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CARDIOVASCULAR PROTEOMICS AND MITRAL VALVE DISEASE IN DOGS: SEARCHING FOR A SEROLOGICAL BIOMARKER

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CHAPTER 1

Introduction

1.1   Introduction

The World Health Organization reports that in 2008 cardiovascular diseases (CVDs) were the leading cause of death, with 17 million deaths and an estimated 23.6 million people will die from CVDs each year by 2030 (www.who.int, 2008) (Figure 1). Cardiovascular-related disorders account for the highest mortality rates in the world, and affect the quality and the quantity of life of patients, creating an economic burden of prolonged therapeutic intervention. (White MY et al., 2007) For these reasons there is a great interest in understanding and study the CVDs from different points of view, trying to uncover the cellular and the molecular alterations that influence the progressions of these pathologies, to improve the diagnostic tools and to optimized the therapeutic interventions. Myxomatous valve disease (MVD) is the most common heart valvular disease in humans in Europe and United States, and it's the primary naturally occurring heart disease in dogs(Lacerda et al., 2009). MVD is pathologically identical in humans and dogs, suggesting a common pathogenesis in these species, and creating an increasing interest in the canine MVD as a model for the human medicine. Nowadays, MVD is one of the most studied CVDs, because of its high prevalence in the clinical practice. In veterinary medicine the prevalence, especially in old, small breed dogs, approaches 100%, thus the incidence of MVD over canine lifetimes in most breeds is close to 100%. Thanks to the research efforts, great diagnostic and therapeutic progresses have been made in the last decades, and continuous improvement are in the making. The diagnostic tools required for MVD assessment are actually large scale used, both in human and in veterinary medicine, allowing an effective clinical management. The therapeutic intervention are day by day more precise, and in human medicine the surgical intervention on the mitral valve is a common practice. In recent years, new technologies have been used by the cardiovascular medicine, and the advancement of proteomic techniques has improved the methods available for investigating CVDs.(Cui Z. et al., 2011). Proteomics is the large-scale study of proteins and its application to uncover the protein function and structure in normal or disease states in the cardiovascular field is called cardiovascular proteomics. (Cui Z. et al., 2011) Through the cardiovascular proteomics the clinician could get important data that can be matched with the classical clinical and diagnostic features, in order to ensure a faster or more precise diagnosis, or a better management of the cardiovascular patient. Even if the use of cardiovascular biomarkers is widespread in both human and veterinary medicine, the application of cardiovascular proteomics to the MVD study has recently begun.
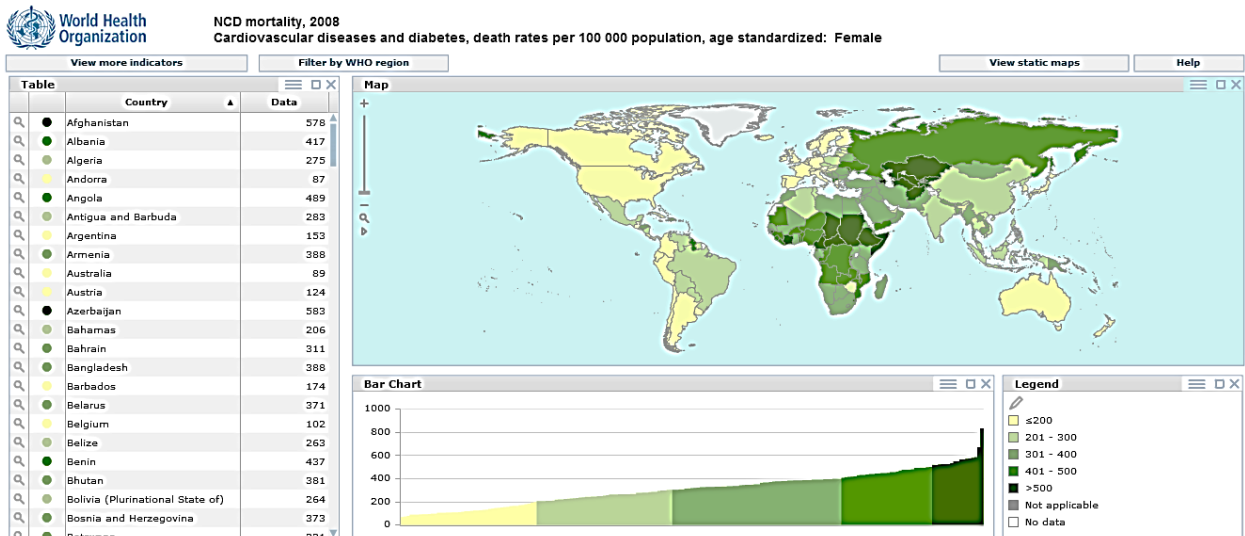
Figure 1 Interactive graph: CVDs and diabetes deaths in 2008. (WHO website)

In the present study we selected a cohort of private-owned dogs recruited from the Cardiologic Service of the Department of Veterinary Science and Public Health. The dogs were selected with precise inclusion and exclusion criteria. Two breeds were considered: Cirneco dell'Etna and Cavalier King Charles Spaniels. The Cirneco dell'Etna breed is a poorly diffuse Italian hunting breed whose CVDs prevalence has never been studied before, while the Cavalier King Charles Spaniel breed is the most studied breed for all the MVD researches, because of the high prevalence of MVD, the early onset and the strong scientifically proved hereditary component. The objective of the present study was to search one or more than one serological biomarkers in dogs affected by MVD, comparing blood samples from the healthy dogs of the groups with blood samples from the dogs affected by different stages of MVD. The proteomics results were then matched with the clinical and echocardiographic data obtained in the clinical trial of all the patients included in the study, to find a connection available in the clinical practice.

CHAPTER 2

Mitral Valve Disease:

Past, Present and Future

2.1 Mitral Valve Disease (MVD)

Mitral valve disease is the most common acquired cardiovascular disease in dogs and the most frequent valvular heart disease of humans in the richest countries (Europe and United States). In the dog MVD represents the 75% of all CVDs, affecting primary the mitral valve, while in the 30% of the affected dogs both mitral and tricuspid valves are involved. (Atkins et al., 2009; Borgarelli & Haggstrogm, 2010). Various other names for the disease include endokarditis valvularis chronica fibrosa (nodosa), chronic valvular endocarditis, chronic valvular disease, billowing sail distortion of the mitral valve, endocardiosis, chronic mitral valve fibrosis, senile nodular sclerosis, mucoid degeneration, chronic myxomatous valve disease, and degenerative mitral valve disease. Chronic MVD in dogs has been known to cause congestive heart failure (CHF) in dogs for more than 100 years. (Borgarelli & Buchanan, 2012)

2.2   Etiology and prevalence

The prevalence of MVD varies with age and whether the study is based on necropsy or clinical findings. Most frequency data are based on auscultation of a mitral insufficiency murmur. The disease is age related and the prevalence, especially in old, small breed dogs, approaches 100% thus the incidence of MVD over canine lifetimes in most breeds is close to 100%. (Fig. 2) (Borgarelli & Buchanan, 2012) The vast majority of the breeds at elevated risk of MVD are small or toy breeds, and males are 1.5 times more represented than females. In human medicine MVD is more prevalent in females than males, but equally to canine MVD, the prevalence of MVD in the human population is associated with age, and the patients with mitral valve prolapse tend to have low body mass index, but the reason for this phenomenon is uncertain. (Aupperle & Disatian, 2012) In human medicine height has been linked to a variety of heart diseases. The first study in 1951. published by the Journal of American Medical Association, examined the physical characteristics of 100 humans that underwent a myocardial infarction, and found that they were 5 cm shorter than the control group. In 2010 a meta-analysis was conducted on a combined dataset of more than 3 million individuals. They showed that the risk of cardiovascular death in this combined group was 50% higher in short men and women than in tall men and women. (Parker & Kilroy-Glynn, 2012) It is interesting to note that the correlation between dog size and canine MVD is

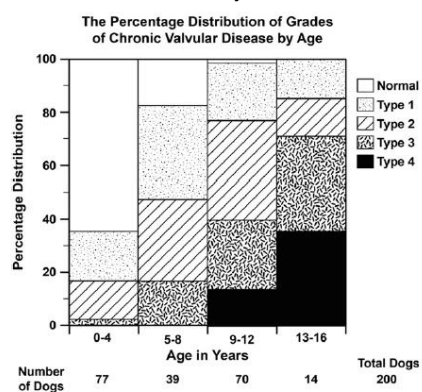**Figure 2 Bar chart showing the percentage distribution of 4 grades of chronic valvular disease by age in 200 dogs. (Whitney, 1967)**

similar to what human medicine discovered about height and cardiovascular diseases. The cause of canine MVD is currently unknown, although a genetic tendency to develop the disease has been proven. Some breeds such as the Cavalier King Charles Spaniel (CKCS) and the dachshund are particularly predisposed to the development of MVD, with a prevalence of the disease in CKCS older than 10 years of more than 90%. In this breed in particular, a polygenic mode of inheritance is suggested by a strong inherited component, and recently two loci have been associated with MVD in CKCS. (Parker & Kilroy-Glynn, 2012; Madsen et al., 2011) In addition poorly defined environmental factors play a role in the rate of onset or severity of the disease. MVD has been considered as a non-inflammatory myxomatous degeneration of the atrioventricular valves, but now a growing interest in the role that serotonin and other inflammatory mediators may play in the pathology is arising. (Rusch, 2009) The report of Rush et al. of 2006 (Rush et al., 2006) revealed a higher concentration of C-reactive protein in dogs affected by MVD, suggesting a possible role of systemic inflammation in the pathogenesis. At the same way, the etiology of human MVD is unknown. The risk factor of mitral valve prolapse may be inherited as an autosomal dominant or a polygenic abnormality and it often displays familial transmission. (Aupperle & Disatian, 2012)

2.3 Histopathological findings

The macroscopic appearance of MVD in dogs varies from small focal thickening in the contact zone, to large areas of smooth surfaced nodular thickening of the distal portion of all leaflets and commissural cusp tissue, depending on the stage of degeneration. In severe cases the thickening extends into proximal segments of the attached primary chordae tendineae. Lengthening and rupture of chordae tendineae are also common in dogs with advanced MVD. (Fig. 3) (Borgarelli & Buchanan, 2012) In the 1974 Whitney presented a classification system of the atrioventricular lesions that is still actual.
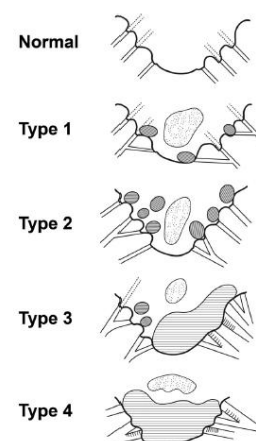

Figure 3 The 4 types of atrioventricular lesions presented by Whitney. (Whitney, 1974)

The method divided the progression of MVD into four types of lesions:

Type I lesion
A few small oedematous nodules are seen in the area of apposition, opposite to the origins of the chordae tendineae. The chordae tendineae themselves are unchanged. There is no valvular incompetence

Type II lesion
Here the oedematous nodules are larger and more numerous and some are greyish-white in colour. These lesions are confined to the area of apposition, the chordae tendineae are unaffected and there is no evidence of valvular incompetence

Type III lesion
Here there are larger greysh-white nodules and plaque-like elevations located in the area of apposition and associated with thickening and irregularity of the proximal portion of the chordae tendineae, where they join the inflow surface of the valve cusp. There is evidence of valvular incompetence in some of these cases

Type IV lesion
Here there are large greysh-white nodules and plaque-like elevations situated in the area of apposition and an associated thickening and irregularity of related chordae tendineae. These chordae may be stretched and ruptured in some cases. There is evidence of valvuar incompetence in the majority of these cases.

The microscopic features of normal canine and human mitral valves are three well-defined layers: the atrialis, the spongiosa and the fibrosa. The atrialis layer is mainly composed of elastic fibers, the spongiosa layer is rich in glycosaminoglycans and the fibrosa layer predominantly consists of densely-packed collagen bundles.
The microscopic aspect of myxomatous valves in humans and in dogs includes increased cellularity and disorganization as well as transdifferentiation of valve endothelial and valve interstitial cells. In canine degenerated valves, the changes occur primarily in the atrialis layer by an accumulation of transformed valve interstitial cells. In the advanced stages, the major structural changes, as glycosaminoglycan infiltration, appear in the spongiosa layer, both in human and canine MVD . The degeneration is most pronounced at the free margins of the leaflets. When the disease progresses, the amount of glycosaminoglycans increases and invades into the other layers. The elastic fibers become fragmented and split, the collagen bundles are disorientated by glycosaminoglycan infiltration, and the collagen fibrils also appear disrupted. Similar changes occur in the chordae tendineae. The anterior leaflet is more frequently involved in canine MVD, while the posterior leaflet is more often and more severely affected than the anterior leaflet in humans. (Aupperle & Disatian, 2012)

2.4   Natural history and physiopathology

"The natural history of MVD in dogs is a story of aging, stress, functional effects and the development of complications. The mitral valve is probably the most abused and stressed tissue in the body because it is intermittently bent, slammed, tensed, shear stressed and stretched, 200 times a minute, 24 h a day, 365 days a year for 10-15 years. The wonder is not that it degenerates but rather how it survives so well." (Borgarelli &Buchanan, 2012) This sentence has been published on an important article that review the natural history of mitral valve disease, and encloses the physiopathology of the disease. Chronic mitral valve disease has a long pre-clinical period characterized initially by morphologic changes observed only on necropsy in the first third of life. The progression to mitral valve insufficiency is observed mainly in the middle third, and it may lead to secondary chamber dilation and hypertrophy in the latter third of life.

During the first stages of MVD the affected valves preserve their competence and no hemodynamic abnormalities are observed. When the morphologic alterations of the leaflets result in mitral regurgitation, the pressure of the left atrium increases, and the cardiac output decreases. Classic compensatory mechanisms (sympathetic nervous system activation, enhanced renin-angiotensin system activity) are activated in an attempt to restore blood pressure and tissue perfusion.

The sympathetic system determines:
- Venous vasoconstriction to increase venous return to the heart
- Arterial vasoconstriction to preserve constant arterial blood pressure
- Increase of heart rate and myocardial contractility to preserve cardiac output

The Renin-Angiotensin system determines:
- Vasoconstriction mediated by II angiotensin
- Retention of water and sodium through the release of aldosterone

These compensatory mechanisms preserve the stroke volume, increasing the total blood volume and arterial blood pressure. By persisting for several months or years, these overextended mechanisms determine excessive vasoconstriction, excessive water retention and increase in blood volume, with myocardial stress. Despite the hemodynamic benefits of these compensations, their chronic activation is maladaptive. The volume overload of the left ventricle and atrium leads to a progressive increase in left atrial pressure. Progression of heart disease and pump dysfunction triggers a spiral of increasing dependence on neurohormonal activity to maintain normal blood pressure and flow. As long as the dilated left atrium remains sufficiently compliant to accept the regurgitant blood volume, cardiac heart failure does not develop, until the time that pulmonary venous pressure increases sufficiently to cause pulmonary edema, or

perfusion of skeletal muscles becomes severely limited. Usually, overt heart failure occurs years after the start of mitral insufficiency, when accompanied by progressive cardiac remodeling and myocardial disease. Recent studies revealed that most cardiac enlargement occurs in the year preceding the onset of congestive heart failure. (Rusch, 2009; Borgarelli & Buchanan, 2012)

2.5   Clinical presentation

The clinical presentation of MVD is characterized by a long pre-clinical phase, when the only clinical finding is a cardiac murmur in the absence of any sign of cardiac decompensation.
The period between first identification of a murmur and onset of clinical signs is generally years. Usually the first symptom is exercise intolerance, while cough and dyspnea appear in the second stage of the pathology (heart failure).
Death due to MVD is most often mediated by congestive heart failure, although sudden death can occasionally occur. Usually, overt heart failure occurs years after the start of mitral insufficiency, when accompanied by progressive cardiac remodeling and myocardial disease. Precipitous events are major chorda tendinea ruptures or the left atrium ruptures. Acute rupture of a chordae tendineae may lead to a catastrophic heart failure and a sudden-onset of pulmonary edema. Acute CHF develops as the left atrium is unable to compensate for an acute increase in the regurgitant volume. Another sudden and frequently fatal event is the left atrial tear and/or rupture. The tear can result from atrial stretch from mitral regurgitation or from endocardial damage caused by the jet of blood chronically impinging on the endocardial aspect of the left atrium. The weakened endocardium can split or several times the entire left atrial wall ruptures, creating fatal hemopericardium. If the left atrial rupture perforates the atrial septum, the acquired atrial septal defect usually does not cause acute left heart failure but leads to right heart failure eventually. Rusch, 2009; Borgarelli & Buchanan, 2012)

2.6   ACVIM consensus

In 2009 the American College of Veterinary Internal Medicine (ACVIM) Specialty of Cardiology consensus panel, convened to formulate guidelines for the diagnosis and treatment of chronic valvular heart disease. Until that moment, the main guidelines considered were the modified New York Heart Association (NYHA) and International Small Animal Cardiac Health Council (ISACHC) functional classification systems. These guidelines were both designed to provide a framework for discussing and comparing the clinical signs of patients in heart failure. These functional classification systems vary in their details, but both serve as semiquantitative schemes for judging the severity of a patient's clinical

signs and to decide whether to treat or not a patient. Such categorization aids in teaching therapeutic protocols and constitutes a basis for stratification of subjects in clinical trials. The problem of these functional classification identified by the ACVIM Cardiology panel was that they were based on subjectively assessments of symptoms that can vary quickly over a short period of time. The goal of the new guidelines was to link the severity of signs to appropriate treatments at each stage of illness. In formulating these guidelines, the consensus panel adapted the 2001 American College of Cardiology/American Heart Association classification system for the treatment of heart disease and failure in human patients to the management of canine chronic valvular heart disease. In this approach, patients are expected to advance from one stage to the next unless progression of the disease is altered by treatment.

The ACVIM classification system describes 4 stages of heart disease and failure (Table 1) and is meant to complement the previous functional classification. (Atkins et al., 2009)

Stage ACVIM		Features
A		Patients at high risk for developing heart disease but that currently have no identifiable structural disorder of the heart (eg, every Cavalier King Charles Spaniel without a heart murmur)
B	B1	Asymptomatic patients that have no radiographic or echocardiographic evidence of cardiac remodeling in response to chronic valvular heart disease.
	B2	Asymptomatic patients that have hemodynamically significant valve regurgitation, as evidenced by radiographic or echocardiographic findings of left-sided heart enlargement
C		Patients with past or current clinical signs of heart failure associated with structural heart disease. Because of important treatment differences between dogs with acute heart failure requiring hospital care and those with heart failure that can be treated on an outpatient basis, these issues have been addressed separately by the panel. Some animals presenting with heart failure for the 1st time may have severe clinical signs requiring aggressive therapy
D		Patients with end-stage disease with clinical signs of heart failure caused by chronic valvular heart disease that are refractory to "standard therapy". Such patients require advanced or specialized treatment strategies in order to remain clinically comfortable with their disease. As with Stage C, the panel has distinguished between animals in Stage D that require acute, hospital-based therapy and those that can be managed as outpatients

Table 1: The ACVIM classification system

The aim of the panelists was to encourage the veterinarian to consider the risk factors and the prerequisites for developing MVD, in order to aid in developing screening programs and identify the symptomatic and the asymptomatic patients.

For each stage they indicate the therapeutic, dietary and diagnostic/screening strategies.

2.7 Evaluation of the asymptomatic patient

Murmur is the typical clinical finding of MVD in an asymptomatic dog. Point of maximal murmur intensity is over the left apex, with radiation dorsally and to the right in most cases. The murmur intensity is classified with a grading scale (I-VI/VI) and is not correlated to the severity of the pathology. When a murmur is auscultated in a dog without clinical signs, baseline testing can be offered to the owner to obtain a starting point for comparison at subsequent examinations. The client should be clearly informed of the presence of the murmur and of the fact that the disease may ultimately progress to cardiac heart failure.
The minimum suggested database for these dogs includes:
-   physical examination
-   thoracic radiographs (to verify the presence/absence of cardiomegaly)
-   echocardiography (to confirm the diagnosis and evaluate heart dimension and function)
-   blood pressure measurement (excluding systemic hypertension)

Chest radiography is recommended in all patients with MVD to assess the hemodynamic significance of the murmur and the comorbidity of respiratory diseases. Cough in dogs with MVD may be related to congestive heart failure, to respiratory disease and/or to the compression of the left mainstem bronchus by an enlarged left atrium.

The electrocardiogram of a dog affected by MVD could be normal or could presents alterations of heart rate, rhythm and waves configuration. Electrocardiographic findings include evidence of left ventricular hypertrophy, widened P waves of left atrial enlargement, and infrequently P pulmonale.

The echocardiography is the gold standard diagnostic tool for MVD. The diagnosis is based on assessment of morphologic alteration of mitral leaflets and valvular insufficiency, if present.
The echocardiographic examination is useful to evaluate:
-       the morphologic aspect of the valvular apparatus and the movements of the leaflets
-       the dimension of the cardiac chambers
-       the mitral regurgitation (entity of the regurgitant blood, direction and velocity of the regurgitant jet)
-       the tricuspid morphologic alteration and tricuspid regurgitation, if present

- the myocardial contractility
- the diastolic function

2.8 Evaluation of the symptomatic patient

Once heart failure develops, a range of clinical presentations are possible, related to the degree and duration of valvular dysfunction. In an acute setting, clinical signs are usually behavioral or pulmonary in origin, and include: cough, tachypnea, retching/gagging, nocturnal dyspnea, orthopnea and sometimes social isolation. Syncope may be the initial sign of heart disease and can occur as a result of significant arrhythmias, secondary to low cardiac out-put, in association with a vasovagal response, or following a coughing spell. (Kirk XIV). Some dogs exhibit decreased exercise tolerance, and weight loss for weeks to months before the onset of heart failure. The most frequent symptoms (cough and dyspnea) are common to respiratory diseases such as acute/chronic inflammatory/infective upper or lower respiratory diseases, neoplasia and cardiopulmonary filariosis. It's therefore important to discriminate between respiratory and cardiologic disease. The physical examination in the symptomatic patient could reveal loud bronchial sounds that can progress to pulmonary crackles with the onset of alveolar edema, apart from a systolic murmur. Often the femoral pulses are easily palpated and prominent. Irregular pulse rate and strength may be noted in association with arrhythmias. Usually heart rate is increased and sinus arrhythmia is absent. If present, a precordial trill may be palpable over the apex. In the most severe cases hepatomegaly, ascites and jugular distension, could be visible in dogs with right-sided cardiac heart failure.

During this phase the instrumental examination recommended are:
- thoracic radiographs (to verify the presence/absence of blood stasis and pulmonary edema)
- electrocardiogram (to screen the patient for possible arrhythmias associated to the MVD)
- echocardiography (to confirm the diagnosis and staging)
- blood pressure measurement (excluding systemic hypertension)

Thoracic radiographs are the first diagnostic tool to use in a patient with respiratory symptoms, because it let the clinician to evaluate the cardiac silhouette, to visualize all the thoracic structures and to appreciate pulmonary venous dilation, if present. The earliest characteristic findings on chest radiographs of a symptomatic dog affected by MVD are mild left ventricular and left atrial enlargement. Left ventricular and atrial enlargement elevate the

trachea and carina on the lateral radiographic projection, with a decrease in the angle between the trachea and the
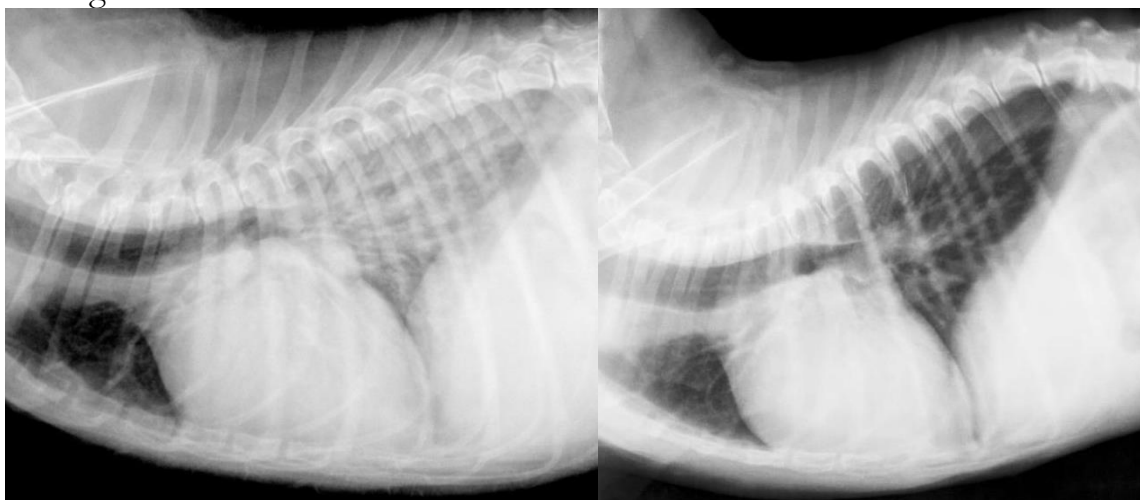

Figure 4 Chest radiography: LL projection of the same dog affected by MVD. On the left: radiographic signs of advanced pulmonary edema; on the right after diuretic therapy mild interstitial opacity and venous distension persist. Generalized cardiomegaly, bulging of the left atrium, elevation of the trachea and compression of the main steam bronchus in both images.

thoracic spine. The left mainstem bronchus may become elevated and compressed in cases of moderate-to-severe left atrial enlargement. Early pulmonary edema is seen as a diffuse increase interstitial density, progressing to fluffy densities and air bronchograms with onset of alveolar edema. (Rusch, 2009)

The electrocardiogram of a symptomatic dog could be unremarkable or could presents some arrhythmias, primarily atrial arrhythmias in the advanced stages could be detected. Sinus rhythm or sinus tachycardia is typical of dogs with MVD and congestive heart failure, because of the activation of the sympathetic system. Conversely, ventricular arrhythmias are relatively uncommon in animals with compensated disease, and even in dogs with congestive heart failure ventricular ectopic beats or ventricular tachycardia are uncommon. Atrial fibrillation develops in some dogs with marked atrial dilation.

The echocardiographic examination of the symptomatic patient is of great importance to assess the secondary lesions and the hemodynamic consequences of the mitral valve insufficiency. With the echocardiographic imagines we can assess the leaflets lesions, the left ventricular overload, the left atrial enlargement, the presence/absence of pulmonary hypertension and the presence/absence of pleural or pericardial effusions. Through the echocardiography the clinician gets objective informations about the cardiac status of the patient that allow to staging the dog and to establish the correct treatment protocol. The

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echocardiographic informations are at the base of a precise follow-up of the MVD dog and they are the foundation of the therapeutic decisions, providing also some great prognostic values.

2.9   Treatment of MVD

To our knowledge no medical therapy has been proven to stop the disease progression. The objective of the medical therapy is to improve the quality of life and the survival time. In the pre-clinical stage no therapy is indicated, although some authors recommend the use of ACE inhibitors, β-blockers and spironolattone. The ACE-inhibitors provide a modest vasodilators effect and an importantly reduction of angiotensin II and aldosterone levels, with a protective effect on the cardiac muscle. Their use in the asymptomatic dogs has been evaluated in two multicentric studies that didn't show any significative difference between treated and not-treated dog, at the beginning of the clinical phase. (Kvart et al., 2002; Atkins et al., 2007; Locatelli, 2013) A latter study underlined a positive effect of ACE-inhibitor on the survival time of dogs affected by MVD. (Locatelli, 2013; Pouchelon, 2008) In the symptomatic patients, the medical therapy objective are to improve the general conditions of the dog, and to decrease the severity of the clinical signs. Diuretics and dietary sodium restriction are crticial for the management of congestive heart failure, in conjunction with ACE inhibitors.

2.10   Mitral valve repair
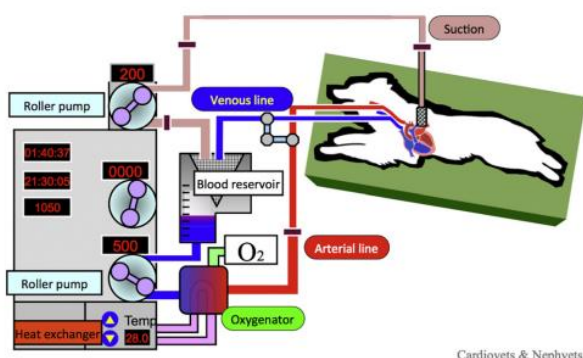
Figure 5 Schematic image of a cardiopulmonary bypass. (Uechi, 2012)

The ideal therapy of MVD would stop the progression of the valvular degeneration and would improve the valvular function. These results could be obtained only with surgical repair of the valvular apparatus, or with a valvular substitution before the beginning of the symptomatic stage.

Nowadays the surgical approach to the valve is not so diffuse in veterinary medicine, because of economic and practical limitations. Surgical treatments for mitral regurgitation include valve replacement and valve repair. While these are standard therapies in human medicine , mitral valve surgery has been reported in comparatively few veterinary patients. The lack between human and veterinary medicine on surgical mitral valve repair is

connected to both economic and practical reasons. For these kind of procedures a cardiopulmonary bypass is required. A successful cardiopulmonary bypass requires a dedicated team of surgeons, pefusionists, anesthesiologists, cardiologists, intensive care specialists and strong veterinary technicians support. The economic aspect is not neglectable: actually the cost of the procedures may be prohibitive for the vast majority of dog owners.

Once these techniques are mastered and refined for veterinary medicine, it seems more probable that surgery developing catheter-based interventions to limit mitral regurgitation will become the preferred treatment for those that can afford the procedure. (Uechi, 2008)

2.11 Genomics and MVD

The central dogma of biology is that DNA provides a template for RNA synthesis, which provides a template for protein synthesis. Changes within the DNA will affect all the downstream processes and can ultimately result in disease. Genomics is the study of an organism's genome, including genes, regulatory sequences, and the non-coding regions of DNA. In both human and veterinary medicine MVD has been studied from a genetic and a genomic point of view. A familial occurrence of MVD has been observed in many studies in the human population. In some human families, MVD has demonstrated autosomal dominant or X-linked inheritance (Monteleone & Fagan, 1969; Devereux et al., 1982). However, there seems to be a high degree of genetic heterogeneity of the disease in the human population, and the inheritance of MVD is now believed to be polygenic. A genome-wide association study would be a powerful approach for discovering genetic causes of mitral valve diseases in humans, but it would require a large number of cases and controls. Due to population bottlenecks in the past, individual dog breeds are genetically much more homogeneous than the human population, and within a predisposed dog breed, complex diseases are most likely caused by the same loci. (Lindblad-Toh et al. 2005). This increases the power to detect association between phenotype and genotype, and aids the identification of key factors in the molecular mechanisms, underlying disease development (Karlsson & Lindblad-Toh 2008).

Canine MVD has been studied from a genetic point of view above all in the Cavalier King Charles Spaniel (CKCS) breed, because of the early onset of the pathology and the high prevalence of MVD among this breed. In 2011 Madsen et all. (Madsen et al., 2011) conducted a genome wide association study on a cohort of Cavalier King Charles Spaniel (139 cases and 102 controls) and they identified two loci associated with the development of MVD, confirming that the dog breed is sufficiently homogeneous with respect to the genetic background for a polygenic disease, to provide convincing evidence for loci

underlying MVD in a fairly small number of cases and controls. (Madsen et al., 2011) In 2012 French et al. (French et al, 2012) conducted a genome-wide association study on 36 CKCS affected by MVD. They divided the group into two subgroups: early and late onset group. They didn't identify any region of highly discrepant homo/heterozygosity in the two groups. Furthermore, there was no evidence for loci associated with mitral valve murmur. The study of French et al. suggested that familial occurrence of mitral valve murmur in the CKCS breed is not due to a single major gene effect.

Further studies are therefore needed to clarify the genetic aspects of MVD according to these preliminary results.

2.12 The importance of the genes into the breeds

As mentioned before some breeds are particularly predisposed to the development of MVD. The breed specificity of MVD incidence is not unusual for a genetic disease in dogs, as the breed structure of the modern domestic dog can lend itself to sustaining a detrimental genetic mutation. Breeds are essentially closed populations, once a malicious mutation develops or is introduced, it can readily expand throughout the population. As a result, the presence of an inherited disease in a small number of breeds is to be expected. What is unexpected in the case of MVD is that the majority of affected breeds display an average adult weight of less than 9 kg. There are three possible sources of common ground for the small breed dogs: morphologic, genetic, and historic ground.

Figure 6 Neighbor joining tree of the breeds at risk of MVD. Breeds are grouped in the following order clockwise from the top and indicated by alternating gray bands: toy, spaniel, scent hound, working, mastiff, terrier, mountain, retriever, herding, and sighthound. The positions of the MVD at risk breeds are indicated by circles on the tree. Red circle ¼ breed with an average adult weight less than 9 kg, purple circle ¼ average adult weight between 9 and 14 kg, blue circle ¼ average adult weight over 14 kg. (Parker & Kilroy-Glynn, 2012)

Morphologic commonalities include overall body size, but might also include skeletal shapes or components that are common in very small animals.

Genetic similarities arise from the mutations that restrict growth. Apart from the genes under selection, MVD-causing mutations may be carried with these genes through what is commonly referred to as "hitchhiking".
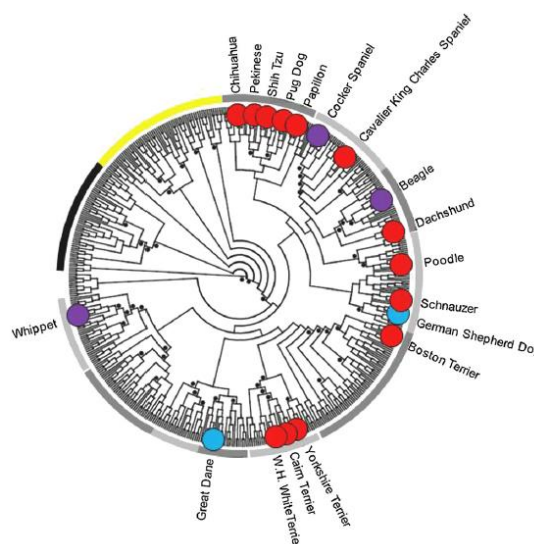
Historically, it is possible that all small dogs that are affected by MVD share a unique common ancestor that is not directly related to size, and that they have inherited a set of mutations from that common source that have increased susceptibility to MVD. If a common ancestor exists, MVD may have been introduced at a very early stage of breed development, and may have reached a relatively high rates due to reductions in the gene pools of most breeds after development. Different genetic studies have identified some cluster of breeds, that largely correspond to specific morphologies, geographical origins or historical occupations that are shared among the breeds in the cluster. (Ostrander EA, Kruglyak, 2000; Parker et al., 2010) The first group that has been identified was the Ancient group, that is comprised of breeds developed in Asia and Africa. The Ancient group is distinct from the Modern group, which is composed mainly by breeds of European origin.
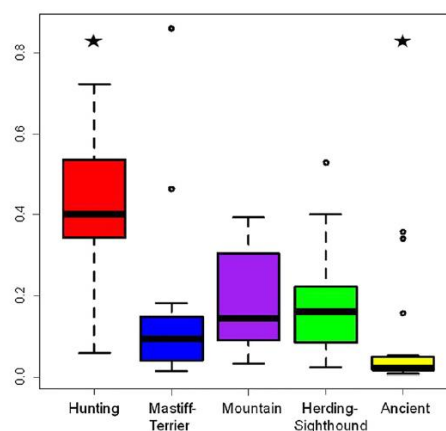

Figure 7 The five primary breed clusters. (Parker & Kilroy-Glynn, 2012)

The Modern group is further divided into four clusters based on the genetic studies:

1. The Herding-Sighthound
2. The Mastiff-Terrier cluster
3. The Mountain cluster
4. The Hunting cluster

The Hunting cluster is the most diverse one, including gun dogs, hounds and a subset of terriers and working dogs. The Toy breeds do not form a separate group, but rather display a mixture of all five groups in different proportions. The majority of them seem to form a cluster within the Modern group, with the closest breeds belonging to the Hunting cluster. Is interesting to notice that all of the breeds predisposed to MVD are part of the Modern cluster. If we look at the five primary breed groups to the high risk breeds, the Hunting group is doubtless the most important contributor with an average of 42% of their genomes coming from this ancestral cluster. (Parker et al., 2012)

2.13 The breeds considered in the present study

Cirneco dell'Etna (CdE): The first information about this breed date back to the V century a.c. It was thought that this breed came from the Egyptian pharaoh dogs, and imported in Italy by the Phoenician merchants, because of its great physical similarity with the dogs represented in that culture. The presence

of dogs similar to the CdE in the ancient Egypt is largely documented by bas-relief and ancient representations of Egyptian hunting dogs really close to the CdE. The subsequent studies demonstrated that the CdE breed is an autochthonous breed of the Italian region Sicilia. The first traces of the presence of this dog in Sicilia date back to 3 millennia ago. CdE

Figure 8 V-II century a.c., Sicilian coins representing the CdE dog

are represented in mosaics, literary compositions, graves and necropolis. The most famous and important representation is that of the Sicilian coins dating to the V-II century a.c., testifying the presence of this dog from very ancient times. The CdE dog is an hunting primitive dog, exploited for the hare hunting. We can therefore collocate this breed in the Hunting cluster. It' is a medium size dog: medium height 46-50 cm for the males and 42-46 cm for the females; medium weight 10-12 kg for the males and 8-10 kg for the females.

Figure 9 A photograph of a Cirneco dell'Etna dog.

The breed is poorly diffuse in Italy and really uncommon out of Italy. No studies about CVDs of this breed has been conducted before and no informations about the prevalence of MVD in CdE dogs exists to the author's knowledge.

Cavalier king Charles Spaniel (CKCS): The Cavalier King Charles Spaniel is one of the most popular breeds in Europe and, above all, in the United Kingdom. The origin of this breed date back to the first half of the 1600 d.c., when it was already present in England. The actual breed is a direct descendent of the small Toy Spaniels seen in paintings during the 16th, 17th and 18th centuries. During Tudor times, Toy Spaniels were quite common as ladies' pets. The breed gained prominence during the reign of King Charles II, because he had a large number of the King Charles Spaniels that followed him everywhere. The King Charles changed drastically its morphology in the late 17th century, when it was interbred with flat-nosed breeds by Queen Mary I and William the Orange. They favored breeds such as the short-nosed Pug and Japanese Chin and passed their characteristics down to the existing court's spaniels by breeding these dogs to King Charles Spaniels. This breeding resulted in a transformation of the King Charles Spaniel. Until the 1920s, the
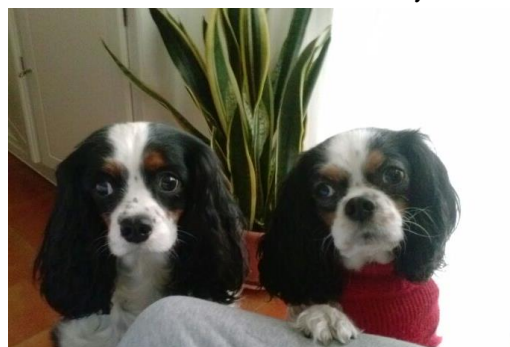

Figure 10 Vicky and Tina, two females of Cavalier King Charles Spaniel, tricolor coat

Cavalier shared the same history as the smaller King Charles Spaniel. In the early 1920's breeders attempted to recreate what they considered to be the original configuration of the breed, a dog resembling Charles II's King Charles Spaniel of the Restoration. In 1928 a small group of breeders of "original type" King Charles Spaniels united and a breed club was formed: the name Cavalier King Charles Spaniel was chosen for the new breed. Today the CKCS is in the genetic cluster of the Hunting Spaniel. Breed standards state that height of a Cavalier should be between 30 to 33 cm with a proportionate weight between 4.5 to 8.2 kg. Because of the high prevalence of MVD, the early onset and the proved hereditary transmission, CKCS is the most studied breed in the MVD field. The prevalence of the MVD in CKCS at the age of 6–7 years has been estimated in 50%, while in dogs older than 10 years is estimated in more than 90%. In this breed a polygenic mode of inheritance is suggested by a strong inherited component. The CKCS is considered, with the dachshund, the breed with the highest incidence of MVD, and the breed with the youngest onset.

2.14 Proteomics

Whilst a genome provides a blueprint of an organism it does not reveal anything about how a gene mutation leads to the disease. Furthermore, genotype–phenotype correlation studies have shown marked variability in the degree of phenotypic expression of known mutant genes, pointing to the existence of modifying factors. These modifications can occur at the DNA, RNA, and/or protein level, and it is therefore becoming clear that the functional complexity of an organism far exceeds that represented by its genome. Genomics is therefore being complemented by the newer discipline of proteomics, which has greater potential to understand the dynamic processes that govern cellular biology. (Lam et al., 2006) Proteomics aims to investigate the protein complement of the genome: the proteome. The term proteome was first coined by Marc Wilkins in 1995 and describes the entire collection of proteins of an organism, including products arising from events such as the processing of mRNA transcripts and post-translational modifications. Proteomics-based studies are focused on the interactions of multiple proteins and their role as part of a biological system rather than the structure and function of one single component. Proteins are directly involved in virtually all cellular activities and as such influence cell phenotype and hence the tissue or organ. The phenotype varies under normal physiological conditions (e.g., cell-cycle stage, differentiation, function, and age) or in response to pathophysiological stresses. Chronic pathologies or acute injuries have a major impact on the cellular environment, changing the proteins and messenger RNA (mRNA) profiles. Thus, the proteome consists of information from protein expression, post translational modifications (PTMs),

processing and turnover, localization and time. The PTMs alter the chemical nature of an amino acid residue, that can induce a modification in the protein structure, biological function and subcellular localization. For this reason the PTMs cannot be inferred from the genome, because they are the result of multifactorial mechanisms. By improving the understanding of the molecular mechanisms that influence the protein profiles, several pathologies may be detected earlier or managed more efficiently. (White & Van Eyk, 2007). The procedure of a proteomic study starts usually with the sample preparation, and continues with protein separation, protein imaging and subsequent identification of proteins and peptide differentially expressed. (Fig.6) (Cui et al., 2011)
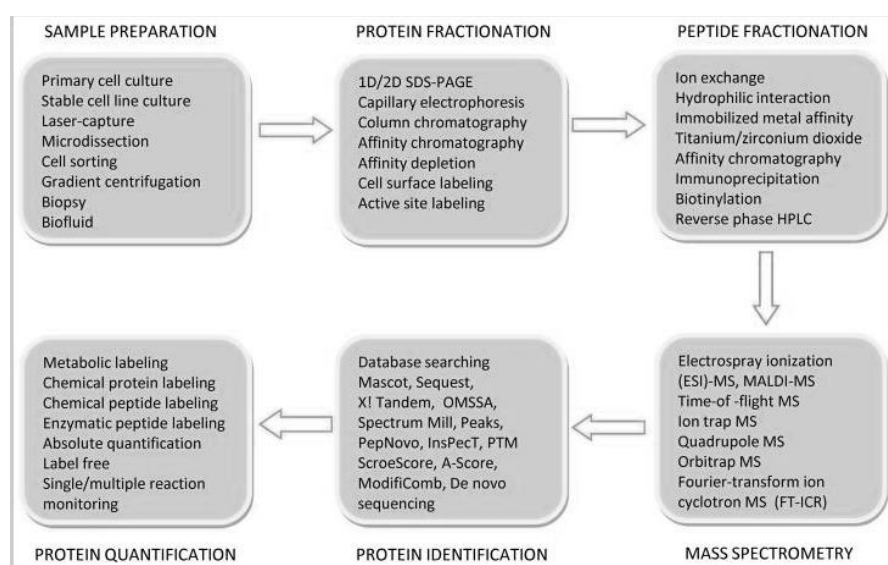

Figure 11 Schematic representation of a proteomic study procedures. (Cui et al, 2011)

Sample preparation is the first and more critical step of a proteomic study, because possible artefacts could be created at this stage and could interfere with the subsequent data analysis. The sample preparation should be performed as rapidly as possible to reduce the artefacts. The procedure of sample preparation and the choice of the chemical agents used is strictly dependent on the type of sample and on the type of proteins of interest.

Protein separation is the basic step for a proteomic study. The ability to separate complex protein mixtures with high resolving power as well as high reproducibility is at the base of a good study. 2-D gel electrophoresis is the most used and effective method for protein separation. This technique separates the proteins with the combination of two different methods: proteins are separated in the first dimension based on their charge properties by isoelectric focusing, and in the second dimension based on their molecular mass. The major limit of the 2-D gel electrophoresis is the low reproducibility and the mass limit that should be in the range between 5 and 500kDa. Other separation methods are capillary electrophoresis and liquid chrompatography, which has been developed

to improve protein separation with respect to biological function or chemical structure.

Protein detection and imaging follow 2-D gel electrophoresis separation. Proteins spots are visualized using standard protein staining methods. A digital image of the protein profile is obtained and it starts the image analysis using specialized software. Usually a control sample is used to compare the gel images of the experimental group and to identify differential protein expression.

Protein identification begins with the use of a photolytic enzyme to produce small peptide fragments. The set of the small peptide is then subject to mass spectrometry (MS), which is the most used tool for protein identification. This technique yields an accurate mass-to-charge ratio of each peptide fragment. There are different MS methods which are classified based on distinct ionization processes. MS could be divided into shot-gun analysis and targeted analysis. The shotgun MS is a discovery-based technique, that is used mainly when no scientific hypothesis has been formulated about what kind of protein will be detected. It's a slow method, that provides a relative quantification of the proteins, and it's mainly used when the samples are abundant in protein content. The targeted MS, conversely, is an hypothesis-based method, that is used when an hypothesis about what kind of proteins will be identified has been formulated. It's a fast MS that provides an absolute protein quantification, and it's used when the sample has a low protein content. (Lam et al., 2006)

2.15 Cardiovascular proteomics

The application of proteomic methods to uncover the protein function and structure in normal or disease states in the CV field is called cardiovascular proteomic. Cardiovascular proteomics can be subdivided into:
   o Mechanistic studies that aim at locating proteins which are directly and causally involved in functional or disease processes.
   o Biomarker studies that have the objective to locate proteins altered in abundance or modified in a predictable manner in response to the disease, with the aim of using these protein as a biomarker for that same CVDs. These studies are less focused on elucidating the mechanism of disease, instead aiming to improve clinical tools relating to the disease state.

The basic procedure of a cardiovascular proteomic study is to compare the protein complements of diseased hearts or sera with controls. Any protein that have altered expression between the two groups can be studied further for their involvement in disease pathogenesis. (Edwards et al., 2008) Many CVDs have been object of cardiovascular proteomic studies with different results. Some pathologies such as dilated cardiomyopathy, atrial fibrillation, ischemia-reperfusion injury and artherosclerosis have been more investigated than others.

These studies have revealed a number of proteins with altered expression during myocardial injury of different nature, based on the premise that death myocytes are released into the blood. Thanks to the identification of proteins coming from human myocardial tissue, a human heart 2-D gel electrophoresis protein database has been created. Proteome databases for canine myocardial tissues have been created too, by identifying and comparing homologous proteins from this specie to human heart 2-D gel electrophoresis database.

2.16 Proteomics and veterinary medicine

Although the application of proteomics in veterinary medicine has lagged behind human medical uses, an increasing and relevant number of studies have been conducted on these items. The investigation on farm animals is the field were proteomics have been more applied. Proteomics research in bovine medicine is mainly focus on bovine mastitis, the most frequent and most costly production disease in dairy cows. In pig medicine the pathogenesis of several viral disease has been explored by proteomics, while in horses the articular disorders and the equine recurrent uveitis are the main items of proteomics research. The use of proteomics in companion animals studies is less extensive than that for farm animals. Infectious and neoplastic diseases are the applications where proteomics approaches have been more exploited.   For the infectious diseases like Leishmania and Rabies, proteomics has been used in an attempt to identify novel antigens for vaccine production and diagnostic development, while for the oncologic field proteomics has been applied in the research of potential biomarkers of neoplastic disease. (Ceciliani et al., 2014)

At the same way, the cardiovascular medicine searched for different type of biomarkers for dogs and cats, producing a remarkable number of studies and identifying a relevant number of cardiovascular biomarkers for the dogs and the cats. In a recent review article Boswood proposed a system for categorizing biomarkers used in congestive heart failure:

1. Markers of myocyte injury
2. Markers of myocyte stress
3. Markers of remodeling
4. Markers of endothelial dysfunction
5. Markers of inflammation
6. Neurohormonal markers

Within the markers of <u>myocite injury</u> the cardiac troponins I and T have been widely used in veterinary medicine and in clinical practice. Troponins I and T are

intracellular proteins that are not normally present in detectable concentrations in the circulation. The presence of high concentrations of troponins I and T in the serum is associated with myocardial injury. Currently, the principal clinical use of concentrations of troponins in human patients is for the detection of myocardial ischemia secondary to coronary vascular disease, primarily atherosclerosis. This is not a condition that occurs commonly in veterinary patients; however troponins have been measured in a multitude of different situations in dogs and cats to evaluate the effects of both cardiac and extracardiac diseases on myocardial integrity.(Boswood, 2009) Among the biomarkers of myocyte stress, the natriuretic peptides are the most studied, both in human and in veterinary medicine. All natriuretic peptides share a common phylogenetic background but, due to differences in receptor-binding affinities, they have evolved into different hormones with clear distinct functions. B-type natriuretic peptide (BNP) is the most studied member of the natriuretic peptide family, and, together with its cleavage equivalent amino-terminal proBtype natriuretic peptide (NT-proBNP), they have emerged as important cardiovascular serum markers. The mechanism at the base of their use as a serological biomarker of myocardial stress is that, after stimulation of cardiomyocytes in cardiovascular diseases, preproBNP production is up-regulated, resulting in an increase of the BNP and NT-proBNP concentrations in the circulation. Accordingly, there has been a great interest in their use for the diagnosis or exclusion of heart failure, as well as for prognostic and therapeutic applications. (Roland et al., 2009)

2.17 Proteomics and mitral valve disease: state of the art

Mitral valve disease is the most common heart valvular disease in humans in Europe and United States and it's the primary naturally occurring heart disease in dogs(Lacerda et al., 2009). According to the great scientific interest that MVD has reached in the last few years, the scientific studies about this item are increasing in number since 1944. Being the disease pathologically identical in humans and dogs, a common pathogenesis has been suggested in these species. For this reason, canine MVD has been used as a model for human medicine. The scientific community is involved in searching new therapeutic approach and new diagnostic tools that could offer a better understanding and a more precise management of the pathology. In this context the first proteomic study on MVD has been conducted in 2009 by Lacerda et al. The objectives of the study were to develop proteomic methodology suitable for analysis of extracellular matrix-rich heart valve tissues, and survey over and under-expressed proteins that could provide mechanistic clues into the pathogenesis of MVD. The mitral leaflets of 12 dogs were obtained post-mortem and classified macroscopically and

microscopically as normal (four dogs), early MVD stage (four dogs) and late MVD stage (four dogs). Epidemiologic data (breed, sex, age, gender) were collected, and then three-way proteome
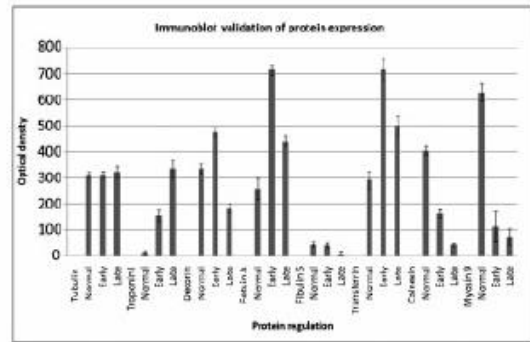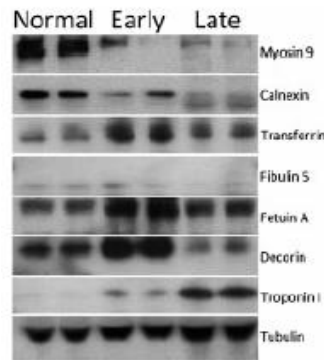

Figure 12 Protein expression in normal, early-stage and late-stage myxomatous valves. (Lacerda et al., 2009)

comparisons of normal, early-stage and late-stage myxomatous valve regions were performed. A shotgun proteomic analysis was used for protein quantification. Proteins were classified based on function, and clustered according to differential expression. More than 300 proteins were identified, and 117 of these proteins were differentially expressed.

Protein profiles showed that early- and late-stages valves were closely related, and this finding suggested that the proteomic changes of the leaflets appear in the eraly onset of the pathology, and persist till late-stage.
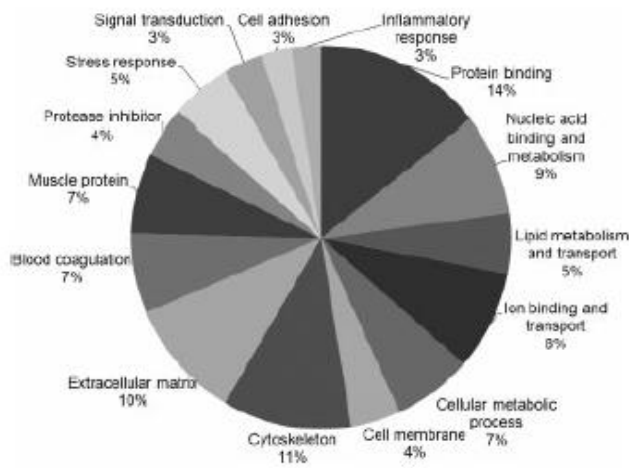
Figure 13 Functional classification of proteins differentially expressed between canine normal and myxomatous mitral valves identified based on homology with human proteins with known function. (Lacerda et al., 2009)

The differentially expressed proteins were:
-structural proteins
-proteins involved in the metabolism
-proteins involved with calcium binding and calcification.

The overall expression patterns were for proteins to be fully downregulated or upregulated in myxomatous valves, or upregulated in early-stage and downregulated in late-stage disease. The degree to which these expression patterns could be explained by differences in cellular events versus relative abundance of cellular/extracellular proteins was unclear. It is doubtless that differentially

expressed proteins identified in this study provided a basis for othere investigations into the pathogenesis of canine and human MVD.

In 2013 the second proteomic study on MVD and the first one conducted on serum samples was published by Tan et al. (Tan et al., 2013) Peripheral venous blood samples were collected from 166 human patients affected by MVD and 66 normal patients, in order to search for clinical biomarkers that may aid in detecting or risk stratifying this condition. All the epidemiological data and the echocardiographic measurements were recorded. The samples were trated and underwent the proteomic procedures to obtain the differential expressed proteins (sample preparation, protein quantitation, labeling of the samples, mass spectrometry, database search of the data and quantitation of the proteins differentially expressed).

Three proteins were identified to be differentially expressed between MVD and not MVD patients: platlet basic protein (PBP), haptoglobin and complement c4 were down-regulated in MVD cases, while fibronectin was up-regulated in MVD patients. Fibronectin up-regulation was not confirmed with ELISA testing and it was tought to be an outlier effect.

Haptoblogin is an hemoglobin-binding plasma protein. Since haptoglobin is not recycled after clearance of the hemoglobin–haptoglobin complex, the storage pool is depleted and low serum levels are characteristic of hemolytic states. Hemolytic anemia hasn't been associated with MVD, but it's possible that the altered leaflets and the increased valvular shear stress lead to hemolysis and hemoglobin release into circulation, depleting the haptoglobin storage pool.

Platelet basic protein (PBP) is a chemokine localized predominantly in platelets, and released upon platelet activation. PBP functions as signaling molecules and as anti-microbial peptides. It is possible that decreased plasma PBP could contribute to the increased risk of infective endocarditis associated with MVD. Reduced PBP may also implicate altered platelet function in MVD patient. The degree of platelet dysfunction may be related to the severity of MR. These thesis has been object of discussion, providing conflicting data on the exact relation between platelet dysfunction and MVD.

Complement component c4b resulted down-regulated in MVD cases. Complement c4b is part of the complement system, the most important component of humoral autoimmunity in the natural defense mechanism. It is possible that chronic infections could create immune complexes over a long period of time, resulting in a natural depletion of its components. The complement system is also involved in myocardial cell injury related to ischemia and reperfusion, and in the pathogenesis of atherosclerosis.

All of these three proteins differentially expressed have a presumptive role in the pathogenesis of MVD, so that further studies are needed to confirm there preliminary results. In this researches appeared clear that it is necessary to

correlate biomarker levels with clinical phenotype and outcomes in larger, more diverse populations, to establish appropriate threshold levels upon which to base clinical decision-making.

CHAPTER 3

Objective

3.1 Objective

The objective of the present study was to assess the prevalence, the clinical and the echocardiographic presentation of MVD in two groups of two different breeds of dogs: Cirneco dell'Etna, a breed never considered before for any CVDs studies, and Cavalier King Charles Spaniel, the most important breed for MVD research.
Furthermore, we tried to identify and study serum proteins of the same patients differentially expressed in healthy dogs and in dogs affected by MVD from mild to severe stages. The proteomics results were then matched with the clinical features, to find a connection available in the clinical practice.

The goals of the present study were:
- To assess the prevalence of MVD in the Cirneco dell'Etna breed
- To provide a global scanning of the serum protein modifications and to find a serological biomarker that could be useful to better define the diagnosis, the therapy, and the clinical presentation of the MVD.

Moreover, taking into account that the canine model is of great significance for the human MVD, the present study, that has been conducted on a well classified canine model, could be an interesting starting point for further studies in human medicine.

CHAPTER 4

Materials and methods

4.1 Materials and methods

This is a prospective study that has been conducted on private-owned dogs recruited from the Cardiologic Service of the Department of Veterinary Science and Public Health, University of Milan, from January 2011 to January 2014, in cooperation with the proteomic laboratory ISILS-UNIMI.

Two groups of dogs have been selected, based on the following inclusion criteria:

- o Healthy dogs of the selected breeds without any detectable pathology at the moment of the inclusion in the study
- o Dogs of the selected breeds affected by MVD, without any other detectable pathology at the moment of the inclusion in the study

The exclusion criteria were:

- o Dogs of any breed different from the breeds selected
- o Mix-breed dogs
- o Dogs affected by any detectable pathology
- o Dogs affected by any other cardiac pathology different from MVD

The breeds selected were:

- o Cirneco dell'Etna
- o Cavalier King Charles Spaniel

4.2 Step 1

From January 2011 to July 2012, we included in the study all the Cirneco dell'Etna dogs that entered the Cardiologic Service of the Department of Veterinary Science and Public Health. Each dog has been subjected to a complete physical examination, an echocardiography and a blood sampling.

Physical examination: it was aimed at evaluating the general physical condition of the dogs, checking the skin, the lymph nodes dimension and texture, the color of the mucous membranes, the thoracic and cardiac auscultation, the abdominal examination and the body temperature. It was paid particular attention to the cardiovascular system.

Cardiovascular system evaluation: the cardiovascular system was evaluated checking the mucous membranes, capillary refilling time, heart rate, heart rhythm, respiratory rate, presence/absence of a murmur, type and intensity of the murmur if present, grade of the murmur(I-VI/VI), type of pulse and pulmonary auscultation.

The anamnesis and the clinical data were collected on a database support for each patient.

The echocardiography was then performed on the awake dogs, according to the echocardiographic guidelines of the American Society of Echocardiography (Thomas, et al. 1993).

The echocardiographs were performed with an Esaote machine MyLab 50gold (Firenze, Italia), with phased array multifrequency probes.

Two-dimensional and M-mode echocardiography were used to define valve morphology and structures, including the type of lesions and the presence/absence of valvular prolapse.

Figure 14 M-mode image of a dog left ventricle at the level of the papillary muscles. Short axis.

The B-mode technique was used to study the anatomy of the valves. With the B-mode images it's possible to identify valvular thickening and valvular prolapse into the atria, from the first stage of disease. With advancing disease the valve becomes progressively thicker, and left atrial and ventricular enlargement is noted. Rupture of a chordae tendineae leads to a flail mitral leaflet with chaotic valve motion and a tip of the leaflet pointing dorsally into the left atrium in systole.

The M-mode technique was used to study the myocardial contractility, the mitral valve motion and to measure the left ventricle dimensions.

Figure 15 B-mode image, right parasternal long-axis view- Color-flow Doppler identified the mild mitral regurgitation

Measurement of chamber size and function help to define both the chronicity and the extent of remodeling.

The assessment of valvular insufficiency was made using all echocardiographic modalities to assess the significance and severity of the leak.

Color-flow Doppler rapidly identifies the presence of regurgitation. Attention to the jet area with respect to the receiving chamber, the width of the jet at its orifice, and the area of flow convergence adds accuracy in assessing the severity of regurgitant volume.

Spectral Doppler helps to measure the regurgitant volume and fraction, and is used to measure regurgitant orifice area.

Pulsed-wave Doppler and Continuous-wave Doppler assess hemodynamic severity by evaluating spectral trace density, velocity of flows, and flow profiles.

The examination consisted of standard views in both right and left parasternal location. The right parasternal location was used to provide the following standard images:

- Right parasternal long-axis view
- Right parasternal long-axis view optimized for the left ventricular outflow tract
- Right parasternal short axis view optimized for the papillary muscles
- Right parasternal short axis view optimized for the mitral valve
- Right parasternal short axis view optimized for the left auricle/aorta ratio (base of the heart)
- Right parasternal short axis view optimized for the pulmonary artery
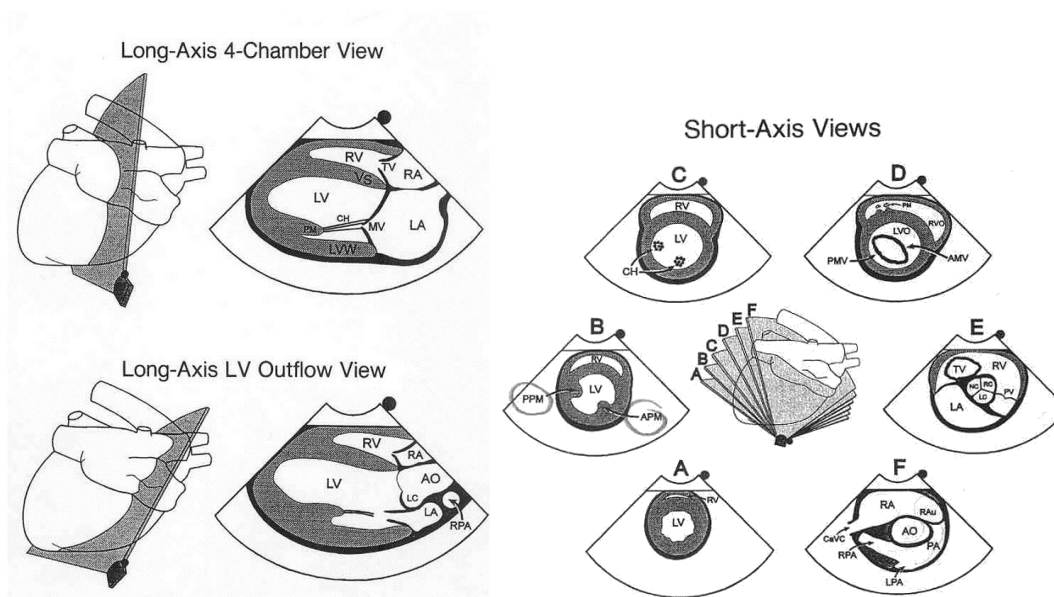
Figure 16 Images from Thomas et al, 1994. On the right: schematic representation of a right parasternal long axis view echocardiographic image. On the right: schematic representation of a short axis right parasternal view at different levels. RV= right ventricle; TV= tricuspid valve; RA= right atrium; LV= left ventricle; MV= mitral valve; LA= left atrium; VS= ventricular septum; LVW= left ventricular wall; CH= chordae tendinae; PM= papillary muscle; AO= aorta; RPA= right pulmonary artery; LPA= left pulmonary artery; LC= left coronary cusp; RC= right coronary cusp; NC= non coronary cusp; APM= anterior papillary muscle; PPM= posterior papillary muscle; Rau= right auricular; PA= pulmonary artery; CaVC= caudal vena cava.

The retrosternal view was used to assess the aortic flow velocity.

The left parasternal location was used to provide the following standard images:
- Left parasternal long-axis two chamber view
- Left parasternal long-axis four chamber view

The spectral Doppler and the colour Doppler were used for the assessment of possible valvular regurgitation and for the quantification and study of the blood flow through the valves.
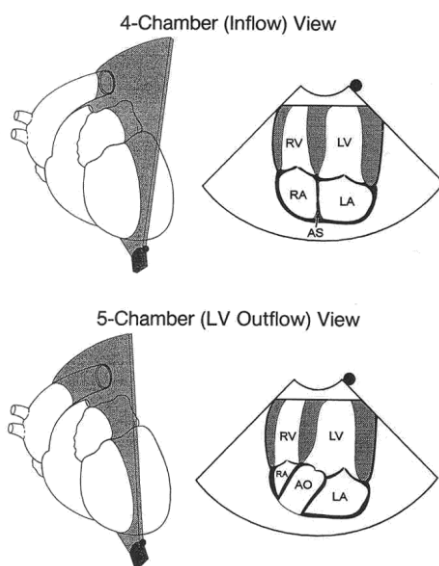

Figure 17 Images from Thomas et al, 1994. Schematic representation of a left apical view and a five chamber left parasternal view. RV= right ventricle; RA= right atrium; LV= left ventricle; LA= left atrium; AS= atrial septum; AO= aorta.

Based on the physical examination data and based on the echocardiographic response, we included in the study all the normal CdE dogs, and all the CdE dogs affected by MVD. All the patient affected by any heart disease different from MVD were exluded. All the patients affected by any systemic disease were excluded.

Each patient included in the study was then allocated in one of the following groups:

Normal dogs (N): no physical and no echocardiographic signs od MVD

Very slightly affected dogs (VS): asymptomatic dogs, with a cardiac murmur of I grade, and a mild Doppler evidence of mitral regurgitation, with mild mitral valve prolapse and without any echocardiographic signs of leaflets thickening (corresponding to ACVIM stage b1)

Mild affected dogs (M): asymptomatic dogs, with a cardiac murmur from II to VI grade, and echocardiographic evidences of MVD, with mitral valve prolapse and valvular thickening (corresponding to ACVIM stage of heart failure b1 and b2)

Severely affected dogs (S): symptomatic dogs, with physical signs of heart failure and echocardiographic evidences of MVD (corresponding to ACVIM stage of heart failure c and d)

Peripheral venous blood samples were taken from each patient after a 12 hour fasting. The samples were collected in a EDTA tube (2.5 ml blood) and in two serum tubes (2.5 ml of blood for each tube). The EDTA tube was used for a cellular blood count of each patient, and it was than eliminated. The samples in the serum tube were separated by centrifugation at 3000 ×g for 5 minutes and the plasma aliquoted. The total protein content and the blood glucose of each sample was immediately evaluated. The remaining plasma was stored at −18 °C until further analysis.

4.2 Step 2

Eight serum samples were selected for the proteomic analysis, in order to have 2 samples for each group. The serum samples were analyzed through 2D electrophoresis followed by Maldi TOF MS analysis for protein identification. Two sera from normal dogs, two from very slightly affected, two from mild affected and two from severely affected dogs were used for proteomic analysis. Briefly, protein concentration in all samples was determined using 2D Quant Kit (GE Healthcare). GE Healthcare immobilized pH gradient (IPG) strips (Immobiline DryStrip pH 3-10NL, 7 cm) were rehydrated overnight in 125µl of buffer containing 7 M urea, 2 M thiourea 2% CHAPS, 1% DTT, 2% ampholine pH 3.5–10. 100 µg of protein sample were loaded on each IPG strip using cup loading at the cathodic side.(20) Isoelectric focusing was performed using an Ettan IPGphor III IEF system (GE Healthcare) at 20° C with a current of 120 µA per strip. For IEF the following protocol was used: 30 V (4 h), 50 V (3 h), 100 V (3 h), 500 V (3 h), 1000 V (3 h), 3000 V (3 h), 4000 V (3 h), 6000 V (3 h) and 8000 V (8 h).(21) After the first dimension, IPG strips were equilibrated twice with a solution containing 6M urea, 2% SDS, 50 mMTris–HCl pH8.8 and 30% glycerol, for 15 min, under gentle stirring. For the first equilibration step was used 1% DTT and for the second 2.5% iodoacetamide. The second dimension was performed using homemade 10% acrylamide vertical SDS-PAGE slab gels on a mini-protean tetracell (BioRad). IPG strips were put on top of the SDS gels which were poured up to 1 cm from the top of the plates and then sealed with 1 ml of a solution containing 0.5% low-melting-point agarose diluted in hot SDS running buffer (25 mMTris–HCl pH 8.3, 192 mM glycine, 0.1% SDS). Molecular weight protein markers (Invitrogen) were applied on one end of the IPG strips. In the second dimension, gels were run at 15 mA per gel, for 20 min and then at 40 mA per gel, until the bromophenol blue front-line came out of the gel. After runs, gels were stained with colloidal Coomassie and digitalized with PharosFX Plus Laser Imaging System (BioRad).

Image and statistical analysis were performed using Progenesis SameSpots (Nonlinear Dynamics).

4.3 Step 3

From September 2012 to January 2014, we included in the study all the Cavalier King Charles Spaniel dogs that entered the Cardiologic Service of the Department of Veterinary Science and Public Health. The dogs underwent the same clinical evaluation of the Cirneco dell'Etna dogs: a complete physical examination, with particular attention to the cardiovascular system, and an echocardiography were performed.

The echocardiography was performed providing the standard images recommended by the echocardiographic guidelines of the American Society of Echocardiography. The following echocardiographic parameters were then taking into account by the present study:

- Left ventricular inner dimension in systole and in diastole (LVIDs e LVIDd)
- End diastolic volume index (EDVI):

$\{[7/ (2,4+LVIDd/10)] \times [(LVIDd/10)^3/BW]\}$ 35

- End systolic volume index(ESVI):

$\{[7/ (2,4+LVIDs/10)] \times [(LVIDs/10)^3/BW]\}$ 35

- Left atrium/aorta root ratio (La/Ao ratio)
- Fractional Shortening (FS%)
  $[(LVIDd-LVIDs)/LVIDd] \times 100$
- Ejection Fraction (EF%)
  $[(End Diastolic Volume – End Systolic Volume) \times 100/EDV$

LVIDs and ESVI are measurement indicative of the systolic dimension of the left ventricle, and they are a reflection of the systolic function. The LVIDd and EDVI are indicative of the diastolic dimension of the left ventricle and they are used to evaluate the presence or absence of left ventricular overload. These measurements reflect maximum ventricular filling when the heart is relaxed. The La/Ao ratio is indicative of the left atrial dilation subsequent to the mitral regurgitation, and is an important prognostic factor. Dogs with La/Ao ratio greater than 1.7 have been found to be a poor prognostic indicator for survival.

The FS is the most common echocardiographic measurement of left ventricular function. It's influenced by the preload, the afterload and the contractility. The EF represents the percent of blood volume ejected from the left ventricle during

systole. The load dependency of EF% and FS% explains why, in the case of MVD, impaired myocardial function may be associated with normal values of these indices. Therefore, most authors agree that normal values for FS% or EF% in dogs with severe MR suggest systolic myocardial failure

The dogs selected were allocated into three categories based on the physical examination and on the echocardiographic data:

- o <u>Normal dogs (N)</u>: no physical and no echocardiographic signs od MVD

- o <u>Mildl affected dogs (M)</u>: asymptomatic dogs, with a cardiac murmur and echocardiographic evidences of MVD (corresponding to ACVIM stage of heart failure b1 and b2)

- o <u>Severely affected dogs (S)</u>: symptomatic dogs, with physical signs of heart failure and echocardiographic evidences of MVD (corresponding to ACVIM stage of heart failure c and d)

The decision to divide the CKCS population in 3 subgroups and not in 4 subgroups was based on the proteomics results obtained from the CdE group: no significative differences were found between the VS group and the other groups, so that it was decide to incorporate the mildly affected subjects in an only one group (M group). Peripheral venous blood samples were taken from each patient after a 12 hour fasting. The sample were collected in two serum tubes (2.5 ml of blood for each tube). The samples were separated by centrifugation at 3000 ×g for 5 minutes and the plasma aliquoted were stored at −18 °C until further analysis.
From the starting group of 24 dogs, an homogeneous group of thirteen dogs was created in order to have a peer distribution of normal, mild and severe affected dogs. It was not possible to have five dogs of each category because just three dogs in the severe stage were recruited, being the dogs with the highest mortality rate.

4.4 Step 4
Six serum samples were selected for the proteomic analysis, in order to have 2 samples for each group. The serum samples were then analyzed through 2D electrophoresis followed by Maldi TOF MS analysis for protein identification. Two sera from normal dogs, two from mild affected and two from severely affected dogs were used for proteomic analysis.

Proteomic analysis was performed as described above in section 4.2.

4.5 Descriptive and statistical analysis

All calculations were done using SAS statistical software (SAS version 11 SW; SAS Institute, Cary, North Carolina, USA). Descriptive statistics were used for age, gender, weight and all the echocardiographic variables considered. Descriptive data are reported as mean ± S.D. or percent as appropriate. Normality of the distributions was examined using the Shapiro-Wilk test (W> 0,05). Differences between the categories of disease severity were determined by analysis of variance (ANOVA) and the Student's t Test for the normal data, and with the Kruskal-Wallis test for non-parametric variables or non-parametric Wilcoxon Each Pair test for the not normally distributed variables.

CHAPTER 5

Results

5.1 Results

Cirneco dell'Etna group

From January 2011 to July 2012, fourteen Cirneco dell'Etna dogs entered the Cardiologic Service of the Department of Veterinary Science and Public Health, University of Milan. Considering the total number of dogs that were examined by the Cardiologic Service (302 dogs) during the same period, the CdE represented the 4.6%. After the clinical and the echocardiographic examination 4 dogs (29%) were classified as normal, nine dogs (64%) were affected by MVD and in one case (7%) it was diagnosed a congenital heart disease (pulmonic stenosis, PS). Considering the dogs affected by MVD, 3 dogs were very slightly affected, 3 were mildly affected and 3 dogs were severely affected: one of these dog died during the study period because of congestive heart failure. From the starting group of 14 dogs, a group of twelve dogs was created for the proteomic and clinical analysis, because the subject affected by SP and the death subject were eliminated.

The Cirneco dell'Etna group was therefore composed of twelve dogs. Seven dogs were female (58%), two were spayed females (17%) and three were males (25%).The medium weight was (kg ± standard deviation) 11.7 kg ± 0,95 while the medium age was (years ± standard deviation) 7.5 years ± 4.6.
All the variables resulted normally distributed (W>0,05).
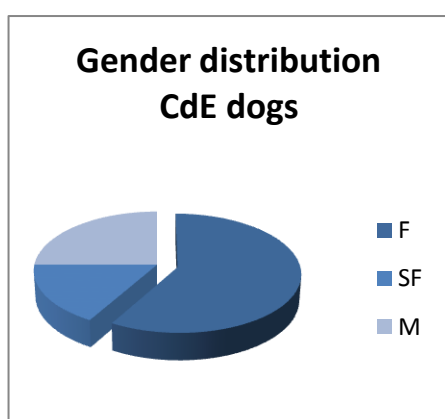

Chart 1 Pie Chart of the gender distribution in the CdE group

Four dogs were allocated in the normal (N) group, three dogs were allocated in the very slightly affected group (VS), three dogs were allocated in the mildly affected (M) group and 2 dogs were allocated in the severely affected (S) group.

ID num.	Sex	Age	Weight	Echocardiographic diagnosis	Group
1	F	1	12	Normal	N
2	M	2	12.3	Normal	N
3	F	6	12	Normal	N
4	F	2	11.5	Normal	N
5	SF	8	13.2	Mild mitral insufficiency	VS
6	F	7	11.8	Mild mitral insufficiency	VS
7	M	6	12.2	Mild mitral insufficiency	VS
8	F	7	12	Mild mitral insufficiency with mitral leaflets prolapse and valve thickening	M
9	F	13	12.8	Mild mitral and tricuspid insufficiency	M
10	M	12	10	Mild mitral and tricuspid insufficiency	M
11	F	12	10	Severe mitral and tricuspid insufficiency	S
12	SF	15	11.6	Severe mitral and tricuspid insufficiency, pulmonary hypertension	S

Table 2 Cirneco dell'Etna group

- **N (Normal) group**
  The N group was composed of 4 dogs: 3 female (75%) and 1 male (25%). The medium age was 2.7 years $\pm$ 2.21 and the medium weight was 11.95 kg $\pm$ 0.33

- **VS (Very slightly affected) group**

  The VS group was composed of 3 dogs: one female (33%), one spayed female (33%) and one male (33%). The medium age was 7 years $\pm$ 1 and the medium weight was 12.4 kg $\pm$ 0.7.

- **M (Mildly affected) group**
  The M group was composed of 3 dogs: 2 female (67%) and 1 male (33%). The medium age was 10,6 years $\pm$ 3.2 and the medium weight was 11.6 kg $\pm$ 1.4.

- **S (Severely affected) group**
  The S group was composed of 2 dogs: 1 female and 1 spayed female. The medium age was 13.5 years $\pm$ 2.1 and the medium weight was 10.8 kg $\pm$ 1.13.
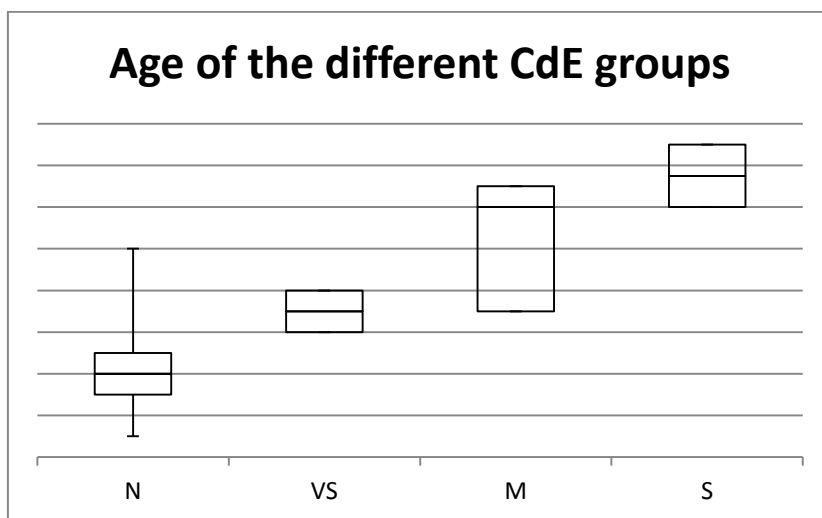
Chart 2 Box-plot of the age distribution in the 4 CdE subgroups. Data are presented as upper and lower quartiles and interquartile range (box), the median (horizontal line), and the maximum and minimum values (crosses).

The ANOVA of the age in the 4 groups was performed (F= **0.0023***) and then the significance of difference between the patient groups of the same variable was tested with the Student's t test.
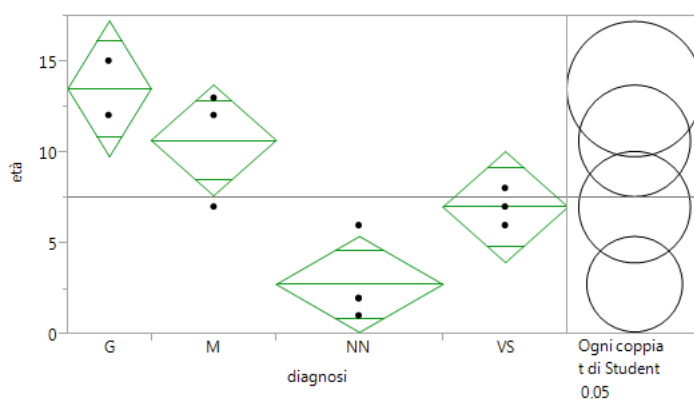
Level	- **Level**	p- value
G	N	**0.0006***
M	N	**0.0019***
G	VS	**0.0144***
VS	N	**0.0411***
M	VS	0.0854
G	M	0.2121

Chart 3 ANOVA and Student's t test graphic representation of the age distribution in the 4 groups of CdE dogs. P values for each couple are reported in the table.

Proteomic analysis was performed in the four different experimental groups as previously described. Two sera from normal dogs, two from very slightly

affected, two from mild affected and two from severely affected dogs were analysed through 2D electrophoresis followed by Maldi TOF MS analysis for protein identification.

Among analysed proteins two of them were found to be differentially expressed and were successfully identified through MS analysis. The first one, spot number 2092, was found to be a protease inhibitor (serpin peptidase inhibitor, alpha-1 antiproteinase, antitrypsin) and its expression was increase according to the severity of the pathology. The detail of the trend for alpha-1 antiproteinase is shown in chart 4. On the other hand it was found a strong downregulation of the representation of complement C3 protein according to the severity of the pathology. This trend is shown in chart 5, both differential representation of this protein was statistically significant with a p-value lower than 0,05.
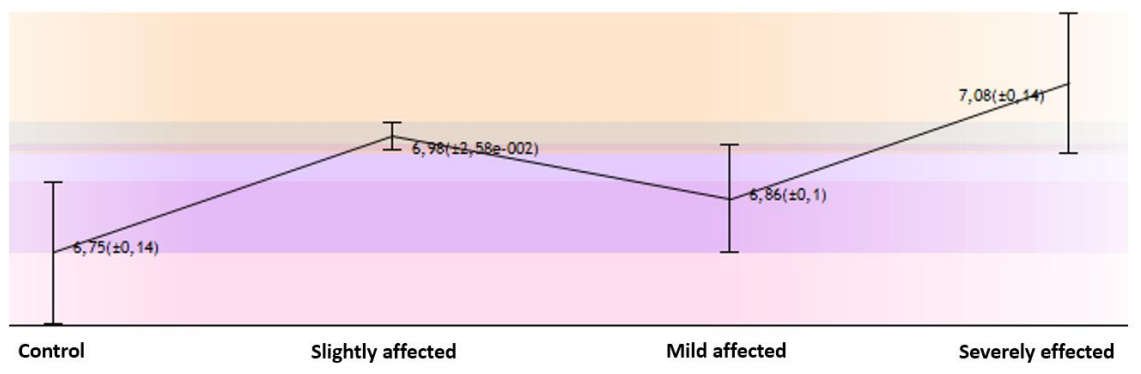

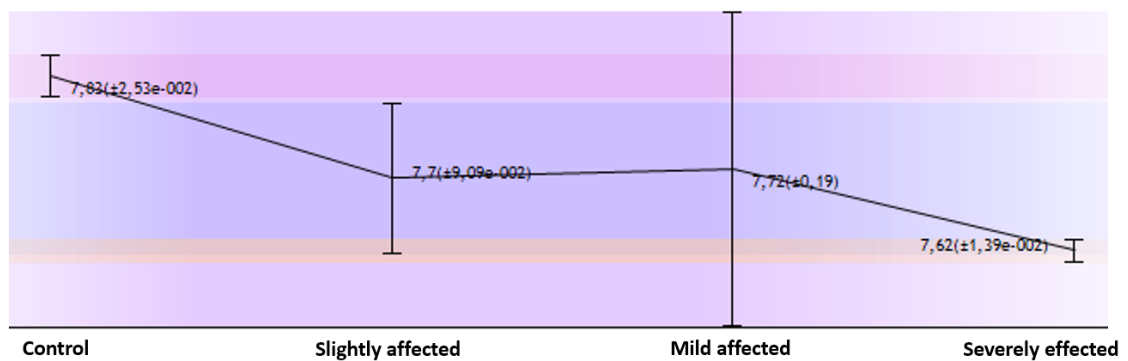**Chart 4: alpha-1 antiproteinase trend in the different stages of pathology**

Chart 5: Complement C3 trend in the different stages of pathology

Cavalier King Charles Spaniel group

From September 2012 to January 2014, 24 Cavalier King Charles Spaniel entered the Cardiologic Service of the Department of Veterinary Science and Public Health, University of Milan. Considering the total number of dogs that were examined by the Cardiologic Service (341 dogs) during the same period, the CKCS represented the 7%. Eight CKCS (33%) were classified as normal after the clinical and the instrumental evaluations, one dog (4%) had a congenital heart disease (patent ductus arteriosus, PDA), and 15 patients (63%) were classified as affected by

Gender distribution CKCS

Chart 6 Pie Chart of the gender distribution in the CKCS group

MVD. The medium age was 4.8 years ± 3.2 and the medium weight was 8.7 kg ± 2.4. Thirteen dogs were male (54%), nine dogs were female (38%) and two dogs were spayed female (8%). Nine of the 15 CKCS affected by MVD were mild affected, and six dogs were severely affected: two of them died during the study period because of congestive heart failure. From the starting group of 23 dogs, an homogeneous group of thirteen dogs was created in order to have a peer distribution of normal, mild and severe affected dogs. It was not possible to have five dogs of each category because just three dogs in the severe stage were recruited after the death of two patients, being the dogs with the highest mortality rate.

The group selected had the following distribution: five dogs were female (38%), two dogs were spayed females (15%) and six dogs were males (46%). The medium weight was 8.8 kg ± 1.8 and the medium age was 4.4 years ± 3.13.
The medium LVIDd was 31.5 mm ± 4.91 and the medium LVIDs was 19.3 mm ± 4.65. The medium EDVI was 88 ± 32 and the medium ESVI was 25 ± 12. The medium La/Ao ratio was 1.48 ± 0.5. The medium FS% was 38 ± 9 and the medium EF% was 68 ± 12.
The normal distribution of all the continuous variables was tested with Shapiro-Wilk test. Age, weight, LVIDd, EDVI, FS% and EF% were normally distributed (W> 0,05), while LVIDs, ESVI and La/Ao were not normally distributed.

Variable	W
LVIDs	0.0016
LVIDd	**0.8552**
ESVI	0.0074
EDVI	**0.3629**
La/Ao	0.0046
FS%	**0.1091**
EF%	**0.3883**

Table 3 W-values for each echocardiographic variable

For the not normally distributed variables non-parametric tests were then used (Kruskal-Wallis and Wilcoxon), while for the normally distributed data parametric tests were used for the analysis of variance (ANOVA and Student's t).

ID num.	Sex	Age	Weight	Echocardiographic diagnosis	Group
1	F	1,5	4,9	Normal	N
2	F	0,8	6,6	Normal	N
3	FS	5	10	Normal	N
4	M	1	8	Normal	N
5	F	3,5	6,8	Normal	N
6	M	6	9,6	Mild mitral insufficiency	M
7	M	5	10	Mild mitral insufficiency	M
8	M	5	14,4	Mild mitral insufficiency	M
9	F	1	4,4	Mild mitral and tricuspid insufficiency	M
10	F	3	12,7	Mild mitral and tricuspid insufficiency	M
11	FS	11	11,1	Severe mitral and tricuspid insufficiency, pulmonary hypertension	S
12	M	6	8,6	Severe mitral and tricuspid insufficiency, pulmonary hypertension	S
13	M	9	8,15	Severe mitral and tricuspid insufficiency, pulmonary hypertension	S

Table 4 Cavalier King Charles group

- **N (Normal) group:**

The N group was composed of five dogs: 3 females, 1 spyed female and 1 male. The medium age was 2.36 years ± 1.8 and the medium weight was 7.2 kg ± 1.8. All the medium echocardiographic data ± SD are reported in the following table.

N group	LVIDd (mm)	LVIDs (mm)	EDVI normal value < 100 ml/m³	ESVI normal value < 30 ml/m³	La/Ao normal value < 1.5	FS% normal value > 20%	EF% normal value > 40%
Medium ± SD	29 ± 2.2	20 ± 6.2	72 ± 13	24 ± 8	1.1 ± 0.2	35 ± 2	66 ± 3

Table 5 Mean echocardiographic variables ± SD for the N CKCS group

- **M (mildly affected) group:**

The M group was composed of five dogs: 3 males and 2 females.
The medium age was 4 years ± 2 and the medium weight was 10 kg ± 3.8
All the medium echocardiographic data ± SD are reported in the following table.

M group	LVIDd (mm)	LVIDs (mm)	EDVI normal value < 100 ml/m³	ESVI normal value < 30 ml/m³	La/Ao normal value < 1.5	FS% normal value > 20%	EF% normal value > 40%
Medium ± SD	30.4 ± 6.2	19.5 ± 4.6	82 ± 38	29 ± 19	1.3 ± 0.3	36 ± 11	64 ± 17

Table 6 mean echocardiographic variables ± SD for the M CKCS group

- **S (severely affected group):**

The S group was composed of 3 dogs: 2 males and 1 spayed female.
The medium age was 8.6 years ± 2.5 and the medium weight was 9.2 kg ± 1.5.
All the medium echocardiographic data ± SD are reported in the following table.

S group	LVIDd (mm)	LVIDs (mm)	EDVI normal value < 100 ml/m³	ESVI normal value < 30 ml/m³	La/Ao normal value < 1.5	FS% normal value > 20%	EF% normal value > 40%
Medium ± SD	36.6 ± 2.2	17.7 ± 1.7	126 ± 6	20 ± 3	2.3 ± 0.7	46 ± 10	77 ± 11

Table 7 Mean Echocardiographic variables ± SD for the S CKCS group

The ANOVA of the age between the 3 groups was performed (F=**0.0056***) and then the significance of difference of the same variable between the groups was tested with the Student's t test. A statistically significant difference was found

between the S and the other two groups, while no difference was found between the M and the N group.
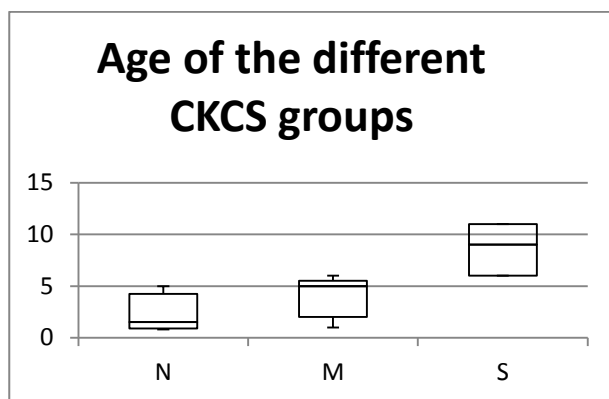
Age		
Level	**- Level**	**p value**
S	**N**	**0.0018***
M	**N**	0.2342
S	**M**	**0.0109**

Chart 7 Box-plot of the age distribution in the 3 CKCS subgroups. Data are presented as upper and lower quartiles and interquartile range 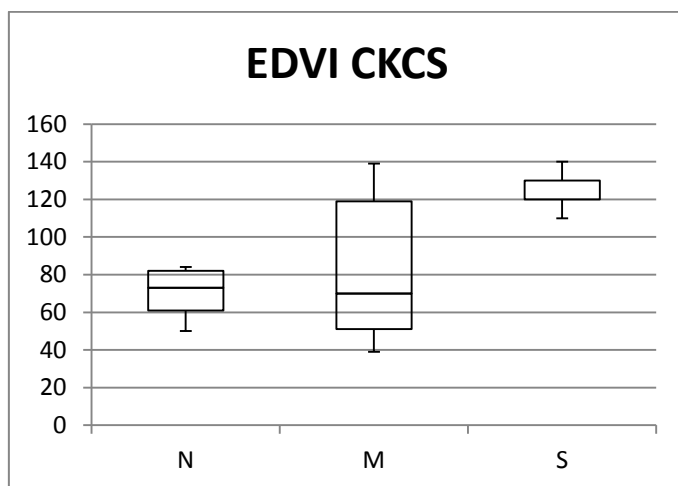(box), the median (horizontal line), and the maximum and minimum values (crosses). p-values for each couple are reported in the correspondent table (Student's t test).

The ANOVA of the variable EDVI between the 3 groups was performed (F=**0,0417***). The significance of difference between patient groups of the variable EDVI was studied with the Student's t test and resulted statistically different only between the N and the S group. No difference was found between the other groups.

EDVI		
Level	**-**	**Level**	**p value**
S		**N**	**0,0369***
M		**N**	0,2330
S		**M**	1,0000

Chart 8 Box-plot of the EDVI values distribution in the 3 CKCS subgroups. Data are presented as upper and lower quartiles and interquartile range (box), the median (horizontal line), and the maximum and minimum values (crosses). p-values for each couple are reported in the correspondent table (Student's t test).

The analysis of variance of the variable La/Ao between the groups was studied using the Kruskal-Wallis test (Chi Q= **0,0472**). The significance of difference between patient groups of the variable La/Ao was studied with the Wilcoxon test and resulted statistically different only between the N and the S group. No difference was found between the other groups.

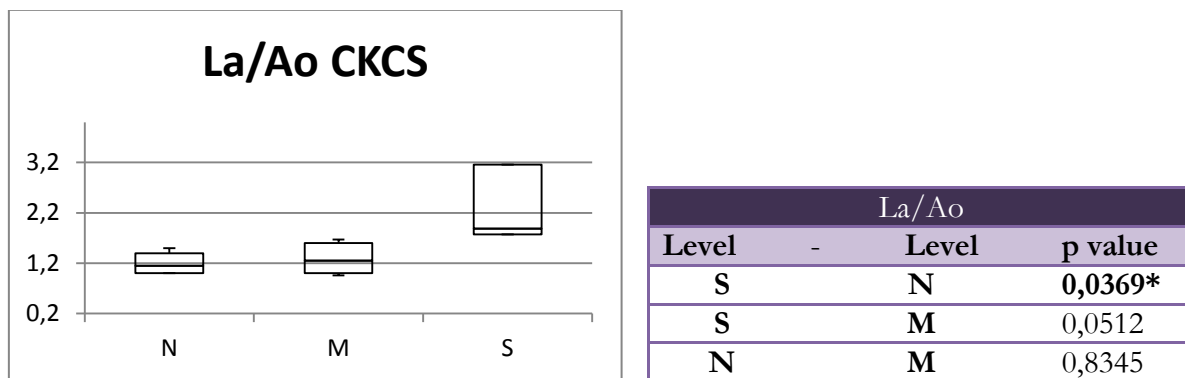| La/Ao			
Level	-	**Level**	**p value**
S		**N**	**0,0369***
S		**M**	0,0512
N		**M**	0,8345

Chart 9 Box-plot of the La(Ao values distribution in the 3 CKCS subgroups. Data are presented as upper and lower quartiles and interquartile range (box), the median (horizontal line), and the maximum and minimum values (crosses). p-values for each couple are reported in the correspondent table (Wilcoxon test).

The analysis of variance of the variable ESVI between the groups was studied using the Kruskal-Wallis test (Chi Q= 0,6974). Therefore the significance of difference of the mean between patient groups of the variable ESVI was studied with the Wilcoxon test and no difference was found between the groups.

The ANOVA of the variable EF% between the 3 groups was performed (F= 0.3536). No statistically significant difference was found between the mean EF of the 3 groups using the Student's t test.

The ANOVA of the variable FS% between the 3 groups was performed (F=0.2471). No differences statistically significant were found between the mean FS% of the 3 groups with the Student's t test.

Proteomic analysis was performed in three different experimental groups as previously described. Two sera from normal dogs, two from mild affected and two from severely affected dogs were were analyzed through 2-D electrophoresis followed by MALDI TOF mass spectrometric analysis for protein identification. Among analyzed proteins three of them were found to be differentially expressed and were successfully identified through MS analysis. The first one,

was found to be serum albumin and its expression was decreasing according to the severity of the pathology. The detail of the trend for serum albumin is shown in chart 11. Conversely it was found a strong up-regulation of some specific types of IgG and IgM according to the severity of the pathology. This trend is shown in chart 11, both the differential expression of those proteins were statistically significant with an anova p-value lower than 0,05.
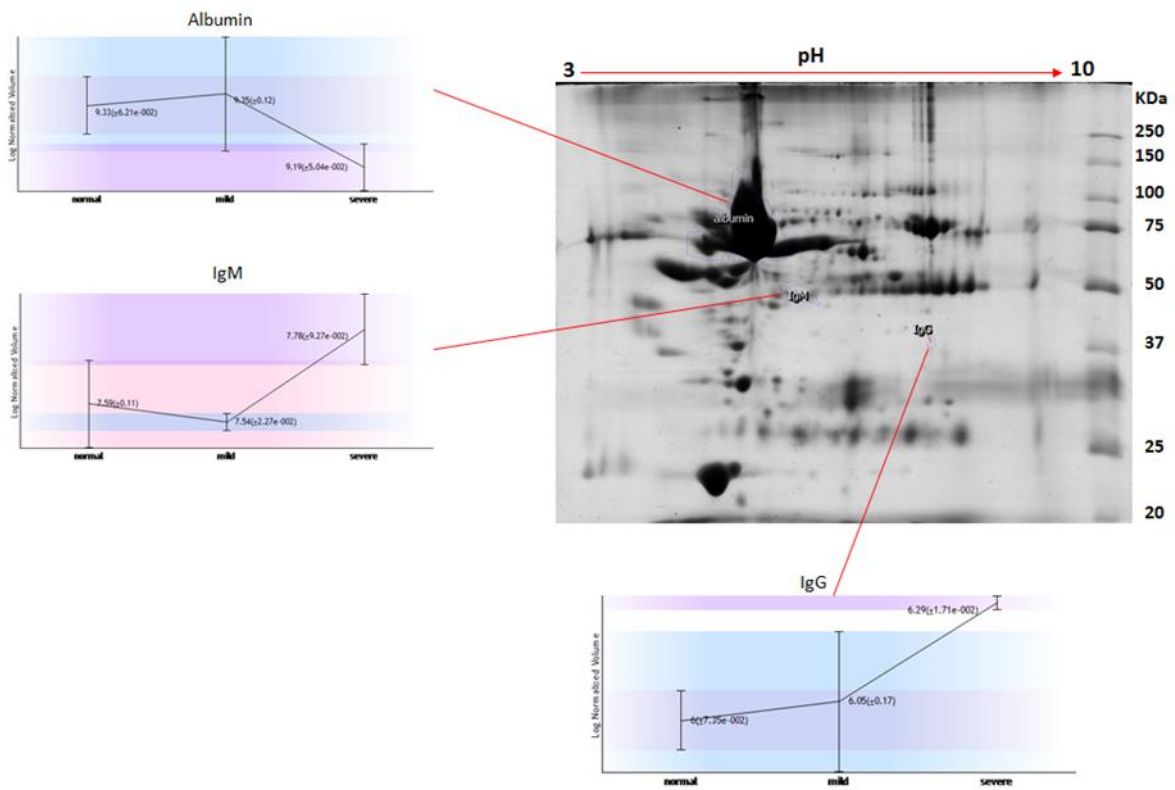

Chart 90: Representative 2-D maps of canine serum showing the differentially expressed proteins between experimental groups

CHAPTER 6

Discussion

6.1 Cirneco dell'Etna group

Cirneco dell'Etna group: The Cirneco dell'Etna breed represented a small part of our population (4.6%). The prevalence of MVD among this breed was high (64%) and a statistical correlation between MVD and age was found: the age of the normal group was statistically different from the age of the other 3 groups. The oldest dogs were those affected by MVD, and all the dogs older than 6 years old had echocardiographic signs of MVD. We expected that MVD, if present in the CdE breed, would have affect the oldest dogs, being a typical pathology of the elderly dogs. An unexpected result was the relatively young median age of the dogs affected by MVD: in particular the VS group had a mean age of 7 years. This result is unexpected, because MVD is defined as a pathology typical of the old, small breed dogs. The CdE breed is a medium size breed, and for its weight the old age is reached at 8 years old. Two of the dogs of the VS group were under the 8 years old and the other one was 8 years old. Therefore we can conclude that the CdE breed is predisposed to the development of MVD, and that the age of onset is relatively low. The membership of the CdE dogs to the Hunting cluster is maybe at the base of the predisposition of this breed to develop MVD. The CdE breed is a primitive hunting dog, that supposedly hasn't been inbreed and manipulated by the human action like other breeds during the centuries. We can therefore imagine that if a common ancestor exists, MVD may have been introduced at a very early stage of breed development. The CdE have then inherited a set of mutations from that common source that have increased susceptibility to MVD, and may have reached a relatively high rates due to reductions in the gene pools of most breeds after development.

The proteomic analysis of the CdE samples gave two important results: the alpha-1-antytripsina (A1AT) was up-regulated in the patients affected by MVD, according to the severity of the pathology, while the complement C3 was down-regulated with the development of MVD, according to the stage of the disease.

Alpha-1-antytripsina (A1AT): A1AT is a glycoprotein, generally known as serum trypsin inhibitor. The protein was called "antitrypsin" because of its ability to covalently bind and irreversibly inactivate the enzyme trypsin in vitro. The term alpha-1 refers to the enzyme's behavior on protein electrophoresis. There are several "clusters" of proteins: albumin, alpha, beta and gamma (immunoglobulins). The non-albumin proteins are referred to as globulins. The alpha region can be further divided into two sub-regions, termed "1" and "2". Alpha 1-antitrypsin is the main enzyme of the alpha-globulin 1 region. The correct name, however, is alpha-1 proteinase inhibitor, because it is a serine protease inhibitor (serpin) inhibiting a wide variety of proteases. A1AT is an

acute-phase protein (APP). The APP are a class of proteins whose plasma concentrations increase (positive acute phase proteins) or decrease (negative acute phase proteins) in response to inflammation (acute-phase reaction also called acute phase response). APP have a wide range of activities that contribute to the host defence: they can directly neutralize inflammatory agents, help to minimize the extent of local tissue damage, as well as participate in tissue repair and regeneration. It protects tissues from enzymes of inflammatory cells, especially elastase, which normally digests damaged or aging cells and bacteria in order to provide for healing. There is a rapid increase in plasma concentration of many complement cascade components, the activation of which ultimately results in the local accumulation of neutrophils, macrophages and plasma proteins. Inflammation is essential for atherogenesis, and many inflammatory markers have been analyzed for their association with short- and long-term outcome in patients with manifestations of coronary artery disease and in apparently healthy subjects. A1AT in human blood can increase to three or four times the normal level to protect tissues in times of infection, pregnancy, or other situations that subject the body to an increase in neutrophil elastase. In its absence, elastase is free to break down elastin , which contributes to the contractility of the lungs, resulting in respiratory complications such as emphysema leading to finally to COPD (chronic obstructive pulmonary disease). In the acute phase reaction, a further elevation is required to "limit" the damage caused by activated neutrophil granulocytes and their enzyme elastase, which breaks down the connective tissue fiber elastin. Because elastin is a major component of vessel wall elastic lamina, and degradation of elastic fibers may be important in the loss of vessel tone and the development of atherosclerosis, this implicates A1AT in the artherogenic process. The association between MVD and inflammation is not a novelty: in the last years there has been a growing interest in the role that inflammatory mediators may play in accelerating the pathology of MVD. There is one report of elevated C-reactive protein concentrations in the serum of affected dogs, suggesting a possible role of low-grade systemic inflammation in the disease progression. (Rush et al, 2006) Another study evaluating genomic expression patterns from the valves of dogs with MVD, confirmed activation of several pathways involved in cell signalling, inflammation, and extracellular matrix activation, with several inflammatory cytokines and serotonin-transforming growth factor identified as contributory to the development of the degenerative process in the valve. (Oyama and Chittur, 2006). The up-regulation of the A1AT in the MVD patients may be related to the state of chronic inflammation of the affected valves. The damaged valves cells may activate the inflammatory cells. Subsequently, the A1AT could play a role as an opposer to the inflammatory agents, helping to minimize the local tissue damage, as well as participating in tissue repair and regeneration. Previous

works have demonstrated that inflammation plays an important role in the development of cardiovascular diseases: in patients with acute coronary syndrome, coronary atherosclerotic plaques are characterized by an abundant inflammatory infiltrate. (Correale ate al., 2012)

Complement C3: The complement system is a group of proteins that made part of the immune system, and is the most important component of humoral autoimmunity in the natural defence mechanism. The complement proteins are activated by inflammation, infection and other pathogenetic mechanisms. The role of the complement is to protect the organism through the removal of the pathogens, or facilitating their control by other biological, serical or cellular mechanisms. The complement system is also involved in myocardial cell injury related to ischemia and reperfusion, and in the pathogenesis of atherosclerosis. Complement C3 (C3) is an acute phase reactant produced by the liver, secreted by activated macrophages at inflammation sites and by adipocytes, and has a central role in the immune system. C3 has been associated with atherosclerosis and cardiovascular risk factors in human medicine. Furthermore the fraction C4b of the complement has been associated recently to MVD: C4b resulted down-regulated in human MVD (Tan et al., 2013). The authors suggested that chronic infections and/or inflammation could create immune complexes over a long period of time, resulting in a natural depletion of its components. The same process could explicate the C3 down-regulation of our patients. Moreover, the complement system is involved in myocardial cell injury and in the pathogenesis of atherosclerosis: it was demonstrated that many MVD hearts of dogs have histopathological lesions in the myocardium, including small foci of myocardial fibrosis and necrosis, as well as widespread intramural coronary arteriosclerosis. These chronic alterations could be a further mechanism of activation of the complement system, with final depletion of its components as the complement C3.

6.2 Cavalier King Charles Spaniel group

Cavalier King Charles Spaniel: The Cavalier King Charles Spaniel group represented a relatively modest part of our population (7%). The prevalence of MVD among the CKCS breed was high (63%). The medium age of the total group was low (4 years). This result was expected, because the CKCS is the breed with the earliest onset of MVD, so that almost all the dogs included in the study were relatively young dogs. All the subjects mildly affected by MVD (M group) were young dogs: the mean age of this group was 4 years old, a very young age for a dog of that body weight. The S group had a mean age

statistically different from the two other groups (8.6 years), including the oldest subjects (6, 9 and 11 years old). This is an expected result, being the MVD a chronic and degenerative pathology, that in the CKCS has an early onset, and a chronic progression, so that the most severe cases are often seen in the oldest dogs. Even so, we have to notice that the mean age of the S group was still a young age if compared with the life expectancy of an healthy Cavalier King Charles Spaniel dog. This results confirm that the CKCS is a breed with a high predisposition to MVD, with a very early onset.

The echocardiographic variables considered were LVIDs, LVIDd, ESVI, EDVI, La/Ao ratio, FS% and EF%. The results obtained in the CKCS group reproduce the echocardiographic trend of MVD. The normal group had mean values in the range of normality. The M group presented values in the range of normality, but an increase in the mean values was found. The ESVI and La/Ao were next to the border line values of normality.

The echocardiographic values of the S group were above the normal limits, in particular EDVI and La/Ao ratio were severely increased, and the mean La/Ao ratio was markedly increased, underling the poor prognosis of the S group dogs. A statistic significant difference of the mean values was found only between N and S group, for the EDVI and La/Ao ratio variables, because of the normal values of the mildly affected dogs, confirming the substantial overlap of the numeric variables of a normal dog with a dog in the first stage of MVD

These results were those expected, because MVD is a chronic pathology with a constant but often slow progression, so that the mildly affected dogs had echocardiographic values in the range of normality, but with an increasing trend if compared with the normal dogs, while the severely affected dogs had values openly above the normal range. The variables considered were those indicative of left ventricular function and left atrial dilation, so that an increase in these variables above the normal range indicates the presence of morphologic and hemodynamic alterations secondary to mitral valve regurgitation, and the passage from ACVIM stage b1 to the ACVIM stages b2,c and d. This results confirm the fact that in the first stages of the pathology no morphologic or hemodynamic alteration are found. For the mildly affected dogs the echocardiographic aspect of the mitral valve apparatus and the Doppler study of the mitral flow will be pivotal to assess the presence/absence of MVD, while for the severely affected dogs the evaluation of the above mentioned variables is mandatory to assess the global heart status.

The proteomic analysis on the CKCS samples emphasized some important differences among the groups: the serum albumin was down-regulated in our samples, according to the severity of the pathology, while it was found a strong up-regulation of some specific types of IgG and IgM, according to the severity of the pathology. The proteomics results of the two breeds were markedly

different to each other. We made some hypothesis about this discrepancy, and it was thought that the CdE samples resulted different, with a worst quality of proteomic analysis results, because they were hemolytic, so that the haemoglobin present in the sera may have affected the proteomic analysis, covering some protein expression. With the CKCS dogs the blood samples were taken with all the possible precautions to avoid the hemolysis, and the sera obtained were of better quality than that of the CdE group. This is a possible explication to the different proteomics results obtained. Anyway, we have to remember that the samples were taken from totally different breeds, and the genetic component of the protein expression may have play an important role.

Serum albumin: The albumins are a family of globular proteins, the most common of which is serum albumin. The albumin family consists of all proteins that are water-soluble, are moderately soluble in concentrated salt solutions, and experience heat denaturation. Albumins are commonly found in blood plasma, and are unique from other blood proteins in that they are not glycosylated. Albumin is the main protein of the blood plasma. It binds water, cations (such as Ca2+, Na+ and K+), fatty acids, hormones, bilirubin, thyroxine and pharmaceuticals. Its main function is to regulate the colloidal osmotic pressure of blood. Serum albumin is the most abundant blood plasma protein and is produced in the liver and forms a large proportion of all plasma proteins. Serum albumins are important in regulating blood volume by maintaining the oncotic pressure (also known as colloid osmotic pressure) of the blood compartment. They also serve as carriers for molecules of low water solubility isolating their hydrophobic nature, including lipid soluble hormones, bile salts, unconjugated bilirubin, free fatty acids (apoprotein), calcium, ions (transferrin), and some drugs. Low albumin (hypoalbuminemia) concentration may be caused by liver disease, nephrotic syndrome, burns, protein-losing enteropathy, malabsorption, malnutrition, late pregnancy, artefact, genetic variations and malignancy. Several processes control plasma albumin concentration, including the absolute rate of albumin synthesis, the fractional catabolic rate, albumin distribution between the vascular and extravascular compartments, and exogenous loss of albumin. The rate of albumin synthesis is affected by both nutrition and inflammation, given that albumin is a negative acute phase protein.
The observation that serum albumin is a negative acute phase protein supports the contention that serum albumin concentration is a marker of inflammation. (Don & Kaysen, 2004) We can therefore suppose that, similar to the A1AT and to the complement C3, the decrease in serum albumin concentration in our MVD patients may be a consequence of the state of chronic inflammation that could play an important role in the myxomatous pathogenesis. Hypoalbuminemia in human patients suffering from CHF is common and it is

considered a strong predictor of death in patients with CHF. Hypoalbuminemia in these patients is not due only to malnutrition, loss of appetite in the chronic patient and to cardiac cachexia. In these patients hypoalbuminemia is caused by chronic inflammation, liver dysfunction from right ventricular failure, hyper catabolism and hemodiluition. (Yamamoto et al., 2012) In our patients the decrease in the serum albumin level is evident above all in the S group (chart 11), that was the group composed by the severely affected dogs with congestive heart failure as a consequence of MVD. For this reason we can suppose that the above mentioned mechanisms that are demonstrated in human medicine, could be established in the canine patient, being the pathogenesis of MVD common to the two species.

IgG and IgM: Immunoglobulins are glycoprotein molecules that are produced by plasma cells in response to an immunogen and which function as antibodies. The immunoglobulins derive their name from the finding that they migrate with globular proteins when antibody-containing serum is placed in an electrical field Immunoglobulin G (IgG) and immunoglobulin M (IgM) are two antibodies isotypes. Antibodies are major components of the immune system. IgG is the main antibody isotype found in blood and extracellular fluid allowing it to control infection of body tissues. By binding many kinds of pathogens, IgG protects the body from infection. IgM antibodies appear early in the course of an infection and usually reappear, to a lesser extent, after further exposure.
Immunoglobulins bind specifically to one or a few closely related antigens. Each immunoglobulin actually binds to a specific antigenic determinant. Antigen binding by antibodies is the primary function of antibodies and can result in protection of the host. Frequently the binding of an antibody to an antigen has no direct biological effect. Rather, the significant biological effects are a consequence of secondary "effector functions" of the antibodies. The immunoglobulins mediate a variety of these effector functions. Such effector functions include fixation of complement and binding to various cell types.
To the author knowledge, no association between IgG, IgM and canine MVD has been made before. Conversely, in human medicine, an increased prevalence of MVD is reported in patients with systemic lupus erythematosus and autoimmune thyroid disease. In 2009 Evangelopoulos et al. examined serum samples from humans MVD subjects for various organ and non-organ specific autoantibodies. IgA and IgG were found at a statistically significant higher incidence in the MVD affected humans, insinuating the presence of subclinical autoimmunity and autoimmune mechanisms involved in the MVD pathogenesis. We can suppose that, playing a role in the complement, the up-regulation of IgG and IgM in our samples could be related, once again, to the state of chronic inflammation of the MVD patients. Furthermore, the MVD patients are more

prone to develop infective endocarditis, a condition that determines a great stimulation of the immune system and an up-regulation of the antibodies.

CHAPTER 7

Limitations of the study

7.1 Limitations of the study

The present study has been conducted on a relatively small number of cases. In particular, the severe groups in both the CdE and the CKCS were composed of a fairy small number of dogs. It was not possible to have five dogs of each category because just two dogs in the severe stage were recruited for the CdE group and just three dogs in the CKCS group, being the dogs with the highest mortality rate. This is a great limitation for all the statistical analysis.
For the future it could be of great interest to increase the number of dogs included in the study, enhancing the statistical powerful

The clinical part of the present study didn't required any economic effort, being a prospective study made on our pool of patients, but all the proteomic procedures are relatively high cost techniques, requiring expensive materials and a proteomic laboratory with prepared and trained team.
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In the present study we didn't consider the anatomic alterations of the valves because we didn't perform any post-mortem examination on our patients. For this reason we didn't compare the autoptical findings with the clinical and proteomic results. For the future it could be interesting to evaluate the proteomic alterations in both mitral valve tissue and sera, comparing the findings with the clinical features.

CHAPTER 8

Conclusions

8.1 Conclusions

Based on our study results, we can conclude that the Cirneco dell'Etna breed is a primitive hunting breed predisposed to the development of MVD, with an early onset of the pathology. All the CdE dogs older than 6 years should be therefore evaluated for MVD, and included in a screening program. To the author's knowledge the CdE breed was never included before among the breeds predisposed to MVD. The membership of the CdE dogs to the Hunting cluster is maybe at the base of the high prevalence of MVD among the breed. For the future it will be of great interest to continue the genetic studies conducted on the breeds clusters, including the CdE in the Hunting cluster.

We confirmed that Cavalier King Charles Spaniel is a breed with a high prevalence of MVD and an early onset too, as previously reported. In our study we observed that the disease is chronic, with a slow progression. In the first stages of the pathology no morphologic or hemodynamic alteration are found and the echocardiographic diagnosis is based on the mitral valve apparatus aspect and on the Doppler identification of the regurgitation: in these dogs the echocardiographic aspect of the mitral valve apparatus and the Doppler study of the mitral flow will be pivotal to assess the presence/absence of MVD, even if a complete echocardiographic exam should be always performed. Conversely, in the advanced stage the echocardiographic variables indicative of systolic function, ventricular overload, atrial dilation and left ventricular function are markedly different from the normal values, and the echocardiographic diagnosis is completed with the evaluation of the altered hemodynamic status and of the morphologic abnormalities.

The proteomic analysis conducted on our samples and correlated to the clinical results, indicate that the MVD is a pathology that is strictly connected to a chronic low inflammation state. The up-regulation of A1AT, IgG, IgM, and the down-regulation of complement C3 and serum albumin are connected with an inflammatory status, that cause a depletion of the components of the complement system, an activation of the acute phase proteins and of the components of immunity response like IgG and IgM immunoglobulins.

The hypothesis that MVD could be related to a chronic inflammation was already speculated in the last years, and, based on the present study results, we think that the analysis of the inflammatory mediators in MVD patients could be a great chance to uncover the pathogenetic mechanism at the base of mitral valve disease.

CHAPTER 9

References

References

1. **Atkins C, Bonagura J, Ettinger S, Fox P, Gordon S, Haggstrom J, Hamlin R, Keene B, Luis-Fuentes V, Stepien R.**(2009) Guidelines for the diagnosis and treatment of canine chronic valvular heart disease. *J Vet Intern Med.* Nov-Dec;23(6):1142-50.

2. **Atkins CE, Keene BW, Brown WA et al.** *(*2007) Results of the veterinary enalapril trial to prove reduction in onset of heart failure in dogs chronically treated with enalapril alone for compensated, naturally occurring mitral valve insufficiency. J Am Vet Med Assoc; Oct 1; 231:1061-1069.

3. **Aupperle H, Disatian** (2012) S. Pathology, protein expression and signaling in myxomatous mitral valve degeneration: comparison of dogs and humans. *J Vet Cardiol.* Mar; 14(1):59-71.
   becomes biology's best in show: genome analyses in the

4. **Borgarelli M, Buchanan JW**. (2012)Historical review, epidemiology and natural history of degenerative mitral valve disease. J Vet Cardiol Mar;14(1):93-101.

5. **Borgarelli M, Crosara S, Lamb K, Savarino P, La Rosa G, Tarducci A, Haggstrom** (2012)J.Survival characteristics and prognostic variables of dogs with preclinical chronic degenerative mitral valve disease attributable to myxomatous degeneration *J Vet Intern Med.* Jan-Feb;26(1):69-75.

6. **Borgarelli M1, Haggstrom J**.(2010) Canine degenerative myxomatous mitral valve disease: natural history, clinical presentation and therapy.*Vet Clin North Am Small Anim Pract.* Jul;40(4):651-63.

7. **Boswood A**.(2009)Biomarkers in cardiovascular disease: beyond natriuretic peptides. *J Vet Cardiol.* May;11 Suppl 1:S23-32.

8. **Ceciliani F1, Eckersall D, Burchmore R, Lecchi C.** (2014) Proteomics in veterinary medicine: applications and trends in disease pathogenesis and diagnostics. *Vet Pathol.* Mar;51(2):351-62. Epub 2013 Sep 17.

9. **Correale M1, Totaro A, Abruzzese S, Di Biase M, Brunetti ND** (2012) Acute phase proteins in acute coronary syndrome: an up-to-date. *Cardiovasc Hematol Agents Med Chem* Dec;10(4):352-61

10. **Cui Z, Dewey S, Gomes AV.** (2011) Cardioproteomics: advancing the discovery of signaling mechanisms involved in cardiovascular diseases. *Am J Cardiovasc Dis.*1(3):274-92. Epub 2011 Sep 10.

11. **Devereux RB, Brown WT, Kramer-Fox R, Sachs I**.(1982) Inheritance of mitral valve prolapse: effect of age and sex on gene expression. Ann Intern Med. Dec;97(6):826-32

12. **Don BR, Kaysen G**. (2004) Serum albumin: relationship to inflammation and nutrition. *Semin Dial.* Nov-Dec;17(6):432-7.

13. **Edwards AV1, White MY, Cordwell SJ.** (2008)The role of proteomics in clinical cardiovascular biomarker discovery. *Mol Cell Proteomics.* Oct;7(10):1824-37 Epub 2008 Jul 30.

14. **Evangelopoulos ME1, Toumanidis S, Sotou D, Evangelopoulos C, Mavrikakis M, Alevizaki M, Dimopoulos MA**. (2009) Mitral valve prolapse in young healthy individuals. An early index of autoimmunity? *Lupus.* Apr;18(5):436-40.

15. **French AT, Ogden R, Eland C, Hemani G, Pong-Wong R, Corcoran B, Summers KM.** (2012) Genome-wide analysis of mitral valve disease in Cavalier King Charles Spaniels. *Vet J.* Jul;193(1):283-6. *Genome Res*;10:1271e1274

16. **Karlsson EK, Lindblad-Toh K.** (2008) Leader of the pack: gene mapping in dogs and other model organisms. *Nat Rev Genet.* Sep;9(9):713-25

17. **Kvart C, Häggström J, Pedersen HDet al.** (2002) Efficacy of enalapril for prevention of congestive heart failure in dogs with myxomatous valve disease and asymptomatic mitral regurgitation. *J Vet Intern Med.*; 16:80-88.

18. **Lacerda CM, Disatian S, Orton EC**. (2009)Differential protein expression between normal, early-stage, and late-stage myxomatous mitral valves from dogs. *Proteomics Clin Appl.* Dec;3(12):1422-9

19. **Lam L1, Lind J, Semsarian C**. (2006)Application of proteomics in cardiovascular medicine. *Int J Cardiol.* Mar 22;108(1):12-9. Epub Feb 8.

20. **Lathrop JT1, Anderson NL, Anderson NG, Hammond DJ** (2003) Therapeutic potential of the plasma proteome. *Curr Opin Mol Ther.* Jun;5(3):250-7.

21. **Lindblad-Toh K et al**. (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature.* Dec 8;438(7069):803-19.

22. **Locatelli C**. (2013*)* Degenerazione mixomatosa della valvola mitrale: la più comune patologia cardiaca nel cane. *Missione Veterinaria,* Anno VII n. 73, Aprile

23. **Madsen MB, Olsen LH, Häggström J, Höglund K, Ljungvall I, Falk T, Wess G, Stephenson H, Dukes-McEwan J, Chetboul V, Gouni V, Proschowsky HF, Cirera S, Karlskov-Mortensen P, Fredholm M.** (2011) Identification of 2 loci associated with development of myxomatous mitral valve disease in Cavalier King Charles Spaniels. J Hered. Sep-Oct;102 Suppl 1:S62-7.

24. **Monteleone PL, Fagan LF.** (1969) Possible X-linked congenital heart disease. *Circulation.* May;39(5):611-4.

25. **Ostrander EA, Kruglyak L.** (2000) Unleashing the canine genome. *Genome Res.* 2000 Sep;10(9):1271-4.

26. **Oyama MA1, Chittur SV** (2006) Genomic expression patterns of mitral valve tissues from dogs with degenerative mitral valve disease. *Am J Vet Res*.Aug;67(8):1307-18.

27. **Parker HG, Kilroy-Glynn P.** (2012) Myxomatous mitral valve disease in dogs: does size matter*? J Vet Cardiol.* Mar;14(1):19-29.

28. **Parker HG, Shearin AL, Ostrander EA.** (2010) Man's best friend: domestic dog. Annu Rev Genet;44:309e336.

29. **Pouchelon JL, Jamet N, Gouni V et al.** (2008) Effect of benazepril on survival and cardiac events in dogs with asymptomatic mitral valve disease: a retrospective study of 141 cases. *J Vet Intern Med* 22:905-914.

30. **Roland R.J. van Kimmenade, MD, James L. Januzzi Jr** (2009) The evolution of the natriuretic peptides Current applications in human and animal Medicine *Journal of Veterinary Cardiology* 11, S9eS21

31. **Rusch J E.** (2009) Chronic valvular disease in dogs. In *Kirk's Current Veterinary Therapy XIV edn*. Eds R.W. Kirk, J.D. Bonagura. W.B. Saunders, Philadelphia. Pp 780-786

32. **Rush JE1, Lee ND, Freeman LM, Brewer B.** (2006) C-reactive protein concentration in dogs with chronic valvular disease. *J Vet Intern Med*. May-Jun;20(3):635-9.

33. **Tan HT, Ling LH, Dolor-Torres MC, Yip JW, Richards AM, Chung MC. Uechi M**.(2012) Mitral valve repair in dogs. J Vet Cardiol. Mar;14(1):185-92

34. **Tan HT1, Ling LH, Dolor-Torres MC, Yip JW, Richards AM, Chung MC.** (2013) Proteomics discovery of biomarkers for mitral regurgitation caused by mitral valve prolapse. *J Proteomics*. Dec 6;94:337-45.

35. **Thomas, W. P., Gaber, C. E., Jacobs et al.** (1993) Recommendations for standards in transthoracic two-dimensional echocardiography in the

dog and cat. Echocardiography Committee of the Specialty of Cardiology, American College of Veterinary Internal Medicine. Journal of Veterinary Internal Medicine 7, 247-252

36. **Uechi M.,** (2012)Mitral valve repair in dogs *J Vet Cardiol.* Mar;14(1):185-92

37. **Van Eyk JE.** (2011) Overview: the maturing of proteomics in cardiovascular research. *Circ Res.* Feb 18;108(4):490-8.

38. **Van Kimmenade RR1, Januzzi JL Jr.** (2009) The evolution of the natriuretic peptides - Current applications in human and animal medicine. *J Vet Cardiol.* May;11 Suppl 1:S9-21.

39. **White MY, Van Eyk JE.** (2007) Cardiovascular proteomics: past, present, and future. *Mol Diagn Ther*.;11(2):83-95. Review.

40. **Whitney J.** C., (1974) Observations on the effect of age on the severity of heart valve lesions in the dog. *J Small Animal Practice*, 15, 511-522

41. www.who.int/gho/ncd/mortality_morbidity/cvd/en/index.html

42. **Yamamoto E1, Sato Y, Sawa T, Fujiwara T, Fujiwara H, Takatsu Y** (2012) Correlation between serum concentrations of B-type natriuretic peptide and albumin in patients with chronic congestive heart failure *Int Heart J.* 53(4):234-7.