Role of inflammation and extracellular matrix remodelling in dogs with cardiac and systemic diseases

Sonja Fonfara

ACADEMIC DISSERTATION

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ABSTRACT

Cardiac diseases are common in dogs and are associated with ventricular dilatation or hypertrophy as well as cardiac dysfunction. In cardiac diseases, activation of the neurohormonal and inflammatory systems contribute to cardiac remodelling through degradation or increased deposition (fibrosis) of the extracellular matrix (ECM). Important factors in this process are cytokines as mediators of inflammation, matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of metalloproteinases; TIMP) as regulators of the myocardial ECM composition. Recently, there was evidence that also leptin plays a role in human cardiac diseases. However, the precise mechanisms that cause pathological cardiac remodelling in both humans and other mammalian species are incompletely understood. Furthermore, functional impairment of the heart and cardiomyocyte damage are observed in human and canine patients with systemic diseases, again without current knowledge on the underlying process.

The aim of the present studies was to investigate cardiac remodelling in canine patients with cardiac and systemic diseases. For this purpose, a quantitative assessment of the transcription of cytokines (IL-1, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α, IFN-γ, TGF-β1, TGF-β2, TGF-β3), MMP (MMP-1, -2, -3, -9, -13), TIMP (TIMP-1, -2, -3, -4) and leptin in the blood of healthy dogs and dogs with cardiac diseases and in the myocardium of dogs with cardiac diseases, dogs with systemic diseases not involving the heart as well as healthy control dogs was obtained.

In comparison to healthy dogs, which constitutively transcribed most markers in blood, dogs with cardiac diseases exhibited a selective increase (IL-1, IL-2, MMP-1, -3, TIMP-3) or reduction (TNF-α, TGF-β1, -β3, TIMP-1, -2) of inflammatory and ECM remodelling markers and an increase of leptin. In contrast, in the myocardium of dogs with cardiac and systemic diseases, the transcription of all markers was significantly higher than in hearts of healthy control dogs. This suggests myocardial inflammation and remodelling not only in association with cardiac diseases, but also with systemic diseases that do not involve the heart. The results also indicate a localised myocardial inflammation and remodelling in dogs with cardiac diseases, not secondary to a systemic inflammatory response. Interestingly, transcription levels of most markers exhibited regional differences in diseased dogs in general, with
significantly higher mRNA levels in atria than in ventricles. This indicates differences in the remodelling processes depending on localisation, which was reflected by more severe histological changes in the atria of dogs with cardiac diseases.

In conclusion, the results of the thesis provide evidence of myocardial inflammation and remodelling with regional quantitative differences in dogs with cardiac and systemic diseases and suggest a role for leptin in canine cardiac disease. The results provide further insights into the complex process of cardiac remodelling, which might influence clinical management and the assessment of prognoses in future.
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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ADAM</td>
<td>A disintegrin and metalloproteinase</td>
</tr>
<tr>
<td>AS</td>
<td>Aortic stenosis</td>
</tr>
<tr>
<td>BCS</td>
<td>Body condition score</td>
</tr>
<tr>
<td>CAMkinase</td>
<td>Calcium-calmodulin dependent protein kinase</td>
</tr>
<tr>
<td>CBC</td>
<td>Complete blood count</td>
</tr>
<tr>
<td>CHF</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>CHIEF</td>
<td>Canine Heart Failure International Expert Forum</td>
</tr>
<tr>
<td>DCM</td>
<td>Dilated cardiomyopathy</td>
</tr>
<tr>
<td>DVD</td>
<td>Degenerative valvular disease</td>
</tr>
<tr>
<td>ECG</td>
<td>Electrocardiography</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GDF-15</td>
<td>Growth differentiation factor 15</td>
</tr>
<tr>
<td>HE</td>
<td>Haematoxylin-eosin</td>
</tr>
<tr>
<td>i.e.</td>
<td>id est</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IP&lt;sub&gt;3&lt;/sub&gt;</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IVS</td>
<td>Interventricular septum</td>
</tr>
<tr>
<td>LA</td>
<td>Left atrium</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle</td>
</tr>
<tr>
<td>Lox</td>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>MAPkinase</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinases</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>Membrane-type MMP</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear factor of activated T cells</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor κ-B</td>
</tr>
<tr>
<td>NP</td>
<td>Natriuretic peptides</td>
</tr>
<tr>
<td>PDA</td>
<td>Patent ductus arteriosus</td>
</tr>
<tr>
<td>PS</td>
<td>Pulmonic stenosis</td>
</tr>
<tr>
<td>RA</td>
<td>right atrium</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone-system</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>RV</td>
<td>Right ventricle</td>
</tr>
<tr>
<td>Smad</td>
<td>Sma- and mad-related proteins</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transduction and activators of transcription</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>TACE</td>
<td>TNF-α convertase</td>
</tr>
<tr>
<td>TD</td>
<td>Tricuspid valve dysplasia</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cells</td>
</tr>
<tr>
<td>VSD</td>
<td>Ventricular septal defect</td>
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INTRODUCTION

Cardiac remodelling is associated with a change in left ventricular geometry and occurs in response to haemodynamic changes in cardiac diseases. Remodelling is an adaptation to volume or pressure overload and presents as eccentric or concentric hypertrophy, depending on the cardiac disease (Weber et al., 1992; Valgimigli et al., 2001; Opie et al., 2006). An important component in this process is the cardiac extracellular matrix (ECM), which is responsible for normal left ventricular geometry and therefore coordinated left ventricular pump function (Weber, 1989; Weber et al., 1994; Spinale, 2007).

In human medicine, involvement of inflammatory processes in cardiac diseases has been reported (Levine et al., 1990; Mann, 2002; Anker and von Haehling, 2004). Cardiac diseases are associated with cardiomyocyte injury and activation of the neurohormonal system, which both initiate an inflammatory reaction (Opie, 2002; Anker and von Haehling, 2004; Oyama, 2009; Frangogiannis, 2012). Cytokines are important mediators of inflammation. By activating enzymes, such as matrix metalloproteinases (MMP) and their inhibitors (tissue inhibitors of metalloproteinases; TIMP), responsible for ECM degradation and deposition, respectively, cytokines are involved in cardiac remodelling (Tsuruda et al., 2004; Graham et al., 2008). Initially, these processes result in degradation of the normal ECM and removal of degenerate cells, which is essential for the resolution of inflammation and the transition to myocardial repair (Dobaczewski et al., 2010; Frangogiannis, 2012). However, with progression of disease, ECM degradation with ventricular dilatation and systolic dysfunction or an increased ECM deposition with ventricular hypertrophy, increased ventricular stiffness and diastolic dysfunction occurs (Weber et al., 1994; Thomas et al., 1998; Kim et al., 2000; Spinale, 2007).

Little is known about the pathogenesis of cardiac remodelling in dogs with cardiac diseases. Several recent studies investigated the pathological changes in the valves and the expression of mediators of ECM remodelling in dogs with naturally occurring degenerative valvular disease (DVD) and in experimental settings (Aupperle et al., 2008; Disatian et al., 2008; Aupperle et al., 2009a; Disatian and Orton, 2009; Aupperle and Disatian, 2012; Lacerda et al., 2012; Orton et al., 2012; Han et al., 2013). However, these studies did not investigate the myocardium and only very few
studies exist that investigate other cardiac diseases (Kukielka et al., 1995; Gilbert et al., 1997; Basso et al., 2004; Oyama and Chittur, 2005; Tidholm and Jonsson, 2005; Lobo et al., 2010).

Leptin, an adipocytokine, was reported to play a role in cardiac diseases in people (Filippatos et al., 2000; Schulze et al., 2003; Schulze and Kratzsch, 2005; Karmazyn et al., 2007). It contributes to a systemic inflammatory state by increasing the levels of circulating catecholamines and inflammatory cytokines (Haynes et al., 1997b; Loffreda et al., 1998; Filippatos et al., 2000). The relevance of obesity for human cardiovascular disease is well known, and also in dogs obesity is an increasing problem (German et al., 2010). Elevated leptin levels are reported in obese dogs and an association of obesity with ventricular hypertrophy and cardiac dysfunction has been suspected (Ishioka et al., 2002; German et al., 2010; Mehlman et al., 2013). However, the potential role of leptin on myocardial inflammation and remodelling in dogs is not known.

Myocardial impairment has been reported in people and dogs with systemic diseases (Parker et al., 1984; Nelson and Thompson, 2006; Merx and Weber, 2007; Serra et al., 2010; Langhorn et al., 2013). Despite a high awareness of the effect of systemic diseases in the heart in human medicine, studies investigating cardiac function and myocardial inflammation in veterinary patients are sparse (Nelson and Thompson, 2006; Langhorn et al., 2013). An increase in troponin I consistent with myocardial cell damage is reported in dogs with systemic diseases (Serra et al., 2010; Langhorn et al., 2013). However, whether cardiac remodelling occurs in these patients is not known.

A better understanding of cardiac inflammation and remodelling and the identification of circulating biomarkers would be helpful for the clinical management and the assessment of prognosis in dogs with cardiac and also systemic diseases. Biomarkers for congestive heart failure and cardiomyocyte damage exist in dogs and single studies reported circulating cytokines and MMP in dogs with DVD and systemic diseases (Oyama et al., 2009; Fonfara et al., 2010; Ljungvall et al., 2011; Zois et al., 2012; Langhorn et al., 2013). However, studies investigating mRNA levels of markers of inflammation and ECM remodelling in the blood and myocardium of dogs with other cardiac or with systemic diseases are very fragmentary (Kukielka et al., 1995; Oyama and Chittur, 2006). Furthermore, the role of leptin in cardiac
inflammation and remodelling might be of importance considering the increase of obesity in the canine population.
2 REVIEW OF THE LITERATURE

2.1 Cardiac diseases in dogs

Cardiac diseases are frequent in dogs and progression into heart failure is a common cause for morbidity and mortality in this species. Cardiac diseases are either present at birth, congenital cardiac diseases, or develop during adulthood, acquired cardiac diseases. The most common congenital cardiac diseases are aortic stenosis (AS), pulmonic stenosis (PS), patent ductus arteriosus (PDA), ventricular septal defect (VSD) and tricuspid valve dysplasia (TD; Tidholm, 1997; Oliveira et al., 2011). These diseases affect either the function of a valve, obstruct cardiac output or led to connections between the systemic and pulmonary circulation. As a consequence, they cause volume or pressure overload of the heart with subsequent cardiac adaptation, which involves remodelling of the extracellular matrix (ECM; see chapter 2.2).

Common acquired cardiac diseases in dogs are degenerative valvular disease (DVD) and dilated cardiomyopathy (DCM) (Serfass et al., 2006; Martin et al., 2009; Wess et al., 2010). DVD is generally caused by progressive myxomatous degeneration of the atrioventricular valves and accounts for 75–80% of canine cardiac diseases (Borgarelli and Buchanan, 2012; Fox, 2012). Its prevalence is strongly age-related and dogs of small to medium size breeds are most commonly affected, with some breeds, such as Cavalier King Charles Spaniels, Dachshunds and Chihuahuas, being over-represented (Borgarelli and Buchanan, 2012). In contrast, DCM is a primary myocardial disease that is characterised by cardiac enlargement, impaired systolic and diastolic function and, in some breeds, ventricular arrhythmias. It is a disease of large- and medium sized breed dogs and Doberman pinschers, Newfoundlands and Great Danes are overrepresented (Tidholm et al., 2001a; Martin et al., 2009). Although canine DCM is described as one disease, it varies in the presenting complaint, clinical evaluation, and rate of progression (Serfass et al., 2006; Martin et al., 2009; Wess et al., 2010). A familial form of DCM has been identified in several breeds and is suspected in others (Tidholm et al., 2001a; Martin et al., 2009; Wess et al., 2010).
Genetic causes are reported for several cardiac diseases, but in many the cause is unknown (Noutsias et al., 2002; Maron et al., 2006; Elliott et al., 2008; Werner et al., 2008; Madsen et al., 2011; Mausberg et al., 2011; Meurs et al., 2012). It is suspected that cardiac inflammation and remodelling play a role in development and observed variations of cardiac diseases. Haemodynamic abnormalities associated with cardiac diseases will further contribute to ECM remodelling and progression of disease.

2.2 Cardiac remodelling

Cardiac diseases are associated with cardiac remodelling, which is a disease-induced change in the composition and function of the heart. To specify, cardiac remodelling has recently been defined as ‘molecular, cellular, interstitial and genomic changes, which are manifested clinically as changes in size, shape and function of the heart following cardiac injury’ (Valgimigli et al., 2001). The process is influenced by haemodynamic load, neurohormonal activation and other factors still under investigation. Components involved in the remodelling process are cardiomyocytes, the interstitium, fibroblasts, collagen and the vasculature (Weber et al., 1992; Valgimigli et al., 2001; Opie, 2002; Anker and von Haehling, 2004).

2.2.1 Cardiac extracellular matrix

The myocardium represents the main component of the heart and is comprised of cardiomyocytes and connective tissue. Although the myocytes represent most of the myocardial mass, they account for only approximately 30-40% of the cell number, and vascular smooth muscle cells, endothelial cells and fibroblasts together represent the majority (Nag, 1980; Weber, 1989; Banerjee et al., 2007). Fibroblasts produce and maintain the connective tissue fibre network, the ECM, which contributes to the tensile strength and stiffness of the heart (Weber, 1989; Weber et al., 1994; Spinale, 2002, 2007).

The ECM consists of a structural network of interstitial collagens to which other matrix components are attached. In mammalian species, 85% of the myocardial collagen is type I collagen and 11% is type III collagen (Weber, 1989; Weber et al., 1994). The remaining components are collagens type IV, V and VI, elastin, the
glycoproteins laminin and fibronectin, glycosaminoglycans and proteoglycans (Spinale, 2002, 2007). Apart from fibroblasts, the myocardium has other non-myocytic resident cells, namely macrophages and mast cells; these are the source of cytokines and growth factors (Nag, 1980; Stewart et al., 2003). Transmembrane proteins, such as integrins and membrane bound matrix metalloproteinases, enable cell to cell communication (Giancotti and Ruoslahti, 1999; Ross, 2002; Ottaviano and Yee, 2011).

The cardiac ECM is critical for the maintenance of the structural integrity of the heart. It translates the force that is generated by the myocytes into an organised ventricular contraction and prevents myofibre slippage (Weber, 1989; Weber et al., 1990; Spinale, 2002, 2007). Furthermore, it affects cell development, proliferation, migration and adhesion, is a reservoir for ECM signalling molecules and is involved in cell to cell signalling (Ross, 2002; Souders et al., 2009). Therefore, the ECM plays an important role in ventricular geometry and function. Accordingly, in cardiac diseases not only cardiomyocyte abnormalities, but also inadequate ECM remodelling processes are important causes for cardiac dysfunction (Weber, 1989; Weber et al., 1990; Spinale, 2002, 2007).

2.2.2 Neurohormonal activation in cardiac diseases

During progression of cardiac diseases, the myocardium adapts to volume or pressure overload, depending on the underlying disease. Initially, it compensates for the primary defect, before the stage of overt myocardial failure. Myocardial contractile failure is the consequence of different initiating mechanisms, i.e. pressure overload in AS and PS, volume overload in PDA, TD and DVD, primary myocardial damage in myocarditis, cardiomyopathy and myocardial infarction (Valgimigli et al., 2001; Opie, 2002). Although the aetiologies of these diseases are different, they share several pathways in terms of molecular, biochemical and mechanical processes that cause cardiac remodelling and are involved in progression of cardiac disease (Valgimigli et al., 2001; Opie, 2002; Tsuruda et al., 2004).

Myocardial failure results in inadequate cardiac output and, therefore, low blood pressure and decreased end-organ perfusion (Valgimigli et al., 2001; Opie, 2002). This is sensed by baro-, mechano- and chemoreceptors and leads to the activation of interrelated neuroendocrine systems, as an attempt to maintain the circulation and
normal organ perfusion. Involved are the sympathetic nervous system (SNS), the renin-angiotensin-aldosterone-system (RAAS), vasopressin, endothelin-1 (ET), natriuretic peptides (NP) and cytokines (Valgimigli et al., 2001; Opie, 2002; Anker and von Haehling, 2004; Oyama, 2009).

Noradrenalin, the main neurotransmitter of the SNS, stimulates cardiac beta 1-receptors located on the sinus and atrioventricular nodes, conducting cells and cardiomyocytes, which results in increased cardiac chrono-, dromo-, lusi- and inotropy; stimulation of peripheral alpha-receptors located on vascular smooth muscle cells results in peripheral vasoconstriction (Katz, 2011). This improves cardiac output and stabilises the blood pressures, however, tachycardia and increased afterload increase the work for the heart and thereby the myocardial oxygen demand and energy expenditure. Furthermore, beta 1-receptor stimulation leads to increased intracellular calcium levels. Cytosolic calcium overload is associated with early and late afterdepolarisations, which are an important cause of premature systoles and tachycardias. Furthermore, calcium contributes to cardiac remodelling via calmodulin and calcineurin activating nuclear transcription factors, i.e. nuclear factor κ-B (NF-κB) and nuclear factor of activated T-cells (NFAT; Fig. 1; Opie, 2002; Oyama, 2009; Katz, 2011).

Reduced renal perfusion and renal beta-receptor stimulation activates the RAAS. Angiotensin (AT) II causes decreased fluid excretion, is a potent peripheral vasoconstrictor and stimulates the secretion of aldosterone, catecholamines, vasopression and ET (Opie, 2002; Oyama, 2009; Katz, 2011). Furthermore, AT II contributes to proliferative and inflammatory responses via activation of AT1-receptors on cardiomyocytes and cardiac fibroblasts, causing activation of phospholipase C and mitogen-activated protein (MAP) kinase pathways (Fig. 1) (Duff et al., 1995; Paradis et al., 2000). Stimulation of central AT1-receptors results in an increased sympathetic outflow. Aldosterone acts on renal tubules to increase sodium reabsorption and excretion of potassium and hydrogen ions, thereby increasing the blood volume. However, in addition, aldosterone stimulates cardiac fibrosis and promotes maladaptive proliferative responses (Fig. 1; Oyama, 2009; Katz, 2011).

The release of vasopressin as the major regulator of the body’s water balance causes increased water reabsorption by the kidney through the binding of vasopressin to its receptor V2 on the basolateral membrane of the principal cells in
the collecting ducts (Nonoguchi et al., 1995). Activation of V2 results in phosphorylation of aquaporin and translocation of the phosphorylated protein to the luminal membrane where it forms water-permeable channels that increase the water influx (Nielsen et al., 1995). Vasopressin also increases thirst via V1a-receptors on neurons in the supraoptic and paraventricular nuclei of the hypothalamus. V1-receptors on vascular smooth muscle cells and cardiomyocytes stimulate G protein \( G_{\alpha q} \), which results in phospholipase C induced release of inositol 1,4,5-triphosphate (IP\(_3\)) and diacylglycerol, an increase of cytosolic calcium and, subsequently, activation of calcium-calmodulin dependent protein kinases (CAMkinases) and calcineurin (Gopalakrishnan et al., 1991; Xu and Gopalakrishnan, 1991; Briner et al., 1992). This results in vasoconstriction and activation of several transcription factors (NF-κB, NFAT) in cardiomyocytes and therefore pathological cardiac hypertrophy (Fig. 1; Katz, 2011).

Endothelin-1 binds to ET\(_A\)- and ET\(_B\)-receptors. Both receptor types are present on cardiomyocytes and vascular smooth muscle cells (Ito et al., 1993; Pollock et al., 1995). Stimulation of ET\(_A\)-receptors results in increased myocardial contractility and vasoconstriction via G protein \( G_{\alpha q} \) and phospholipase C activation (Pollock et al., 1995). It also stimulates proliferation via the activation of several MAPkinase pathways (Fig. 1; Ito et al., 1993; Yamazaki et al., 1996). The response to ET via binding to the ET\(_A\)-receptor dominates over that evoked by binding to the counter-regulatory ET\(_B\)-receptors (Katz, 2011).

Natriuretic peptides, atrial and brain NP, inhibit the sympathetic nervous system, the RAAS and ET release and are therefore counter regulatory to the above mentioned mechanisms (Fujisaki et al., 1995; Levin et al., 1998; Brunner-La Rocca et al., 2001; Potter et al., 2006). NP are released by atrial cardiomyocytes in response to atrial stretch and by ventricular cardiomyocytes in heart failure and bind to specific receptors, NP receptors A and B, which can be found in cardiomyocytes, fibroblasts and endothelial cells in the heart, and in several other organs (de Bold et al., 1981; Levin et al., 1998; Magga et al., 1998; Yamane et al., 2011). NP receptors are G protein coupled receptors synthesising cyclic guanine monophosphate and stimulating protein kinase G, which results in reduced intracellular calcium release and attenuated MAPkinase activity and therefore inhibition of cellular hypertrophy.
and proliferation (Fig. 1; Potter et al., 2006). However, the beneficial effects of NP are markedly blunted in heart failure (Levin et al., 1998).

The neurohormonal activation in cardiac diseases improves cardiac function and stabilises blood pressure, however, this occurs at the expense of increased cardiac work (increased pre- and afterload), intracellular calcium imbalances, proliferative responses and endothelial cell and cardiomyocyte membrane damage (Valgimigli et al., 2001; Opie, 2002; Anker and von Haehling, 2004; Katz, 2011). As a result, the myocardial oxygen demand increases and defects in the myocardial contraction-relaxation cycle, arrhythmia and myocardial dysfunction develop (Valgimigli et al., 2001; Opie, 2002; Martin et al., 2009; Katz, 2011).
Figure 1: Simplified signal transduction in cardiac remodelling.
Myocardial hypertrophy is an adaptation to volume or pressure overload. Myocardial stretch, the hormones mentioned above, cytokines and growth factors released due to cardiomyocyte damage as well as neurohormonal stimulation contribute to myocardial hypertrophy and fibrosis (Weber et al., 1994; Opie, 2002; Chatterjee, 2005; Heineke and Molkentin, 2006). In physiological amounts collagen might help to limit ventricular dilatation, but an increased proportion results in excessive chamber stiffness and diastolic dysfunction (Spinale et al., 1991; Weber et al., 1994; Spinale, 2002, 2007). On the other hand, increased collagen degradation results in myocyte slippage and cardiac chamber dilatation, thereby, it can cause systolic dysfunction and ventricular rupture (Weber et al., 1994; Spinale et al., 1996; Thomas et al., 1998; Ducharme et al., 2000; Kim et al., 2000; Spinale, 2002, 2007). Apart from the alteration of fibrous elements of the ECM, cardiac remodelling involves cellular remodelling, i.e. changes in cardiomyocyte size and shape (Opie et al., 2006; Dobaczewski et al., 2010; Frangogiannis, 2012; Koitabashi and Kass, 2012). Progressive cardiac remodelling results therefore in ventricular hypertrophy or dilatation, deforms left ventricular geometry and impairs cardiac function. However, the precise molecular mechanisms that underlie the transformation from compensated left ventricular hypertrophy to pathological remodelling and cardiac failure remain incompletely understood.
2.3 Role of inflammation in cardiac diseases

Several studies report on the role of the immune system and ECM remodelling in the progression of cardiac diseases (Levine et al., 1990; Spinale, 2002; Anker and von Haehling, 2004; Spinale, 2007; Chen et al., 2008; Graham et al., 2008). Myocarditis, but also myocardial infarction, inflammatory DCM as well as heart failure with reduced or normal ejection fraction are associated with inflammatory processes in the myocardium (Levine et al., 1990; Anker and von Haehling, 2004; Maron et al., 2006; Dobaczewski et al., 2010; Frangogiannis, 2012). It is commonly believed that besides an activation of neurohormones, pro-inflammatory cytokines contribute to the progression of heart failure, therefore, the latter is now regarded as a state of chronic inflammation (Pagani et al., 1992; Anker and von Haehling, 2004; Chen et al., 2008; von Haehling et al., 2009; Hedayat et al., 2010; Tamariz and Hare, 2010). Cardiac inflammation is associated with myocardial infiltration of inflammatory cells, the production of cytokines, endothelial cell activation and myocardial cell damage and degeneration (Mann, 2002; Anker and von Haehling, 2004; Wei, 2011; Frangogiannis, 2012). In people with DCM and congestive heart failure (CHF), activation of endothelial cells with upregulation of endothelial cell adhesion molecules induces recruitment of inflammatory cells, such as macrophages, neutrophils and lymphocytes, into the myocardium (Devaux et al., 1997; Noutsias et al., 1999; Noutsias et al., 2002; Noutsias et al., 2003; Anker and von Haehling, 2004; Wei, 2011). These cells are involved in initiating cardiac regeneration and repair. Alongside damaged myocardial cells, cells of the ECM, i.e. fibroblast, mast cells and infiltrating inflammatory cells, contribute to the production of cytokines and growth factors (Nag, 1980; Stewart et al., 2003; Souders et al., 2009; Wei, 2011; Frangogiannis, 2012). However, control of the inflammatory state by anti-inflammatory and pro-fibrotic factors is crucial.

Different populations of T helper (Th) subpopulations are reported to play a role in progression of cardiac diseases. Four major lineages, Th1, Th2, Th17 and T regulatory (Treg) cells are known (Mosmann et al., 1986; Aggarwal et al., 2003; Zhu et al., 2010). These T cell subsets are involved in fibrosis in chronic cardiac injury, while Th2 polarised responses promote fibrosis, Th1 cells might be anti-fibrogenic (Marra et al., 2009; Wei, 2011). Inflammatory events trigger a Th1 response and Th1 cytokines are believed to participate in the initiation of fibrosis, whereas the chronic
disease processes are usually driven by a Th2 response, resulting in fibroblast activation and proliferation, myofibroblast differentiation and ECM accumulation. In human heart failure patients, an immune response of Th1 predominance and Th1/Th2 imbalance has been reported (Cheng et al., 2009). Th17 cells are involved in the initiation or progression of inflammatory diseases, promoting degradation of type I and type III collagens and contributing to myocardial fibrosis (Feng et al., 2009; Wei, 2011). On the other hand, Treg cells constitute an anti-inflammatory and pro-fibrotic lineage of T cells that secrete IL-10 and TGF-β (Huber et al., 2006; Wei, 2011; Tang et al., 2012). However, Treg cells might also be involved in limitation of fibrogenesis, since they can suppress an excessive immune activation (Kvakan et al., 2009). Depending on the primary insult, the underlying condition and the activated cell populations cardiac regeneration and repair or a progressive cardiac disease can develop.

2.3.1 Cytokines

In cardiac disease, cytokines are produced by resident myocardial cells, such as damaged cardiomyocytes, fibroblasts, mast cells, and infiltrating inflammatory cells, for example macrophages and T cells (Kuhl et al., 1996; Noutsias et al., 2002).

Cytokines are highly potent endogenous mediators of intercellular communication (Klesius, 1982; Trotta, 1991). They bind to specific receptors and act as gene-regulatory proteins through the activation of transcription factors, such as NF-κB, sma- and mad-related proteins (Smad) and signal transduction and activators of transcription (STAT) proteins (Schutze et al., 1992; Briscoe and Guschin, 1994; Nakao et al., 1997; Anker and von Haehling, 2004).

Based on their effect, cytokines were originally classified as pro- and anti-inflammatory. The pro-inflammatory cytokines of highest relevance in the progression of CHF in humans and in animal models are interleukin (IL)-1, IL-6, IL-8 and tumour necrosis factor (TNF)-α, whereas IL-10 and transforming growth factor (TGF)-β, for example, are anti-inflammatory cytokines and considered to be cardioprotective (Pagani et al., 1992; TorreAmione et al., 1996; Bolger et al., 2002; Chen et al., 2008; von Haehling et al., 2009). Their different cytokine expression patterns characterise Th subpopulations, Th1 cells produce IFN-γ, whereas Th2 cells produce IL-4, IL-5 and IL-13 and Th17 cells produce IL-17, IL-21, IL-22 (Mosmann et
al., 1986; Aggarwal et al., 2003; Zhu et al., 2010). Treg cells can be differentiated into Treg type 1 cells which secrete IL-10, and Th3 cells which secrete TGF-β (Huber and Schramm, 2006; Wei, 2011; Tang et al., 2012).

2.3.1.1 Pro-inflammatory cytokines

The role of IL-1, IL-6 and TNF-α in human cardiac diseases has been studied extensively. Elevated circulating and myocardial levels were found in patients with various cardiac diseases and in CHF and have been correlated to disease severity and mortality (Torre-Amione et al., 1996; Deswal et al., 2001b; Torre-Amione, 2005; von Haehling et al., 2009). Pro-inflammatory cytokines have negative inotropic effects, which are mediated through down-regulation of cardiac beta-receptors, myocardial nitric oxide production and impaired myocardial energy production (Finkel et al., 1992; Pagani et al., 1992; Zell et al., 1997). These cytokines play a role in cardiac remodelling by activating transcription factors and stimulating enzymes involved in ECM degradation and deposition (Sivasubramanian et al., 2001; Bradham et al., 2002b; Siwik and Colucci, 2004). Furthermore, they stimulate the expression of adhesion molecules, activate inflammatory cells and contribute to the production of cytokines and reactive oxygen species, which maintain the inflammatory process (Kukielska et al., 1993; Braun et al., 1995; Kukielska et al., 1995; Deswal et al., 2001a; Yndestad et al., 2003; Torre-Amione, 2005; Castellano et al., 2008; Chen et al., 2008; Hedayat et al., 2010; Zhang et al., 2011). The resulting myocardial impairment, cardiac remodelling and persistent inflammatory response are suspected to contribute to the progression of CHF. The harmful effects of pro-inflammatory cytokines and the increased levels in patients with CHF were the rationale behind several clinical trials that blocked TNF-α in human heart failure patients (reviewed by Pagani et al., 1992; Mann, 2002). However, the results of these trials were disappointing, since no improvement of clinical signs and reduced survival times were observed (Anker and von Haehling, 2004; Torre-Amione, 2005; Chen et al., 2008). This suggests that pro-inflammatory cytokines do not only exert detrimental effects, but might also be involved in cardiac regeneration in patients with CHF (Chen et al., 2008; Hedayat et al., 2010).
2.3.1.2 Anti-inflammatory cytokines

Anti-inflammatory cytokines, such as IL-10 and TGF-β, inhibit pro-inflammatory cytokines (Tsunawaki et al., 1988; de Waal Malefyt et al., 1991; Bolger et al., 2002; Anker and von Haehling, 2004; Kaur et al., 2009). IL-10 is capable of down-regulating numerous inflammatory pathways by suppressing cytokine, adhesion molecule and matrix metalloproteinase production and by regulating growth and differentiation of lymphocytes (Fiorentino et al., 1991; Mosmann, 1994; Song et al., 1997; Moore et al., 2001; Anker and von Haehling, 2004; Frangogiannis, 2012).

TGF-β is a powerful immunosuppressive and profibrotic cytokine (Edwards et al., 1987; Dobaczewski et al., 2011; Kapur, 2011). It inhibits pro-inflammatory cytokine and endothelial cell adhesion molecule expression and thereby reduces the rolling and emigration of neutrophils and lymphocytes (Gamble and Vadas, 1988, 1991; Smith et al., 1996; Frangogiannis, 2012). TGF-β has three isoforms, TGF-β1-3. Of these, TGF-β1 and TGF-β3 are major stimulators of fibroblasts during normal ECM homoeostasis and tissue repair (Lim and Zhu, 2006; Creemers and Pinto, 2011; Dobaczewski et al., 2011). In cardiac diseases, they are considered to play a role in the resolution of inflammation and the transition to fibrosis. However, by inducing fibrosis and cardiomyocyte hypertrophy TGF-β promotes the structural remodelling of the heart and might therefore contribute to systolic and diastolic dysfunction in cardiac diseases (Nakao et al., 1997; Song et al., 1997; Lim and Zhu, 2006; Creemers and Pinto, 2011; Dobaczewski et al., 2011; Westermann et al., 2011; Frangogiannis, 2012). TGF-β2 is important for the fetal development of the heart and might be involved in activation of the fetal gene programme in the failing myocardium (Lim and Zhu, 2006).

2.3.2 Growth differentiation factor 15

Growth differentiation factor 15 (GDF-15), a member of the TGF-β superfamily, regulates inflammatory and apoptotic pathways needed for tissue development, differentiation and repair (Kempf and Wollert, 2009). A weak basal expression of GDF-15 is present in most tissues, with a marked increase in its expression in response to tissue injury and inflammation (Kempf and Wollert, 2009). In the heart, GDF-15 is produced by cardiomyocytes, endothelial cells, smooth muscle cells, adipocytes and macrophages (Kempf and Wollert, 2009) and is used as a biomarker
for disease progression and prognosis in people with acute and chronic heart failure (Kempf et al., 2007; Kempf and Wollert, 2009; Lok et al., 2012). In myocardial ischaemia, GDF-15 is considered to exert a protective role, because of its anti-apoptotic and anti-inflammatory effects. It might also be involved in cardiac fibrosis, as increased GDF-15 levels in cardiac diseases appear to correlate with the degree of myocardial fibrosis (Kempf et al., 2006; Kempf and Wollert, 2009; Lok et al., 2012). Nonetheless, people with DCM have been shown to exhibit only weak myocardial GDF-15 mRNA and protein production (Lok et al., 2012). Canine GDF-15 has been isolated from and characterised in an osteosarcoma cell line (Yamaguchi et al., 2008), but its transcription in the myocardium of healthy dogs and dogs with cardiac diseases has so far not been investigated.

2.4 Extracellular matrix remodelling in cardiac diseases

2.4.1 Matrix metalloproteinases and their inhibitors

ECM remodelling is regulated by a family of proteolytic enzymes, the matrix metalloproteinases (MMP), along with their natural tissue inhibitors (tissue inhibitors of metalloproteinases; TIMP) (Woessner, 1991; Nagase and Woessner, 1999; Spinale, 2002; Visse and Nagase, 2003; Spinale, 2007; Graham et al., 2008).

The MMP family comprises more than 25 zinc-dependent ECM-degrading endopeptidases that degrade the ECM under both physiological and pathological conditions (Visse and Nagase, 2003). There are two principal types of MMP, those that are secreted into the extracellular space and represent the majority of MMP species, and those that are membrane bound (membrane-type MMP; MT-MMP). The secreted MMP are synthesised and released as inactive zymogens and are activated by enzymatic cleavage to exert their proteolytic activity, whereas MT-MMP undergo intracellular activation through a pro-protein convertase pathway and are proteolytically active once inserted into the cell membrane (Somerville et al., 2003; Visse and Nagase, 2003). Activated MMP can degrade all ECM components; they stimulate other MMP and release growths factors (Nagase and Woessner, 1999; Nagase et al., 2006). Their activity is regulated by TIMP-1-4 (Gomez et al., 1997; Nagase and Woessner, 1999; Visse and Nagase, 2003). TIMP bind to the catalytic
domain of active MMP and prevent their access to substrates (Gomez et al., 1997; Nagase and Woessner, 1999; Visse and Nagase, 2003). TIMP-1, -2 and -4 are soluble, whereas TIMP-3 interacts with the ECM and might therefore exert a more localised, potent and prolonged effect (Gomez et al., 1997). By inhibiting MMP activity, TIMP prevent ECM degradation, are profibrotic and potentially anti-inflammatory. TIMP are also involved in myofibroblast activation, increased collagen synthesis, fibroblast apoptosis and inhibition of angiogenesis (Gomez et al., 1997; Vanhoutte and Heymans, 2010). Additionally, TIMP-3 has been demonstrated to inhibit the a disintegrin and metalloproteinase (ADAM-17), also named TNF-α convertase (TACE), and might therefore have a fundamental role in the control of inflammation through the regulation of TNF-α signalling (Kassiri et al., 2005; Vanhoutte and Heymans, 2010).

Previous studies have shown that MMP and TIMP are expressed in the mammalian myocardium: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3 and MMP-7), membrane-type MT1-MMP and TIMP-1-4 have been identified in the major cell types of the normal myocardium, i.e. cardiac myocytes, fibroblasts, smooth muscle cells and endothelial cells (Woessner, 1991; Spinale, 2002; Visse and Nagase, 2003; Spinale, 2007; Vanhoutte and Heymans, 2010).

Myocardial MMP induction is influenced by mechanical stimuli, neurohormones (including AT II, ET, noradrenalin, corticosteroids), growth factors and cytokines, such as IL-1 and TNF-α (Tyagi et al., 1993; Bradham et al., 2002b; Spinale, 2002, 2007; Tsuruda et al., 2004; Koskivirta et al., 2010; Vanhoutte and Heymans, 2010; Lacerda et al., 2012). TIMP-2 is constitutively expressed within the heart, whereas TIMP-1 and TIMP-3 are induced by pro-inflammatory cytokines (Li et al., 1999). During cardiac remodelling in health and disease, collagenases degrade interstitial type I, II and III collagens, whereas gelatinases act in particular on basement membranes and partially degraded collagen. Stromelysins have a broad substrate specificity including proteoglycans, laminin, fibronectin, gelatine and basement membrane collagens (Spinale, 2002; Visse and Nagase, 2003; Spinale, 2007). Besides matrix degradation, MMP activate other MMP (Nagase et al., 2006; Vanhoutte and Heymans, 2010). Increased MMP activity may result in collagen degradation, increased inflammatory response, ECM remodelling as well as left
ventricular dilatation and dysfunction (Thomas et al., 1998; Chow et al., 2007; Sivakumar et al., 2008). On the other hand, increased TIMP activity can result in excessive collagen deposition and, therefore, increased left ventricular stiffness and dysfunction (Spinale, 2002; Visse and Nagase, 2003; Spinale, 2007; Vanhoutte and Heymans, 2010). An equilibrium between deposition and degradation of ECM components is therefore required for efficient left ventricular geometry and cardiac function. Accordingly, an imbalance is a major cause of pathological ECM remodelling (Thomas et al., 1998; Spinale, 2002, 2007; Graham et al., 2008). In humans different expression patterns are reported depending on the cardiac disease, with increased myocardial MMP-1, MMP-2, TIMP-1 and TIMP-2 and variably altered MMP-9 transcription in the failing myocardium (Thomas et al., 1998; Siwik and Colucci, 2004; Picard et al., 2006; Batlle et al., 2007; Spinale, 2007; Graham et al., 2008; Sivakumar et al., 2008).

2.4.2 Lysyl oxidase

Lysyl oxidase (lox) is an extracellular, copper dependent amino oxidase that catalyses lysine-derived crosslinks between collagen and elastin, resulting in the deposition of insoluble collagen fibres (Smith-Mungo and Kagan, 1998). Apart from lox, four lox-like (loxl-1, -2, -3, -4) proteins exist (Lopez et al., 2010). In the heart, lox is the most abundant of the group of proteins and the only one that uses collagen as substrate (Lopez et al., 2010). It might also be involved in fibroblast motility and migration as well as the regulation of cell adhesion (Nelson et al., 1988; Giampuzzi et al., 2005). Lox is produced by fibroblasts and myofibroblasts and its transcription is stimulated by hypoxia-inducible factor-1α, TNF-α and TGF-β1 (Smith-Mungo and Kagan, 1998; Lopez et al., 2010; Voloshenyuk et al., 2011). Increased lox levels, associated with excessive fibrillar collagen cross linking and fibre deposition, have been reported in human patients with enhanced myocardial stiffness, left ventricular dysfunction and heart failure (Sivakumar et al., 2008; Lopez et al., 2009; Lopez et al., 2010; Kasner et al., 2011). Lox might also play an important role in atrial fibrosis and development of atrial fibrillation (Adam et al., 2011).
2.5 Role of leptin in cardiac inflammation and remodelling

Recent studies have shown that adipose tissue is not only an energy storage organ, but also exerts important endocrine and immune functions (Karmazyn et al., 2008; German et al., 2010; Sweeney, 2010; Ouchi et al., 2011). The latter are mediated through the release of adipocytokines, which activate neutrophils, monocytes and lymphocytes, thereby mediating the production of pro-inflammatory cytokines and promoting T helper 1 responses. Centrally, leptin activates the sympathetic nervous system (Loffreda et al., 1998; Karmazyn et al., 2008; Knudson et al., 2008; Fernandez-Riejos et al., 2010). Therefore, obesity is now considered to be accompanied by a systemic inflammatory state (Loffreda et al., 1998; Fernandez-Riejos et al., 2010).

Leptin belongs to the group of adipocytokines, which includes also resistin, adiponectin, visfatin and more classical cytokines, such as TNF-α, IL-1 and IL-6 (Rondinone, 2006; Karmazyn et al., 2008). Leptin is the product of the Ob gene and was first shown to be secreted by white adipocytes (Zhang et al., 1994; Igel et al., 1997). It has a profound impact on body weight control and its deficiency and receptor defects are associated with obesity (Zhang et al., 1994; Igel et al., 1997). While leptin regulates energy homoeostasis by reducing appetite and increasing the basal metabolic rate, obesity is associated with hyperleptinaemia, which develops most probably as a consequence of leptin resistance (Igel et al., 1997). Circulating leptin levels are correlated with pro-inflammatory cytokine levels and increased leptin concentrations were found in several chronic diseases, including cardiac diseases and CHF (Faggioni et al., 1998; Loffreda et al., 1998; Schols et al., 1999; Filippatos et al., 2000; Schulze et al., 2003; Schulze and Kratzsch, 2005; Conde et al., 2010; Fernandez-Riejos et al., 2010; Sweeney, 2010; Ouchi et al., 2011).

In the heart, cardiomyocytes, endothelial cells and smooth muscle cells produce leptin and its receptor and the production is increased by AT II and ET, suggesting local auto- and paracrine effects (Sierra-Honigmann et al., 1998; Rajapurohitam et al., 2003; Purdham et al., 2004; Schulze and Kratzsch, 2005; Karmazyn et al., 2007). Furthermore, localised depots of epicardial or perivascular fat might play a significant physiological or pathological role (Cheng et al., 2008; Knudson et al., 2008; Sweeney, 2010). The physiological response of cardiomyocytes to leptin is a negative inotropic function mediated by endogenously produced nitric oxide.
(Karmazyn et al., 2008). Leptin induces cardiomyocyte hypertrophy and collagen synthesis through stimulation of MAPkinase and RhoA/Rho kinase pathways, it protects cardiomyocytes from apoptosis induced by chronic ischaemia and modulates the fatty acid metabolism in the heart, resulting in an increased rate of fatty acid oxidation and thereby increased myocardial oxygen consumption (Atkinson et al., 2002; Rajapurohitam et al., 2003; Sharma and McNeill, 2005; Karmazyn et al., 2007, 2008; Schram et al., 2010; Sweeney, 2010). The latter might contribute to decreased cardiac efficiency in cardiac diseases. In heart failure, leptin is involved in the neurohormonal activation, due to its central sympathoexcitatory effects and is associated with high levels of catecholamines and pro-inflammatory cytokines (Haynes et al., 1997a). Furthermore, the leptin induced increased metabolic rate results in a progressive catabolic syndrome, and high leptin blood levels have been shown to be associated with unfavourable outcomes in human patients (Toth et al., 1997; Schulze and Kratzsch, 2005). However, controversial observations exist and in mice with leptin receptor deficiency, activation of leptin pathways have been reported to decrease cardiac hypertrophy, apoptosis and inflammation (McGaffin et al., 2010).

2.6 Cardiac inflammation and remodelling in the dog

Progressive cardiac remodelling is known to be associated with concentric or eccentric hypertrophy of the left and/or right ventricle in dogs with acquired and congenital cardiac diseases. Myocardial function and the degree of hypertrophy can be investigated using echocardiography and thoracic radiography and is used to assess the severity and progression of cardiac diseases in dogs (Oyama and Thomas, 2002; Van Israel et al., 2002; Hori et al., 2008; Yamane et al., 2008; Martin et al., 2009; Lord et al., 2011).

In canine DVD, volume overload results in eccentric left ventricular hypertrophy and atrial dilation and is suspected to be associated with loss of collagen and cardiomyocyte degeneration (Zheng et al., 2009; Pat et al., 2010). Normal mitral valve leaflets consist of four layers, the atrialis, spongiosa, fibrosa and ventricularis, and represent a thin, translucent structure without nodules or thickening (Fox, 2012), whereas mitral valve leaflets of DVD patients are diffusely thickened and distorted, due to increased ECM deposition in the spongiosa and disruption of the collagen layers in the fibrosa (Pomerance and Whitney, 1970; Aupperle and Disatian, 2012;
Fox, 2012; Lacerda et al., 2012; Orton et al., 2012). The distorted mitral valve leaflets cause impaired mechanical valvular function and thereby mitral regurgitation and left sided volume overload.

In canine DCM, a primary myocardial disease is present associated with attenuated wavy fibre cardiomyocyte degeneration and/or fatty-fibrous degeneration, this causes eccentric left ventricular hypertrophy (Basso et al., 2004; Tidholm and Jonsson, 2005; Lobo et al., 2010).

Activation of SNS, RAAS and NP during progression of the disease is reported in dogs with acquired cardiac diseases, but less is known about the presence and type of inflammatory process (Koch et al., 1995; Pedersen et al., 1995; Tidholm et al., 2001b; Uechi et al., 2002; Sisson, 2004; Marcondes Santos et al., 2006; Fujii et al., 2007; Oyama, 2009; Ettinger et al., 2012). In canine DVD, inflammation is not suspected to play an important pathogenic role (Oyama and Chittur, 2006; Aupperle and Disatian, 2012; Orton et al., 2012) and an increase in IL-6 transcription (Oyama and Chittur, 2006), but no myocardial inflammatory infiltration was detected in dogs with DVD (Aupperle and Disatian, 2012; Fox, 2012). Also, Zois et al. reported decreased circulating IL-2, IL-7 and IL-8 levels with increased severity of DVD (Zois et al., 2012).

In human medicine, an inflammatory form of DCM has been reported (Kuhl et al., 1996; Richardson et al., 1996; Noutsias et al., 2002; Westermann et al., 2011), this is also suspected in dogs such as Doberman pinschers with DCM (Gilbert et al., 1997). However, this has so far not been further investigated, for example by studying cytokine expression. In a dog model, cytokine stimulation and ischaemia-reperfusion injury was shown to upregulate the cardiac IL-8 and ICAM-1 production (Kukielska et al., 1993; Kukielska et al., 1995; Oyama and Chittur, 2006).

There is evidence that the cardiac remodelling observed in dogs with DVD is influenced by TGF-β, MMP and TIMP. Down-regulation of TGF-β1 transcription was seen in a dog model of mitral regurgitation (Zheng et al., 2009), whereas an increase of valvular TGF-β1 and TGF-β3, but not TGF-β2 protein expression was detected in dogs with DVD (Aupperle et al., 2008). Furthermore, increased MMP-1, MMP-3, TIMP-1, -2 and -3, reduced MMP-2 and variably altered MMP-9 production was observed in dogs with DVD and the dog model of mitral regurgitation (Oyama and
Chittur, 2006; Aupperle et al., 2009a; Zheng et al., 2009; Ljungvall et al., 2011; Obayashi et al., 2011; Aupperle and Disatian, 2012). It is hypothesised that ECM remodelling in canine DCM is influenced by upregulation of MMP-9 and/or TIMP-1 (Gilbert et al., 1997; Oyama and Chittur, 2005).

Obesity is increasingly recognised in veterinary patients, obese dogs have been shown to exhibit increased leptin levels (Ishioka et al., 2002; German et al., 2010) and a recent study suggested an association of obesity with ventricular hypertrophy and cardiac dysfunction in dogs (Mehlman et al., 2013). However, the role of leptin in development and progression of canine cardiac disease and CHF has so far not been investigated. Congenital cardiac diseases that cause pressure overload present with concentric hypertrophy (Oyama and Thomas, 2002), but studies investigating the presence of inflammation or cardiac remodelling in these patients have so far not been undertaken.

2.7 Effect of systemic, non-cardiovascular diseases on cardiac inflammation and remodelling

Systemic diseases are known to result in cardiac damage and impaired cardiac function in humans and dogs (Parker et al., 1984; Nelson and Thompson, 2006; Merx and Weber, 2007; Flynn et al., 2010; Serra et al., 2010; Langhorn et al., 2013). In dogs, an association of cardiac function and outcome is suspected (Nelson and Thompson, 2006; Bulmer, 2011). It has been shown that circulating endotoxins, a systemic inflammatory response with increased circulating catecholamines and cytokine levels and the RAAS trigger myocardial inflammation and cause cardiomyocyte damage (Chen et al., 2008; Sciarretta et al., 2009; Flynn et al., 2010; Scruggs et al., 2010). The innate immune system and resultant inflammatory response can be activated by pathogens, i.e. bacteria, but also by tissue destruction (Kaczorowski et al., 2009; Cohen, 2010; Bulmer, 2011). Especially Toll-like receptors promote the transcription of numerous cytokines via the NF-κB pathway, play a central role in myocardial dysfunction in sepsis and are involved in cardiac remodelling (Cuenca et al., 2006; Kaczorowski et al., 2009; Cohen, 2010; Bulmer, 2011). As reported above, it has been shown in animal models that cardiomyocyte damage results in cytokine, chemokine and adhesion molecule expression which
induces leukocytes recruitment and cardiac inflammation as well as ECM degradation (Dobaczewski et al., 2010). Despite a potential marked effect of systemic diseases on cardiac function and remodelling and, therefore, morbidity and mortality, studies investigating the effect of systemic diseases on the heart in animals are sparse (Nelson and Thompson, 2006; Serra et al., 2010; Bulmer, 2011; Langhorn et al., 2013). Several systemic diseases in dogs have been shown to be associated with increased circulating cardiac troponin I levels, suggesting myocardial cell damage (Serra et al., 2010; Langhorn et al., 2013). Nelson and Thompson reported a reduced systolic function in dogs with severe systemic illness, such as sepsis and cancer, and 75% of these dogs died (Nelson and Thompson, 2006). In contrast, dogs that had been discharged showed reversible myocardial depression and improved myocardial function at revisit investigations (Nelson and Thompson, 2006; Dickinson et al., 2007). In people with septic shock, increased ventricular volumes and reduced ejection fraction resolved in those patients that survived (Parker et al., 1984).

In human patients with malignant neoplastic diseases and metastases, activation of the inflammatory system contributes to clinical signs, progression of disease and cardiac cachexia (Seruga et al., 2008). In dogs with lymphoma, increased blood levels of markers for remodelling, such as MMP-9, reduced TGF-β1 and increased TNF-α levels were detected; these returned to normal in cases of tumour remission (Hofer et al., 2011; Aresu et al., 2012). However, the potential effect on cardiac function was not investigated in these studies.
3 AIMS OF THE STUDY

The aims of the current study were

I to investigate the role of inflammation and ECM remodelling in the heart of dogs with cardiac diseases and systemic diseases by

a. Investigating quantitative pro- and anti-inflammatory cytokine and GDF-15 transcription in the blood and myocardium as markers for cardiac inflammation;

b. Investigating quantitative MMP, TIMP and lox mRNA expression in the blood and myocardium as markers for ECM remodelling in the heart;

c. Investigating potential quantitative differences in inflammatory and ECM remodelling processes in different cardiac regions;

d. Investigating the constitutive cytokine, GDF-15, MMP, TIMP and lox transcription in the blood and myocardium of healthy control dogs.

II to investigate the role of leptin in dogs with cardiac diseases by

a. Investigating quantitative leptin transcription in blood and myocardium of dogs with cardiac and systemic diseases;

b. Investigating regional differences in leptin transcription in the myocardium of dogs with cardiac and systemic diseases.
4 MATERIAL AND METHODS

Dogs with cardiac diseases (see 4.1, Table 1), systemic diseases (see 4.2, Table 2) and healthy control dogs (see 4.3, Table 3) were included in the PhD project. Tables 1, 2 and 3 provide the information on the dogs and the studies of the PhD project to which they contributed.

4.1 Dogs with cardiac diseases (18 dogs)

All dogs with cardiac diseases were patients presented to the Small Animal Teaching Hospital, University of Liverpool, apart from two dogs that had been investigated and diagnosed by a cardiologist [Simon Swift, MA, VetMB, CertSAC, DipECVIM-CA (Cardiology)] in another practice and had been referred. The most frequent breed in this group were Boxer dogs (n=5), followed by Labradors and Doberman (n=3 each; Table 1). A physical examination was performed and complete blood count (CBC), blood biochemistry, blood pressure measurement, electrocardiography (ECG), echocardiography and thoracic radiographs were obtained in all dogs. Thoracic radiographs were taken, if the dogs were stable enough for sedation and positioning. A Holter monitor (Lifecard CF, Spacelabs Healthcare Ltd, Hertford, UK) was applied to confirm the diagnosis and significance of arrhythmias. Dogs with various cardiac diseases and without clinical evidence of systemic disease were included in this group. Congestive heart failure was confirmed by physical examination, echocardiography and/or thoracic radiographs. The Canine Heart Failure International Expert Forum (CHIEF) heart failure classification scheme was applied (Strickland, 2008; Table 4). Two dogs were in heart failure class B, 11 dogs in heart failure class C3 and five dogs in heart failure class D. Five dogs had congenital and 13 dogs acquired cardiac diseases. The most frequent acquired cardiac diseases were DCM (n=5) and DVD (n=4). Three dogs with DCM (dogs 10, 11, 13) and one dog with DVD (dog 17) had atrial fibrillation. Dogs that were found to be in CHF were stabilised with heart failure medication, including furosemide, nitroglycerine, pimobendan, benazepril and spironolactone. The treatment of the dogs was individualised and subject to the discretion of the attending cardiologist, following the recommendations for the treatment of dogs with acute CHF and according to the individual response to treatment.
Table 1: Dogs with cardiac diseases, breed, age, sex, disease, heart failure classification, body weight, body condition score, study into which they were included and samples taken.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease</th>
<th>CHIEF</th>
<th>BW (kg)</th>
<th>BCS (/9)</th>
<th>Study</th>
<th>Samples taken (Bl, My)</th>
<th>Circ marker</th>
<th>Myoc</th>
<th>Myoc</th>
<th>ECM</th>
<th>leptin</th>
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<td>M</td>
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<td>Arrh C</td>
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<td>X</td>
<td>X</td>
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</tr>
</tbody>
</table>

4.2 Dogs with systemic diseases (10 dogs)

All dogs in this group had been patients of the Small Animal Teaching Hospital, University of Liverpool. A physical examination was performed, and CBC and blood biochemistry were obtained (Table 2). Further investigations were undertaken to obtain a diagnosis, including combinations of urinalysis, ultrasonography, radiography, computed tomography, magnetic resonance imaging, fine needle aspirates and/or biopsies when indicated. Only dogs with systemic diseases that did not involve the heart were included in the study. Among these, lymphomas (n=4) and brain tumours (n=2) were most frequent.
Table 2: Dogs with systemic diseases, breed, age, sex, disease, heart failure classification, body weight, body condition score, study into which they were included and samples taken.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease</th>
<th>CHIEF</th>
<th>BW (kg)</th>
<th>BCS (/9)</th>
<th>Study</th>
<th>Samples taken (Bl, My)</th>
<th>Circ marker</th>
<th>Myoc cyto</th>
<th>Myoc ECM</th>
<th>leptin</th>
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<td>II, III, IV</td>
<td>My</td>
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<td>X</td>
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</tr>
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<td>Cocker</td>
<td>8</td>
<td>NF</td>
<td>Brain tumour</td>
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<td>14.4</td>
<td>5</td>
<td>II, III, IV</td>
<td>My</td>
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<td>X</td>
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<td>NF</td>
<td>Lymphoma</td>
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<td>II, III, IV</td>
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<td>X</td>
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<td>NM</td>
<td>Lymphoma</td>
<td>-</td>
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<td>6</td>
<td>IV</td>
<td>My</td>
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<td>Lymphoma</td>
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<td>36.7</td>
<td>3</td>
<td>IV</td>
<td>My</td>
<td>X</td>
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<tr>
<td>24</td>
<td>D de B</td>
<td>1.5</td>
<td>F</td>
<td>Lymphoma</td>
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<td>41.5</td>
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<td>IV</td>
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</tbody>
</table>

4.3 Healthy control dogs (16 dogs)

Eight dogs of this group were recruited from the cohort of healthy dogs that were screened for DCM at the Small Animal Teaching Hospital, University of Liverpool as part of LUPA (www.eurolupa.org) and eight dogs were control dogs from a pharmaceutic company (Pfizer; Table 3). Dogs screened for DCM underwent physical examination, and CBC, blood biochemistry, blood pressure measurement, ECG and echocardiography were obtained. In four Great Danes, the thyroid function (T4, TSH) was investigated. Animals from the pharmaceutic company were clinically healthy. The data and myocardial samples had been collected and were provided by the company.
Table 3: Healthy control dogs, breed, age, sex, disease, heart failure classification, body weight, body condition score, study into which they were included and samples taken.

<table>
<thead>
<tr>
<th>Dog</th>
<th>Breed</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Disease</th>
<th>CHIEF</th>
<th>BW (kg)</th>
<th>BCS (/9)</th>
<th>Study</th>
<th>Samples taken (Bl, My)</th>
<th>Circ marker</th>
<th>Myoc cyto</th>
<th>Myoc ECM</th>
<th>leptin</th>
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Table 4: CHIEF heart failure classification (Strickland, 2008).

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<thead>
<tr>
<th>Class</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>A</td>
<td>Risk of heart disease, but currently no identifiable structural disorder of the heart</td>
</tr>
<tr>
<td>B</td>
<td>Structural heart disease documented but never developed clinical signs of heart failure</td>
</tr>
<tr>
<td>C1</td>
<td>Past or current clinical signs of heart failure associated with structural heart disease Past no clinical signs of heart failure (stabilized/past heart failure)</td>
</tr>
<tr>
<td>C2</td>
<td>Current mild-moderate heart failure</td>
</tr>
<tr>
<td>C3</td>
<td>Current severe heart failure</td>
</tr>
<tr>
<td>D</td>
<td>End-stage cardiac disease with clinical signs of heart failure refractory to standard therapy</td>
</tr>
</tbody>
</table>

4 METHODS

The PhD project comprised four studies. Study I investigated circulating markers of inflammation and ECM remodelling in blood samples of dogs with cardiac diseases, studies II and III analysed myocardial markers of inflammation and ECM remodelling in dogs with cardiac and systemic diseases and study IV investigated circulating and myocardial leptin mRNA levels in dogs with cardiac diseases. For all studies owner consent was obtained and dogs were assigned arbitrary numbers as identifiers in order to maintain confidentiality.

4.4 Circulating markers of inflammation and ECM remodelling

As part of the general investigation, EDTA blood samples for CBC analysis were collected from dogs shown in tables 1 and 3 prior to administration of any medication. From these blood samples, 0.3 mL was immediately taken and frozen at -20°C for 8-24 h within 10 min after sampling, then stored at -80°C until further processing.
4.5 Myocardial markers of inflammation and ECM remodelling

Myocardial samples were collected from carcasses of dogs euthanased upon owner’s request, due to end-stage systemic or cardiac diseases and poor prognosis, or in the case of one dog with recurrent ventricular tachycardia that had developed ventricular fibrillation (dog 6), after spontaneous death (Tables 1 and 2). The hearts were removed within one hour after death and grossly examined for any pathological changes. Myocardial samples of approximately 3 mm$^3$ were collected from the interventricular septum (IVS), right atrium and ventricle (RA, RV) and left atrium and ventricle (LA, LV) for RNA extraction and stored in RNAlater (Life Technologies Ltd, Paisley, UK) at -20°C until use. Hearts were subsequently fixed in 10% formalin and samples from the same sites as those for RNA extraction were prepared and routinely paraffin wax embedded for the histological examination (see 4.7).

The myocardial samples of control dogs were donated from a pharmaceutical company (Pfizer; Table 3). These dogs had been euthanased and grossly examined on site at the pharmaceutical company. Samples from the LA, RA, LV and RV had been collected and immersed in RNAlater (Life Technologies Ltd) immediately after death.

4.6 PCR analysis of blood and myocardium (I-IV)

4.6.1 Primer design (I-IV)

Primers for cytokines, growth differentiation factor-15 (GDF-15), leptin and lysyl oxidase (lox) were designed using the Primer Express software (Life Technologies Ltd). They were selected to span predicted exon boundaries, where possible (Table 5a). BLAST searches were performed to confirm the gene specificity. Target and reference gene primers were synthesised by Eurogentec (Southampton, UK). For the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH), matrix metalloproteinase (MMP)-1, -2, -3, -9 and tissue inhibitor of metalloproteinase (TIMP)-1-4 previously reported published sequences were used (Table 5b) (Clements et al., 2006; Garvican et al., 2008; Clements et al., 2009). Primers were
validated by standard curve of eight serial dilutions and primer efficiencies were between 96% and 120% (Table 5a, b).

Table 5a: Primer sequences, position, accession number of the National Center for Biotechnology Information and efficiency used for quantitative PCR (Studies I, II, IV).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Position</th>
<th>Accession number</th>
<th>Efficiency %</th>
</tr>
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<td>AF187322</td>
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<td>reverse: GGAGCTGCCCCCTCAGTCT</td>
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<tr>
<td>IFN-γ</td>
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<td>03174.1</td>
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<td>Lox</td>
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<td>reverse: TCGCAGCTTGGAGGAGTCTA</td>
<td>288-269</td>
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</table>

Table 5b: Primer sequences, their references and efficiency used for quantitative PCR (Studies I-IV).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ – 3’)</th>
<th>Reference</th>
<th>Efficiency %</th>
</tr>
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<td>CTGGGGCTCAGTTGAAAGGCAAACATGGGGGCATCAG</td>
<td>Clements et al 2006</td>
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<td>MMP-1</td>
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<td>GGTGAAGGAAGTGCAAGTTCTGATAGCTCTCTACTTTGGAAGAGGACTTTCTCTCT</td>
<td>Garvican et al 2008</td>
</tr>
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<td>MMP-2</td>
<td>forward reverse</td>
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</tr>
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<td>MMP-3</td>
<td>forward reverse</td>
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<td>Garvican et al 2008</td>
</tr>
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<td>MMP-9</td>
<td>forward reverse</td>
<td>CACGCATGACATCTCCAGT</td>
<td>Clements et al 2009</td>
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<td>MMP-13</td>
<td>forward reverse</td>
<td>CGCGACCTTTATCTCTCATCT</td>
<td>Clements et al 2006</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>forward reverse</td>
<td>TGCATCTGCTGTTGCT</td>
<td>Clements et al 2006</td>
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<td>TIMP-4</td>
<td>forward reverse</td>
<td>GCAGAGAGAAGTGCTGAGCTCATCA</td>
<td>Clements et al 2006</td>
</tr>
</tbody>
</table>

GAPDH: glyceraldehyde 3-phosphate dehydrogenase, MMP: matrix metalloproteinase, TIMP: tissue inhibitor of matrix metalloproteinase.

4.6.2 RNA isolation

4.6.2.1 RNA isolation from blood samples (I, IV)

A commercially available kit (RiboPure blood kit, Life Technologies Ltd) was used, following the manufacturer’s protocol. To prepare the solutions for RNA isolation, 56 mL 100% ethanol was added to Wash Solution 2/3 and the Elution Solution was heated to 75ºC in an RNase-free tube.

For RNA isolation, 800 µL Lysis Solution and 50 µL Sodium Acetate Solution was added to 0.3 mL defrosting blood which was then thoroughly mixed by vortexing until the solution was homogenous. 500 µL of Acid-Phenol-Chloroform, taken from beneath the overlying layer of aqueous buffer was added to the cell lysate. The fluid was thoroughly mixed for 30 s by vortexing. The mixture was stored for 5 min at room temperature (RT), followed by centrifugation for 1 min at 10,000 x g at RT to
separate the aqueous and organic phases. The aqueous (upper) phase was transferred into a new 2 mL collection tube, the lower phase was discarded. 600 µL (½ of the recovered volume) of 100% ethanol was added and the fluid briefly and thoroughly mixed. 700 µL of the sample was transferred to a filter cartridge assembly and centrifuged for 5-10 s at 10,000 x g to pass the liquid through the filter. The flow through was discarded and another 700 µL were loaded onto the same filter, centrifuged for 5-10 s at 10,000 x g and the flow-through was again discarded. This step was repeated with the remaining sample. 700 µL Wash Solution 1 was added onto the filter, the tube was centrifuged for 5-10 s at 10,000 x g and the flow through was discarded. 700 µL Wash Solution 2/3 was added onto the filter, the tube was centrifuged for 5-10 s at 10,000 x g and the flow-through was discarded. This step was repeated. The tube was then centrifuged for 1 min to remove residual fluid from the filter. The filter was transferred into a collection tube and 40 µL preheated Elution Solution was added to the centre of the filter. The solution was incubated at RT for 20 s and the tube centrifuged for 30 s at 13,000 x g. Another 40 µL of preheated Elution Solution was added and the tube centrifuged for 1 min at 13,000 x g to collect the RNA into the same tube. The eluted RNA (total volume of 80 µL) was stored at -80ºC until further use.

For DNase treatment, 4 µL 20 X DNase Buffer and 1 µL DNase was added to the eluted RNA (80 µL) and the sample gently and thoroughly mixed, followed by incubation for 30 min at 37ºC. 16 µL DNase Inactivation Reagent was added and the sample briefly vortexed to thoroughly mix DNase Inactivation reagent and RNA. Whilst incubating at RT for 2 min, the sample was briefly vortexed once or twice, then centrifuged for 1 min at maximum speed to pellet the DNase Inactivation reagent. The RNA was transferred into a new RNase-free tube. The RNA content of each sample was measured using a spectrophotometer (ND-1000, Nanodrop Technologies, Thermo Scientific, Wilmington, USA) and the samples were stored at -80ºC until further use.

4.6.2.2 RNA isolation from myocardial samples (II-IV)

A commercially available kit (RNA minikit, Qiagen Ltd, Manchester, UK) was used and the manufacturer’s protocol was followed. To prepare the solutions for RNA isolation, 10 µL β-mercaptoethanol was added to 1 mL RLT buffer, and 4 volumes of ethanol (96-100%) was added to buffer RPE prior to first use. Myocardial samples
frozen in RNaLater were removed from the RNaLater, placed in a 1.5 mL Eppendorf tube and snap frozen in liquid nitrogen. The frozen tissue was placed into a new 1.5 mL Eppendorf tube with 300 µL RLT buffer and was disrupted with a pestle. 590 µL RNase free water and 10 µL Proteinase K (Qiagen Ltd) were added, and the sample thoroughly mixed and incubated for 10 min at 55°C. The tube was centrifuged for 3 min at 10,000 x g at RT and the supernatant transferred into a new Eppendorf tube. The equivalent of ½ of the volume (450 µL) of 70% ethanol was added and the fluid immediately mixed by pipetting. 700 µL of the fluid was transferred to an RNeasy spin column that was placed in a 2 mL collection tube. The lid was closed and the tube was centrifuged for 15 s at 10,000 x g. The flow through was discharged and the step repeated with the remainder of the sample. 350 µL RW1 buffer was added and the fluid centrifuged for 15 s at 10,000 x g. The flow through was discarded. 10 µL DNase 1 stock solution was added to 70 µL RDD and gently mixed. This mixture was transferred to the spin column and incubated for 15 min at RT. 350 µL RW1 buffer was added to the spin column. The tube was centrifuged for 15 s at 10,000 x g and the flow through discarded. 500 µL RPE was added, the tube centrifuged for 15 s at 10,000 x g and the flow through discarded. 500 µL RPE was added and the tube centrifuged for 2 min at 10,000 x g. The RNeasy spin column was removed and placed into a new 2 mL collection tube, then centrifuged at maximum speed for 1 min. The old collection tube with the flow through was discarded. The RNeasy spin column was placed in a new 1.5 mL collection tube, 30 µL RNase free water was added and the tube centrifuged for 1 min at 10,000 x g. Another 40 µL RNase free water was added and the tube centrifuged for 1 min. The RNA content was measured and the sample (total volume of 70 µL) stored at - 80°C until further use.

4.6.3 Synthesis of cDNA (I-IV)

200 ng and 500 ng of total RNA isolated from blood and myocardium, respectively, was used to synthesise cDNA, using Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK) and primed with random hexamer oligonucleotides (Promega) in a 25 µl reaction. The RNA was incubated with 0.5 µg random hexamers and RNase free water (to a volume of 13 µL) at 70°C for 5 min. This was added to the Mastermix consisting of 5 µL 5X reaction buffer, nucleotides (dATP, dCTP, dGTP, dTTP, 0.5 mM each), RNase inhibitor (1 U/µL) and reverse transcriptase (2.5 U/µL). The mixture was incubated at 25°C for 5 min, which
was followed by incubation at 37°C for 60 min. Inactivation of the reverse transcriptase was achieved by incubation at 93°C for 5 min. cDNA was stored at -80°C until use in the quantitative PCR.

4.6.3 Quantitative PCR (I-IV)

Aliquots (1 μL) of the cDNA were amplified in duplicates by PCR on an ABI 7700 Sequence Detector (Life Technologies Ltd) using the SYBR Green PCR mastermix (Life Technologies Ltd). Each assay well had a 20 μL reaction volume consisting of 10 μL 2X SYBR Green PCR Mastermix, 0.4 μL each of 10 μM forward and reverse primers and 1 μL of sample cDNA (templates) or water (negative controls). The amplification was performed according to a standard protocol with 2 min incubation at 50°C, and initial denaturation for 10 min at 90°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. The PCR was followed by a dissociation programme, represented by incubation for 1 min at 95°C and subsequent 41 cycles during which the temperature was increased for 1°C at each cycle, starting at 55°C and ending at 95°C. All PCR reactions exhibited one well-defined melting-curve peak. Real-time data were analysed by the Sequence Detection Systems software, version 2.2.1 (Life Technologies Ltd). Relative expression levels were normalised to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

4.7 Histological examination (II-IV)

For the histological examination, two consecutive 3-5 μm thick sections were prepared from the formalin-fixed, paraffin-embedded myocardial samples of all diseased dogs. The first section was stained with haematoxylin-eosin (HE) for the assessment of any pathological changes. To the second section the Masson trichrome stain was applied for the evaluation of the amount and distribution of collagen in the myocardium. The histological examination was undertaken by Dr. Udo Hetzel (Dr.rer.nat., Dr.med.vet., FTA Pathologie, MRCVS), Docent in Veterinary Ultrastructural Pathology, University of Helsinki who recorded all histopathological changes. The most relevant findings [i.e. lipomatosis cordis, cardiac (interstitial, subendocardial or subepicardial) fibrosis, leukocyte infiltration, muscular hypertrophy of small arteries, cardiomyocyte necrosis] were scored semiquantitatively. Healthy
control hearts were used as reference, and the observed changes in the hearts of diseased dogs graded as mild (+), moderate (++) or severe (+++).

4.8 Statistical analysis

Real time PCR data were entered into Excel spreadsheets (Microsoft Corporation, Reading, Berkshire, UK) and the statistical analysis was performed, using Minitab 15 or 16 (Minitab Inc, State College, PA, USA). Basic descriptive statistics (mean, median, variance, standard deviation, interquartile range, confidence interval) were obtained. Following these, a number of variables were transformed to improve normality and model assumptions. IL-1β, IL-4 IL-6, IL-10, TNF-α, TGF-β2, TGF-β3, GDF-15, MMP-2, MMP-9, TIMP-1, -2, -3, -4, and leptin transcription values were log transformed and the inverse square root was applied to IL-2, IL-8, TGF-β1, MMP-1, -3, and MMP-13, whereas the square root was used for lox and the inverse expression for IFN-γ.

The age and body condition score (BCS) of the dogs from which blood samples were examined (Studies I and IV) were not normally distributed and a Mann Whitney U test was used to compare these parameters in dogs with and without cardiac disease or CHF. All other parameters were normally distributed or normalised and the one-way ANOVA test was applied. The weight of the dogs, and the cytokine, growth factor, MMP and TIMP mRNA levels of control and CHF dogs in study I were compared using a one-way ANOVA test. In study IV, the weight between groups and the differences in leptin RNA levels between sex, dogs with and without cardiac disease or CHF, heart failure stage, different regions of the heart and different cardiac disease groups were compared using a one-way ANOVA test. The relation between leptin and body weight and BCS was examined with scatterplots and then tested with the Pearson correlation test.

In study II and III the amount of cytokine and GDF-15 (Study II) and of MMP, TIMP and lox (Study III) mRNA in myocardial samples from various heart regions were compared in control dogs, dogs with systemic diseases and dogs with cardiac diseases. To investigate the degree of myocardial MMP, TIMP, and lox mRNA expression of dogs with CHF and DCM, the data collected from the myocardium of these dogs were grouped and compared with those from dogs with cardiac diseases other than CHF and DCM, control dogs and dogs with systemic diseases. Data
generated from atria and ventricular samples of dogs with atrial fibrillation were compared with those of dogs with cardiac diseases without atrial fibrillation and dogs with systemic diseases. Results are displayed as range and median or mean and standard deviation as appropriate.

Cluster analysis was performed with normalised and non-normalised data to explore the relation between cytokines, MMP, TIMP in blood samples of dogs with CHF (Study I), between cytokines and GDF-15 (Study II) and between MMP, TIMP, lox (Study III) in dogs with cardiac diseases, using the correlation matrix and a Ward linkage dendrogram. Individual correlations were examined with the Pearson correlation test. A trend for statistical significance was defined as p<0.1, statistical significance was defined as p<0.05.
5 RESULTS

The results presented here are a summary of the detailed results that are presented in the four manuscripts that are included into the thesis.

5.1 Dogs included in studies I–IV

In study I, blood samples of 8 dogs with cardiac diseases and 8 healthy control dogs were compared. Study II was conducted on myocardial tissue of 7 dogs with systemic diseases, 7 dogs with cardiac diseases and 8 healthy control dogs, and study III comprised myocardial samples of 6 dogs with systemic diseases, 9 dogs with cardiac diseases and 8 control dogs. Study IV was undertaken on blood samples of 8 dogs with cardiac diseases and 8 healthy control dogs, and on myocardial samples of each 10 dogs with cardiac and systemic diseases. In this study, the dogs with systemic diseases served as control, since samples of healthy control dogs were not available. Information on the dogs included in studies I-IV is presented in tables 1, 2 and 3.

5.2 Pathological changes in the heart of dogs with cardiac and systemic diseases (II-IV)

A gross and histopathological examination was performed on all dogs with cardiac and systemic diseases to confirm or exclude the diagnosis of cardiac disease, respectively, and to investigate the myocardium for any pathological changes in dogs with systemic diseases (Studies II-IV). The gross pathological evaluation confirmed the clinical diagnosis in all dogs with heart disease. The histological examination revealed that dogs with DCM had features consistent with the fibrous fatty type described in the literature (Tidholm and Jonsson, 2005). Dogs with other cardiac diseases exhibited only minor changes, except for dog 18, which had suffered from pyogranulomatous epicarditis and myocarditis. The gross examination did not identify any significant changes in dogs with systemic diseases. However, three dogs with lymphoma (dogs 22 - 24) showed neoplastic infiltrates in the myocardium and were therefore excluded from studies II and III. The histological examination of all other dogs with systemic diseases did not yield any significant findings.
5.3 Role of inflammation in dogs with cardiac and systemic diseases (I, II)

To investigate the role of inflammation in dogs with cardiac and systemic diseases, blood and myocardial samples of dogs with cardiac and systemic diseases were investigated for the transcription of pro-inflammatory cytokines IL-1, IL-2, IL-6, IL-8, TNF-α, IFN-γ and anti-inflammatory and profibrotic cytokines IL-4, IL-10, TGF-β1, TGF-β2 and TGF-β3. Based on the transcription of IL-4, IL-10, IFN-γ and TGF-β, a potential predominance of a T helper subpopulation would be identified. The results were compared with the mRNA expression in blood and myocardial samples of control dogs.

5.3.1 Circulating markers of inflammation in dogs with cardiac diseases (I)

In most blood samples, constitutive transcription of IL-1, IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ, TGF-β1 and TGF-β3 was identified, whereas IL-4 and TGF-β2 mRNA was not detected. In comparison to control dogs, dogs with cardiac diseases exhibited a significant increase in the transcription of IL-1 (p=0.015) and IL-2 (p=0.043), whereas the TNF-α (p<0.001), TGF-β1 (p=0.098) and TGF-β3 (p=0.006) mRNA expression was reduced (Fig. 2). No difference was detected for IL-8 (p=0.146), IL-6 (p=0.52), IL-10 (p=0.94) and IFN-γ (p=0.47).
Figure 2. Study I; relative TNF-α, TGF-β1 and TGF-β3 mRNA expression in the blood of dogs with cardiac disease (CD; n=8) and of healthy control dogs (Control; n=8). For all three cytokines transcriptions were higher in control dogs. Intervalplot showing 95% confidence interval. TNF: tumour necrosis factor, TGF: transforming growth factor.

5.3.2 Myocardial markers of inflammation in dogs with cardiac and systemic diseases (II)

In control dogs, only TNF-α, TGF-β1, and TGF-β3 were transcribed in the myocardium, with higher levels in the male dogs (p<0.001; Fig. 3). These transcription levels were higher than in male dogs (p<0.005) and lower than in female dogs (p<0.016) with cardiac and systemic diseases, comparing the respective sexes.
All markers of inflammation were found to be transcribed in the myocardium of dogs with cardiac and systemic diseases, with significantly higher transcription levels for IL-1 (p=0.001), IL-6 (p=0.007), IL-8 (p<0.001), IL-10 (p=0.001), TNF-α (p=0.004) and IFN-γ (p=0.008) in dogs with cardiac than dogs with systemic diseases (Fig. 4). The expression of IL-2 (p=0.008) and IFN-γ (p=0.042) was significantly higher in both atria and of IL-1 (p=0.012) and TGF-β1 (p=0.048) in the right atrium, compared with findings for the ventricles of dogs with cardiac diseases (Fig. 5).
Figure 4. Study II, relative IL-1, IL-6 and IL-10 mRNA expression in the myocardium of dogs with cardiac disease (CD; n=7) and systemic diseases (SD; n=7). For all three cytokines, transcription levels were significantly higher in dogs with cardiac diseases. Interval plot showing 95% confidence interval for the mean. IL: interleukin.

![Interval plot showing 95% confidence interval for the mean.](image)

Figure 5. Study II, relative IL-1, IL-2 and IFN-γ mRNA expression in atrial (A) and ventricular (V) samples of dogs with cardiac disease (n=7). For all three cytokines, transcription levels were significantly higher in the atria. Results for IL-2 and IFN-γ were inversed for normalization. Interval plot showing 95% confidence interval for the mean. IL: interleukin, IFN: interferon.

![Interval plot showing 95% confidence interval for the mean.](image)
A comparison of the cytokine expression in atria and ventricles of dogs with cardiac and systemic diseases showed significantly higher IL-1 (p=0.031), IL-8 (p<0.001), TNF-α (p=0.048), TGF-β1 (p=0.03) and TGF-β3 (p=0.022) mRNA levels in the atria and significantly higher IL-8 (p=0.033), IL-10 (p=0.002), TNF-α (p=0.044) and IFN-γ (p=0.004) mRNA levels in the ventricles of dogs with cardiac diseases.

5.4 Role of ECM remodelling in dogs with cardiac and systemic diseases (I, III)

The presence, type and degree of ECM remodelling was investigated by the quantitative assessment of MMP-1, -2, -3, -9 and -13 and TIMP-1, -2, -3 and -4 transcription in dogs with cardiac and systemic diseases in comparison to control dogs.

5.4.1 Circulating markers of ECM remodelling in dogs with cardiac diseases (I)

MMP-1, -3, -9 and TIMP-1, -2, and -3 were constitutively transcribed in the blood of all control dogs, whereas MMP-2 and TIMP-4 mRNA mRNA was not detected. Dogs with cardiac disease had significantly higher MMP-1 (p=0.031), MMP-3 (p=0.061) and TIMP-3 (p=0.012) and lower TIMP-1 (p=0.015) and TIMP-2 (p=0.011) mRNA blood concentrations than control dogs (Fig. 6). No difference was observed for MMP-9 (p=0.65).
5.4.2 Myocardial markers of ECM remodelling in dogs with cardiac and systemic diseases (III)

In the control dogs, only mRNA for TIMP-3 and TIMP-4 was detected, and at significantly higher concentrations in male than in female dogs (p<0.001). In myocardium of dogs with cardiac and systemic diseases all MMP and TIMP were transcribed, and the mean TIMP-3 and TIMP-4 transcription levels were significantly higher in diseased than in control dogs (p<0.001). While there were overall no significant differences between dogs with cardiac diseases and those with systemic diseases, dogs with CHF and DCM showed significantly higher myocardial MMP-2 (CHF p=0.01; DCM p=0.019) and TIMP-1 (CHF p=0.024; DCM p<0.001) than dogs with other cardiac and systemic diseases; dogs with DCM had additionally elevated myocardial TIMP-2 (p=0.028) mRNA levels (Fig. 7).
Figure 7. Study III, relative MMP-2, TIMP-1, and TIMP-2 mRNA expression in all dogs with cardiac diseases (CD; n=9), dogs with dilated cardiomyopathy (DCM; n=4), and dogs with systemic diseases (SD; n=6). For all three markers, transcription levels were significantly higher in dogs with DCM. Interval plot showing 95% confidence interval for the mean. MMP: matrix metalloproteinase, TIMP: tissue inhibitor of matrix metalloproteinase.

The comparison of cardiac regions in diseased dogs identified significantly higher transcription levels for MMP-1 (p=0.002), MMP-2 (p=0.006), TIMP-2 (p=0.014) and TIMP-3 (p=0.032) in the atria than the ventricles. Especially dogs with atrial fibrillation showed a significantly greater degree of MMP-2 (p=0.024), MMP-13 (p=0.046) and lox (p=0.005) transcription than dogs with systemic diseases (Fig. 8).
Figure 8. Study III, relative MMP-2, MMP-13 and lysyl oxidase (Lox) mRNA expression in atrial samples of dogs with atrial fibrillation (AF; n=4) in comparison to dogs with systemic diseases (SD; n=6). For all three markers, transcription levels were significantly higher in atria of dogs with atrial fibrillation. Intervalplot showing 95% confidence interval for the mean. MMP: matrix metalloproteinase, Lox: lysyl oxidase.

5.5 Role of leptin in dogs with cardiac diseases (IV)

To investigate the role of leptin in canine cardiac diseases, its transcription in blood and myocardium of dogs with cardiac disease was compared with blood samples of healthy control dogs and myocardial samples of dogs with systemic diseases, respectively.

5.5.1 Circulating and myocardial leptin transcription in dogs with cardiac and systemic diseases (IV)

Leptin transcription was detected in all blood and myocardial samples investigated. Dogs with CHF exhibited significantly higher transcription levels (3.54, standard deviation ± 0.76) in the blood than healthy control dogs (2.52 ± 0.73; p=0.013). In the myocardium highest mRNA levels were found in dogs with acquired cardiac diseases (1.74 ± 0.78) and lowest levels in dogs with congenital cardiac
diseases (0.66 ± 0.86), and the levels were significantly higher (p=0.035) and lower (p=0.016), respectively, than in dogs with systemic diseases (control group; 1.32 ± 0.92). Among dogs with cardiac diseases, those with heart failure stage D (1.72 ± 0.91) showed higher leptin mRNA levels than those in heart failure stage C3 (1.18 ± 1.24) and B (1.02 ± 0.53; p=0.031). Also, in dogs with cardiac diseases (Table 6) transcription levels were higher in atria than in ventricles. Furthermore, in dogs with cardiac diseases, female dogs had significantly higher myocardial leptin mRNA levels than male dogs (p=0.001).

Table 6. Study IV, relative leptin expression in different cardiac regions of dogs with acquired and congenital cardiac diseases, and dogs with systemic diseases. Results are displayed as mean with standard deviation. The p-value is the difference between cardiac regions for each group.

<table>
<thead>
<tr>
<th>Cardiac region</th>
<th>Acquired cardiac disease (n=7)</th>
<th>Congenital cardiac disease (n=3)</th>
<th>Systemic diseases (controls, n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventricular septum</td>
<td>0.96 ± 0.83</td>
<td>0.02 ± 0.67</td>
<td>0.65 ± 0.49</td>
</tr>
<tr>
<td>Left atrium</td>
<td>2.37 ± 0.59</td>
<td>1.45 ± 0.38</td>
<td>1.18 ± 0.85</td>
</tr>
<tr>
<td>Left ventricle</td>
<td>1.36 ± 0.76</td>
<td>0.03 ± 1.14</td>
<td>1.68 ± 1.14</td>
</tr>
<tr>
<td>Right atrium</td>
<td>2.28 ± 0.54</td>
<td>0.88 ± 0.42</td>
<td>0.71 ± 0.52</td>
</tr>
<tr>
<td>Right ventricle</td>
<td>1.73 ± 0.84</td>
<td>0.74 ± 0.56</td>
<td>1.74 ± 0.77</td>
</tr>
<tr>
<td>p=0.004</td>
<td>p=0.075</td>
<td>p=0.001</td>
<td></td>
</tr>
</tbody>
</table>

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6 DISCUSSION

Cardiac remodelling is a complex process and involves an inflammatory system and an imbalance of ECM components. To investigate cardiac remodelling in dogs, three studies (Studies I, II and III) of the PhD project investigated the transcription of markers for inflammation (pro- and anti-inflammatory cytokines) and ECM remodelling (MMP and TIMP) and the fourth study investigated the mRNA expression of leptin in the blood and myocardium of dogs. For all markers, the relative expression was quantified in different groups of dogs, i.e. dogs with cardiac diseases, dogs with systemic diseases not involving the heart, and healthy control dogs. The latter served to establish the constitutive expression pattern and degree of the investigated markers. As expected, transcription levels were generally lowest in healthy control dogs and highest in dogs with cardiac diseases. Interestingly, also dogs with systemic diseases not involving the heart showed increased myocardial transcription of the investigated markers. The results of the PhD study provide further details relevant for the pathogenesis of cardiac inflammation and remodelling in dogs with cardiac and systemic diseases.

6.1 Markers of inflammation and ECM remodelling in dogs with cardiac diseases

Activation of the inflammatory system in association with an imbalance of ECM remodelling has been reported in human patients with cardiac disease and CHF (Bradham et al., 2002a; Anker and von Haehling, 2004; Chen et al., 2008; Graham et al., 2008), whereas its role in canine cardiac diseases is not known. In the presented work, the expression of inflammatory and ECM markers was studied in dogs with cardiac diseases, both in the blood (Study I) and in the myocardium itself (Studies II and III). Dogs with cardiac diseases exhibited significantly higher circulating IL-1, IL-2, MMP-1, -3 and TIMP-3 mRNA levels than healthy control dogs, but significantly reduced TNF-α, TGF-β3, TIMP-1 and TIMP-2 levels, while no differences were seen for IL-6, IL-8, IL-10 and IFN-γ (Study I). In contrast, the expression of all markers of inflammation and ECM remodelling was significantly increased in the myocardium of dogs with cardiac and systemic diseases in comparison to controls, and a further increase of the inflammatory markers IL-1, IL-6,
IL-8, IL-10, TNF-α, IFN-γ and the ECM remodelling markers MMP-2, TIMP-1 and TIMP-2 was observed in dogs with cardiac diseases versus those with systemic diseases (Studies II and III). These results confirm those of previous studies that suggest that cardiac diseases are associated with an inflammatory state of the myocardium and cardiac remodelling (Anker and von Haehling, 2004; Chen et al., 2008). Cardiac remodelling was reflected by the gross post mortem results and clinically by ventricular dilatation or hypertrophy and myocardial dysfunction. The lack of inflammatory marker upregulation in the blood alongside the increase of inflammatory markers in myocardium suggests primary myocardial activation of the inflammatory system and not a myocardial response to circulating inflammatory mediators. Furthermore, the histological examination did not reveal substantial myocardial inflammatory cell infiltration (Studies II and III), which renders cardiomyocytes and cells of the ECM the likely source of the markers (Tyagi et al., 1993; Kuhl et al., 1996; Li et al., 1999; Frangogiannis, 2008; Souders et al., 2009). The stimulation of cytokine, MMP and TIMP expression in cardiac diseases, which further stimulates their own and each other’s expression through auto- and paracrine loops, might be caused by haemodynamic abnormalities that result in increased cardiac stretch and activation of the neurohormonal system (Weber et al., 1994; Opie, 2002; Heineke and Molkentin, 2006; Dobaczewski et al., 2010; Frangogiannis, 2012). IL-1, IL-2, MMP-1, -3, and TIMP-3 mRNA levels were elevated in both blood and myocardium of dogs with cardiac diseases, and also in the myocardium of dogs with systemic disease. This might indicate a generalised increased transcription of these markers in diseases with cardiac remodelling.

Dogs with DCM showed a further increase in myocardial MMP-2, TIMP-1 and TIMP-2 transcription, when compared with dogs suffering from other cardiac diseases. This was also reflected by the more severe cardiac histological changes in the heart. However, as these dogs were also in the most advanced disease stages (heart failure class D, in contrast to class B and C3 of dogs with other cardiac diseases), association of marker expression and of histological changes with the severity of the disease instead of the actual disease type appears likely.

Previous investigations found increased myocardial IL-6 transcription (Oyama and Chittur, 2006) as well as increased valvular TGF-β1, TGF-β3, MMP-1, MMP-3, TIMP-1, -2 and -3, reduced MMP-2 and variable MMP-9 production in dogs with
DVD (Oyama and Chittur, 2006; Aupperle et al., 2008; Aupperle et al., 2009b; Ljungvall et al., 2011; Obayashi et al., 2011). The levels of circulating IL-2, IL-7 and IL-8 protein were reduced in dogs with DVD (Zois et al., 2012). In contrast to these results, studies I and III revealed elevated circulating IL-2 and myocardial MMP-2 mRNA levels in dogs with cardiac diseases. These differences in results are consistent with different material (valves versus myocardium) and diseases investigated, and the measurement of protein versus mRNA. The expression of TIMP-1 and TIMP-2 was increased in valvular tissue of dogs with DVD (Oyama and Chittur, 2006; Aupperle et al., 2009b) and in myocardial samples of dogs with DCM (Study III), which suggests that fibrosis might be an important component in both diseases.

Interestingly, not only pro-inflammatory markers, but also the anti-inflammatory cytokine IL-10 and the immune modulator IFN-γ were elevated in dogs with cardiac diseases (Study II). This suggests limitation of the inflammatory reaction by IL-10, since it inhibits the production of pro-inflammatory cytokines (Fiorentino et al., 1991; Mosmann, 1994; Krishnamurthy et al., 2009). IL-10 is also known as a Treg cell product, and Treg cells are anti-inflammatory which reduces fibrogenesis (Kvakan et al., 2009; Tang et al., 2012). IFN-γ is associated with a Th1 cell polarisation, and Th1 cells are also suspected to limit fibrosis (Mosmann et al., 1986; Zhu et al., 2010; Wei, 2011). Accordingly, IL-10 and IFN-γ might have a cardioprotective function. However, in the end-stage of cardiac diseases, the marked inflammatory reaction might override these regulatory components, which would indicate that the increase in IL-6, TNF-α and IL-10 observed in dogs in study II predicts a poor outcome, as reported in people with decompensated heart failure (Miettinen et al., 2008). On the other hand, the anti-fibrotic effects of IL-10 and IFN-γ might also reflect progressive cardiac remodelling.

In studies II and III, the expression levels in the different cardiac regions were compared. In dogs with cardiac and systemic diseases, atria generally showed higher transcription levels than ventricles. These differences matched with the observed extent of pathological changes identified by the histological examination in dogs with cardiac diseases (leukocyte infiltration, lipomatosis cordis, cardiac fibrosis, muscular hypertrophy of small arteries, cardiomyocyte necrosis) and confirm previous reports that suggested differences in the remodelling processes in atria and
ventricles (Hanna et al., 2004; Brundel et al., 2005). Furthermore, dogs with cardiac disease showed significantly higher atrial IL-1, IL-8, TNF-α, TGF-β1 and TGF-β3, and dogs with atrial fibrillation higher MMP-2, MMP-13 and lox transcription, than dogs with systemic diseases (Studies II and III). This increase in inflammatory and pro-fibrotic marker expression in the atria of dogs with cardiac diseases might contribute to atrial dilatation, atrial arrhythmia, cardiac dysfunction and therefore progression of disease (Khan et al., 2004; Khan and Sheppard, 2006; Mukherjee et al., 2006). In dogs with atrial fibrillation (which had been diagnosed in three dogs with DCM and one with DVD in the present study), MMP-2 and MMP-13 might cause ECM degradation which could allow the observed left atrial dilatation, and lox might cause increased collagen crosslinking (Mukherjee et al., 2006; Lopez et al., 2010), both factors known to contribute to the development of atrial fibrillation (Mukherjee et al., 2006; Adam et al., 2011).

6.2 Markers of inflammation and ECM remodelling in dogs with systemic diseases

It has been shown in people and dogs that systemic diseases can lead to myocardial dysfunction and damage (Parker et al., 1984; Nelson and Thompson, 2006; Serra et al., 2010; Langhorn et al., 2013). To investigate, whether systemic diseases induce cardiac inflammation and remodelling, dogs with systemic diseases not involving the heart were included in studies II and III. The histological examination did not reveal any remarkable pathological changes in the heart of these dogs. Nonetheless, the transcription of all markers for inflammation and ECM remodelling was significantly elevated in comparison to controls. In particular the increase of inflammatory markers might be due to generalised activation of the inflammatory system in the course of the dogs’ neoplastic and systemic diseases (Seruga et al., 2008; Flynn et al., 2010; Langhorn et al., 2013). Unfortunately, this could not be further investigated, since circulatory transcription levels were not measured in these dogs. Inflammatory cytokines affect cardiac function, facilitate cardiomyocyte hypertrophy and apoptosis and are involved in remodelling processes by stimulating MMP and TIMP production (Bradham et al., 2002b; Siwik and Colucci, 2004; Chen et al., 2008; von Haehling et al., 2009). The latter are major determinants of pathological ECM remodelling and contribute further to an
inflammatory response (Spinale, 2007; Sivakumar et al., 2008). Therefore, the combination of an increased myocardial cytokine expression with dysregulation of MMP and TIMP expression could affect ECM composition and cardiac function and thereby morbidity and mortality in these patients (Studies II and III). However, the absence of histological changes in the heart that would indicate ongoing inflammatory and/or remodelling processes suggest that the marker upregulation might be a component of the end-stage disease only, leading to acute functional changes that had been absent earlier (Nelson and Thompson, 2006). Since detailed morphological or functional myocardial investigations, such as echocardiography, had not been performed on these patients, it is not possible to further comment on the latter as part of the present study. However, the apparent inflammatory state of the myocardium of dogs with diseases not affecting the heart is of interest and suggests that functional changes and generalised cardiac remodelling processes occur in end-stage systemic diseases. It needs further investigations to determine whether these are indeed acute and reversible or whether they might contribute to morbidity and mortality in these cases.

6.3 Constitutive transcription of markers of inflammation and ECM remodelling in healthy control dogs

To identify the constitutive expression of inflammatory and ECM remodelling markers, studies I, II and III included control dogs. Blood samples of healthy control dogs in study I showed constitutive mRNA expression of several pro-inflammatory (IL-1, IL-2, IL-6, IL-8, TNF-α, IFN-γ) and anti-inflammatory cytokines (IL-10, TGF-β1, TGF-β3), and of both anti-fibrotic (MMP-1, -3, -9) and pro-fibrotic markers (TIMP-1, -2, and -3), albeit at generally lower levels than in diseased dogs in the same study. This finding indicates that circulating leukocytes are generally slightly activated; on the other hand, it does not mean that the proteins are indeed translated or even released into the blood. In contrast, in the myocardium of the healthy control dogs, only TNF-α, TGF-β1, TGF-β3, TIMP-3 and TIMP-4 were transcribed (Studies II and III), suggesting that cells in the myocardium only express a set of markers that maintain the structural balance in the heart and need an (external) stimulus to change their transcription pattern. Baseline expression of TGF-β1, TGF-β2, TGF-β3, MMP-2, MMP-9 and TIMP-3 mRNA has previously been reported in the heart of
dogs (Aupperle et al., 2008; Aupperle et al., 2009b). However, these studies focussed on the mitral valve leaflets and did not investigate the expression of inflammatory cytokines. Heart valves consist mainly of interstitial cells, glycosaminoglycans, proteoglycans, collagen and elastic fibres and are exposed to different strains than the myocardium (Fox, 2012; Lacerda et al., 2012), which is the likely reason for the observed differences in ECM marker transcription. Nonetheless, the transcription of profibrotic markers TGF-β1, TGF-β3 and TIMP-3 in valves and myocardium (Study 3) suggest a balance towards anti-inflammation and regeneration and/or fibrosis in the healthy canine heart (Aupperle et al., 2008; Aupperle et al., 2009b).

Interestingly, there is an apparent sex difference in the level of constitutive myocardial marker transcription, which was not observed in the blood (Study I, data not shown) of control dogs. The myocardial transcription levels of TNF-α, TGF-β1, TGF-β3, TIMP-3 and TIMP-4 were higher in male than in female control dogs (Studies II and III). However, in male dogs with cardiac and systemic diseases the myocardial transcription of TNF-α, TGF-β1, TGF-β3 was lower than in male control dogs, whereas in female dogs with disease an increase of these markers was present (Study II). These findings in male control dogs and female dogs with cardiac disease suggests either cardioprotection due to their anti-inflammatory effects and the stimulation of regulatory T cells (Shah and Qiao, 2008; Kvakan et al., 2009; Li et al., 2010), or increased cardiac fibrosis, associated with either higher regenerative capacity or progression of disease (Lim and Zhu, 2006; Aupperle et al., 2008). Lower levels in female control dogs and male dogs with cardiac disease on the other hand might indicate a pro-inflammatory response or lesser fibrosis. Despite reported sex differences in clinical signs and progression of canine DCM and DVD (Serfass et al., 2006; Wess et al., 2010), the potential influence of sex on the development of cardiac diseases is usually not taken into account, because most dogs are neutered, as they were in the present studies. However, differences between sexes might be underestimated in development, presentation and progression of canine cardiac diseases.
6.4 Leptin in cardiac and systemic diseases

Similar to several of the inflammatory and ECM remodelling markers, leptin transcription was significantly elevated in the blood and myocardium of dogs with cardiac diseases and was associated with disease severity (Study IV). It is now known that leptin is not only an important hormone in obesity, regulating energy homeostasis, but is also involved in inflammation (Igel et al., 1997; Faggioni et al., 1998; Loffreda et al., 1998; Fernandez-Riejos et al., 2010). The results of study IV showed that leptin is produced by the myocardium with higher transcription in dogs with cardiac diseases, and in particular in atria of these diseased dogs. These results are in accordance with those reported from human patients with cardiac disease, where leptin blood concentrations were found to correlate with the degree of heart failure (Schulze and Kratzsch, 2005). The elevated leptin mRNA levels in both blood and myocardium of dogs with cardiac diseases suggest a generalised increased transcription of leptin.

The increased leptin transcription in cardiac diseases observed in study IV and the simultaneous elevation of inflammatory and ECM remodelling markers (Studies II and III), suggest an involvement of leptin in cardiac inflammation and remodelling, thereby further supporting findings in human patients with cardiac diseases (Filippatos et al., 2000; Schulze and Kratzsch, 2005; Sharma and McNeill, 2005; Karmazyn et al., 2007; Schram et al., 2010). The simultaneous increase of these markers might also be the consequence of a higher catabolic state and the development of cardiac cachexia, which is observed in human and canine cardiac patients (Toth et al., 1997; Schulze et al., 2003). However, this possible correlation was not investigated further in study IV. The higher myocardial leptin transcription in female dogs with cardiac diseases might be due to the generally higher fat content of female dogs (German et al., 2010). Increased leptin transcription might contribute to cardiomyocyte hypertrophy and increased collagen deposition (Rajapurohitam et al., 2003; Karmazyn et al., 2007; Schram et al., 2010), which is supported by the increased transcription of profibrotic and anti-inflammatory markers TGF-β1 and TGF-β3 that were detected in female, but not in male dogs with cardiac diseases (Study II). Leptin might therefore play an important role in development and progression of canine cardiac diseases, as recently suggested (Mehlman et al.,
2013), and might contribute to sex differences in progression and clinical presentation.

6.5 Limitations of the studies

The studies included in the thesis are limited by the small number of animals that were investigated and the inhomogeneity of the groups. The main reason for the latter is the very limited access to myocardial samples, therefore, no available dog with cardiac disease was excluded from the study and only dogs with end-stage diseases could be included. Larger sample sizes and the collection of samples from specific disease groups would have strengthened the significance of the results and would have enabled the comparison of different diseases. Furthermore, since the examination was restricted to the assessment of mRNA expression, comments on translation and release of the respective proteins cannot be made. A direct conclusion can also not be drawn about the presence of active enzymes, in particular also since TGF-β and MMP are secreted in a latent form and require cleavage before they become activated.

6.6 Implications for further studies

1. The analysis of IL-1, IL-2, MMP-1, -3, and TIMP-3 mRNA and protein (these were increased in the blood and myocardium of diseased dogs) in blood samples of dogs in different stages of cardiac diseases and its correlation with echocardiographic investigations that assess myocardial morphology and function would detect changes in progression of disease and identify potential biomarkers for cardiac remodelling.

2. Further characterisation of myocardial remodelling by investigating the protein expression of inflammatory and ECM remodelling markers, the presence of fibrosis, myocardial degeneration and apoptosis, activation of endothelial cells, infiltration of inflammatory cells and the presence of cardiac stem cells would reveal further details of the pathogenesis of cardiac diseases.

3. Increasing the sample size of one cardiac disease, i.e. DCM, and the correlation of clinical findings and echocardiographic results with parameters of cardiac
inflammation and remodelling would strengthen the significance of the results and possibly identify clinical parameters of cardiac inflammation and remodelling.

4. The correlation of echocardiographic results with the expression of inflammatory and ECM remodelling markers in the blood and, if available, the myocardium of dogs with systemic diseases and the association of these results with survival of these dogs would allow further assessment of the importance and the time course of cardiac impairment and remodelling in dogs with systemic diseases.

5. Further investigation of the role of leptin in cardiac cachexia and in obese dogs and its association with cardiac function are of interest and would reveal the real significance of leptin in canine cardiac diseases. Furthermore, the investigation of other adipocytokines, such as adiponectin, resistin, visfatin, which were reported to be involved in human cardiac diseases, would give information on their role in canine cardiac diseases.
7 CONCLUSIONS

I Studies investigating the role of inflammation and ECM remodelling in dogs with cardiac and systemic diseases in comparison to healthy controls (Studies I, II and III) led to the following conclusions.

1. The significantly higher expression of all markers of inflammation and ECM remodelling in the myocardium of dogs with cardiac diseases in comparison to hearts of healthy control dogs, whilst only single markers were elevated in the blood of dogs with cardiac diseases, suggest primary myocardial inflammation and ECM remodelling in dogs with cardiac diseases and not a myocardial response to circulating inflammatory mediators.

2. The higher transcription levels of most markers of inflammation and ECM remodelling in atria than ventricles of dogs cardiac and systemic diseases, which matched with the observed extent of pathological changes in dogs with cardiac diseases, suggest different remodelling processes depending on localisation. These differences in inflammation and ECM remodelling are likely to contribute to progression of disease, cardiac dysfunction and development of arrhythmias.

3. The increased expression of inflammatory and ECM remodelling markers in the myocardium of dogs with systemic diseases not involving the heart, in the absence of histological changes in the heart, might indicate acute functional changes in end-stage diseases. The apparent inflammatory state of the myocardium of dogs with systemic diseases might be acute and reversible or might contribute to morbidity and mortality of these dogs.

4. The constitutive expression of profibrotic markers in the myocardium of healthy control dogs suggests a regenerative and profibrotic potential of the healthy canine myocardium.

II The increased transcription of leptin in the blood and myocardium of dogs with cardiac diseases (Study IV) and the simultaneous elevation of inflammatory and ECM remodelling markers (Studies II and III) suggest an involvement of leptin in cardiac inflammation and remodelling in dogs with cardiac diseases.
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