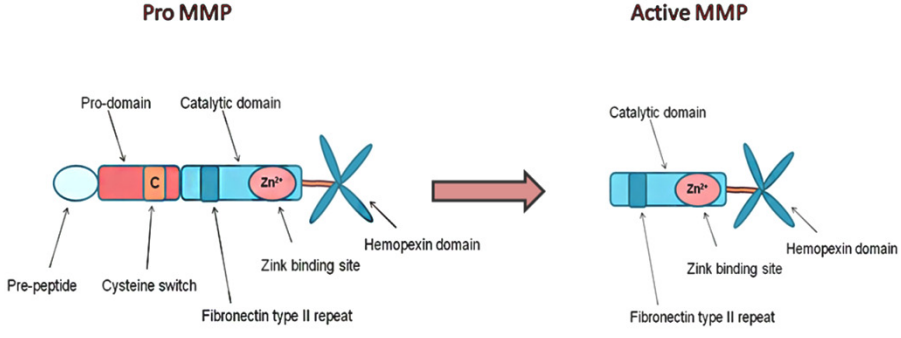

Figure 8. Schematic illustration of the matrix metalloproteinase (MMP) showing structural domains of pro MMP and active MMP (MMP-2 and MMP-9), and the transformation of the latent pro form into the active form. Modified from Vu & Werb (2000).

The MMPs contain zink at their active site, they need calcium for stability, and they are known to be activated at neutral pH. Presence of disease can stimulate activity through a number of enzymatic pathways, resulting in excessive breakdown of extracellular components (Spinale, 2002; Thomas *et al.*, 1998). The biological activation of MMPs is, however, still incompletely understood. Mast cell secretory products, such as chymases, have been suggested capable of inducing myocardial MMP activation in volume-overload states in dogs with experimentally induced chronic MR (Stewart *et al.*, 2003; Dell'Italia *et al.*, 1995). In addition, MMP activation can be induced by the membrane-bound MMPs (MT-MMPs), the extracellular matrix metalloproteinase inducer (EMMPRIN), or by various cytokines (Visse & Nagase, 2003; Spinale *et al.*, 2000; Nagase, 1997). Of the large family of MMPs, which includes stromelysins, collagenases,

gelatinases, and membrane-type MMPs, the gelatinases MMP-2 and -9 have been frequently reported involved in cardiac remodeling processes (Spinale *et al.*, 2002). The MMP-2 and -9 possess the capacity to degrade a number of interstitial proteins, including basement membrane components, collagenes, and laminin (Vu & Werb, 2000; Nagase & Woessner, 1999; Ries & Petrides, 1995). An increased expression of genes encoding MMP-1, -2, -9 and -13 has been shown in myxomatous mitral valves in humans (Togashi *et al.*, 2007; Rabkin *et al.*, 2001; Soini *et al.*, 2001). This is in contrast to findings in myxomatous mitral valves in dogs where no up-regulation of genes encoding MMP-2 and -9 has been shown (Aupperle *et al.*, 2009c; Oyama & Chittur, 2006). Furthermore, the immunohistochemical expression of MMP-2 in myxomatous mitral valves in dogs has been shown to decrease with increasing disease severity (Aupperle *et al.*, 2009b). Human valve samples might have been collected earlier during disease progression; at the time of surgical valve replacement, and not in severe end-stage disease; making assessment of alterations of MMP expression during disease progression difficult in humans. Additionally, this complicates comparisons of MMP results between human and canine studies. Few studies have been published investigating MMP changes in other myocardial tissue structures than valve leaflets in people and dogs with naturally acquired mitral valve disease.

3.3.3 Echocardiography

Ultrasounds are sounds of frequencies higher than 20 kilohertz (kHz), which cannot be perceived by the human auditory system. Ultrasounds are created when piezoelectrical crystals in an ultrasonographic transducer system transform electrical oscillations (of varying voltages) into mechanical oscillations (sounds). When performing an echocardiographic examination (ultrasonographic examination of the heart); sound signals are transmitted by the piezoelectrical crystals into the thorax. Sound waves are reflected back to the transducer when they encounter acoustic interfaces; generating electrical signals which are analyzed by the ultrasonographic unit. The resultant monitor image provides information about cardiac dimensions and function, as well as valvular structure and motion. In addition, Doppler technology allows determination of the velocity and direction of blood flow, which is of value when evaluating potential valve leakages and intracardiac pressure gradients.

Left ventricular anatomical dimensions and function can be assessed subjectively or by using various echocardiographic quantitative techniques. Traditional clinical echocardiographic assessments of the LV rely on 1-

dimensional (M-mode) and two-dimensional (2D) images. These assessments can, however, be flawed by assumptions of LV geometry, and by LV foreshortening due to image plane positioning; potentially leading to inaccuracies in measurements. These technical limitations might be pronounced when LV morphology is changed due to presence of cardiac diseases (Lang *et al.*, 2005; Kupferwasser *et al.*, 1997).

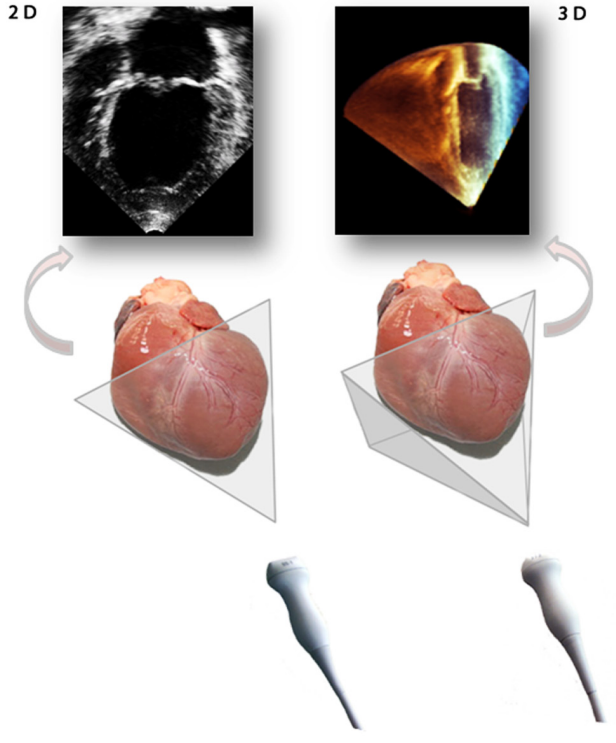

Figure 9. Illustration of the difference between 2D and RT3D image acquisition of the heart in the four-chamber left apical view. In the 2D mode (left), the image is acquired as a two dimensional slice of the heart, whereas the RT3D modality (right) allows acquisition of the image as a three dimensional pyramid. The benefit with the RT3D modality is that the volume can be rotated and cropped to visualize specific anatomic parts in three dimensions, which means that acquisition is less angle dependent than the 2D mode. The modality also allows RT3D casting of the left ventricle.

The first real-time three-dimensional (RT3D) echocardiographic system was introduced in the early 1990ies (Sheikh *et al.*, 1991), and further

improvements in design and engineering have led to the recent commercialization of RT3D echocardiographic systems. Modern RT3D echocardiographic systems utilize high-frequency transducers that consist of more than 3000 individual crystals, which simultaneously acquire data in a 3D pyramidal fashion in real time. The technique, which when including time can be referred to as four-dimensional, allows for superior anatomical delineation of the LV in real time. Due to the ability to manipulate the plane to align the true short- and long-axes of the LV, the problems with chamber foreshortening and oblique imaging planes can be reduced; thus providing more anatomically correct views than conventional 2D echocardiography (Lu *et al.*, 2008; Jacobs *et al.*, 2006; Lang *et al.*, 2006b). All voxels (i.e. volumetric picture elements) representing intensity of echocardiographic reflections at a particular point in space are used in the LV border detection process when creating a global LV RT3D volume dataset. The RT3D dataset can further be divided into 17 regional segments by sectioning the LV from base to apex, perpendicular to the LV long-axis; thereby allowing regional volume assessment (Cerqueira *et al.*, 2002). Abnormal changes in LV shape, accompanying LV dilation, can be assessed by an echocardiographically derived sphericity index (Di Donato *et al.*, 2006; Monaghan, 2006). A 3D echocardiographically derived sphericity index has been demonstrated an earlier and more accurate predictor of remodeling compared to other echocardiographic variables following acute myocardial infarction in human patients (Mannaerts *et al.*, 2004). The sphericity index is usually not included in the routine echocardiographic protocol when assessing LV remodeling in MMVD dogs, and this index might have a potential when investigating progression of remodeling in dogs with MMVD.

4 Aims of the thesis

The general aim of this thesis was to study LV remodeling and function in dogs with different severities of naturally acquired MMVD using both recently developed and previously established diagnostic techniques in order to further explore the complex pathophysiology of MMVD. Increased knowledge of the pathophysiological processes occurring during disease progression could potentially impact both clinical management and prediction of outcome for the individual dog in the future.

The specific aims were to:

- Investigate whether linear and nonlinear signal analyses of cardiac sounds could be used to assess MR severity.
- Investigate whether plasma concentrations of cTnI and CRP were associated with disease severity.
- Investigate whether plasma activities of MMP-2 and -9 were associated with disease severity.
- Investigate how the LV changes in shape and volume in response to increasing disease severity using RT3D echocardiography.

5 Materials and methods

This section summarizes and comments on the material and methods used in the separate papers included in this thesis. More detailed descriptions of the procedures performed are presented in the separate papers.

5.1 Dogs

All studies included in this thesis were approved by the Local Ethical Committee in Uppsala, Sweden. Client-owned dogs were prospectively recruited at the cardiology unit of the Faculty of Veterinary Medicine and Animal Sciences in Uppsala, and informed owner consent was obtained. The number of dogs occurring in more than one of the included papers is summarized in Table 1.

	I *n=77*	**II** *n=81*	**III** *n=75*	**IV** *n=65*
I		53	49	(9)
II			75	(12)
III				(12)
IV				

Table 1. Number of dogs included in the different studies, and shared by two studies of the present thesis. Numbers within brackets represent dogs which were reexamined in study IV, but had been included and examined in previous studies approximately two years earlier.

As inclusion criteria for the studies, dogs had to either have evidence of MMVD or be free from physical and echocardiographic evidence of cardiac disease. Dogs of all breeds were allowed into the study provided that they fulfilled the inclusion criteria. Dogs with congenital heart disease, other acquired cardiovascular disorders or significant organ-related or systemic diseases were not included in the studies. Dogs in need of heart failure therapy in order to prevent clinical signs were allowed into the studies. Furthermore, included dogs had to have a body weight less than 15 kg: Occasionally, large-breed dogs have coexisting MMVD and dilated cardiomyopathy (DCM), and excluding larger dogs from the studies limits the risk of mixing multiple cardiac diseases in the study populations. In addition, some echocardiographic variables might be dependent on body size (Bonagura & Schober, 2009). Hence, interpretation of results was considered easier and more reliable in a more homogenous group of dogs.

A majority of dogs in paper II were also included in paper III. However, pregnant bitches, dogs treated with glucocorticoids, and dogs with detectable neoplasms were excluded from study III due to a possible influence on MMP results (Schafer-Somi *et al.*, 2005; Loukopoulos *et al.*, 2003; Dollery *et al.*, 1995); hence six dogs from study II were excluded from enrolment in study III due to ongoing glucocorticoid therapy or presence of small mammary tumors.

5.2 Methods of examinations

All examinations were performed without sedation in a quiet examination room. Dog-owners were present during all examination procedures in order to keep the dogs comfortable and calm. The procedures included (presented in the order of which they were performed in the studies) an owner-interview (paper I-IV), blood pressure measurement (paper II-IV), physical examination including cardiac and pulmonary auscultation (paper I-IV), electrocardiographic (ECG) and PCG recordings (paper I), venous blood collection (paper II-III), and echocardiographic examinations (paper I-IV)

5.2.1 Blood pressure measurement (paper II-IV)

Blood pressure measurement was performed at the beginning of the examination protocol; after the dog had been adapted to the environment for approximately 10-15 minutes; all in order to reduce the influence of stress on the blood pressure results. Blood pressure was indirectly measured using automated oscillometric technique. In paper II-III, a standard

oscillometric device (Oscillometric Krutech VET420A, Jorgen Kruuse A/S, Marslev, Denmark) was used, whereas a high definition oscillometric (HDO) device (Vet HDO monitor, S +B medVet GmbH, Babenhausen, Germany) was used in paper IV. For all measurements, dogs were minimally restrained in a standing position, and an appropriate neonatal cuff, with a width of approximately 40% of the tail circumference, was applied to the base of the tail with the artery marker placed ventrally. Once reliable consecutive readings were obtained, the mean of five consecutive blood pressure measurements was calculated (Brown *et al.*, 2007).

5.2.2 Assessment of heart sounds and murmurs (paper I-IV)

Cardiac auscultation (paper I-IV)

Cardiac auscultation was conducted in a quiet examination room with the dog in a standing position. A Welsh Allyn Meditron sensor-based electronic stethoscope (Meditron ASA, Medi-Stim ASA, Oslo, Norway) was used for auscultation in all papers. The audibility of detectable heart murmurs (which all had to have the point of maximal audibility located over the mitral valve area; at the costochondral junctions between the fifth and sixth intercostal spaces on the left side of the chest wall) was graded on a scale from I-VI in accordance with established guidelines (Gompf, 1988): (Grade I murmur is very faint and only heard with special effort, while a grade VI is extremely load and accompanied by a palpable thrill on the thoracic surface).

Signal analysis of heart sounds and murmurs (paper I).

The electronic stethoscope was connected to a laptop computer (Dell latitude D800 laptop, Dell Computer Corp, Limerick, Ireland) with accompanying acquisition software (Meditron analyzer, version 4.0V, Welch Allyn Meditron ASA, Medi-Stim ASA, Oslo, Norway), for recording of the PCG signals. During recording, the flat acoustic sensor of the electronic stethoscope was placed firmly over the point of maximal audibility over the mitral valve area to provide the loudest and clearest heart murmur possible. An *ECG* (lead II) was recorded simultaneously with the PCG. Each recording lasted for 10 seconds. Background noise was minimized, and the mouth was gently closed during the recording in panting dogs; to reduce ventilation artifacts.

All recorded phonocardiographic signals were manually segmented using the ECG recordings as an aid for timing of the heart sounds. Four markers (beginning of S1, end of S1, beginning of S2 and end of S2) were determined for each heart cycle. Noisy or corrupted signal segments (as

determined by visual inspection of the data) were excluded from further analyses. All processing of PCG signals was performed by use of a mathematical computer program (MATLAB, version 7.3, The MathWorks Inc, Natick, Mass., USA), which was programmed to automatically derive seven sound variables from the segmented PCG signals. While regular frequency sounds can be investigated using linear techniques, non-linear techniques are required when investigating the complexity of sounds: Both linear and non-linear techniques were used in paper I. In brief: 1) The *first frequency peak* represents the dominant frequency component in the signal. 2) The *murmur energy ratio* quantifies the percentage of higher frequencies in the spectrum (Nygaard *et al.*, 1993), and is defined as the energy between 50-500 Hz divided by the energy between 20-50 Hz. 3) The *murmur duration>200 Hz* measures the duration of sounds exceeding 200 Hz as a fraction of the length of systole. 4) The *sample entropy* is a complexity measure useful for investigating dynamics of time series (Richman & Moorman, 2000). 5) The *auto mutual information function* represents the predictability of a signal 6) The e*nergy ratio of S1* represents the normalized energy within the S1 segment. Since it is impossible to measure an absolute sound audibility, the variable was normalized against the energy in diastole as outlined by Durand *et al* (1990). 7) The *energy ratio of S2* represents the energy within the S2 segment normalized against the energy in diastole.

5.2.3 Analysis of circulating cardiac biomarkers (paper II-III)

Blood sampling (paper II and III)

Blood was collected by jugular venipuncture into 5-mL tubes containing EDTA, and the plasma was separated by centrifugation within 30 minutes of collection. Plasma was transferred to 1.5 mL plastic cryotubes, and all samples were stored at -80°C for batched analyses. The frozen plasma was thawed slowly at room temperature prior to analysis.

Cardiac troponin I (paper II and III).

Concentrations of cTnI were analyzed in duplicate using a recently refined, enzyme-linked immunosorbent assay (Access® Systems AccuTnI® Assay, Beckman Coulter, Inc., Fullerton, California, USA), according to the manufacturer's instructions. The troponin amino acid sequence is highly conserved across species, allowing the use of human immunoassays for analysis of canine samples (O'Brien *et al.*, 2006;Oyama & Sisson, 2004). In addition, an in-house validation was performed by a trained laboratory

technician, and the tested dilutional parallelism of canine plasma confirmed linearity within the assay system. The lower limit of detection for the assay used in the present study was 0.001 ng/mL, which is an approximately 10-100 times higher sensitivity compared to most assays used in clinical settings today.

C-reactive protein (paper II and III)

Concentrations of CRP were analyzed in duplicate using a previously validated (Kjelgaard-Hansen *et al.*, 2003) commercially available canine CRP ELISA assay (Tridelta PhaseTM Range CRP – Canine Assay, Tridelta Development Ltd, County Wicklow, Ireland), according to the manufacturer's instructions. An in-house validation confirmed dilutional parallelism linearity within the assay system.

Matrix metalloproteinase 2- and -9 (paper III).

Plasma MMP activity was analyzed using gelatin zymography, as previously described (Rajamäki *et al.*, 2002). Zymography is an electrophoretic technique that includes a substrate copolymerized with a polyacrylamide gel for detection of enzyme activity. After preparation and incubation, the zymogram was stained and washed, and the gelatinolytic activity was revealed as clear bands against a darkly stained background (where the enzyme had degraded the substrate). For quantification of gelatin degradation, the gels were scanned, background grey values were subtracted, and the densitometric results were measured. Due to the risk of variation between each gel, the densitrometric results of each band were assessed by comparison with the activity of the pro MMP-2 band in standard lane on each gel. All zymograms were analyzed for pro- and active MMP-2 and -9 forms.

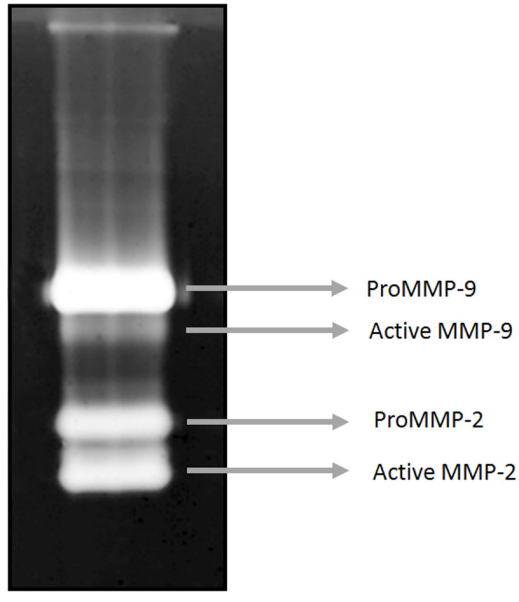

Figure 10. A zymogram from a dog showing gelatinolytic activity of the matrix metalloproteinases pro MMP-2 and -9 and active MMP-2 and -9, which are revealed as clear bands against a darkly stained background. (Courtesy of Minna Rajamäki).

5.2.4 Echocardiographic examinations (paper I-IV)

Dogs were gently restrained in both right and left lateral recumbency on an ultrasound examination table. Echocardiographic examinations were performed by use of a GE Vivid 3 ultrasonographic unit (General Electric Co, Stockholm, Sweden) equipped with a 5-MHz transducer in paper I-III, and a Philips iE33 ultrasonographic unit (Philips Ultrasound, Bothell, WA, USA) equipped with a 5-1 MHz transducer (for M-mode and 2D), and a 7-2 MHz matrix transducer (for 2D and RT3D) in paper IV. Continuous ECG (lead II) monitoring was performed during the echocardiographic examinations in all studies.

M-mode, 2D and Doppler examinations (paper I-IV)

M-mode and 2D loops of standardized views (Thomas *et al.*, 1993) were digitally stored in all studies. Screening of potential regurgitations through the mitral, tricuspid, aortic, and pulmonic valves was performed using color Doppler echocardiography. Assessment of mitral valve structures and MR severity was conducted from the right parasternal long-axis view and the left apical four-chamber view. Demonstrated MR on the echocardiogram was subjectively assessed as the area of regurgitant jet relative to the area of the

left atrium, as previously described (Olsen *et al.*, 2003; Pedersen *et al.*, 1996). The left atrial to aortic root (LA/Ao) ratio was measured as previously described (Hansson *et al.*, 2002). M-mode measurements of the LV were performed using standard techniques (Bélanger, 2005) on images obtained from the right parasternal short axis view. The LV internal dimensions were related to body size by allometric scaling of body weight; and the values for the percent increases of end-diastolic left ventricular internal dimension ($LVIDd_{inc}$) and end-systolic left ventricular internal dimension ($LVIDs_{inc}$) were calculated as follows: [observed dimension – expected normal dimension]/expected normal dimension x100. Expected normal dimensions were calculated as previously described (Cornell *et al.*, 2004): LVIDd (body weight$^{0.294}$ × 1.53), and LVIDs (body weight$^{0.315}$ × 0.95).

Real-time three-dimensional echocardiography (paper IV).

Real-time three-dimensional (RT3D) datasets were acquired from the left parasternal apical four-chamber view. Series of four to seven consecutive ECG R-wave triggered cycles were acquired, from which four sub-volumes were automatically derived and integrated into one pyramidal volume; thereby providing a RT3D full-volume dataset of the entire LV.

The RT3D data analyses were performed off-line using a commercial software (QLAB advanced quantification, version 7.0, Philips Ultrasound, Bothell, WA, USA), which displays the RT3D volume data in three different orthogonal planes; the two-and four-chamber views, and the short-axis view. The orthogonal planes were all aligned interactively by use of color-coded conventions, according to the manufacturer's instructions, in order to obtain the most anatomically correct apical views for optimal border delineation of the LV. Anatomic landmark definition was performed manually at the endocardial border in the LV cavity in the end-diastolic volume (EDV) frame. Automatic endocardial border tracing created a cast of the LV cavity, and the LV cavity border detection was then verified for accuracy in each view, and manually adjusted as required. The procedures described for the EDV frame were repeated on the end-systolic volume (ESV) frame. Finally, the traced borders were processed to automatically calculate the EDV and ESV by an algorithm model in the software. The cardiac volumes were indexed to body weight (volume); EDV/kg and ESV/kg, based on the presumption of a linear relationship between cardiac volumes and body weight (Bonagura & Schober, 2009; Cornell *et al.*, 2004). The RT3D long-axis length was measured in the four-chamber view as the distance from the endocardial apex to the mid-point of the mitral valve.

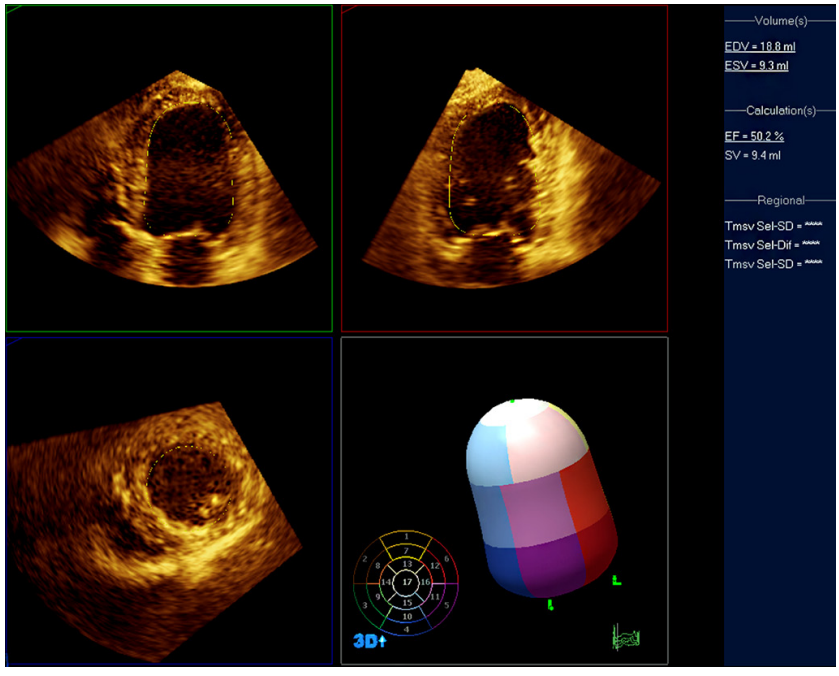

Figure 11. Example of a real-time three-dimensional (RT3D) volume dataset from a dog displayed in three orthogonal planes; the two- (upper right) and four-chamber views (upper left), and the short axis view (lower left), from which endocardial borders of the LV cast were identified, and end-diastolic and end-systolic LV volumes were obtained. The left ventricular 3D cast is automatically divided into 17 segments according to established guidelines (lower right). For the purpose of this thesis, the 17 segments were further joined into three major segments: basal, mid and apical segments.

Seventeen segments, defined by the American Society of Echocardiography (Cerqueira *et al.*, 2002), were automatically calculated from the LV cast, allowing regional RT3D LV volume assessment (*Figure 2*). The different segments were identified on the LV cast as fractions of the LV long axis length. The 17 segments were further joined into three major segments: Basal EDV and ESV included segments 1-6; mid EDV and ESV included segments 7-12; apical EDV and ESV included segments 13-17. Percentage contributions to the global EDV and ESV of each of the major regional segments, were calculated (basal, mid and apical EDV % and ESV %).

Sphericity index was calculated as the RT3D-EDV divided by the volume of a sphere, with the sphere volume calculated as $1/6\ \pi \times L^3$ (where L is equal to the LV long-axis length) (Di Donato *et al.*, 2006).

5.3 MMVD classification systems (paper I-IV)

Criteria for the diagnosis of MMVD included characteristic valvular lesions of the mitral valve apparatus (thickened and/or prolapsing mitral valve leaflets) and demonstrated MR on color Doppler echocardiogram
All dogs included in the four papers were classified using an echocardiographic classification system: Estimation of disease severity was based on the obtained LA/Ao ratio and the MR jet size, and dogs were classified as follows: Healthy; LA/Ao < 1.5 and no MR jet, mild; LA/Ao ≤ 1.5 and MR jet < 30%, moderate; LA/Ao > 1.5 and < 1.8 and MR jet < 50%, and severe; LA/Ao ≥ 1.8 and MR jet > 50%.

The echocardiographic classification system used in paper IV differed slightly from the above described classification system. There is a problem in interpretation of small MR jets on the color echocardiogram: Some are likely to represent early stages of MMVD, whereas others may be trivial nonpathologic jets (Nakayama *et al.*, 1994; Perry & Bouchard, 1990). Improvements of the Doppler technique in new ultrasonographic units might increase the likelihood of detecting minimal MR signals. Hence, the use of the new ultrasonographic unit in paper IV might have increased detection of with minimal MR of which a proportion most likely was trivial nonpathologic jets. Hence, dogs with minimal MR and LA/Ao < 1.5 were allowed into the healthy classification group in paper IV. Because minimal jets could create sounds influencing the sound signal variables investigated in paper I; dogs having minimal MR jets were classified as mild in this study. Some of the dogs included in paper I were also included in paper II and III, and the same echocardiographic classification system was applied in these papers. The murmurs were analyzed by sound signal analysis techniques in paper I, and it was therefore of interest to relate the results obtained using this technique to traditional auscultation assessments performed by veterinarians experienced in veterinary cardiology. Hence, an auscultatory classification system was also applied (in addition to the echocardiographic classification system) in paper I. For the auscultatory classification, dogs were divided into the following groups: normal (no audible cardiac murmur), low audibility (grades I-II), moderate audibility (grades III-IV) and high audibility (grades V-VI) murmurs.

5.4 Statistical analyses

Data are presented as medians and interquartile ranges (IQR). A value of $P < 0.05$ was considered significant for the analyses, unless otherwise indicated.

The Cuzick test for ordered groups (paper I) and Kruskal-Wallis test (paper II-IV) were used to investigate overall associations between the investigated variables (sound signal variables, circulating biomarkers and RT3D echocardiographic variables) and the four MMVD severity groups. In variables in which a significant association was detected, a pair-wise comparison was also performed by use of the Mann Whitney U-test with Bonferroni adjustment: Comparing four groups to each other involves six comparisons, resulting in a significant *P*-value of < 0.008.

Unilinear (paper I-IV) and multiple regression (paper I-III) analyses were used to evaluate associations between the variables of interest (sound signal variables, circulating biomarkers and RT3D echocardiographic variables), and dog characteristics, HR obtained from the echocardiogram, systolic arterial pressure (SAP), and M-mode and 2D echocardiographic measurements. In the multiple regression model, analyses were performed in a backward stepwise manner (Bland, 1995), starting with all variables included in the model and then removing the variable with the highest *P*-value until all the remaining variables had a value of $P < 0.05$. All variables were assessed only as main effects; no interaction terms were considered in the model. The adjusted R^2 is defined as the percentage of the total sum of squares that can be explained by the regression and it also considers the degrees of freedom for variables added.

In paper I, linear discriminant analysis (Fischer, 1936) was used to investigate whether a combination of sound variables could be used to distinguish severe MR from the other three severity groups. The diagnostic efficacy of the optimal combination of sound variables (which were obtained from the linear discriminant analysis) was further evaluated by use of receiver operating characteristic (ROC) curves. In particular, sensitivity, specificity, and area under the curve (AUC) were investigated.

6 Results

This section summarizes the results from the separate papers included in the thesis. More detailed descriptions of the results are presented in the separate papers.

6.1 Signal analysis of heart sounds and murmurs (paper I)

The box and whisker plots in *Figure 12* show the sound variables evaluated against the auscultatory and echocardiographic classification systems. In brief: The first frequency peak, and consequently the frequency spectrum, was shifted towards higher frequencies with more severe MR. More severe MR produced a murmur with "harsher" quality, as shown by higher murmur energy ratio. Analysis of murmur duration > 200 Hz showed that murmurs shifted from early or late systolic to holosystolic with increasing MR severity. More severe MR showed more irregularity in flow behavior, as indicated by higher values of sample entropy. Lower values of auto mutual information were found in dogs with severe MR, reflecting decreasing predictability of the sound signal with increasing signal complexity. The energy of S1 decreased with increasing murmur audibility, but showed no association with MR severity using the echocardiographic classification system. The energy of S2 was found to decrease with increasing MR severity.

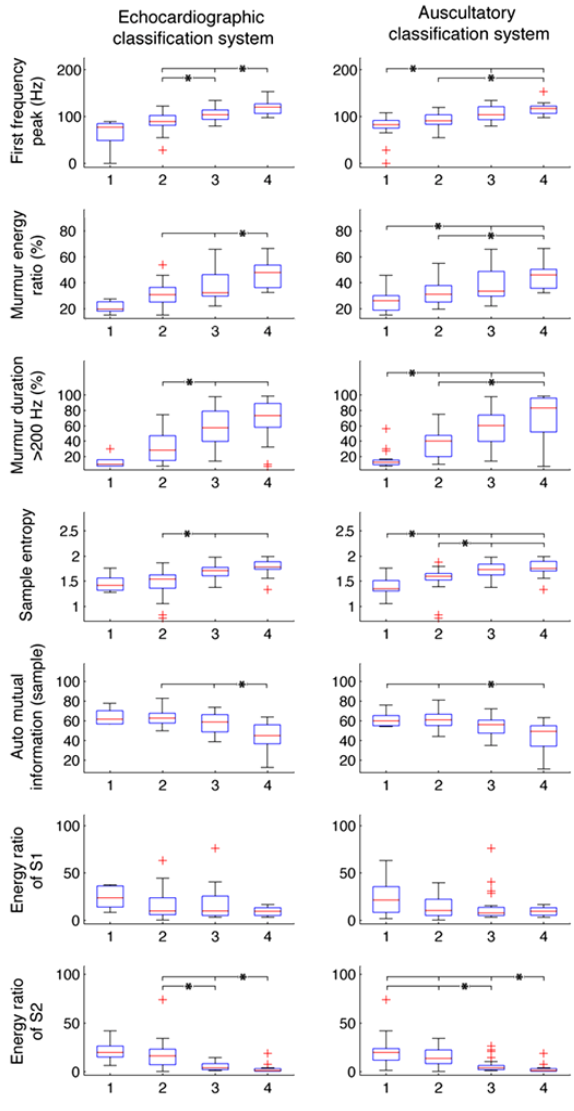

Figure 12. Box-and-whisker plots of seven sound variables evaluated against the auscultatory (left column) and echocardiographic (right column) classification systems. The upper and lower limits of each box represent the lower quartile and upper quartile values, respectively; the horizontal line within each box represents the median. The whiskers represent the extent of the data (1.5 times the interquartile range). Outliers are indicated (plus signs). Because of the low number of dogs in the clinically normal group using the echocardiographic classification system, these dogs were excluded from the multiple group-wise comparison tests. ⋆Values indicated by brackets differ significantly ($P < 0.008$).

Using the seven sound variables as dependent variables and dog characteristics (age, gender, breed and body weight), heart rate and LA/Ao-ratio as independent variables in a multiple regression model, confirmed a major effect of the LA/Ao on the sound variables, but showed an absence of effect of the other variables included in the model. The first frequency peak was the sound variable giving the highest model R^2 ($R^2 = 0.40$, $P < 0.001$).

The linear discriminant analysis demonstrated that the optimal combination of sound signal variables (smallest variable set with the largest possible amount of correct detections) was the selection of energy ratio of S2, auto mutual information and first frequency peak, resulting in a sensitivity of 88% a specificity of 82% (using the echocardiographic classification system). The ROC curve, which summarizes the diagnostic performance of a test, had an AUC of 0.89 for this combination.

6.2 Analysis of circulating biomarkers (paper II-III)

6.2.1 Cardiac troponin I (paper II-III).

Detectable concentrations of cTnI were found in 67 % of the included dogs. Plasma cTnI showed either barely detectable concentrations, or concentrations below the lower limit of detection for the assay in the healthy dogs, but cTnI concentration increased with increasing MMVD severity. Uni- and multiple regression analyses showed a major effect of age, CRP concentration, HR, and $LVIDd_{inc}$% on cTnI concentration, and the final multiple regression model had an adjusted R^2 of 0.63 ($P < 0.0001$). Age was the variable most strongly associated with cTnI in the unilinear analyses ($R^2 = 0.50$, $P < 0.0001$). Because of the strong association between age and cTnI concentration, further investigations were performed within the four different MMVD groups: Concentration of cTnI increased significantly with increasing age in the mild MMVD group ($R^2 = 0.47$, $P < 0.0001$), which included nearly 50% of the dogs in the study population, and in the severe MMVD group ($R^2 = 0.26$, $P = 0.018$).

6.2.2 C-reactive protein (paper II-III)

Detectable concentrations of CRP were found in all included dogs.
No significant difference was shown between CRP concentration and the four MMVD severity groups, but CRP concentration was associated with cTnI concentration, breed, and systolic blood pressure in the multiple regression analysis. However, the regression model had a comparably low model R^2 ($R^2 = 0.24$, $P < 0.0001$), and slight modifications in the order the

variables were removed in the backward analysis process had a comparably large effect on the outcome, indicating an unstable model.

6.2.3 Matrix metalloproteinase 2- and -9 (paper III).

Zymography of the plasma samples revealed gelatinase expression of pro MMP-2 and pro MMP-9 activities in all included dogs, and active MMP-9 was found in 85 % of the dogs. Active MMP-2 could not be detected in the study population. No overall significant differences were found between MMP activity, and the four MMVD severity groups.

Uni- and multiple regression analyses showed that pro MMP-9 decreased with decreasing SAP, and was higher in male dogs than in female dogs. However, the final multiple regression model was weak with an adjusted R^2 of 0.14. Systolic arterial pressure (SAP) was the variable most strongly associated with pro MMP-9 in the unilinear analyses ($R^2 = 0.10$, $P < 0.0037$).

Uni- and multiple regression analyses showed that active MMP-9 decreased with increasing $LVIDs_{inc}$, and with decreasing cTnI, SAP, and age (the latter did only reach a significant level in the unilinear analysis). The final multiple regression model had an adjusted R^2 of 0.29. Left ventricular end-systolic dimension was the variable most strongly associated with active MMP-9 in the unilinear analyses ($R^2 = 0.11$, $P = 0.0039$).

Pro MMP-2 activity was not significantly associated with any of the investigated variables in the uni- or multiple regression analyses.

6.3 Assessment of left ventricular volume and shape (paper IV).

Dogs with severe MMVD had higher values of EDV, ESV, long-axis length, and sphericity index, compared to dogs in the other MMVD severity groups *(Figure 13)*. Dogs with moderate MMVD had higher contribution of the mid EDV segment to the global EDV, compared to values in dogs with mild MMVD.

Unilinear regression analyses showed that global and regional EDV and ESV, long-axis length, and sphericity index increased with increasing disease severity, as indicated by increasing LA/Ao ratio, $LVIDd_{inc}$ %, and $LVIDs_{inc}$ %. In addition, SAP decreased with increasing EDV and ESV. Even though all three regional LV segments contributed to the increase in global EDV and ESV with increasing MMVD severity, the mid EDV contributed the most to the global EDV increase. Assessing regional contribution to changes in LV shape; sphericity index was associated with decreasing percentage

contribution of basal EDV, and increasing percentage contribution of apical EDV to global EDV.

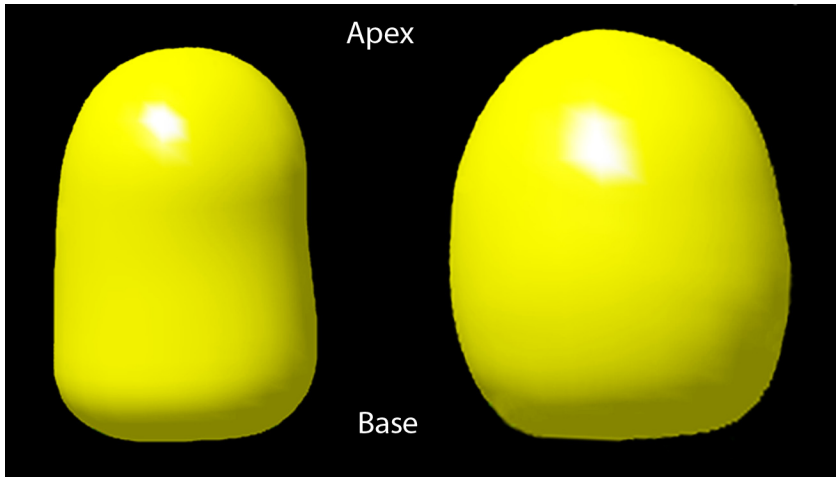

Figure 13. Left ventricular end-diastolic casts obtained from the RT3D dataset from a healthy dog (left) and a dog with severe MMVD (right). The diseased heart has a more rounded left ventricular appearance (and thereby an increased sphericity index), in addition to a globally increased left ventricular volume.

7 General discussion

The cardiac remodeling process in dogs with MMVD is highly complex, with different processes occurring simultaneously, and most likely with varying degrees of contribution during disease progression. Some results from the studies included in the present thesis might primarily be important for understanding the pathophysiological changes occurring during the LV remodeling process, whereas others could potentially be more directly valuable for improving the assessment of MMVD severity; and hence, the clinical management of affected dogs.

7.1 Changes in left ventricular morphology and function

7.1.1 Heart sounds and murmurs

The murmur-audibility has previously been shown to increase with severity of MMVD (Häggström *et al.*, 1995), but signal processing of murmurs has, to the best of the author's knowledge, never been applied for assessment of MR in any species. Quantification of MR can provide valuable information about LV remodeling status and function in MMVD dogs. In paper I, duration of systolic frequency contents exceeding 200 Hz was nearly nonexistent in normal dogs (recorded cardiac acoustic signals can be obscured by respiratory sounds, rumbling sounds from the stomach, friction rubs, and ambient sounds; explaining the existence of detectable sound signals in systole; even in the absence of a systolic murmur), but increased in duration with increasing MR severity: Dogs with moderate to severe MMVD had long systolic durations of frequencies exceeding 200 Hz. These findings indicate a maintained energy in the MR jet in late stages of the disease. In the typical MMVD dog, the maximal systolic flow velocity of the MR remains comparably constant until late stages of the disease when it might decrease (Olsen *et al.*, 2010); likely as a consequence of altered

intracardiac pressure gradients due to LV systolic dysfunction and increased intraatrial pressure. But even though LV systolic function worsens in late stages of the disease (Borgarelli *et al.*, 2007), sufficient function remains to eject a large MR volume into the LA; thus generating a holosystolic high frequency murmur. The large retrograde stroke volume in dogs with more severe MR creates a murmur of "harsher" quality and with higher frequency content, indicated by an increase in the first frequency peak, and murmur energy ratio, in these dogs. In addition, the results from the non-linear investigations using sample entropy and auto mutual information indicate that more severe MR has more irregularity in the flow behavior, and thus less predictability in the signal.

The intensity of S1 has commonly been regarded to increase with increasing disease severity in dogs with MMVD. Remarkably, in paper I, the energy of S1 was shown to decrease with increasing murmur audibility, and showed no association with MR severity using the echocardiographic classification system. Merging of the S1 with the forceful murmur might give an impression of increased audibility of the S1 when subjectively assessed by cardiac auscultation, possibly explaining previous reports of increased S1 audibility with increasing MR severity (Gould *et al.*, 1968). The relative intensity between S1 and S2 has been shown to change with increasing MMVD severity when manually measured on PCG recordings (Häggström *et al.*, 1995), but an objectively performed study of potential heart sound alterations caused by MR has, to the author's knowledge, never been published. A possible mechanism explaining the decrease in S1 energy with increasing murmur audibility is that the degenerated valve might influence the vibrations involved in the origin of S1. A reduction in LV systolic function with disease progression could potentially also influence the S1. The energy of S2 was found to decrease with increasing MR severity, which is in accordance with a previous PCG study in MMVD dogs (Häggström *et al.*, 1995). The intensity of S2 is reported to primarily depend on the rate of change in the pressure gradient across the aortic valve at closure (Sabbah & Stein, 1976), and a diminished forward stroke volume (Kittleson & Brown, 2003) might explain the decreased energy ratio of S2 seen in dogs with increasing severity of MR.

7.1.2 Circulating cardiac biomarkers

Cardiac troponin I

To the best of the author's knowledge, this was the first study exclusively designed to investigate the association between cTnI concentration and

MMVD severity in dogs. Potential effects of CRP and dog characteristics on cTnI concentration were controlled for in the study. Plasma cTnI concentration was found to increase with increasing MMVD severity, which indicates ongoing myocyte injury in a chronic remodeling process. However, the exact cause of such processes remains unknown. Intramural myocardial arteriosclerosis; with loss of compliance in the arterial vascular walls due to luminal narrowing, has been described in dogs in CHF due to MMVD (Falk *et al.*, 2010; Falk *et al.*, 2006). These changes might affect the vascular supply to the myocardium; ultimately promoting regional hypoxia and myocyte death (Sabbah *et al.*, 1995; Detweiler *et al.*, 1968). The arteriosclerotic changes have furthermore been shown associated with myocardial fibrosis in MMVD dogs (Falk *et al.*, 2010; Falk *et al.*, 2006; Detweiler, 1989; Jönsson, 1972; Detweiler *et al.*, 1968) supporting the hypothesis of arteriosclerosis-induced myocyte death as a causative factor in development of myocardial fibrosis in MMVD dogs. Yet, a reversed scenario is possible: Remodeling of the ECM might increase the amount of myocardial fibrosis, which by reducing the capillary density in the myocardium; and thereby, the oxygen diffusion distance, damage myocyte integrity (as seen in dogs with experimentally induced CHF) (Sabbah *et al.*, 1995). The oxygen demand might furthermore be increased in the hypertrophied myocardium; potentially lowering the threshold for myocardial hypoxia (Piano *et al.*, 1998). Such a scenario, if present, is likely to occur intermittently or be of subclinical character in the clinical situation because most dogs with MMVD do not present typical changes indicative of hypoxia on the ECG. Myocyte loss can also be a result of programmed cell death (apoptosis) in the hypertrophied myocardium in dogs (Sharov *et al.*, 1996), potentially due to increased levels of aldosterone, norepinenephrine, AII, and various inflammatory mediators (Ferrari *et al.*, 1998; Sabbah *et al.*, 1995): However, it is not established if their concentrations are sufficiently high to promote direct myocyte necrosis in the failing heart in dogs with MMVD.

Paper II described that the cardiac release of cTnI started in the early stages of MMVD in dogs. The true biological half-life of cTnI has been reported to be approximately 70 min in dogs (Jaffe *et al.*, 1996). Thus, the increase in cTnI concentration was most likely due to an ongoing release of cTnI caused by a continuous remodeling process, rather than an acute process. Minimal myocardial cTnI loss might be of minor importance for contractile function in the short term. However, chronic myocardial degradation and long-term loss of myocardial troponins has been suggested to affect LV contractile function (Van der Laarse, 2002).

A strong association between age and cTnI concentration was found, indicating that age causes myocardial changes leading to cTnI leakage. In fact, age was the variable most strongly associated with cTnI concentration. An association between age and cTnI has previously been reported in healthy dogs (Oyama & Sisson, 2004), people (Venge *et al.*, 2003), and rats (O'Brien *et al.*, 2006). Intramural arteriosclerotic changes have been shown associated with normal aging in dogs, potentially causing defects in the oxygenation potential in the myocardium in elderly dogs; and thereby myocyte injury or loss (Whitney, 1976; Jönsson, 1972; Detweiler & Patterson, 1965). A marked myocyte loss has been reported to occur with age in humans (more evident in males) potentially explained by ischemic injuries (Olivetti *et al.*, 1995; Olivetti *et al.*, 1991). A certain degree of cardiac fibrosis has also been seen in the aging heart in various species (Lakhan & Harle, 2008; Thomas *et al.*, 2000; Villari *et al.*, 1997; Klima *et al.*, 1990; Mukherjee & Sen, 1990), but the actual prevalence of myocardial fibrosis in elderly dogs is not known. Because changes associated with normal aging might be difficult to separate from those caused by cardiac diseases, the effect of age on cTnI needs consideration when evaluating this biomarker in dogs. Hypothetically, age-related changes in cardiovascular structure and function might reduce the ability to compensate for cardiac diseases in elderly individuals.

C-reactive protein

Concentration of CRP was not associated with MMVD severity in the examined dogs. All dogs had detectable circulating concentrations of CRP, but based on previous reported normal variations in healthy dogs (Mischke *et al.*, 2007; Kjelgaard-Hansen *et al.*, 2003) dogs with clinically important systemic inflammation were not represented in the study population; as all dogs had CRP concentrations within the normal range. A multiple regression model showed significant associations between CRP concentration and cTnI concentration, breed, and SAP. However, a comparably low model R^2, in combination with the knowledge that CRP can increase due to tissue destruction or inflammatory stimuli in other organs, suggest that CRP is not a sensitive biomarker for evaluation of MMVD remodeling in dogs. Yet, in order to further evaluate the role of inflammation in the pathogenesis of MMVD, further investigations, including other markers of inflammation, are needed.

Matrix metalloproteinase 2- and -9

Changes in the myocardial structure with loss of the fine collagen weave surrounding the myocytes might occur due to selective induction of MMPs (Spinale, 2002; Woessner, 1991). Previous reports on MMPs in dogs with naturally acquired MMVD have focused on changes in the mitral valve leaflets (Aupperle *et al.*, 2009a; Aupperle *et al.*, 2009c; Oyama & Chittur, 2006). To the best of the authors's knowledge, paper III is the first study exclusively designed to investigate circulating activity of MMPs in different severities of MMVD in any species. Circulating activity of pro– and active MMP-2 and -9 were investigated, and activity of MMP-9 was shown to decrease with increasing $LVIDs_{inc}$ % and decreasing SAP. Hence, activity of MMP-9 was, in contrast to cTnI, linked to variables reflecting systolic function, but not to variables reflecting LV dilation; indicating that MMP-9 and cTnI might reflect different aspects of the complex cardiac remodeling process in MMVD dogs. The MMVD severity classification system used in this thesis did primarily reflect degree of left-sided cardiac dilation; possibly explaining the lack of significant differences between MMP activity and MMVD severity groups. Changes in activity could, moreover, possibly be intermittently up- or down-regulated during the disease progression (potentially counterbalanced by the TIMPs), which could create a unique MMP profile in the single dog at a given time, not reflected in group-wise comparisons including many dogs.

Down-regulation of MMP-9 has previously been suggested involved in the pathogenesis of naturally occurring MMVD in dogs (Aupperle *et al.*, 2009c). A normalization or down-regulation of MMP activity has furthermore been described to occur in dogs with sustained volume overload after experimentally induced cardiac failure (Khan *et al.*, 2004; Nagatomo *et al.*, 2000). Such a down-regulation might protect against uncontrolled progressive dilation, by reducing extracellular matrix breakdown and enhance fibrosis development (Gill *et al.*, 2006; Anne *et al.*, 2005; Peterson *et al.*, 2001; Blaustein *et al.*, 1995; Dollery *et al.*, 1995); a hypothesis strengthened by the finding of decreased activity of MMP-9 with increasing amounts of fibrosis in atrial appendages in human patients with mitral valve disease (Anne *et al.*, 2005). An increased expression of genes encoding MMP-1 and -9, in combination with decreased expression of genes controlling synthesis of ECM components, was suggested responsible for the loss of collagen within the ECM seen in a dog model four months after MR induction (Zheng *et al.*, 2009). As stated by the authors of that study; a time point of four months post MR induction might have been early in the time course, and fibrosis (as commonly seen in dogs with severe

MMVD of naturally acquired origin (Falk *et al.*, 2010; Falk *et al.*, 2006)) and a changed MMP profile might have ensued at a later stage of MR (Zheng *et al.*, 2009).

Active MMP-9 was in paper III shown most strongly associated with the echocardiographic variable $LVIDs_{inc}$, which (based on the finding in the present thesis and in previous published articles) has been shown to increase in dogs with more severe MMVD; indicating systolic dysfunction (Borgarelli *et al.*, 2007). An increased amount of myocardial fibrosis might influence the Frank-Starling mechanism (Komamura *et al.*, 1993), and disable an optimal transduction of contractile force generated by the myocytes in systole. This could possibly explain the association between systolic dysfunction and MMP activity. However, identification of systolic dysfunction is challenging in dogs with MMVD: The retrograde ejection of LV stroke volume, which starts already in early systole (Lord, 1974; Eckberg *et al.*, 1973); reduces afterload, whereas the increased volume load leads to an increased preload (O'Gara *et al.*, 2008). Depending on severity of MR, these changes lead to normal to hyperdynamic LV contraction, even in the presence of intrinsic myocardial dysfunction. Many of the commonly used echocardiographic indices for evaluating systolic function, such as the ejection phase indices (ejection fraction and shortening fraction) are, besides being dependent on intrinsic contractility, also known to be influenced by hemodynamic load and sympathetic tone, which potentially mask significant myocardial dysfunction in dogs with MR. Assessment of LV end-systolic dimension has been suggested to better reflect systolic dysfunction in the presence of MR (Borgarelli *et al.*, 2007; Borow *et al.*, 1980). The end-systolic dimension increases as the systolic function declines; despite increasing retrograde LV stroke volume into the low resistance LA (O'Gara *et al.*, 2008). But if the LV contractile function is preserved, the fully compensated LV will shorten to an almost normal end-systolic dimension (Bonagura & Schober, 2009). The more recently introduced tissue doppler imaging (TDI) technique has been considered comparably independent of loading conditions, but recent studies suggest that this technique is affected by loading condition and sympathetic tone activity to a greater extent than previously expected, which might limit the additional informative value obtained from this technique, compared to when using $LVIDs_{inc}$(Tidholm *et al.*, 2009). Although myocardial systolic function declines with progression of disease, the remodeling process allows the LV to retain a relatively well preserved forward cardiac pump function even in advanced MMVD (Kittleson *et al.*, 1984): Increased pulmonary blood volume, and not decreased forward stroke volume, has been shown to be the main cause of

abnormal cardiopulmonary function in dogs with MMVD (Eriksson *et al.*, 2010). This finding corresponds with the clinical observation that dogs with severe MR suffer more commonly from pulmonary congestion and edema (which cause respiratory signs) than signs caused by reduced forward cardiac output (lethargy, weakness, exercise intolerance) (Olsen *et al.*, 2010).

Activity of MMP-9 decreased with decreasing SAP in paper III. Reduced forward stroke volume due to MR (Kittleson & Brown, 2003) and LV systolic dysfunction (Borgarelli *et al.*, 2007), might contribute in lowering SAP in dogs with more severe MMVD (as SAP is determined by cardiac output, which is the product of stroke volume and systemic vascular resistance). However, all dogs in paper II-IV had SAP within, or close to, normal reference ranges, indicating that the LV forward systolic function does not decline dramatically, and that regulatory mechanisms contribute in maintaining acceptable SAP even in the severe stage of the disease. The SAP did not differ significantly between dogs in the different MMVD severity groups in paper III, although dogs with more severe disease tended to have lower SAP than dogs with less severe disease. Such a tendency has previously been described in dogs with MMVD (Moonarmart, 2008). Significantly lower SAP (using a conservative P value; $P < 0.008$) in dogs with severe MMVD compared to dogs in the other MMVD severity groups were shown in paper IV. Data obtained by the HDO device used in paper IV might be more accurate and precise than data obtained by a standard oscillometry device (Schmelting *et al.*, 2009). In addition, a faster acquisition time of the HDO device could have reduced the influence of stress on the results in paper IV. All in all; these improvements in technology might have uncovered differences in SAP between MMVD severity groups in paper IV, which could not be shown in paper III using standard oscillometry.

Circulating activities of MMP-2 and -9 have been reported to increase in human patients with acute cardiovascular diseases (Hojo *et al.*, 2001; Inokubo *et al.*, 2001), which contrasts the findings in the MMVD dogs in paper III. Hence, type, degree, and duration of extracellular stimuli in different cardiac diseases likely affect the MMP profile within the failing myocardium, as previously suggested (Spinale, 2002). Pro MMP-2 was not significantly associated with any of the investigated variables in paper III, and active MMP-2 could not be detected in the study population. Consequently, based on these results, and previously published results (Aupperle *et al.*, 2009c; Oyama & Chittur, 2006), MMP-2 is possibly not playing a central role in the progression of naturally acquired MMVD in dogs.

7.1.3 Left ventricular volume and shape

To the best of the authors' knowledge, this is the first RT3D echocardiographic study designed to investigate how the LV changes in global and regional volume and shape in response to different severities of naturally acquired mitral valve disease in any species. The cardiac remodeling process progresses slowly, but inexorably, over years as the degree of MR worsens (Lord *et al.*, 2010). However, the results from paper IV clearly suggest that a large LV volume expansion does not occur before dogs have reached the more severe stage of the disease: Group-wise comparisons of global and regional EDV and ESV (using a conservative *P* value; $P < 0.008$) could only separate dogs with severe MMVD from other MMVD severity groups. The regurgitant fraction (the percentage of stroke volume ejected into the LA), which is regarded the major determinant factor of cardiac size and disease severity, is higher in dogs with severe disease compared to in dogs with mild to moderate disease (Kittleson & Brown, 2003); likely explaining the more pronounced LV dilation in dogs with severe MMVD in paper IV. This result is in accordance with previous studies using radiography, or circulating B-type natriuretic peptide (BNP) (which is released from the myocytes in response to increased LV myocardial stress) to investigate the progression of volume overload before onset of CHF in dogs with MMVD (Lord *et al.*, 2010; Tarnow *et al.*, 2009). The results reflect a more drastic increase in volume overload in dogs with close proximity to CHF. The rate of change of LV volumes in individual dogs could not be assessed in paper IV because the dogs were not followed over time.

The LV mid segment was the regional segment increasing the most in EDV and ESV with increasing disease severity in dogs included in paper IV. Potentially, LV anatomy allows more pronounced myocardial stretch in the mid segment, while the apical and basal segments are more restricted to LV expansion, due to supporting structures such as the AV annular ring in the basal segment. In addition, the LV has a smaller diameter in the apical region than in the mid-segment, and because the pressure within the LV at a given moment is the same, regardless location, wall stress is smaller in the apical than in the mid segment, according to the law of LaPlace. This, in turn, could lead to lesser tendency for dilatation of the apex. The percentage contribution of the mid segment to global EDV was only significantly higher in dogs with moderate MMVD compared to values in dogs with mild MMVD. The lack of significant differences between severe MMVD dogs and other severity groups suggests a change in LV shape in the

moderate stage; leading to increased percentage contribution of the mid segment.

Results from paper IV on LV sphericity index indicate that the LV shape changes from elliptical to more globular in response to chronic volume overload. An increase in global LV sphericity might allow myocardial adaptation to abnormal regional wall stress. However, an increase in LV sphericity might also disrupt normal mitral annular geometry; and as a result; increase the production of secondary MR (Hung *et al.*, 2004; Lapu-Bula *et al.*, 2002; Otsuji *et al.*, 1997; Kono *et al.*, 1992). Accordingly, a more globular LV shape might, to some degree, protect the myocardium from the abnormal wall stress caused by the LV volume overload, but at the same time contribute to a further increase in volume overload by stimulating secondary MR.

Assessing regional contribution to changes in LV shape, sphericity index was shown associated with decreasing percentage contribution of basal EDV to global EDV and increasing percentage of apical EDV. Rounding of the LV base with increasing MMVD severity, results in a better fit of the basal EDV into the sphere; and this change in LV shape leads to a reduced percentage contribution of the basal EDV to the global EDV. The apical EDV segment in the elliptical LV has less contact with the theoretical sphere, compared to in the spherical LV; resulting in increased percentage contribution of the apical EDV to the global EDV with increasing sphericity index. Although the mid segment was the segment increasing the most with increasing LV dilation, no association was shown between sphericity index and percentage contribution of mid EDV to the global EDV; most likely reflecting that only minor changes occur in the LV mid segmental outlining with increasing MMVD severity. These findings, in combination with the result that the long-axis length increased with increasing disease severity, suggest that although the LV changes into a more globular shape with increasing MMVD severity, the shape does not end up as an absolute sphere.

7.2 Possible clinical implications

7.2.1 Heart sounds and murmurs

The sound signal content of the murmur changes with severity of MR, which leads to increased audibility of the murmur. Hence, a typical dog (a small-breed dog, which at mature age has developed a systolic murmur of maximal audibility over the mitral valve area) previously diagnosed with MMVD by echocardiography, might be monitored by murmur assessment

until a high-audibility murmur has been diagnosed or overt signs of CHF have developed. Correct identification of severe MR would be of particular interest for clinicians when performing risk assessment or when deciding if special care and more extensive examinations are required. Because characterization of acoustic findings has been shown highly dependent on the experience of the examiner, and a considerable inter-observer variation exists (Höglund *et al.*, 2004; Pedersen *et al.*, 1999; Rajakumar *et al.*, 1999; Kinney, 1988); improvement in sound analysis tools is desirable. A majority of the sound variables investigated in paper I was dependent on MR severity, which demonstrates a diagnostic potential. The different investigated sound variables reflect different fractions of the heart sounds and murmurs; and a combination of variables was shown to optimize detection of severe MR.

The traditional auscultation technique will remain important in the near future because clinically practical tools for mathematical sound analyses procedures do not yet exist. Advances in the technical devices, making signal analysis techniques clinically applicable, are warranted: A future introduction of an innovative "intelligent stethoscope" with decision support abilities (Ahlstrom *et al.*, 2006), could hopefully offer a simple and cost-effective method for monitoring dogs with MMVD.

7.2.2 Circulating cardiac biomarkers

Both cardiac and extracardiac diseases have the potential to induce myocyte injury, and due to the non-specific origin of myocardial damage; assessment of circulating concentrations of cTnI is unlikely to perform well when establishing the diagnosis of MMVD. Although the circulating cTnI concentration increased with increasing MMVD severity, a considerable overlap existed between the different severity groups; hence, cTnI concentration alone cannot effectively be used for MMVD severity assessment. Establishing prognosis in a single dog with MMVD is difficult, and myocardial changes reflected by high sensitivity cTnI analyses might provide prognostic information. High sensitivity assays have shown previously immeasurable troponin concentrations in humans with cardiovascular diseases, to be highly predictive of future cardiac events (Eggers *et al.*, 2009; Schulz *et al.*, 2007). Increased cTnI concentrations have furthermore been shown linked to a worse outcome in dogs with cardiomyopathy (Oyama & Sisson, 2004). The studies in papers II and III were not designed to evaluate the prognostic value of the circulating biomarkers, and future longitudinal studies are needed for that purpose. Different biomarkers can provide information about different aspects of

cardiovascular diseases, and a combination of troponin analyses with analyses of other biomarkers, such as BNP, has the potential to further improve the prognostic assessment of the individual cardiac patient (Latini *et al.*, 2007; Jernberg *et al.*, 2002). However, effect of age on cTnI needs consideration when assessing cTnI in canine cardiac patients.

Concentration of CRP has previously been reported associated with severity of CHF (Pye *et al.*, 1990), and shown capable of predicting acute cardiovascular events in human patients (Sakkinen *et al.*, 2002). The lack of association between CRP concentration and MMVD severity in the examined dogs in paper II indicates a lack of diagnostic value of CRP analyses in MMVD dogs. However, a potential value of CRP (preferably when used in combination with other biomarkers) for predicting outcome in MMVD dogs, cannot be excluded.

Whereas established cardiac biomarkers; such as the natriuretic peptides, primarily reflect ventricular wall stretch (and thereby indirectly volume overload), MMP-9 was shown associated with variables reflecting systolic function in paper III. Hence, MMP-9 might provide additional information to other biomarkers used when trying to explore the complex cardiac remodeling process in MMVD dogs. However, the significant associations found between MMP-9 activity and some of the investigated variables were rather weak and circulating activities of MMP-2 and -9 cannot be regarded as valuable diagnostic biomarkers in clinical practice based on the results from paper III. Identification of the specific portfolio of MMPs and TIMPs expressed within the failing myocardium could potentially improve future treatment strategies for individuals with chronic cardiac diseases; aiming to prevent or slow down the disease progression, instead of only improving clinical signs at the end of the remodeling process.

7.2.3 Left ventricular volume and shape

The results from the RT3D LV volume assessment of dogs in paper IV showed prominent LV volume expansions only in dogs with more severe MMVD. Left heart chamber enlargement has been characterized by a slow phase of steadily progressing MMVD until about 6 to 12 months before onset of CHF, when rate of change of enlargement is fast (Lord *et al.*, 2010). Routinely performed volume assessment could potentially improve prediction of outcome for individual dogs; allowing risk-detection of forthcoming CHF at an earlier stage. The study design of paper IV did not include longitudinal follow-up studies; hence, the value of RT3D measurements in predicting outcome could not be evaluated. In order to use RT3D LV volumes in clinical practice, normal reference values need to

be established. However, although clinical use of modern 3D systems is boosted by advances in computer technology; reducing acquisition and post processing time, a shortcoming of the RT3D technique still lies in the fact that it is more time consuming compared to traditional echocardiographic techniques.

8 Conclusions

- Linear and nonlinear analyses of cardiac sounds can be used to assess MR severity, as shown by the associations found between many of the investigated sound variables and MR severity: More severe MR produces a murmur of "harsher" quality, longer duration, and with more complexity in the signal. The energy of S1 was not associated with MR severity (assessed by echocardiography), whereas the energy of S2 decreased with increasing MR severity.

- Circulating cTnI concentration increased with increasing disease severity, however, the effect of age on cTnI needs consideration when evaluating chronic cardiac remodeling in dogs using a high sensitivity cTnI assay. Circulating CRP concentration was not associated with disease severity.

- The MMP-2 and -9 activities were not associated with MMVD severity groups (which were mainly based on severity of volume overload). However, MMP-9 activity decreased with worsening systolic function. Based on the findings from this thesis, MMP-2 might play a minor role in MMVD in dogs.

- Chronic volume overload in dogs with MMVD changes LV geometry. The RT3D examinations showed prominent LV volume expansions only in dogs with more severe MMVD. The mid LV segment contributed the most to the global volume increase. The LV shape changed from elliptical to more globular in response to increasing volume overload, with the basal and apical segments contributing the most to the increase in sphericity.

9 Implications for future research

- Further studies, investigating the potential of signal analysis technique for analysis of cardiac sounds in dogs with various cardiac diseases, are warranted.

- High sensitivity cTnI assays, capable of detecting subtle myocardial changes, could be valuable for future prospective studies. Preferably, cTnI could be combined with other biomarkers in order to better predict long-term outcome.

- Further investigations, including other markers of inflammation than CRP, could be conducted in order to further evaluate a potential role of inflammation in the pathogenesis of MMVD and CHF development.

- An important future direction is to characterize the specific portfolio of MMPs and TIMPs in the myocardium, and furthermore, investigate their interactions in dogs with MMVD.

- Assessment of LV volume and shape could potentially allow early detection of dogs at risk for rapid progression into congestive heart failure. Longitudinal follow-up studies are needed to investigate whether RT3D echocardiography could improve prediction of outcome in dogs with MMVD compared to conventional echocardiography.

10 Populärvetenskaplig sammanfattning

Kronisk hjärtklaffsdegeneration är den vanligaste hjärtsjukdomen hos hund, och den drabbar framför allt hundar av små till medelstora raser från medelåldern och uppåt i ålder. Sjukdomen leder till att hjärtklaffen mellan vänster förmak och kammare (mitralisklaffen) förändras i struktur och får ett onormalt rörelsemönster. Sjukdomen liknar mitralisprolaps-syndromet hos människor. Eftersom klaffen inte längre kan sluta tätt, börjar blod läcka tillbaka upp i förmaket när hjärtat kontraheras, istället för att enbart pumpas ut i stora kroppspulsådern. Turbulens i blodet uppstår i samband med klaffläckaget, vilket ger upphov till ett blåsljud som kan höras när man lyssnar på hjärtat med stetoskop. Graden av turbulens styrs både av graden av klaffläckage och av vänster kammares funktionsduglighet. För att kompensera klaffläckaget ökar volymen i vänster förmak och kammare, liksom vänster kammares muskelmassa. Hundar med kronisk hjärtklaffsdegeneration kan ha sjukdomen under flera år utan att visa sjukdomstecken. Hos vissa hundar blir dock återflödet till förmaket så kraftigt att hjärtat inte längre klarar att kompensera för otätheten, och hundarna drabbas av hjärtsvikt.

Syftet med avhandlingen var att beskriva hur vänster kammare förändras hos hundar med olika långt framskriden hjärtklaffsdegeneration genom att använda digital analys av blåsljud och hjärtljud, specifika markörer som kan mätas i blodet och modern ultraljudsteknik.

I avhandlingen användes för första gången hos något djurslag digital signalanalysteknik för att undersöka förändringar i blåsljudets och hjärttonernas karaktär vid olika långt framskriden kronisk hjärtklaffsdegeneration. Tekniken möjliggör även analys av ljudsignaler som inte kan uppfattas av människans hörsel. Resultaten visade att hundar med kraftigt klaffläckage hade blåsljud med högre frekvensinnehåll, duration och

komplexitet, jämfört med hundar med mildare klaffläckage. Även hjärttonernas energiinnehåll påverkades av graden av klaffläckage.

Förändringar i hjärtats volym och vikt bidrar till att olika substanser frisätts ut i blodet. Dessa substanser kan användas som biologiska markörer (biomarkörer) för hjärtsjukdom och fungerar som ett komplement till mera traditionella undersökningsmetoder. Troponiner är proteiner som reglerar muskelcellernas kontraktionsförmåga. Cardiac troponin-I (cTnI), som endast finns i hjärtmuskulatur, läcker i samband med skador på hjärtmuskelceller ut i blodet. En ny, känslig analysmetod, med kapacitet att upptäcka mycket små cTnI nivåer i blodet användes för att undersöka hjärtmuskelskada hos hundar med olika grad av kronisk hjärtklaffsdegeneration. Resultaten visade att cTnI ökade med ökad sjukdomsgrad och en ökning förelåg redan hos hundar med lindrig sjukdom. Sambandet mellan inflammationsmarkören C-reaktivt protein (CRP) och kronisk klaffdegenerationsgrad undersöktes också, och resultaten tyder på att CRP inte är kopplad till grad av kronisk hjärtklaffsdegeneration hos hund. Vid kronisk hjärtklaffsdegeneration uppstår förändringar i hjärtats stödjevävnad, vilket kan påverka hjärtats funktion. Matrix metalloproteinaser (MMP) är enzymer som anses spela en viktig roll för att reglera hjärtats stödjevävnad hos hundar med kronisk hjärtklaffsdegeneration. Resultat från avhandlingen tyder på att det finns ett samband mellan MMP nivå i blodet och hjärtats sammandragningsförmåga hos hundar med kronisk hjärtklaffsdegeneration.

Modern tredimensionell (3D) ultraljudsteknik kan förbättra utvärderingen av vänster kammares volym och form. För första gång har modern ”real-time” tredimensionell (RT3D) ultraljudsteknik använts för att utvärdera kammarens geometri hos hundar med olika långt framskriden kronisk hjärtklaffsdegeneration. Redan på ett tidigt stadium av sjukdomsutvecklingen kunde en liten ökning av vänster kammares volym ses, men först vid långt gången sjukdom kunde en påtaglig volymsökning påvisas. Vänster kammare uppvisade dessutom en mera rundad form hos hundar med långt gången sjukdom.

I avhandlingen presenteras nya resultat som ökar kunskapen om de komplexa förändringar som sker i hjärtat hos hundar med kronisk hjärtklaffsdegeneration; kunskap som förhoppningsvis kommer att bidra till förbättrat omhändertagande av hjärtsjuka hundar i framtiden.

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