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***“CARDIORENAL SYNDROME-ANEMIA” COMPLEX
IN SMALL ANIMAL MEDICINE:
RESEARCH IN DIAGNOSTIC***

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ABSTRACT

Cardiorenal syndrome (CRS) can be defined as a pathophysiologic disorder of the heart and kidneys whereby acute or chronic dysfunction of one organ may induce acute or chronic dysfunction of the other. The most common acquired heart disease affecting old dogs and leading to congestive heart failure (CHF) is myxomatous mitral valve disease (MMVD). The worsening of cardiac performances and reduction of renal perfusion contribute to the development of chronic kidney disease (CKD) (CRS type 2). In cats the most common cardiac disease is hypertrophic cardiomyopathy (HCM), a primary myocardial disorder characterized by increased cardiac mass and a hypertrophied, not dilated, left ventricle. Anemia is often associated with heart failure and renal insufficiency in an unfavorable triad called “CRS-anemia” complex. Iron deficiency can be present, alone or in combination with anemia, worsening quality of life and shortening survival.

The aim of the thesis was to describe cardiorenal syndrome in small animal medicine through the identification of general comorbidities and particular novel diagnostics tools to improve fast diagnosis and the medical approach to the cardiovascular diseases in small animal medicine.

Firstly, iron status in dogs with MMVD was evaluated; although not as frequent as in humans, iron deficiency was recognized in almost the 20% of dogs with MMVD, with a 6.3 higher risk of being included in a higher ACVIM class.

Symmetric dimethylarginine, a novel biomarker of glomerular filtration rate, was evaluated on sera of dogs with MMVD and proved to be not influenced by the presence of the heart condition and can thus be considered a reliable biomarker in these dogs.

Creatine-kinase was measured in sera of healthy cats and cats with different forms of cardiomyopathy to highlight its role in the early diagnosis of the disease; the results suggest that feline macro-CK1 may have a different structure compared with other species and a potential role of CK-MB in the evaluation of feline cardiomyopathies.

Accuracy and reliability of D-Heart, the first portable, multiple lead smartphone electrocardiograph in the canine patient, was evaluated, for rapid diagnosis of arrhythmias. The device proved effective and accurate recording of ECG in the canine patient.

Finally, the selected ELISA kit to detect aldosterone in canine urine was found to be accurate and effective, constituting a simple, safe and economical alternative to the radioimmunoassay method. Comparison between healthy dogs and dogs with MMVD (B1) showed no statistically significant difference in urinary aldosterone:creatinine (UAldo:C) ratio. The mean values of UAldo:C ratio in the present study were greater than those reported in literature, suggesting the need of a deep study to re-evaluate the normality threshold set by the literature (1.0 µg/g), which does not seem to be valid in the population of the present study.

ABSTRACT (Italian version)

La sindrome cardiorenale (CRS) può essere definita come un disordine fisiopatologico di cuore e reni in cui la disfunzione acuta o cronica di un organo può indurre una disfunzione acuta o cronica dell'altro.

La cardiopatia acquisita più comune che colpisce i cani anziani e che porta a insufficienza cardiaca congestizia (CHF) è la degenerazione mixomatosa della valvola mitrale (MMVD). Il peggioramento della performance cardiaca e la riduzione della perfusione renale contribuiscono allo sviluppo della malattia renale cronica (CKD) (CRS tipo 2). Nei gatti la più frequente malattia cardiaca è la cardiomiopatia ipertrofica (HCM), un disturbo miocardico primario caratterizzato da un aumento della massa cardiaca ipertrofia ventricolare sinistra. L'anemia è spesso associata a insufficienza cardiaca e insufficienza renale in una triade sfavorevole denominata "complesso sindrome cardiorenale - anemia". La carenza di ferro può essere presente, da sola o in combinazione con l'anemia, con peggioramento della qualità della vita e riduzione della sopravvivenza.

Lo scopo della tesi era di descrivere la sindrome cardiorenale nella medicina dei piccoli animali attraverso lo studio delle comorbidità e degli strumenti diagnostici innovativi per migliorare la diagnosi rapida e l'approccio medico alle malattie cardiovascolari.

In primo luogo, è stato valutato lo stato di ferro nei cani con MMVD; sebbene non così frequente come negli esseri umani, la carenza di ferro è stata riconosciuta in quasi il 20% dei cani con MMVD,

con un rischio di 6,3 maggiore di essere incluso in una classe ACVIM più alta per cani con basse concentrazioni di ferro.

La dimetilarginina simmetrica, un nuovo biomarker di velocità di filtrazione glomerulare, è stata valutata su sieri di cani con MMVD e si è dimostrata non influenzata dalla presenza della malattia cardiaca, potendo quindi essere considerata un biomarker di funzionalità renale affidabile in questi cani.

La creatin-chinasi è stata misurata in sieri di gatti sani e gatti con diverse forme di cardiomiopatia per evidenziare il suo eventuale ruolo nella diagnosi precoce della malattia; i risultati suggeriscono che la macro-CK1 felina può avere una struttura diversa rispetto a quella di altre specie e che, fra le frazioni espresse, CK-MB potrebbe avere un ruolo nella diagnosi delle cardiomiopatie feline.

La precisione e l'affidabilità di D-Heart, il primo elettrocardiografo portatile a più derivate utilizzabile nel paziente canino, sono state valutate, per la diagnosi rapida delle aritmie. Il dispositivo ha dimostrato una registrazione efficace e accurata dell'ECG nei cani inclusi nello studio.

Infine, il kit ELISA selezionato per rilevare l'aldosterone su campioni di urina di cane è risultato essere accurato ed efficace, costituendo un'alternativa semplice, sicura ed economica al metodo RIA.

Il confronto tra cani sani e cani con MMVD (B1) non ha mostrato differenze statisticamente significative nel rapporto aldosterone urinario:creatinina (UAldo:C). I valori medi del rapporto UAldo: C nel presente studio erano maggiori di quelli riportati in letteratura, suggerendo la necessità di uno studio approfondito per rivalutare la soglia di normalità stabilita dalla letteratura (1.0 µg/g), che non sembra essere valida nella popolazione del presente studio.

STATE OF ART

Cardiovascular diseases in dogs

The study of the cardiovascular system has been a challenge for researchers of all ages. Aristotle (384 - 322 BC), considered the first expert in comparative anatomy, began his research with the dissection of animal bodies, since it was not possible to dispose of human cadavers. After that came Galen (129 - 217 AD), Leonardo da Vinci (1452 – 1519) and Andreas Vesalius (1514 - 1564), that, in different times, added knowledge to this intricate puzzle. It was from the works of the Italian surgeon and anatomist Girolamo Fabrizzi (1537 - 1619) that the valves structures and possible function were first identified.

William Harvey (1578 - 1657) first postulated that blood could be in continuous movement inside the body, guaranteed by the heart as a pump, and undertook open chest experiments in different animals, even dogs, to confirm his theory. At the time, arteries were believed to actively dilate during diastole. The first heart catheterizations were performed in the early 1900.

Franz Hutyra (1860 - 1934) and Josef Marek (1868 - 1952), from the Budapest Royal Veterinary College, are considered pioneers of the veterinary medicine and first describers of several techniques to study the heart of living animals, like percussion or auscultation. David K. Detweiler (1920 - 2009) is actually considered “Father of Veterinary Cardiology” due to his tireless work in analysing thousands of dogs and describing his findings in the most reliable and rigorous way possible. Another pivotal moment surely was the introduction of echocardiography in the late 70’s, still considered the most useful, non-invasive method for diagnosis and study of the cardiovascular structure and function.

Myxomatous mitral valve disease (MMVD) is the most common acquired heart disease diagnosed in dogs and has been recognized for over 100 years. The first epidemiologic studies were conducted in the 1960s and revealed greater frequency in small breed dogs, in particular among Cocker spaniels, Dachshunds and Poodles [Buchanan, 2013].

MMVD is considered the most common cause of congestive heart failure in canine population. The 30% of animals diagnosed with MMVD develop mitral valve regurgitation and, with the progression of the disease, heart failure. [Parker, 2012].

In the worldwide canine population, the disease is recognized in a number of patients between the 3 and the 7%. [Borgarelli, 2012] and, in the only United States, with an estimated dog population of 77.7 million, this percentage translates into among 2.3 and 3.5 million affected animals. [Borgarelli, 2012].

The breed most affected by MMVD is nowadays the Cavalier King Charles Spaniel, followed by the German Dachshund and the Poodle. [Parker, 2012] Sex is also an important variable: males tend to develop the pathology earlier than females; [Borgarelli, 2010] most of the affected animals do not exceed 9 kg in weight. [Fleming, 2011; Parker, 2012].

Endocardiosis affects in the 62% of cases only the mitral valve, in 32.5% the mitral valve and the tricuspid valve, and in 1.3% only the tricuspid valve. [Haggstromm, 2004].

Histologically, the disease is characterized by a degenerative dystrophic process affecting the connective tissue, within which the number of glycosaminoglycans (GAG) increases.

Microscopically, the spongy component of the valve is protruding, due to the increase in GAG, and cellular abnormalities and cytoskeleton disorganization can be observed. Endothelial cells, in some areas, are absent, with exposure of the underlying tissue layers. [Haggstromm, 2004].

The etiopathogenesis of MVD is still partially unknown, although several hypotheses have been advanced. Orton advocated a mechanical stretch and tension that induce collagen remodeling of the inner valvular layers, and mitral leaflet deformation [Orton, 2012]. Oyama and Ljungvall speculate on humoral/circulating factors as responsible for valvular degeneration [Oyama, 2010; Ljungvall, 2013]. Inflammatory pathways are also up regulated in dogs with MVD and a dysregulation of the ECM (extracellular matrix) of the mitral inner valvular layers has been found. [Oyama, 2006]

The development of endocardiosis causes a decrease in the ability of the valve leaflets to work correctly, with loss of elasticity and ability to close properly. This, associated with the positive

pressure inside the left ventricle developed during the systole, causes the prolapse of the leaflets, with displacement beyond the valvular annulus [Borgarelli, 2008].

This represents the basis for the appearance of a blood flow from the left ventricle to the left atrium (speaking of mitral valve), defined pathological because the blood moves in the opposite direction to normality: the "mitral regurgitation".

Prolapse increases the mechanical stress to which the valve is subjected, while regurgitation is responsible even for traumatic endothelial injuries that can further nourish the inflammatory chronic status and predispose to the development of endocarditis in presence of known comorbidities. Furthermore, collagen and other components of the extracellular matrix are exposed to blood flow in areas where the endothelium disappears, and this may promote the appearance of thrombotic phenomena. [Borgarelli, 2010].

The pathophysiological changes that occur in the animal are imputable to the volumetric overload of the left side heart once the valve incompetence is established. [Nelson – Couto, 2010].

Valve regurgitation usually develops over a prolonged period of time, so the trend of the disease is slow. In the initial phase, no apparent changes in myocardial function or cardiac volume indices are induced, the flow rate is conserved, and the regurgitation is supported by the left atrium. [Nelson – Couto, 2010]. As the disease progresses, the regurgitation volume increases and compensatory mechanisms, both cardiac and non-cardiac, are established. [Tilley, 2007]. The more blood flows back to the atrial level, the less enter into the aorta; consequently, the tissue perfusion is decreased. This activates the sympathetic nervous system baroreceptors. In addition, hypoxemia and hypoperfusion are sensed by the kidneys, stimulating the activation of the Renin - Angiotensin - Aldosterone system (RAAS), a physiological compensatory mechanism.

Cardiovascular diseases in cats

Feline cardiomyopathy was recognized in 89% of cats with arterial thromboembolism in the 1950s and 1960s; was then described as “chamber enlargement, endocarditis, and myocarditis or infarction” [Buchanan, 2013].

Myocardial diseases, or cardiomyopathies, are currently considered the most common heart disease diagnosed in cats. Cardiomyopathies are defined by the World Health Organization (WHO) as diseases of myocardium associated with cardiac dysfunction. Feline cardiomyopathies are currently classified following the WHO guidelines adapted for the feline patients, although this classification is not exempt from limitations, mostly attributable to the mixing of anatomical (i.e. hypertrophic) and functional (i.e. restrictive) terms [Ferasin, 2009]. Moreover, different cardiomyopathies with different onset and trend, can converge towards similar or identical patterns in their advanced stage, due to the substantial overlapping of compensatory mechanism, making it difficult to differentiate and formulate the correct diagnosis. [Ferasin, 2009]

Hypertrophic cardiomyopathy (HCM) is currently considered the most common heart disease in cats, accounting for around the 75% of the heart disease diagnosed in this species. It is characterized by a symmetric – asymmetric increase in left ventricle wall thickness and diastolic dysfunction with preserved ejection fraction; the disease can be silent for a long period of time, depending on the severity of the hypertrophy, but can lead to heart failure. Hypertrophy can develop as a consequence of an inherited condition, in which a mutation of the genome, mainly in the areas designated for sarcomeric protein codification (primary HCM), or as a consequence of ventricular pressure overload, like in systemic hypertension, of hyperthyroidism or hypersomatotropism. The presence of diastolic dysfunction progressively increases the filling pressure, that will be compensated at the beginning by left atrium enlargement and the same compensatory mechanism explained for the MMVD will activate; the exhaustion of compensatory mechanism and progressive increases in left atrium pressure will finally results in congestive heart failure [Tilley, 2007].

THE CARDIORENAL SYNDROME

Definition and classification

Cardiorenal syndrome is a clinical complex condition that involves two main actors: the heart and the kidney, both concurring to its pathophysiology. It was defined in 2008 by the Acute Dialysis Quality Group as “disorders of the heart and kidneys whereby acute or chronic dysfunction in one organ may induce acute or chronic dysfunction of the other” [Ronco, 2008]. The effective classification proposed by the Group used to divide CRS into four type, according to the first organ implied, and sub staged as acute or chronic. A fifth type is referred to a systemic disease that affects both organs at the same type [Ronco, 2008].

The consensus classification includes:

- Type I (acute cardiorenal), where acute heart failure (HF) is directly associated with acute kidney injury (AKI). It occurs in around the 25% of human patients hospitalized for acute HF.
- Type II (chronic cardiorenal), in which chronic heart failure is associated with chronic kidney disease (CKD). In human, close to 50% of patients with chronic heart failure have a glomerular filtration rate (GFR) $< 60\text{ml/min/1.73m}^2$ and, during follow up, worsening renal function is a strong independent predictor of negative outcome [Di Lullo, 2017].
- Type III (acute renocardiac), where acute kidney injury (AKI) is associated with acute heart failure.
- Type IV (chronic renocardiac), in which the driving factor of CKD is associated with chronic heart failure.
- Type V (secondary cardiorenal), where there is concomitant development of both kidney and heart failure due to an underlying systemic disorder (diabetes, hypertension, amiloidosis).

In 2015, a veterinary consensus statement published by Pouchelon et al. proposed a veterinary definition and classification of CRS, based on the human model but with some slight adjustments [Pouchelon, 2015].

The definition proposed by the Cardiovascular-Renal Axis Disorders (CvRDs) Consensus Group is “disease-induced, toxin-induced, or drug-induced structural and/or functional damage to the kidney or to the cardiovascular system leading to disruption of the normal interactions between these systems, to the ongoing detriment of one or both applies to different clinical presentations” [Pouchelon, 2015].

The Consensus Group decided to rename CRS as CvRD (= Cardiovascular Renal Disorder), and to classify it as follows:

- CvRD-H, in which renal impairment is caused by a primary cardiovascular pathology
- CvRD-K, in which a kidney disease is causing heart dysfunction
- CvRD-O, characterized by a concomitant heart and kidney dysfunction, due to an underlying systemic disorder, drug assumption or toxin activity.

These three categories can be further divided based on the clinical presentation of the patient in stable condition (S) or unstable condition (U).

Epidemiology

The exact nature and prevalence of cardiorenal syndrome is still unclear.

In human medicine, recent studies estimated that around 1 of every 3 adults in the United States has experienced some form of cardiovascular disease (hypertension, coronary heart disease, heart failure (HF), stroke, or congenital heart disease), and almost 13% of the US population has some form of CKD [Rosner, 2011]. As reported by Bongartz in 2005, cardiovascular diseases are cause of the 43.6% of all deaths in patients with end-stage renal disease (ESRD) and death from cardiac causes is 20 times more common in patients with CKD than in general population [Bongartz, 2005].

In veterinary medicine, few studies have been published. The prevalence of azotemia in dogs with heart disease varies between 7 and 25%. Martinelli et al in 2016 published an original paper on the cardiorenal disorders in dogs affected by MMVD.

This retrospective paper analysed the prevalence of azotemia and anaemia in a population of dogs with MMVD, confirming a prevalence of 25% for CKD and the presence of a direct correlation by the stage of the kidney disease, represented by the international renal interest society (IRIS) and selected echocardiographic parameters like left atrium/aorta ratio, suggesting a direct connection between cardiac remodeling and renal impairment. In particular the paper reported a prevalence of azotemia in patients with asymptomatic MMVD (ACVIM B1) of the 12 %, suggesting the presence of primary and early renal damages [Martinelli, 2016].

“The Cardiorenal Connection”

This term was introduced in 2005 by Bongartz and colleagues to group all the mechanism that can explain how volume and blood pressure are controlled in the body and, by extension, how cardiorenal syndrome can be generate when there is a derangement [Bongartz, 2005].

It is based on the RAAS, the balance between NO and reactive oxygen species (ROS), inflammation, and the action of sympathetic nervous system (SNS), as cornerstones of the connection between the heart and the kidneys. When there is an imbalance of one of these elements, the others can be disturbed as well, and the result of disequilibrium and synergies can finally result in cardiac and renal structural and functional damages [Bongartz, 2005].

➤ The RAAS

The renin-angiotensin-aldosterone system is a neurohormonal cascade that begins at renal level and helps restore blood pressure and volume as a defense against hypoperfusion of vital organs, during, for example, hemorrhages. Stimuli such as hypovolemia, hypotension, hyponatremia and sympathetic nervous system activation promotes renin secretion by the juxtaglomerular apparatus and, subsequently, the conversion of angiotensinogen, a prohormone released by the liver, into angiotensin I. The Angiotensin-converting Enzyme (ACE) present in the circulation converts, by a cleavage action, angiotensin I into angiotensin II. Angiotensin II and vasopressin in turn regulate the production of renin with a negative feedback. Angiotensin II is a potent vasoconstrictor and stimulate the release of aldosterone, a steroid hormone produced by the glomerular area of the adrenal cortex. Aldosterone

mediates the reabsorption of sodium from the urine, increases the reabsorption of water and, therefore, increases the volemia. It is also able to induce the sense of thirst. In heart failure (HF), the activation of this system is due to the hypoperfusion mediated by inadequate heart function; at first, this mechanism has short term positive effects in maintaining hemodynamic stability but results in long term detrimental effects like volume overload, increased in systemic and pulmonary resistances and decreased in heart contractility. Volume retention is mainly imputable to the reabsorptive action of angiotensin II (Ang II), with further congestive effects as a consequence. Inappropriate activation of the RAAS is not only prerogative of HF but also of kidney diseases and this can further complicate this scenario [Nelson Couto, 2010; Kittleson, 1998]. Besides the dysregulation of fluid volume and vasoconstriction, the RAAS can have further deleterious effects in connections with the other pathophysiological mechanism. In fact, Ang II activate NADPH-oxidase, leading to the production of reactive oxygen species (ROS) in various districts, like endothelial cells, vascular smooth muscle cells, renal tubular cells and cardiomyocytes. A study from Heymes and colleague documented an increase activity of the NADPH oxidase enzyme in heart tissue of patients affected by HF [Heymes, 2003]. Moreover, Ang II is implicated in changing of the cell's oxidative status, causing vascular inflammation. Detrimental effects of the inadequate activation of the RAAS can be counterbalanced with drugs like ACE inhibitors, that could attenuate angiotensin conversion enzyme activity and inhibit the cascade; the ACE inhibitors can also reduce the production of ROS and his release in circulation, increase the bioavailability of nitric oxide (NO) and reduce the hyperactivity of SNS [Bongartz, 2005].

➤ The balance between NO and ROS

Nitric oxide (NO) is a molecule produced from L-arginine and oxygen in a reaction catalyzed by the enzyme nitric oxide synthase (NOS). It has an important role as a messenger in biological systems, in particular in the cardiovascular, nervous and immune system. The main site of its synthesis is the endothelium and, from there, NO diffuses to underlying smooth muscle cells causing vasodilation, increases in blood flow, decreases in blood pressure, and, in the renal district, natriuresis and

desensitization of tubuloglomerular feedback. Nitric oxide's role in dilating blood vessels makes it an important controller of blood pressure. It has a fundamental role in the regulation of blood pressure and its levels tend to fall in systemic hypertension. On the other hand, his balance with ROS has to be considered, since when the ROS levels rises, as in cardiorenal syndrome, there is less bioavailability of NO. In patients with renal failure, NO reacts with ROS and asymmetric dimethyl arginine (ADMA) released in the blood circulation and this cause NO-deficiency. Also, in HF increased oxidative stress has been demonstrated and decreased antioxidant status has been associated with progression of the disease. Oxidative stress is strongly connected to the inflammatory status, since it can lead to overproduction of pro-inflammatory cytokines, in particular IL-1, IL-6 and TNFalpha [Bongartz, 2005].

➤ Inflammation

Several pro-inflammatory and inflammatory mediators were found over expressed in HF and KD, being on one hand the expression of the inflammatory status mediated by the presence of a chronic pathology and, on the other hand, contributing to the pathogenesis of the cardiorenal syndrome. In chronic KD, the levels of C reactive protein, IL-1 β , IL-6 and TNFalpha can predict atherosclerosis. In HF, plasma and myocardial levels of TNFalpha and IL-6 are predictors of the trend of the syndrome; IL-18 is associated with myocardial dysfunction [Bongartz, 2005].

The interconnections within biological systems are so close that this constant, low grade, inflammation can generate ROS production and contribute to imbalance between ROS and NO; moreover, IL and ROS enhance renin secretion and the expression of the receptor 1 for angiotensin (AT1), providing evidence of a further, possible link between chronic inflammatory status and RAAS [Bongartz, 2005].

➤ Sympathetic nervous system (SNS)

The activation of the SNS is subordinated to the function of aortic and carotid baroreceptors, which, once sensed the hypoperfusion, causes an increase in the discharge frequency of the sinus node, with a positive chronotropic effect, and the activation of the β_1 myocardial receptor, with a positive inotropic effect, in order to provide the preservation of the cardiac output. When the heart rate is too high, however, the systole becomes less effective, decreasing the cardiac output and leading to apoptosis in cardiomyocytes, myocardial hypertrophy and focal necrosis. Furthermore, the activation of the SNS causes reflex vasoconstriction at the peripheral level (abdominal viscera and skin). The kidney, not protected by vasoconstriction, will respond to hypoperfusion by increasing renin release. Peripheral vasoconstriction also causes an increase in the afterload [Ettinger, 2017; Kittleson, 1998]. SNS is the first line response when it comes to hypovolemia. Excesses in SNS activation can promote apoptosis of the cardiomyocytes, myocardial necrosis and left ventricle hypertrophy, partially imputable to the direct effect of catecholamines, with involvement of ROS. Moreover, the chronic, hyperactivation of SNS causes a progressive loss of sensitivity of the beta adrenoceptor, responsible for positive chronotropic, dromotropic and inotropic effects. This can lead to progressive reduction in heart rate variability and tendency to arrhythmia. Also, Renin release can be significantly increased by the chronic activation of the SNS, having an effect on promoting the growth of the intrarenal blood vessels; this mechanism is mediated by ROS production [Bongartz, 2005].

Pathophysiology of CvRDH (S)

Different elements concur to the development of the syndrome. Chronic heart failure constantly reduces renal perfusion. The fluid overload induces chronic renal congestion. The continuous activation of SNS lead to overproduction of epinephrine, angiotensin, endothelin, and release of natriuretic peptides and nitric oxide [Di Lullo, 2017].

Diuretics drugs, together with ACE inhibitors, are used to reduce volume overload and congestion, but can have side effects due to possible hypovolemia and hypotension, increasing in turn kidney damage. In MMVD, the valve incompetence causes systolic regurgitation in the left atrium, and

decreasing stroke volume [Borgarelli, 2010; Petric, 2015]. In an attempt to restore blood pressure and tissue perfusion, compensatory mechanisms, supported by the actions of sympathetic nervous system and renin-angiotensin-aldosterone system (RAAS), are activated. Nevertheless, the worsening of the cardiac performance and the hemodynamic consequences of reduced cardiac output (CO) determine low renal perfusion, nephrons loss, and contribute to the development of renal insufficiency in the setting of heart failure [Buglioni, 2015]. The continuous activation of RAAS plays a prominent role in initiating renal dysfunction, salt and water retention, and venous congestion as well [Borgarelli, 2010].

A study from Hillege et al investigated the renal function as a predictor of outcome in patients with different heart condition and reported that the presence of renal impairment constitutes an independent predictor of outcome and increased mortality [Hillege, 2006].

Pathophysiology of CvRDK (S)

Chronic kidney disease (CKD) has long term deleterious effects on systolic function and can induce left ventricular hypertrophy. Anemia, that can be found in association to CKD due to the reduction of circulating erythropoietin, iron and folic acid deficiency and chronic inflammation, can in turn reduce left ventricular systolic dysfunction. Moreover, the management of HF in patients with CKD is particularly challenging due to the fact that the proposed treatments often act on opposite sides and can result in undertreatment of volume overload and congestion in order to reduce further kidney damages. In human patients, since early stage of CKD echocardiography report the occurrence of impairment in ejection fraction and increases in left ventricular end systolic and diastolic diameters [Di Lullo, 2017].

Some of the mechanism proposed to explain this progression are pressure and volume overload, cause and effect of the decrease in glomerular filtration rate. With the progression of the disease, systemic hypertension can occur, increasing left ventricular hypertrophy and, consequently, ischemic damages [Di Lullo, 2017].

AIMS

Aims of the thesis

General aim of the project was to describe cardiorenal syndrome in small animal medicine through the identification of general comorbidities and particular novel diagnostics tools to improve on one hand the diagnosis and on the other hand the medical approach to the cardiovascular diseases in small animal medicine.

Specific aims were:

- I. to determine the iron status in dogs affected by MMVD, analysing whether any difference in serum iron values was present between the American College of Veterinary Internal Medicine (ACVIM) classes, between symptomatic and non-symptomatic patients for HF and with respect to selected echocardiographic parameters. Moreover, we investigated the possible existence of any difference in serum iron concentration among patients presenting MMVD and azotemia as a model of cardiorenal syndrome. A final aim was to evaluate the presence of an association between low serum iron concentration and patient's survival time.
- II. to determine the reliability of serum SDMA as a novel biomarker of early renal impairment in dogs affected by MMVD in different American College of Veterinary Internal Medicine (ACVIM) classes, with serum creatinine within the normal range of values, as an animal model of cardiorenal syndrome type 2.
- III. to investigate the presence of changes in percentage or activity of CK-MB, determined through the electrophoretic separation of isoenzymes, in serum of cats affected by myocardial disease compared to healthy cats. The investigation on the total activity of CK and its macro and isoenzymes in serum of cardiopathic cats may contribute to elucidate the specific pattern of distribution of CK macro and isoenzymes in this species in order to

understand if CK can be a suitable biomarker of occult heart disease in cats and especially in cats with CKD as an animal model of cardiorenal syndrome type 4.

- IV. to evaluate the accuracy and reliability of D-Heart Vet as the first portable, multiple lead smartphone electrocardiograph in the canine patient, compared to the standard ambulatory 6-lead ECG.
- V. to evaluate the performances of a non-species-specific ELISA kit available on the market for the measurement of urinary aldosterone in dogs and to evaluate the urinary aldosterone:creatinine ratio in a population of healthy dogs and dogs affected by MMVD.

DESCRIPTION OF STUDIES

Iron status in dogs with myxomatous mitral valve disease

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Anaemia and Iron deficiency

In human patients, CHF comes with several comorbidities, that have been identified over the years. The most important so far was anemia, that can be defined as a reduction of red blood cells (RBC) number, hemoglobin or haematocrit values [Weiss, 2010]. Although the causal relationship between HF and anemia remains poorly defined, there are multiple potential mechanisms by which the CHF syndrome could contribute to the development of anemia; these include hemodilution, renal dysfunction, production of proinflammatory cytokines, decreased perfusion of the bone marrow, and drug therapy [Arora, 2014]. In reality, it is likely that several of these mechanisms are active simultaneously, and that anemia in CHF is the result of a complex interaction between cardiac performance, neurohormonal and inflammatory activation, renal dysfunction and bone marrow responsiveness [Arora, 2014]. In human medicine, recent studies have highlighted the role of iron deficiency (ID) in heart failure (HF) patients. In fact, anemia is a well-known comorbidity in human patients affected by CHF, with a prevalence from clinical studies reported to be around 40% [Arora, 2014; Fitzsimmons, 2015; Klip, 2013; Okonko, 2011] and considered an independent prognostic factor in cardiologic patients. Dysregulation of iron metabolism was generally considered relevant only in association with anaemia, which have a prevalence of approximately 50% (Okonko, 2011; Arora, 2014).

Nevertheless, recent studies have found that the prevalence of serum iron deficiency (SID) in HF can be higher than the prevalence of anaemia and varies from 37% to 61% (Wong, 2016). In these

patients, ID can also be present in the absence of anaemia and is associated with the worst symptoms (Klip, 2013, Rangel, 2014, Fitzsimmons, 2015). ID represents an increased risk of mortality and morbidity itself (Klip, 2013, Rangel, 2014, Fitzsimmons, 2015). Moreover, ID is responsible for exercise intolerance, reduction in quality of life, and can promote cardiac remodelling, as shown in murine animal models (Nayto, 2009, Wong, 2016). Myocytes and cardiomyocytes are in fact cells with elevated energy demand, for which the maintenance of a correct iron metabolism is fundamental. Alterations in this metabolism can promote impairment in oxidative and cellular energy metabolism (Arora, 2014).

The pathogenetic hypotheses proposed to explain ID in HF are: insufficient dietary iron intake, poor gastrointestinal iron absorption due to intestinal mucosa oedema, hepcidin overexpression due to a chronic inflammatory state caused by HF, and the use of certain drugs such as anticoagulants or ACE inhibitors that can reduce erythropoietin production (Van der Meer, 2004, Opasich, 2005, Kazory, 2009).

ID can be defined as absolute or functional and both can be found in patients affected by heart diseases (Jankowska, 2013). In fact, impaired absorption from the gastrointestinal (GI) tract, chronic blood loss and decrease of dietary intake can mediate absolute ID, but since HF is a chronic condition that promotes inflammatory status of the organism, functional ID due to cytokines and hepcidin overexpression can be present (Wong, 2016).

To assess iron status, in human medicine, ferritin can be used. Indeed, ferritin has an important role in the inflammatory cycle and represents both a well-known marker of iron stores as well as an important biomarker of inflammation, that could be present in chronic diseases (Bohn, 2015). It has been reported that ferritin levels can be high in the presence of several pathologies such as acute respiratory distress syndrome, coronary artery disease, hypertension and myocardial infarction (Kell, 2014). Moreover, immunoassays for human serum ferritin cannot be used in other species, so individual assays must be developed and calibrated for each species (Andrews, 2010).

Material and methods

This is a retrospective study performed on medical records and stored serum samples collected between January 2015 and June 2016 from dogs admitted to the Cardiology Unit of the Department of Veterinary Medicine (DIMEVET) of the University of Milan.

The inclusion criteria were as follows: a diagnosis of MMVD and a complete medical record including physical examination, chest X-ray and echocardiographic evaluation, complete blood count (CBC) and serum biochemical panel. All dogs affected by congenital or acquired heart diseases different from MMVD, as well as by systemic diseases, except chronic kidney disease (CKD), were excluded.

A full echocardiographic examination was performed using standardized thoracic imaging planes according to the recommendations of the American Society of Echocardiography [Thomas, 1993; Bonagura, 2000]. From 2D view we obtained: Aortic root (Ao) and Left atrial (LA) dimension (right parasternal short-axis view). Mitral valve inflow (E peak velocity, E/A ratio) and peak velocity of mitral and tricuspid regurgitations (MR and TR) were obtained through colour and spectral Doppler. We then calculated: left atrial-to-aortic root ratio (LA/Ao), End systolic volume index (ESVI), End diastolic volume index (EDVI), according to the Teichholz formula normalized to body surface area (BSA). The MMVD patients were categorized based on the ACVIM classification [Atkins, 2009]. As recommended by the ACVIM guidelines, stage B included dogs affected by MMVD without signs of CHF (namely, in stage B1, dogs without evidence of cardiac enlargement; in B2, dogs with signs of cardiac remodelling as LA and/or LV enlargement); stage C dogs with clinical signs of CHF due to MMVD, and stage D subjects presenting both clinical signs of CHF and refractoriness to standard medical treatments [Atkins, 2009; Boswood et al. 2016].

Doppler blood pressure (BP) measurements were performed in all patients as reported in the ACVIM guidelines and was considered normal when ≤ 150 mmHg [Brown, 2007].

The International Renal Interest Society (IRIS) guidelines were used to classify patients affected by CKD, and dogs were considered azotemic if their serum creatinine (sCr) values were ≥ 1.4 mg/dl. According to IRIS, patients were classified as IRIS 2 (sCr between 1.4 and 2 mg/dl), IRIS 3 (sCr

between 2.1 and 5.0 mg/dl) and IRIS 4 (sCr >5.0 mg/dl). Sub staging was performed based on BP and the presence of proteinuria. Proteinuria was investigated through urine creatinine to protein ratio (UPC) and considered present when $UPC \geq 0.5$ [IRIS 2015].

Blood samples were collected from the brachiocephalic vein during routine clinical evaluation. For CBC, EDTA was used as anticoagulant, whereas for biochemical evaluation, the blood was placed in tubes without anticoagulant and centrifuged at $3250 \times g$ for 5 minutes. The CBC was performed using an automated laser haematology analyser (Sysmex XT-2000iV; Sysmex corporation; Japan). For every CBC, red blood cells (RBC), haematocrit (Ht), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red distribution width (RDW) and platelet count were considered. Simultaneous evaluation of the blood smear was performed and the degree of anisocytosis and poikilocytosis was reported, together with a description of the main erythrocytes' alterations. Anaemia was defined as a haematocrit value $\leq 37\%$ [Tvedten, 2010]. Routine biochemistry was carried out with an automated chemistry analyser (Cobas Mira; Roche diagnostics, Switzerland) with reagents provided by Hagen diagnostic system (Italy). The serum samples were kept frozen ($-20^{\circ} C$) until the iron analyses were performed.

As described by Zaldivar-Lopez and colleagues, iron status was evaluated by measuring SIC (normal value 90 - 200 mcg/dL) and total iron-binding capacity (TIBC, normal value 270-496 $\mu g/dL$) [Zaldivar-Lopez, 2015]; the TIBC was calculated by adding the unsaturated iron binding capacity (UIBC) to the SI. Percentage transferrin saturation (%SAT, normal value $> 23\%$) was obtained using the formula $\%SAT = SI/TIBC \times 100$ [Andrews, 2010].

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 23 [Release 23.0.0.0]. Normality of the distribution was tested using the nonparametric Shapiro Wilk test. Baseline descriptive statistics were presented as the mean and standard deviation for normally distributed continuous variables, whereas non-normally distributed variables were presented as median and interquartile (IQ) range.

Differences in SIC, UIBC, TIBC and %SAT among ACVIM classes, between symptomatic and non-symptomatic patients, between IRIS classes and between azotemic and non-azotemic patients were analysed by using One-Way ANOVA tests (normal distribution). Moreover, One-Way ANOVA tests (normal distribution) was used to determine the presence of statistically significant differences among ACVIM classes, between symptomatic and non-symptomatic patients, between IRIS classes and between azotemic and non-azotemic for selected echocardiographic parameters.

The possibility for SID (dichotomous variable) to be a risk factor for dogs to be classified in a higher ACVIM classes was evaluated using an ordinal logistic regression by GLMs (Type I SS). The same model was performed for IRIS classes, whereas to evaluate SID when associated to azotemia, a binary logistic regression model was performed. The models were adjusted for age (continuous, computed in years), sex and size, which was considered as a dichotomous variable (small dogs ≤ 10 kg or medium > 10 kg).

To verify the association between low SIC and selected echocardiographic parameters, other GLMs Type I were run. Each echocardiographic parameter was inserted in a model (linear distribution) and SID used as predictive variable. Models were adjusted for age, gender and size.

Survival was calculated as days between diagnosis and death (all causes of mortality) or between diagnosis and last contact with the owners (control visit or phone call). Subjects lost to follow-up were included in the survival analysis up until the last time at which they were known to be alive and then were thereafter censored in the analysis. The Kaplan-Meier method was used to estimate the survival function and plot time to event curves in the different groups; the equality of survival distributions was tested using the Log-Rank method, and then univariate and multivariate Cox proportional hazard analysis were used to evaluate the influence of different variables on survival. The variables considered were: presence/absence of clinical signs of CHF (dichotomous variable), age, sex, body weight, ACVIM class, RBC, Hb, Ht, RDW, SIC, low SIC (dichotomous variable), sCr, IRIS class, ESVI, EDVI, MR peak velocity, TR peak velocity, presence/absence of pulmonary hypertension (dichotomous variable), La/Ao, E/A, peak velocity of E wave.

$P < 0.05$ was set to indicate statistical significance.

Results

Two hundred seventy-six privately owned dogs were admitted to the Cardiology Service of the Department of Veterinary Medicine (DIMEVET) of the University of Milan between January 2015 and April 2016; among these, 116 (42%) were diagnosed with MMVD. Fifty-four of these (46%) fulfilled the inclusion criteria and were thus enrolled in the study.

The mean age of the dogs was 11 years (± 3.2 SD), and median body weight was 11 kg (IQR 6 – 22). Most of the patients included were intact males (n=23, 43%), followed by neutered females (n=17, 31%), neutered males (n=8, 15%) and intact females (n=6, 11%). The most represented breeds were mongrels (n=24, 44%), Dachshund (n=4, 7%) and Cavalier King Charles (n=3, 6%). Breeds with less than three dogs were grouped and listed as “others” (n=21, 39%). Twenty-two dogs were classified as ACVIM B1 (41%), 14 as ACVIM B2 (26%), 15 as ACVIM C (28%) and 3 as ACVIM D (5%). Sixty-seven % (n=36) of the dogs were asymptomatic for CHF (ACVIM classes B1 and B2), and 33% were symptomatic (ACVIM classes C and D) (n=18). Most of the dogs were classified as IRIS class 1 (80%, 44/54). Ten dogs presented azotemia with sCr levels above the references, 7 were classified as IRIS class 2 (14%) and 3 as IRIS class 3 (6%). No dogs were classified as IRIS class 4. Non-azotemic dogs represented the 80% of the population (n=44), whereas 20% presented azotemia (n=10). Of these, 2 dogs (2/10, 20%) were classified as ACVIM B1, 2 (2/10, 20%) as ACVIM B2 and 6 (6/10, 60%) as ACVIM C.

The mean values of SIC, TIBC, UIBC and %SAT for different ACVIM classes and in symptomatic or non-symptomatic patients for CHF are reported in table 1.

	Non-symptomatic			Symptomatic	
	ALL	ACVIM B1	ACVIM B2	ACVIM C	ACVIM D
SIC (mg/dL)	135.7 ± 49.84	140.86 ± 49.71		123.83 ± 53.38	
		144.18 ± 44.63	135.64 ± 58.21	134.53 ± 51.18	135.19 ± 51.11
TIBC (µg/dL)	423.06 ± 88.70	436.64 ± 74.79		395 ± 110.521	
		430.15 ± 71.12	446 ± 83.23	384.25 ± 125.56	427 ± 88.7
UIBC (µg/dL)	287.36 ± 78.55	261.22 ± 215.53		284.94 ± 252.79	
		207.75 ± 243.64	350.33 ± 120.49	270.6 ± 274.76	356.67 ± 73.87
%SAT	32.33 ± 10.39	31.8 ± 10.65		26.562 ± 10.04	
		32.71 ± 10.7	30.67 ± 10.95	28.97 ± 9.28	16.92 ± 7.63

Table 1. Mean (\pm SD) values of serum iron concentration (SIC), total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and percentage of saturation (%SAT) for different ACVIM classes and symptomatic vs. non-symptomatic patients for CHF.

The mean values of SIC, TIBC, UIBC and %SAT for different IRIS classes are reported in table 2.

	IRIS 1	IRIS 2	IRIS 3
SIC (µg/dL)	141.26 ± 53.87	111.43 ± 29.9	105.33 ± 38.28
TIBC (µg/dL)	426 ± 80.29	416.83 ± 142.93	406.5 ± 19.09
UIBC (µg/dL)	265.92 ± 245.7	332.43 ± 121.4	165.67 ± 211.47
%SAT	30.69 ± 11.71	26.34 ± 4.96	30.02 ± 10.80

Table 2. Mean (\pm SD) values of serum iron concentration (SIC), total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and percentage of saturation (%SAT) for different IRIS classes.

The mean values of SIC, TIBC, UIBC and %SAT for azotemic and non-azotemic dogs are reported in table 3.

	Non-azotemic	Azotemic
SIC (µg/dL)	141.26 ± 53.87*	111.45 ± 29.58*
TIBC (µg/dL)	426 ± 80.29	415.22 ± 113.32
UIBC (µg/dL)	265.92 ± 245.696	283.36 ± 153.74
%SAT	30.68 ± 11.71	27.51 ± 5.74

Table 3. Mean (\pm SD) values of serum iron concentration (SIC), total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC) and percentage of saturation (%SAT) for azotemic and non-azotemic dogs.

* p-value = 0.02 (One-Way ANOVA, Brown and Forsythe Test)

The prevalence of SID in our population of dogs with MMVD was 18% (10/54). Among the dogs non-symptomatic for CHF (ACVIM B1 and B2), 11% presented SID (n=4), whereas in the symptomatic group for CHF (ACVIM C and D), the prevalence of SID was 33% (n=6). All the dogs classified as ACVIM D (3/3, 100%) presented low SIC.

Eight out of ten dogs (80%) with SID were classified as IRIS class 1.

TIBC was within or above the reference range in 6/10 of SID dogs (60%), and %SAT was below the minimum level in 4/10 of them (40%).

No significant differences in the SIC, UIBC, TIBC and %SAT values between the ACVIM classes or IRIS classes nor between the symptomatic and non-symptomatic patients were found, nor any differences according to drugs administered to the patients, basically diuretics, ACE inhibitors or inodilator, aimed to control HF. Between the azotemic and non-azotemic patients the difference in mean SIC values was statistically significant (p-value=0.02); on the contrary, no significant differences in UIBC, TIBC and %SAT were observed.

Only 1 patient (2%) presented low SIC and concurrent anaemia. The prevalence of anaemia in the whole MMVD population was 6% (3/54). Analysis of the blood smear revealed no substantial alterations in dimension or morphology of the erythrocytes, except microcytosis in 2/10 (20%) dogs with low SIC. The only patient presenting a mild degree of poikilocytosis with presence of codocytes and schistocytes was the dog with low SIC and concurrent anaemia.

MCV was below the reference interval in 1/54 dogs (2%), with concurrent microcytosis highlighted at the blood smear analysis. MCHC was in the reference range for all the dogs included. RDW was above the reference interval for 3/54 dogs (5%), one of them presenting low SIC and concurrent anaemia.

Biochemical analysis was unremarkable for all the dogs included in the study, except for the dogs included as azotemic, that all presented elevated sCr. Only 1 dog over 10 presenting low SIC (10%) had concurrent hypoproteinaemia, that can affect SIC (Bohn et al. 2015).

Statistical analysis by GLM revealed that dogs with SID were at higher risk of being included in a higher ACVIM class when compared with dogs with normal levels of iron (OR=6.383, p-value=0.014). The data were adjusted for age, sex and body size (table 4).

	Category	OR (95% CI)	P
	O (= No deficiency; reference)	1	
SID	1 (= deficiency)	6.38 (1.46-27.94)	0.014
Age (continuous)		1.25 (1.02-1.53)	0.032
Body size (dichotomous)	Medium (> 10 kg) (reference)	1	
	Small (\leq 10 kg)	4.26 (1.33-13.65)	0.015
Sex (dichotomous)	Females (reference)	1	
	Males	3.82 (1.13-12.95)	0.031

Table 4. Evaluation of SID as a risk factor for a worst ACVIM classification in dogs through a GLM (Type I SS) ordinal logistic regression (adjusted for age, body size and sex).

In contrast, SID (adjusted for age, sex and size) was neither associated with an increase in IRIS classes nor considered a risk factor for azotemia.

Mean values for different echocardiographic parameters of the dogs included in the study are presented in table 5.

	ACVIM B1	ACVIM B2	ACVIM C	P
ESVI (ml/m²)	28.06 ± 8.90	26.50 ± 12.03	27.23 ± 15.97	
EDVI (ml/m²)	88.81 ± 30.72*	124.47 ± 27.91	153.21 ± 30.24*	0.00
La/Ao ratio	1.18 ± 0.19*	1.95 ± 0.45	2.23 ± 0.40*	0.00
E/A	1.13 ± 10.7*	1.2 ± 0.45	1.66 ± 0.72*	0.01
MR (Vmax)	5.63 ± 0.51	5.45 ± 0.56	5.88 ± 0.78	
TR (Vmax)	1.96 ± 0.48*	2.77 ± 0.62	2.78 ± 0.69*	0.03

Table 5. Mean (\pm SD) values in selected echocardiographic parameters for different ACVIM classes;
* p-value < 0.05 (One-Way ANOVA, Brown and Forsythe Test)

ANOVA revealed that statistically significant differences were present between different ACVIM classes and between symptomatic and non-symptomatic patients for: EDVI (ml/m²), La/Ao ratio, E/A and TR peak velocity. No statistically significant differences were found in echocardiographic parameters between IRIS classes and between azotemic and non azotemic patients.

Statistical analysis by GLMs revealed that dogs with SID were at higher risk of have certain echocardiographic parameters altered, including a higher La/Ao ratio (OR=1.50, p=0.018) and TR peak velocity (OR=1.88, p=0.023), and a lower MR peak velocity (OR=0.49, p=0.002). Log-rank analysis showed shorter survival in MMVD dogs with SID (p-value=0.020). The median survival times (MST) were 201 days for dogs presenting low SIC and 296 days for dogs with normal SIC. Uni- variate Cox analysis showed statistically significant results in determining survival for some of the considered variables. Hazard ratios are reported in table 6.

	HR (95% CI)	P
Age (continuous)	1.406 (1.11-1.78)	0,004
Body weight (continuous)	0.92 (0.85-0.99)	0.023
RDW	1.22 (1.02-1.45)	0.027
EDVI (ml/m²):	1.021 (1.00-1.03)	0.000
La/Ao ratio:	4.77 (1.8-12.63)	0.002
E wave peak velocity:	5.01 (1.32-19.02)	0.018
Pulmonary Hypertension (dichotomous)	3.216 (1.16-8.90)	0.025
Clinical symptoms of CHF (dichotomous)	13.827 (3.10-61.61)	0.001
Ejection fraction (EF)	1.12 (1.05-1.2)	0.001
sCr	3.03 (1.22-7.51)	0.016

Table 6. Single hazard ratios (HRs) presented in Cox proportional-hazards survival univariate analysis.

Multivariate Cox analysis revealed that only age and the presence of CHF symptoms could affect the dogs' survival (table 7).

	HR (95% CI)	P
Age (continuous)	1.7 (1.15-2.5)	0,008
Clinical symptoms CHF (dichotomous)	8.31 (1.64-42.09)	0.011

Table 7. Single hazard ratios (HRs) presented in Cox proportional-hazards survival multivariate analysis.

Discussion

In our population, dogs diagnosed with MMVD and presenting low SIC had a prevalence of 18%. The prevalence of SID among dogs presented with HF due to MMVD (ACVIM C and D) was 33%. The 100% of the dogs admitted with acute decompensated CHF presented SID.

No significant differences in mean SIC between ACVIM classes and between symptomatic and non-symptomatic patients were found, although the 60% of dogs with low SIC were symptomatic for HF. Moreover, based on GLM analysis, dogs presenting with low SIC have a 6.3-times higher risk to be included in a higher ACVIM class when compared with dogs presenting normal SIC.

Low SIC should then be considered a risk factor for disease progression, together with age, sex and size, that reached statistical significance.

Old, male, small size breed dogs with low SIC are at higher risk of being included in a higher ACVIM class. It is true that the prevalence of low SIC in affected dogs is lower than the percentage reported for human patients affected by HF, where the value stands at approximately 50% [Klip, 2013, Fitzsimmons, 2015], but most of the studies on humans are performed on hospitalized patients, who present more severe conditions than those found in our canine population, in which only 3 subjects belonged to ACVIM class D. Nevertheless, the 33% of dogs classified as symptomatic for HF, had low SIC. If we then compare the prevalence of SID between symptomatic canine patients and the human population affected by HF, the percentages are quite similar.

The pathogenetic hypotheses proposed to explain ID in HF are, among others, insufficient dietary iron intake, poor gastrointestinal iron absorption due to intestinal mucosa oedema and hepcidin overexpression related to chronic inflammatory state in HF [Van der Meer, 2004; Opasich, 2005; Kazory, 2009].

In veterinary medicine, anorexia, like it is seen at late stage of HF, is a quite concerning topic for owners and a study from Mallery et al. (1999) reported that is one of the most common reasons for owners to choose euthanasia during late-stage HF [Mallery, 1999]. Anorexia leading to low SIC is not something common in veterinary medicine. Moreover, dogs are normally fed balanced diets, which help to prevent any kind of dietary insufficiency. Dogs' diets are usually rich in meat products,

making dietary haeme, an important iron source released from dietary myoglobin and haemoglobin, more bioavailable [Harvey, 2008].

Even the iron metabolism is different between humans and dogs. Normally, ferric iron ions (Fe^{+3}) derived from diet are reduced to ferrous iron ions (Fe^{+2}) so that they can be absorbed by duodenal enterocytes [Harvey, 2008]. This is certainly necessary for humans but not fundamental for dogs, where the absorption of the two valence forms is equal [Moore, 1944; Harvey, 2008].

Oedema of the GI tract and hepatic stasis are common in HF and can be associated with poor iron absorption from dietary sources. The different pathway of absorption between dogs and humans could offer a partial explanation why dogs affected by HF could have less problems related to iron absorption.

On the other hand, anorexia in symptomatic CHF, in conjunction with GI oedema and the inflammatory status that is related to the presence of CHF could offer a possible explanation for SID in dogs symptomatic for HF, where is more prevalent. It is known that HF is a chronic condition that promotes inflammatory status of the organism, and this can alter iron metabolism inducing functional ID, due to cytokines and hepcidin overexpression. TIBC and %SAT values were used to this purpose, and the results seem to suggest that, in our dogs, ID is more frequently absolute (primary) than functional (secondary to inflammatory conditions). In fact, in real iron deficiency, TIBC is usually normal or slightly over the reference interval, and %SAT is lower, in functional iron deficiency both TIBC and %SAT are lower than the reference intervals [Harvey, 2008; Andrews, 2010].

It is stated that, in human medicine, ID can be present in the absence of anaemia and is associated with the worst symptoms. With respect to anaemia, only one patient in our sample presented anaemia and SID; therefore, SID can effectively be present without anaemia in dogs with HF, as reported in literature regarding human patients [Okonko, 2011; Klip, 2013; Arora, 2014; Rangel, 2014; Fitzsimmons, 2015]. In fact, anaemia is commonly considered a late-onset condition that can be preceded by ID [Harvey, 2008].

Although a statistically significant difference was found in the median iron values between azotemic and non-azotemic patients, it is important to highlight that the 80% of dogs that presented SID were

classified as IRIS class 1; SID does not appear to be an exclusive finding of renal complication of cardiovascular disease (cardiorenal syndrome), and we should consider it as possible comorbidity in HF alone [Rangel, 2014; Fitzsimmons, 2015].

Regarding survival, MST, calculated through Kaplan-Meier, were significantly different in dogs with or without SID. Univariate Cox regression revealed the impact of changes in echocardiographic variables related to severity of MMVD in shortening survival, in particular increase in EDVI, La/Ao ratio, EF and E wave peak velocity and presence of pulmonary hypertension. Moreover, increase in age and in sCr were associated with shorter survival. Finally, presence of clinical symptoms of HF decrease survival times. SIC was not associated with shorter survival, but RDW was, suggesting that alteration in RBC dimensions, that can be mediated by low SIC, and consequent reduced oxygen transport can alter energetic metabolism and heart work and shorten patients' survival. Still, in a multivariate Cox analysis, only increase in age and the presence of clinical symptoms are related to shorter survival. It is important to remind that survival results should be interpreted with caution due to the small number of dogs included in the study and the small percentage of dogs that experienced death in our sample.

With respect to therapeutic options, oral supplementation with ferrous sulphate is the safest and least expensive way of treating SID in dog [Plumb, 2008; Kerl, 2014], but its intestinal absorption is limited in healthy animals, and even more in HF due to intestinal mucosal oedema. However, no specific side effects are reported for iron oral therapies. Therefore, the authors speculate that investigation of iron status in dogs affected by MMVD in order to introduce an iron supplementation if needed seems legitimate.

Intravenous (IV) iron therapy showed promising results in human patients affected by HF in improving exercise capacity, left ventricle function and quality of life [Gutzwiller, 2013, Toblli, 2015]. No studies are reported in veterinary medicine.

There are several limitations in our study. We are aware that the measurement of serum iron represents a small fraction of the total body iron and therefore we decided to also measure transferrin (TIBC) and percentage of saturation. Indeed, the main limitation can be found in the impossibility, at

the moment, to measure ferritin and hepcidin, which are fundamental in the diagnostic algorithm and can be useful markers to characterize SID. Ferritin in particular is considered one of the diagnostic cornerstones of all the study about iron deficiency in cardiovascular diseases in human medicine. Moreover, limitations have to be searched in the retrospective nature of the study. Finally, the limited sample numbers, in particular for the most severe ACVIM class (D), make it harder to draw conclusions for this class of patients. Strict inclusion criteria were applied to improve reliability, that decreased sample size. Small sample size and underrepresentation of ACVIM class C and D could have biased some of the results.

In conclusion, SID is a relatively frequent condition associated to MMVD in dogs; it is present in almost 20% of patients affected by MMVD, but the percentage raise to the 33% in symptomatic patients and to 100% in patients presenting acute decompensated CHF. SID represents a risk factor for dogs affected by MMVD: low SIC is associated with a 6.3-times higher risk of being included in a higher ACVIM class. Moreover, dogs presenting low SIC have a 1.5 times higher risk of presenting an increase in La/Ao ratio.

The 14% of ACVIM B2 dogs presented low SIC: in the authors opinion, monitoring the iron status in dogs with preclinical MMVD over time could provide information about SID as a comorbidity in HF and, maybe, also about the trend of the pathology.

Oral supplementation of iron could be an effective and safe way to restore iron levels in these dogs, although its efficacy could be affected by lack of intestinal absorption. Further studies are needed in a larger population with evaluation of iron storage (i.e., ferritin levels, hepcidin) as well as the feasibility of IV iron supplementation, especially in acute decompensated HF.

Reliability of symmetric dimethylarginine in dogs with myxomatous mitral valve disease as kidney biomarker

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Diagnosis of renal impairment

An early diagnosis of renal impairment in patients affected by cardiovascular disease is very important. In medicine, the more common test to assess kidney function are glomerular filtration rate (GFR), serum urea nitrogen (BUN), serum creatinine (sCr) and proteinuria [Dobre, 2012].

Glomerular filtration rate (GFR) is currently considered the single most useful and sensitive test of renal function, because any decrease in GFR normally means that kidney disease is occurring or progressing [Orvalho, 2017]. Azotemia is common in dogs with cardiac diseases but is mostly mild to moderate and not related to changes in cardiovascular variables [Nicolle, 2007; Martinelli, 2016]. Nicolle et al. in 2007 reported that GFR in 24 dogs with MMVD was significantly lower in upper classes (1.7 ± 0.7 mL/min/kg) than in lower classes (3.1 ± 0.8 mL/min/kg) of MMVD. Only 1/15 dogs classified as mild MMVD had $GFR < 2$ mL/min/kg and only 2/9 dogs classified as severe MMVD had $GFR > 2$ mL/min/kg [Nicolle, 2007]. GFR is indeed an expensive and complex procedure and is not routinely performed on dogs and cats. In fact, it requires the intra venous injection of a marker of filtration, followed by multiple blood samples at determined time, making the process expensive and time consuming [Yerramilli, 2016; Relford, 2016].

Currently, the cornerstone in the diagnosis of CKD in veterinary practice is the measurement of serum Creatinine (sCr), that is the most common marker of GFR. Indeed, the use of sCr has some limitations, since its concentration tends to rise when almost the 75% of the renal activity is lost. Moreover, sCr levels are affected by muscle mass, making the evaluation of renal function more difficult in cachectic and geriatric animals [Relford, 2016; Orvalho, 2017].

Symmetric dimethylarginine (SDMA) has raised, in the last few years, as the most promising novel biomarker in the early detection of kidney failure. SDMA is one of the methylated forms derived from the arginine metabolism, with asymmetric dimethylarginine (ADMA) and monomethylarginine (MMA) [Kakimoto, 1970; Orvalho, 2017].

While ADMA is largely cleared by enzymatic hydrolysis, SDMA is primarily eliminated by renal excretion, suggesting its role as endogenous marker of GFR [McDermott, 1976; Kielstein, 2006; Schwedhelm, 2011]. SDMA increases earlier than creatinine and can identify a GFR decline as early as a 30% [Polzin, 2016]. A large meta-analysis from Kielstein et al. showed that SDMA strongly correlated with inulin clearance and sCr [Kielstein, 2006]. A study from Relford et al. reported that age and lean body mass cannot influence SDMA concentration in small animals [Relford, 2016].

Major non-renal systemic disease, including hepatic and endocrine diseases have not been consistently identified as reasons of elevation in serum SDMA concentrations, neither in humans nor in animals, and the effects of both heart and kidney failure on SDMA elimination are still undefined [Schwedhelm, 2011; Veldink, 2013; Bum-Sul, 2017].

Materials and methods

This was a retrospective case-control study on a clinical population of dogs affected by MMVD (cases) vs healthy dogs (controls). The animals enrolled belonged to the population of dogs referred to the Cardiology Service of our institution between February 2015 and July 2016.

Inclusion criteria were: diagnosis of MMVD, absence of any other concurrent pathology and no hematological alteration compatible with CKD (sCr < 1,4 mg/dl). Dogs with other heart or systemic diseases were excluded. Control group was composed by healthy dogs that were evaluated at the veterinary teaching hospital for periodic screening and then enrolled. For all dogs, informed consent for every procedure signed by the owner was obtained, as routinely done in our clinical practice.

Both groups underwent a complete physical evaluation, chest x-ray, echocardiographic examination, complete blood count (CBC) and serum biochemical panel. A complete echocardiographic examination was performed in both groups using standardized thoracic imaging planes, according to

the recommendations of the American Society of Echocardiography [Thomas, 1993]. Dogs without any clinical, radiographic and echocardiographic sign of cardiac disease were enrolled as controls. Echocardiography was performed using an Esaote MyLab50 Gold cardiovascular, with phased array multifrequency probes (7.5-10 Mhz and 2.5-3 Mhz).

The investigators performing the echocardiographic exams were all focused on specialty cardiology practice (AS, CL, PGB). Dogs diagnosed with MMVD were subdivided in 4 classes, from B1 to D, in line with the ACVIM classification. As recommended by the ACVIM guidelines, stage B included dogs affected by MMVD without signs of CHF (namely, in stage B1, dogs without evidence of cardiac enlargement; in B2, dogs with signs of cardiac remodeling as left atrium (LA) and/or left ventricle (LV) enlargement); stage C included dogs with clinical signs of CHF due to MMVD, and stage D included subjects presenting both clinical signs of CHF and refractoriness to standard medical treatments [Atkins, 2009; Boswood, 2016].

All dogs underwent indirect systemic blood pressure evaluation by Doppler flow meter as reported in ACVIM guidelines [Brown, 2007]. Based on the International Renal Interest Society (IRIS) guidelines, dogs were classified in 5 classes, from 0 to 4. No azotemic patients (normal sCr) were enrolled, and all the dogs were classified as IRIS class 0 (dogs at risk for the development of CKD). An IRIS substage of CKD based on blood pressure and on the presence of proteinuria was performed, when possible, as reported in the IRIS guidelines [IRIS, 2016].

Blood samples were collected from the cephalic vein during routine clinical evaluation, and CBC and selected biochemical parameters were performed. For CBC, EDTA was used as anticoagulant, while, for biochemical evaluation, blood was placed in tubes without anticoagulant and centrifuged at 3250 x g for 5 minutes. CBC was performed using an automated laser hematology analyzer (Sysmex XT-2000iV, Sysmex corporation, Kobe, Japan), while biochemistry was carried out with an automated spectrophotometer (Roche Cobas Mira Classic Biochemistry Analyzer). The biochemical panel performed was composed by: glucose, urea, sCr, total proteins, sodium, potassium, chloride, alanine transaminase, alkaline phosphatase.

An aliquot of serum (at least 300 µl) was frozen and sent to IDEXX laboratories for SDMA determination with the EMIT (Enzyme Multiplied Immunoassay Technology). SDMA in dog was considered normal when < 14 µg/dL [Nabity, 2015]. Urine were collected via spontaneous micturition or cystocentesis and complete urinalysis with sediment and Urine Protein to Creatinine Ratio (UPC) was performed.

Statistical analysis

Normality of data distribution was tested with Shapiro - Wilk test. For baseline descriptive statistics, normally distributed continuous variables were presented as mean and standard deviation, non-normally distributed variables as median and interquartile range (IQR). Data were analyzed with IBM SPSS Statistics 23 [Release 23.0.0.0] using a multivariate generalized linear model (GLM) with backward elimination, applied on ACVIM class (compared with breed, sex, age, weight, SDMA values, Urea values and sCr values) and on SDMA (compared with breed, sex, age, weight, MVD presence or absence, ACVIM class, presence of clinical symptomatology, pharmacological therapy, Urea values and sCr values).

To apply the model, a transformation of ACVIM classes into numbers categories was made, with ACVIM class B1 corresponding to 1, ACVIM B2 to 2, ACVIM C to 3 and ACVIM D to 4. We evaluated if severity of MMVD, CHF symptomatology or pharmacological treatment can influence a single measurement of SDMA levels in dogs affected by the MMVD. $P < 0.05$ was set to indicate statistical significance.

Results

An overall number of 367 privately owned dogs were admitted to the Cardiology Service of DIMEVET of the University of Milan between February 2015 and July 2016; among these, 148 (40%) were diagnosed with MMVD. The medical archives were reviewed in order to include only patients with complete medical and laboratory information.

Thirty-one dogs were enrolled, 24 in the case group and 7 in the control group.

The median age of dogs enrolled in case group was 11.5 years (± 2.83 SD), and the median body weight was 13.8 kg (± 9.91 SD). Most of the dogs included were neutered females (n=11; 46%), followed by intact males (n=8; 33%), intact females (n=3; 12%) and neutered males (n=2; 9%). The most represented breeds were Mongrels (n=8; 33%), Cavalier King Charles (n=2; 8%), Chihuahua (n=2; 8%) and Maltese dogs (n=2; 8%). Breeds with less than two dogs were grouped and listed as others (51%). In this group were also listed Dachshund and Jack Russel terrier.

The median age of the control group was 8.2 years (± 1.92 SD), and the median body weight was 21 kg (± 9.17 SD). Most of the controls included were neutered females (n=5; 71%), followed by intact males (n=2; 29%). The most represented breed were mongrels (n=3; 43%). Thirteen dogs over 24 were classified as ACVIM B1 (54%), 8 as ACVIM B2 (33%) and 3 as ACVIM C (13%). Symptomatic and non-symptomatic dogs for CHF were respectively 13% (n=3) and 87% (n=21). The 3 dogs classified as ACVIM class C were all under standard triple therapy, composed by furosemide, an ACE inhibitor, and pimobendan.

Renal function was tested measuring serum urea, sCr and SDMA. sCr was normal in all dogs, as it was an inclusion criterion. Serum urea (NV= 20 – 60 mg/dL) was over the reference interval in 8 of the cases (33%). None of the controls presented alterations in urea values. Minimum, maximum and mean values are reported in table 8.

	N	Min	Max	Mean	SD
SDMA	31	3	16	11,42	3,28
Age	31	4	17	10,90	3,23
Serum urea (mg/dL)	31	19	115	46,48	21,90
Weight	31	2,3	36,3	15,3	10,2
sCr (mg/dL)	31	,47	1,26	,92	,17

Table 8. Information on the continuous variables

The 25% (n=2) of dogs classified as ACVIM B1, the 24% (n=3) of ACVIM B2 and the 100% of ACVIM C presented urea values above the reference interval. Only medical records of dogs presenting urinalysis were retrospectively included. Urine specific gravity (USG) was normal (NV=1020 - 1060) in 87% of cases (21/24) and in 100% of the controls for which urinalysis was available. For the case group, 2/3 dogs presented USG lower than reference interval (66%), while one presented USG over the reference interval (34%). Both of the dogs with low USG were classified as ACVIM C and were assuming furosemide. No proteinuria was detected in any of the dog considered (UPC < 0.5) (IRIS 2016). SDMA value was normal (< 14 µg/dL) in the 75% (n=18) of cases, and over the reference interval in the 25% (n=6). The 31% of the ACVIM B1 dogs (4/13) and the 25% of the ACVIM B2 (2/8) presented SDMA values over the reference interval. None of the dogs classified as ACVIM C had SDMA above the references. SDMA was above the reference intervals in 4/7 of controls (57%).

According to the new IRIS guidelines, the 25% of MMVD dogs should be shifted from IRIS class 0 to IRIS class 1, while the 57% of dogs classified as controls switched form IRIS class 0 to IRIS class 1. Once set SDMA as dependent variable in the GLM, the results (table 9) showed no statistically significant difference for each variable considered (breed, age, sex, weight, ACVIM class, healthy/affected, presence of CHF symptomatology, pharmacological therapy, sCr, urea).

		IC 95%			
Variable	Factor	OR	INF	SUP	P
Sex	F	1,661	,153	18,07	,677
	FC	1,072	,166	6,93	,942
	M	1,397	,212	9,20	,728
	MC (ref)	1			
Breed	Mongrel	1,036	,357	3,01	,948
	Pure (ref)	1			

Age (years)	Continuous Variable	1,032	,855	1,25	,740
Weight (Kg)	Continuous Variable	,994	,937	1,05	,848
sCr (mg/dL)	Continuous Variable	4,865	,070	339,04	,465
Serum urea (mg/dL)	Continuous Variable	,997	,963	1,03	,862
ACVIM	Continuous Variable	,804	,321	2,01	,641
CHF symptoms	No	1,241	,052	29,90	,894
	Yes (ref)	1			
Pharmacological treatment (CHF)	ACE-inhibitor	1,361	,231	8,013	,733
	ACE-inhibitor, diuretic	1,317	,052	33,426	,868
	ACE-inhibitor, inotropic agent	2,219	,025	198,372	,728
	ACE-inhibitor, inotropic agent, diuretic	1,681	,101	27,989	,717
	No treatment (ref)	1			

Table 9. Linear generalized univariate model analysis with binomial negative response; dependent variable: SDMA

Results after backward elimination confirmed lack of statistically significant differences in the variable considered in the statistical analysis.

		IC 95%			
	Factor	OR	INF	SUP	P
sCr (mg/dL)	Continuous Variable	2,143	,242	19,001	,494

Table 10. Linear generalized multivariate model analysis with binomial negative response [after backward elimination]; dependent variable: SDMA

Once set ACVIM class as dependent variable in the GLM, the results (table 11) only showed a statistically significant difference in the variable age, after backward elimination, for higher ACVIM classes (C) (P = 0.014; OR = 1.3).

		IC 95%			
	Factor	OR	INF	SUP	P
Sex	F	10,015	,175	573,395	,265
	FC	,458	,024	8,880	,606
	M	15,623	,754	323,923	,076
	MC (ref)	1			
Breed	Mongrel	1,270	,214	7,542	,793
	Pure (ref)	1			
Age (years)	Continuous Variable	1,300	,924	1,829	,132
Weight (Kg)	Continuous Variable	,929	,843	1,023	,135
sCr (mg/dL)	Continuous Variable	15,479	,502	477,462	,117
Serum urea (mg/dL)	Continuous Variable	1,036	,975	1,101	,253

Table 11. Linear generalized univariate model analysis with ordinal logistic response; dependent variable: ACVIM

Results after backward elimination are presented in table 12.

Variable	Factor	IC 95%			P
		OR	INF	SUP	
Age (years)	Continuous Variable	1,323	1,058	1,656	,014

Table 12. Linear generalized multivariate model analysis with ordinal logistic response; dependent variable: ACVIM

Discussion

The population of dogs affected by MMVD enrolled in this study presented the typical characteristics reported in literature. In fact, the dogs were mainly of medium age and body weight, mongrels or pure small breed dogs [Borgarelli, 2012].

Most of the dogs were asymptomatic for CHF (87%). Age resulted correlated with ACVIM classes, as reported in literature [Borgarelli, 2012]. Interestingly, multivariate analysis showed that for each year of life of the dog, the risk of belonging to a higher ACVIM class increases of 1.3 times.

According to literature, the prevalence of azotemia in dogs with heart diseases ranges between 7.4 and 25% [Pouchelon, 2015; Nicolle, 2007; Guglielmini, 2013; Martinelli, 2016].

In our population, all dogs presented normal sCr values. No statistically significant differences in urea values have been found among different ACVIM classes, although a positive increasing trend is evident and the totality of dog in ACVIM class C presented urea values above the reference interval. The progression of MMVD and the development of CHF reduce renal perfusion and can result in urea increase [Aronson, 2004; Kazory, 2010]. Also, the activation of the renin angiotensin aldosterone system (RAAS), which increases the absorption of sodium and water, can contribute [Aronson, 2004; Kazory, 2010]. The increase may also be due to the administration of diuretic drugs, prescribed for ACVIM class C patients [Atkins, 2009; Pouchelon, 2015].

The 75% of patients at any stage of MMVD presented normal values of SDMA. SDMA has been compared with all the variables considered in our study, and none of them gave statistically significant result. SDMA appears as a promising early biomarker of kidney function in patients affected by any

stage of MMVD, since the presence of this cardiovascular disease doesn't result in influences on the parameter. According to literature, our study showed that SDMA is not influenced by lean body mass and weight, differently from creatinine [Hall, 2015]; therefore, those data cannot be considered as confounding factors. Moreover, SDMA is not influenced by age: this is an important aspect since the canine population affected by MMVD is usually in the old age. Pedersen and colleagues reported also that SDMA levels were not altered by the presence of asymptomatic mitral regurgitation, but the study did not consider any dog presenting CHF and was conducted only on Cavalier King Charles Spaniels dogs [Pedersen, 2006].

More recently, Bum-Sul and colleagues reported that SDMA levels in dogs with MMVD, mostly symptomatic, were not correlated to age or body weight, but closely correlated to the severity of HF and some echocardiographic markers, like LA/Ao ratio [Bum-Sul, 2017]. Based on our population, since SDMA is considered a biomarker of GFR [Nabity, 2015], our data seems to suggest that the 25% of our population could present a reduction of GFR despite the normal sCr values. Even the comparison between SDMA and creatinine didn't result statistically significant, differently from what reported in literature [Nabity, 2015] but this is likely due to the inclusion criteria used in this study. In fact, all the patients presented normal creatinine values.

It can be noted that, in the control group, there was a greater percentage of patients with SDMA values higher than normal compared to the group of cases (25% vs 57%): this result is of difficult interpretation since, in veterinary medicine, few studies have been made about influences other than CKD on SDMA values and due to the lack of information about abdominal ultrasound. However, all control dogs in our population presented normal urinary specific gravity and no proteinuria, so kidney damage seems less likely. Many different causes have been investigated to justify high levels of SDMA and ADMA concentration in human patients, like in diabetes [Krzyzanowska, 2007] or sepsis [Koch, 2013], but all the principal diseases that can alter SDMA values have been excluded from our population. We cannot exclude the presence of pre-analytical errors in SDMA determination. Moreover, a recent study reported the presence of a small percentage of animals with elevated SDMA but normal sCR in which kidney function must be assessed [Polzin, 2016].

Although no agreement is present on the therapeutic options in dog presenting alteration of SDMA without other signs of renal impairment, the evaluation of this parameter could be important from a clinical point of view, and allows us to suggest for these patients a more targeted diagnostic procedure with a full assessment of kidney function, including second evaluation of SDMA within one month, complete biochemical renal profile examination (creatinine and urea), urinalysis with UPC ratio and ultrasound renal evaluation, as recommended by IRIS guidelines [IRIS, 2016]. Moreover, according to Hall and colleagues the administration of a renal diet to dogs currently classified as IRIS-Stage 1 CKD can result in reduction of blood concentration of kidney biomarkers, in particular SDMA and sCr, besides improving patient's quality of life [Hall, 2018]. A previous longitudinal study on geriatric dogs fed with commercial renal protective food showed that dogs consuming test food showed significant reduction in the concentrations of serum SDMA, sCr, and BUN. Moreover, serum sodium concentrations were seen to be statistically significant lower in dogs assuming this kind of diets, that can be of help in dogs with heart disease in which restriction of sodium is indicated. Finally, a kidney protection food containing more biologically available protein sources with respect to other commercially available dry foods could be of help in preventing cardiac cachexia, loss of muscular masses and mobility in patients affected by MMVD at various gravity levels [Hall, 2016]. This is a retrospective study and, as such, has some inherent limitations. Firstly, the abdominal ultrasound was not systematically assessed in our population of dogs. Secondly, the small number of animals included in higher ACVIM class C and the single determination of the parameter could affect the statistical significance and must be considered in future studies. In fact, a single determination of SDMA, not followed by a second evaluation within one month or abdominal imaging, cannot be considered diagnostic of CRvD. In conclusion, our study showed that SDMA is free from correlation with breed, age, sex, weight, presence/absence of MMVD, presence of CHF symptoms and pharmacological therapy as well. SDMA can be actually considered a reliable parameter for evaluation of renal function in dogs affected by MMVD, especially in those patients with a non-advanced stage of disease (ACVIM class B2), for which an early diagnosis of the onset of kidney failure is fundamental in order to plan a diuretic therapy.

SDMA repeated measurements over time, as recommended by IRIS guidelines, are necessary (IRIS, 2016), because one determination does not allow us to exclude definitely a later onset of the renal impairment and then to be considered diagnostic in order to highlight a possible onset of CRS.

Heart type creatine kinase isoenzyme (CK-MB) in serum of cats with cardiomyopathy: preliminary investigation using the electrophoretic fractionation method

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Early diagnosis of subclinical heart diseases

Echocardiography represent the gold standard in the diagnosis of heart diseases in cardiology, and in particular in cats, in which studies showed that a heart murmur is often absent despite the presence of pathologic status, like in hypertrophic cardiomyopathy (HCM) [Fuentes, 2017] Nevertheless, echocardiography has high costs and requires well trained operators to be performed, so is normally not performed in absence of clinical signs of heart disease, like in cats with chronic kidney disease (CKD), that are however at risk for developing systemic hypertension and, thus, HCM. A study from Payne et al. in 2015 report a cardiomyopathy prevalence of 14.7% in 780 apparently healthy cats from shelters [Payne, 2015]. Moreover, CKD is a very common condition in geriatric cats, being present in around 50% of cats at any stage, and with around 20% of cats presenting concurrent CKD and systemic hypertension [Syme, 2002].

The presence of systemic hypertension is a well know prodromic condition to the development of left ventricular hypertrophy and, eventually, HF.

Creatinkinase (CK) catalyzes the reversible reaction of creatine and ATP to form phosphocreatine and adenosine diphosphate, a crucial reaction for cellular energy generation and metabolism [Walliman, 1992]. The CK enzyme is a dimer of two polypeptide chains, encoded by two genes and translated separately; the CK-M and CK-B monomers form the three dimers CK-MB, CK-MM and CK-BB. Since heart muscle expresses the B gene at a higher rate than the skeletal muscle, the CK-MB isoenzyme exhibits a high cardiac specificity, and it may be measured by immunoenzymatic methods in people [Burgener, 2006; Bodor, 2016].

The availability of automated CK-MB immunoassays and its high diagnostic specificity made CK-MB measurement the “gold standard” for the diagnosis of acute myocardial infarction in people, until the troponins replaced it [Bodor, 2016].

To our knowledge, there is limited information about CK isoenzymes in domestic animals, mostly dealing with laboratory animals [Boyd, 1988; Yasuda, 1990; Evans, 1991]. The comparison of CK isoenzyme profiles within preclinical studies has shown utility for comparing the extent or severity of myocardial injury in animal models [Walker, 2006]. Few reports in dogs are focused on CK isoenzymes [Burgener, 2006; Schober, 1999; Cury, 2005], and almost no activity of the CK-MB has been detected in canine serum [Paltrinieri, 2010]. Compared with troponins, the diagnostic sensitivity of CK-MB for cardiac diseases in dogs is much lower [Preus, 1989; Diniz, 2007; Bakirel, 2009; Fredericks, 2001; Carreton, 2013], since the CK-MB isoform in canine myocardium represents only 4%-13% of the total cardiac CK activity, compared to 40% in people. Furthermore, CK-MB in dog is also expressed in other tissues, thus reducing its cardiac specificity [Aktas, 1993; Dolci, 2006]. Nevertheless, shifts in CK isoenzyme profiles within heart muscle tissue has been used for investigating changes in myocardial metabolism associated with chronic cardiac injury in animals [Sharkey, 1991; Hironaka, 2003]. Aroch et al. reported that increases of total CK activity are common in cats, especially in the case of cardiac diseases, trauma, bite wounds, systemic bacterial infections [Aroch, 2010]. This makes total CK activity an inaccurate outcome predictor. To our knowledge, no reagents to accurately measure CK-MB activity in cats are available and only one study described the electrophoretic distribution of CK isoenzymes in healthy cats [Paltrinieri, 2010]. According to this latter study CK-MM is the predominant electrophoretic fraction, followed by Macro-CK1 and Macro-CK2, whereas CK-MB and CK-BB are virtually absent.

Materials and methods

In this retrospective study, all the cats enrolled were referred to our Institutions for routine diagnostic procedures or wellness visits and were sampled upon informed consent of the owners.

Therefore, according to the Ethical Committee of the University of Milan (decision number 2/2016) formal approval of the Institutional ethical committee was not necessary. Samples collected at the external clinic were sent to the Veterinary Teaching Hospital of the University of Milan in cold chain. Inclusion in the study required: complete clinical record (signalment, anamnesis, clinical findings), measurement of the systolic blood pressure (Doppler sphygmomanometers Huntleigh Vettex Duo and Riester R1), a complete M-mode, B-mode and Doppler echocardiographic examination (Esaote MyLab50 Gold cardiovascular and Esaote MyLab Gamma with phased array probes 7,5 – 10 Mhz) [Ferasin, 2009; Coté, 2011], laboratory data including CBC (performed using Sysmex XT-2000iV, Sysmex Co., or ProCyte Dx, IDEXX Laboratories), a biochemical panel (performed using the automated instruments Cobas Mira, Roche Diagnostics, or Mindray BS-380, Shenzhen Mindray Bio-Medical Electronics Co., Ltd) and serum T4 determination (IDEXX Laboratories), the presence of frozen serum aliquot for total CK measurement and electrophoresis performance.

Exclusion criteria were the presence of congenital heart disease or concurrent diseases or conditions that clearly imply muscular damages such as muscle tissue neoplasia, trauma, cachexia or prolonged recumbency, and samples that were grossly hemolyzed, lipemic or hyperbilirubinemic.

The diagnosis of cardiomyopathy (CM) performed by veterinarians confined to cardiology specialty practice were conform to the guidelines of the literature [Ferasin, 2009]. Cats affected by CM (*cardio tot*) were divided in two groups according to presence (*secondary CM*) or not (*primary CM*) of concurrent diseases (hypertension, hypertiroidism, kidney disease) [Spalla, 2015]. Cats were defined as healthy based on the clinical and echocardiographic examinations, and on the laboratory data, and were thus enrolled as control group. All sera were frozen and maintained at -20°C until total CK activity assessment (performed within 6 months using the CK-NAC method on the Cobas Mira analyzer, Roche Diagnostics) and electrophoresis performance. In order to assess tissue distribution of macro- and isoenzymes in the cat, an electrophoretic separation of CK isoenzymes was performed

on supernatants of homogenized muscular tissues. Slices of skeletal and cardiac muscle of approximately 1 cm³ each were collected during a routine necropsy from a cat referred to the Pathology unit of the Veterinary Teaching Hospital of the University of Milan and dead on the same day for causes unrelated to heart diseases. Slices were immediately placed in 1 mL of saline solution, manually homogenized, and centrifuged at 2500g for 15 minutes. The electrophoresis was performed immediately after centrifugation on the supernatants at different dilutions (1:1, 1:4, 1:8, 1:10) using the procedure described below.

Total CK activity measurement was performed after thawing using the CK-NAC method on the Cobas Mira analyzer (Roche Diagnostics). In 8 cases, total CK activity could not be determined because of depletion of samples. The electrophoretic separation of CK isoenzymes were performed on all frozen sera and homogenized tissues, after thawing, within 6 months from the collection. Electrophoresis was performed according to the manufacturer's instructions using a commercially available kit (Hydragel ISO-CK, Sebia Italia Srl) and an automated apparatus (Hydrasys, Sebia Italia Srl) equipped with specific accessories (Standard Mask Accessories for ISO-CK/LD). Briefly, 100 µL of serum or supernatant from homogenized tissues were mixed with 1 µL of activating solution containing β-mercaptoethanol and incubated for 10 minutes at 20°C. Ten µL of activated serum or supernatant were placed in the wells of the applicator provided with the kit. Agarose gels (8%, pH 8.40±0.05) and applicators were placed in the migration chamber, and the automated migration program was then selected. After migration (10–20W, 27V h, 20°C), CK substrate containing chromogenic solution was applied, then the reaction was stopped using a blocking solution. Gels were then washed, dried by heating, and placed on the scanner provided with the instrument.

Scanned images were analyzed using the software Phoresis (Sebia Italia Srl) and visually inspected for possible errors in separation, which, if present, were manually corrected. Intra- and interassay precision of the method, were < 6%, except for the fractions with negligible quantities (CK-BB and CK-MB) [Paltrinieri, 2010]. Based on total CK activity and on the percentage values recorded after densitometric analysis of the gels, the absolute values (in U/L) of each electrophoretic fraction were calculated, except for the 8 cases on which total CK activity was not determined, as described above.

Statistical analyses

Statistical analysis was performed using an Excel spreadsheet (Microsoft Corp.) and Analyse-it software (Analyse-it Software Ltd.). After determining that data were not normally distributed, a Mann-Whitney *U* test was used to assess the differences for each parameter among cats with different type of *primary CM* (HCM, restricted, dilated, unclassified) and then between cats with *primary CM* and *secondary CM*. The same test was used to compare the whole group of cats with CM (*cardio tot*) and healthy cats (*healthy*), and to further compare healthy cats and cats with each type of CM (HCM, restricted, dilated, unclassified). Differences were considered as significant if $P < 0.05$.

Results

Thirty-nine cats of different breeds from June 2016 to December 2016 fulfilled the inclusion criteria and were retrospectively included in the study.

12 were clinically healthy (31%), 7 males and 5 females with a median age of 8.5 years (min-max age: 1-14 years), and 27 were affected by cardiomyopathy (69%), 16 males and 11 females with a median age of 13.5 years (min-max age: 1-18 years). No significant difference in terms of age was found between the two groups.

Breeds are reported in table 13.

	Healthy	Cardio
	N=12	N=27
Domestic shorthair	6 (50%)	17 (63%)
Maine Coon	4 (33.3%)	2 (7.4%)
Persian	1 (8.3%)	3 (11.1%)
Chartreux	-	2 (7.4%)
Birman	-	1 (3.7%)
British shorthair	-	1 (3.7%)
Exotic shorthair	1 (8.3%)	-
Canadian Sphynx	-	1 (3.7%)

Table 13 Breed distribution of the 39 cats included in the study

The types of cardiomyopathy in the *cardio tot* group (*primary CM* and *secondary CM*) were the following: HCM ($n = 15$; 55.6% of the *cardio tot*), dilated cardiomyopathy ($n = 5$; 18.5%), restricted cardiomyopathy ($n = 3$; 11.1%); unclassified cardiomyopathy ($n = 4$; 14.8%). The *primary CM* group consisted of 19 cats (70.4%), while among the 8 cats with a *secondary CM* (29.6%), 5 had chronic kidney disease (CKD), in 3 cases associated with hypertension, 2 were affected by hyperthyroidism and one case presented hypertension with immune-mediated hemolytic anemia.

The electrophoretic profile obtained from homogenized tissue supernatants (Fig. 1) showed that CK-MM and Macro-CK1 were the predominant fractions both in skeletal and cardiac muscle. In the figure, an electrophoretic gel was obtained by homogenized skeletal muscle (1-5) and cardiac muscle (6-10) with serial dilution (undiluted, 1:2, 1:4, 1:8, 1:10). Serum from a cat with cardiomyopathy and a healthy cat were placed in position 11 and 12 respectively



Figure 1

The undiluted samples of both muscles also showed a weak band of CK-BB; supernatants from the homogenized cardiac tissue expressed strong bands of CK-MB, which were instead weak in the homogenized skeletal muscle. Bands relative to Macro-CK2 were not detectable in both tissues.

Results regarding absolute and percentage values of total CK and of iso and macroenzymes activities in sera from cats of the different groups are reported in table 14.

	Primary CM	Secondary CM	Cardio	Healthy	HCM
	N=19[†]	N=8[†]	N=27[†]	N=12[†]	N=15[†]
CK tot (U/L)	175.0 (112.3-230.0)	162.0 (105.7-515.0)	170.0 (106.7-231.5)*	96.0 (71.3-154.2)	162.0 (101.7-226.0)
CK-BB (%)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
CK-MB (%)	0.3 (0.0-1.7)	0.5 (0.0-1.8)	0.3 (0.0-1.7)	0.0 (0.0-0.3)	0.7 (0.0-2.15) *
Macro-CK1 (%)	34.7 (19.5-63.5)	48.7 (22.3-66.8)	40.4 (19.5-65.2)	62.1 (18.5-71.3)	38.8 (18.3-49.9)
CK-MM (%)	63.8 (32.7-78.4)	49.7 (33.2-77.3)	58.1 (30.9-78.4)	38.0 (28.5-80.3)	60.4 (47.7-79.7)
Macro-CK2 (%)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
CK-BB (U/L)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
CK-MB (U/L)	0.0 (0.0-2.4)	0.7 (0.0-2.3)	0.1 (0.0-2.3)	0.0 (0.0-0.5)	2.07 (0.0-4.4)
Macro-CK1 (U/L)	61.6 (32.2-75.8)	52.8 (44.0-121.4)	57.2 (35.3-79.4)	55.4 (17.7-57.3)	44.2 (36.7-103.2)
CK-MM (U/L)	82.3 (26.2-155.1)	55.6 (32.1-486.6)	81.9 (28.4-158.5)	41.2 (22.4-92.7)	81.4 (37.0-142.4)
Macro-CK2 (U/L)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)

Table 14 Median values and (between parenthesis) I and III quartile recorded for total and fractioned CK in the different groups of cats included in this study. † Number of cases for total CK activity and for the absolute value of CK iso- and macroenzymes were: 7 (cardio + other), 13 (cardio alone), 20 (cardio tot), 11 (healthy), 9 (HCM).

* = P < 0.05 vs healthy cats

In clinically healthy cats, CK-MM and Macro-CK1 were the main fractions, a low percentage of CK-MB was found only in 4 cats, and CK-BB and Macro-CK2 were never detected. Cats with CM were characterized by the predominance of CK-MM and Macro-CK1, equally to the healthy group. However, CK-MB and CK-BB were found in 16/27 cases and in 3/27 cases, respectively. The analysis of individual data showed that most of the cats with a clearly detectable CK-MB band were affected by an HCM ($n = 10$; 59.2% of the cats with CK-MB), while only one patient for each group of dilated and restricted cardiomyopathy showed bands related to CK-MB. The remaining 4 cases with a clearly detectable CK-MB band were cats with unclassified CM. Of the 3 cases with detectable CK-BB, 2 were affected by HCM and one by restricted CM.

Statistical analysis did not reveal significant differences between cats with different types of *primary CM*, and between the whole *primary CM* group and *secondary CM* group, thus these two groups were merged together into a single group of CM (*cardio tot*). Total CK activity was significantly higher in the *cardio tot* group than in clinically healthy cats ($P = 0.030$). No significant differences were found between these two groups for the CK electrophoretic fractions, both in percentage and absolute values. A huge individual variability was found in cats with cardiomyopathy. Figure 2 shows the comparison of absolute and percentage values of CK-MB between cats with CM in presence (*Cardio + other*) or not (*Cardio alone*) of concurrent diseases, between the whole group of cats with CM (*Cardio tot*) and healthy cats, and between cats with HCM and healthy cats. The boxes indicate the I-III interquartile range (IQR), the horizontal line the median value. and the whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR. The grey dots indicate the near outliers (i.e. values higher than the quartile III plus 1.5 x IQR); the white dots indicate the far outliers (i.e. values higher than the quartile III plus 3.0 IQR).

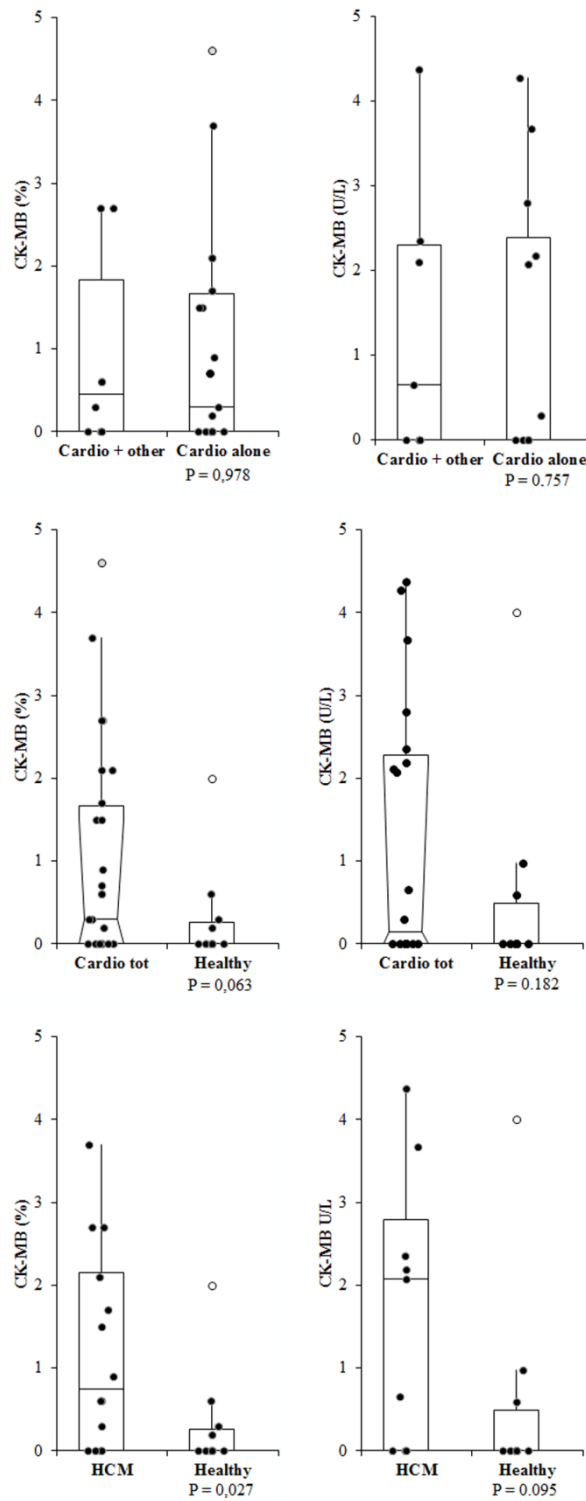


Figure 2

A significantly higher percentage of CK-MB was found in cats with HCM compared with healthy cats ($P = 0.027$). Again, a huge individual variability was detected.

Discussion

This preliminary study was aimed to assess whether the serum concentration of CK-MB, that in people is a marker of heart damage [Burgener, 2006; Bodor, 2016], increases in cardiopathic cats. Being a preliminary study, cats with cardiomyopathy were analysed irrespective of the type and stage of cardiomyopathy. Unfortunately, reagents to directly measure feline CK-MB are not available as well as information on the analytical performances of reagents used in other species [Walker, 2006; Diniz, 2007]. Therefore, we quantified serum CK-MB using the electrophoretic separation of CK iso- and macroenzymes, recently validated in cats [Paltrinieri, 2010].

In the current study, the total CK activity in healthy cats, fell within the range reported for clinically healthy cats in other studies [Aroch, 2010; Fascetti, 1997], although it was lower than that reported in a previous study performed with the same method and instrument [Paltrinieri, 2010]. A huge individual variability was detected for total CK activity in cats with CM; this variability could depend on the co-morbidity with other diseases that cause a secondary muscle involvement [Neumann, 2005]. This hypothesis is supported by the fact that the highest values of total CK were found among cats with a *secondary CM*. Anorexia, possibly secondary to hyperthyroidism or other diseases, may induce a negative energy balance that leads to a transient rhabdomyolysis and increases serum CK levels [Fascetti, 1997; Cardoso, 2014]. Anyway, since statistics did not show differences in total CK activity between cats with primary and secondary CM, we decided to gather the two groups into a single CM group (*cardio tot*). Total CK activity in *cardio tot* group was significantly higher than in healthy cats; nevertheless, for the large majority of cats, it was still included within the range reported in the literature [Aroch, 2010].

The interpretation of electrophoretic bands was driven by the results obtained from homogenized tissues, which, as already reported [Paltrinieri, 2010], confirmed that CK-MB is present in the heart, but it is virtually absent in the skeletal muscle, thus supporting the rationale of this study. Hence, the leakage of CK-MB from damaged myocardiocytes should induce an increase of serum CK, and CK-MB may work as an indicator of cardiomyopathy in cats as in other species.

As further support to this hypothesis, CK-MB was virtually absent from serum of clinically healthy cats, where CK-MM and Macro-CK1 were the predominant isoforms both in percentages and absolute values, although with a high individual variability. This agrees with the previous study [Paltrinieri, 2010] that detected also small amount of CK-BB and Macro-CK2 that were not detectable in our study. This was likely due to the analytical variability of the method, ranging between 2% and 8% [Paltrinieri, 2010].

Interestingly, this study confirmed the abundance of Macro-CK1 in feline serum. This is somehow surprising, since Macro-CK1 in other species is composed of antibodies and CK-BB [Lee, 1994; Chun-Yu, 2010], that was virtually absent in serum. This result suggests that feline Macro-CK1 may have a different structure compared with other species: it has been speculated that Macro-CK1 may be a CK-MM-immunoglobulin complex [Yuu, 1980; Kanemitsu, 1981; Lang, 1982] or a CK-MM with a different electrophoretic mobility. Further researches are needed to clarify the real composition of feline Macro-CK1. However, Macro-CK1 may not be relevant in cats with CM, since its percentage or activity was not significantly higher compared with clinically healthy cats. Conversely, the number of animals showing detectable CK-MB bands in serum was higher in cats with CM than in clinically healthy cats. However, the CK-MB activity was quantitatively low, confirming that the CK-MB isoform represents only a small amount of total cardiac CK, as already reported in dogs [Aktas, 1993; Dolci, 2006] and here demonstrated by the analysis of homogenized cardiac tissue. Moreover, it should be noted that in people, where CK-MB is a marker of myocardial infarction [Bodor, 2016], the isoenzyme is released in blood after the death of myocytes, while most of the CM detectable in this study were not primarily associated to cell necrosis.

The difference between the percentage of CK-MB in serum of clinically healthy cats and of cats with CM did not reach significance. This may depend on the high individual variability of the phenotype within the cardiopathic group, in turn due to its heterogeneous composition in terms of type of disease. This hypothesis is supported by the analysis of individual data that evidenced CK-MB bands in most of the cats with HCM, while CK-MB bands were almost absent in other types of CM. It is interesting to note that 100% of the unclassified CM revealed clearly detectable CK-MB bands (4 out of 4 cases),

although the number of unclassified CM cases in the present study is too low to lead to any conclusion. The pathogenesis of unclassified CM is unclear, but it has been reported that this condition could represent an early or late stage of another recognized CM [Ferasin, 2009].

Increased CK-MB may thus be a distinctive feature of HCM in cats, as in people [Hamada, 2016].

This hypothesis is supported by statistical analysis, that revealed a significantly higher percentage of CK-MB in cats with HCM compared to healthy animals. However, as in the whole population of cats, no significant differences were found in terms of absolute values, likely because the amount of CK-MB released from cardiac muscle is quantitatively minimal, although sufficient to induce percentage variations.

Even though the study design does not allow the investigation of the cause of the increased CK-MB in serum of cats with HCM, it may be postulated that CK-MB is released from hypertrophic myocytes, that in feline HCM are affected by ultrastructural changes, including myofibrillar lysis [Van Vleet, 1980] or mitochondrial depletion [Van Vleet, 1980; Christiansen, 2015], which in turn may reduce the energy supply necessary to maintain the integrity of cell membranes. However, additional studies based around measurement of cTnI and histopathological analysis would be required to evaluate this hypothesis further. Independently on the mechanism responsible for this increase, the higher CK-MB percentage found in cats affected by HCM draws the lines for a perspective research on the role of CK-MB in the evaluation of feline HCM, based on a more standardized case selection and on the stratification of patients according to the extent or severity of the disease.

Hospital Grade Smartphone multiple-lead ECG for the canine patient: results of a pilot comparative analysis with standard 6-lead electrocardiograph

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Cardiorenal syndrome and arrhythmias

In human medicine, the development of arrhythmias in patients affected by CKD is a well-known issue, because of the possible electrolyte alterations, acid-base status, rapid and frequent changes in blood pressure and volume and aldosterone activity. The most common arrhythmias identified are ventricular arrhythmias and atrial fibrillation. Moreover, in end stage CKD, one in two deaths for cardiovascular reasons are attributable to arrhythmic events [Di Lullo, 2017].

On the other hand, MMVD increases the risk of arrhythmias, mainly atrial, due to progressive atrial enlargement. Nevertheless, ventricular arrhythmias could be present, because of the progressive ventricular stiffness and aging, that can damage the conduction system.

Arrhythmias can contribute to the pathogenesis of CRS type 2. In fact, renal perfusion can be influenced by poor cardiac output caused by conduction disorders.

The diagnosis of cardiac arrhythmias through standard 6 lead electrocardiography began over 100 years ago [Drew, 2002]. In the last decade, there has been an exponential increase in the number of smartphone users, and the number of products and applications dedicated to pet owners and veterinarians have increased as well (ie. weight control, physical activity, health information storage, etc.) [Devi, 2015]. Mobile health (mHealth) technologies are revolutionizing the practice of cardiovascular medicine in humans thanks to the global diffusion of smartphone devices [Haberman, 2015]. Profound changes in diagnostics and monitoring have been made, as much relevant data could now be generated locally by the provider on the territory rather than centrally by hospital [Hickey, 2016; Chow, 2016]. For this reason, smartphone-based systems that allow fast and reliable recording of heart rhythm with different supports have emerged. General practitioners and small referral clinics

may benefit from the possibility of performing ECGs in the primary care setting in order to promote tele cardiology as a good clinical practice [Devi, 2015]. Up to now, Vezzosi et al. in 2016 conducted a study about diagnostic accuracy of a single lead smartphone electrocardiograph in the canine patient [Vezzosi, 2016]. However, fast, portable and easy to use electrocardiograph that record ECG on multiple leads is still lacking. D-Heart Vet portable ECG device has been recently developed with this purpose, allowing the acquisition of 6 peripheral leads ECG tracing and sending it live using low energy Bluetooth to the smartphone [Maurizi, 2017].

Materials and methods

A prospective cohort study was carried out at the Cardiology Unit of the Department of Veterinary Medicine (DIMEVET) of the University of Milan from July 2016 and July 2017. The study was reviewed and approved by the Committee for Animal Welfare of the University of Milan (approval number 97/2016; date of approval 26 May 2016). One hundred and fifty-two adult dogs were included in the study. The dogs were referred for a cardiologic evaluation or assessment prior to surgery/anesthesia or were selected among healthy dogs owned by university staff or students. For every dog, informed consent was obtained from owners before every procedure.

For each subject, a standard 6-lead ECG (Cube click-ECG, Cardioline) was recorded for 5 minutes in standard position (unsedated dog, gently restrained in right lateral recumbency). Flattened alligator clips were used as surface electrodes and placed attached to the skin, cranial to the olecranon and the patella. Alcohol was applied in order to maintain electrical contact. The standard ECG was immediately followed by a 6-lead D-Heart ECG recording. The ECG was recorded for at least 120 seconds. A good quality frame and every arrhythmia were saved and stored, with an anonymous ID, on a cloud storage of a dedicated smartphone. The same electrode placement and dog position were used for the two ECG recordings. Patient's data were recorded and collected in a protected database. For each dog, ECG tracings obtained with the two devices were printed at a paper speed of 50 mm/s and a gain of 10 mm/mV. The data analysis was blindly performed by two experienced and independent observers (AS and CL). In case of disagreement, a third impartial observer (PGB)

adjudicated the tracing. Electrocardiographic complexes were measured in lead II for both ECGs. The following variables were measured: mean HR (beats per min, bpm), calculated as the number of QRS complexes recorded in 15 cm and multiplied by twenty; Mean Electrical Axis (MEA); P wave amplitude (mV) and duration (ms); PR interval duration (ms); QRS complex amplitude and duration; T wave amplitude and duration. Reference values for the variables considered were reported by Santilli and colleagues [Santilli, 2009].

Severity of ECG abnormalities was defined by a binary semi-quantitative score based on the sum of 8 criteria, modified by Maurizi et al [Maurizi, 2017]: presence of any arrhythmia, presence of wandering pacemaker, $PR \geq 0,13$ ms, $QRS < 1$ mV, $QRS > 3$ mV, $QRS \geq 0,07$ ms, MEA +40/+100, concordance P – QRS. Each parameter counted as 1 if abnormal. Four ECG groups were identified: normal (≤ 1 criteria); mildly abnormal (2 criteria); moderately abnormal (3 criteria); markedly abnormal (> 4 criteria).

The D-Heart device was conceptualized by one of the authors (NM) and it is constituted by a battery-powered device for ECG measurement on multiple leads (6 peripherals) connected through the use of low energy Bluetooth to a smartphone. The front end is constituted by 3 Sigma Delta modulators able to sample the ECG signal and then filter the signal in a digital way. The module Bluetooth Low Energy is able to send data to the smartphone or Tablet deputy to the ECG signal display, whereas Lithium battery ensures the functionality during measurement. A dedicated and certified App (D-Heart Vet) allows recording, visualization, sharing and categorization of patient's data and ECGs. The App is available for the two main mobile operating systems.

Statistical analysis

Continuous variables are expressed as mean \pm standard deviation or median and interquartile range (IQR), when appropriate, and categorical variables as proportions. The concordance between D-Heart and standard 6 leads electrocardiographs was assessed by: the weighted kw-Cohen index, with its relative significance, taking as the endpoint variable the ECG group; the Bland-Altman method, with a 95% confidence level, for the P wave, PQ, PR and QRS interval measurements.

Since differences between the two measurements did not follow a normal distribution, a non-parametric approach (median value and 2.5° and 97.5°percentiles) was used to determine the limits of agreement. P values were two-sided and considered significant at the 0.05 level. All analyses were performed using SPSS/for Windows, version 20 (Armonk, NY: IBM Corp).

Results

One-hundred sixty-four dogs were enrolled. Only paired ECG that were acceptable for interpretation have been considered for the analysis. Twelve patients (7%) were excluded from the ECG analysis because of: excess of background noise/artifact (N=1, 0.6%), poor cooperation of the animal (N=2, 1.2%), technical errors of the operator in saving the track (N=9, 5.5%). Median age and body weight of the 152 dogs included was 8 (2 - 11) years and 22 (6 – 28), respectively. Population characteristics are displayed in the table 15.

	Total Screened (n=152)	Small Breed (0.5-5 kg) (n=12)	Medium Breed (5-20 kg) (n=69)	Large Breed (> 20 kg) (n=71)	P
Age (years)	8 [2; 11]	4 [1; 10]	9 [3; 13]	7 [2; 11]	0.03
Sex					
Male Intact	63 (41%)	6 (50%)	38 (51%)	22 (31%)	0.07
Male Neutered	12 (8%)	2 (50%)	4 (51%)	6 (31%)	0.32
Female	25 (16%)	3 (50%)	1 (51%)	16 (31%)	0.11
Female Neutered	52 (35%)	1 (50%)	20 (51%)	27 (31%)	0.67
Breeds					
Mixed breed	21 (14%)	3 (25%)	6 (9%)	12 (17%)	0.1
Golden Retrievers	12 (8%)	0	4 (6%)	8 (11%)	0.09
Labrador Retrievers	11 (8%)	0	8 (9%)	3 (4%)	0.6
Dachshunds	7 (5%)	1 (8%)	4 (6%)	2 (3%)	0.7
Poodles	6 (4%)	2 (16%)	2 (3%)	2 (3%)	0.12
Boxer dogs	5 (5%)	1 (9%)	3 (4%)	1 (2%)	0.32

Reason for referral						
Healthy Screening	83 (55%)	6 (50%)	27 (39%)	50 (70%)	0.7	
Known Cardiac Disease	59 (39%)	3 (25%)	38 (55%)	18 (25%)	0.78	
Pre-surgical screening	10 (6%)	3 (25%)	4 (6%)	3 (5%)	0.09	

Table 15 - Baseline characteristics of the screened dog cohort. Patients are divided based on their body size.

Main breeds represented in the cohort have been displayed for clarity

Agreement between the two operators was obtained in 147/152 (97%) cases with D-Heart tracings and in 149/152 (98%) cases with standard 6-lead ECGs.

ECG readings

Concordance between D-Heart ECG readings and standard 6-lead ECG was high for sinus rhythm adjudication, sinus arrhythmia, sinus tachycardia and identification of atrioventricular blocks, right axis deviations, P wave morphology, negative and biphasic T waves. Cohen's kappa coefficients and relative p values are reported in table. There was a 100% concordance for rhythm (sinus rhythm and atrial fibrillation) and ST segment description.

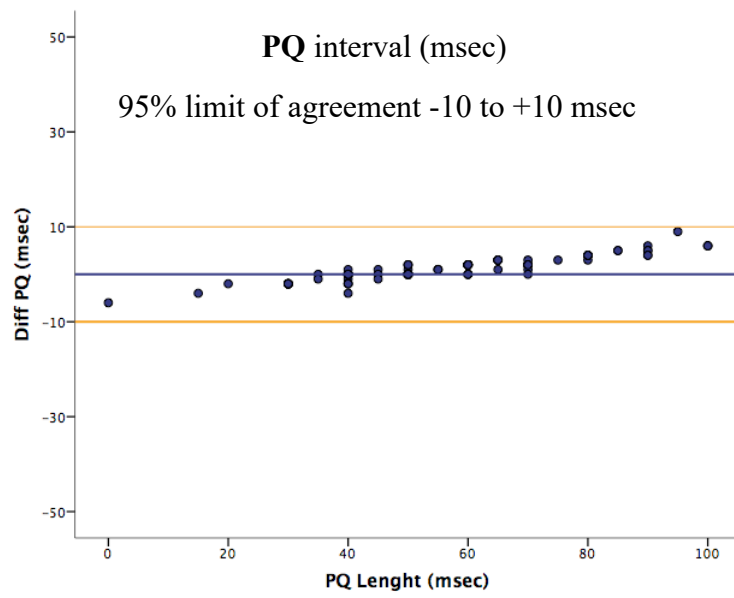
	Standard 6-leads ECG (n=152)	D-Heart device (n=152)	Agreement (Kappa)	P
Sinus Rhythm	41 (27%)	40 (26%)	0.978	<0.01
Sinus Arrhythmia	93 (62%)	98 (63%)	0.910	0.02
Sinus Tachycardia	3 (2%)	2 (1%)	0.899	0.01
Sinus Bradycardia	6 (4%)	6 (4%)	1	
Atrial Fibrillation	2 (1%)	2 (1%)	1	
Atrioventricular Blocks (n)	6 (4%)	5 (5%)	0.989	0.01
Right Axis Deviation (n)	28 (18%)	22 (14%)	0.954	0.01

P wave morphology (positive)	150 (99%)	147 (98%)	0.971	0.01
Isoelectric ST segment (n)	149 (98%)	149 (98%)	1	
Negative T wave morphology (n)	48 (32%)	46 (30%)	0.929	0.01
Biphasic T wave morphology (n)	46 (30%)	48 (32%)	0.918	0.01

Tab. 16 Agreement between D-Heart tracings and standard 6-leads ECG tracings in the adjudication of ECG rhythm and morphology

Standard 6-Lead ECG and D-Heart tracings were respectively classified as normal: 118 (78%) vs 126 (83%); mildly abnormal: 28 (18%) vs 20 (12%); moderately abnormal: 5 (3%) vs 4 (3%); markedly abnormal: 1 (1%) vs 1 (1%). Cohen’s weighted kappa (kw) test demonstrated a concordance of 0,912 (p= 0,03, agreement 95,19%).

PQ and QRS intervals and T waves duration comparison with Bland-Altman method showed good accuracy for D-Heart measurements (95% limit of agreement ± 10 ms for PQ, ± 35 ms for QRS and ± 5 ms for T waves duration). The Bland-Altman graphs are reported as figure 3.



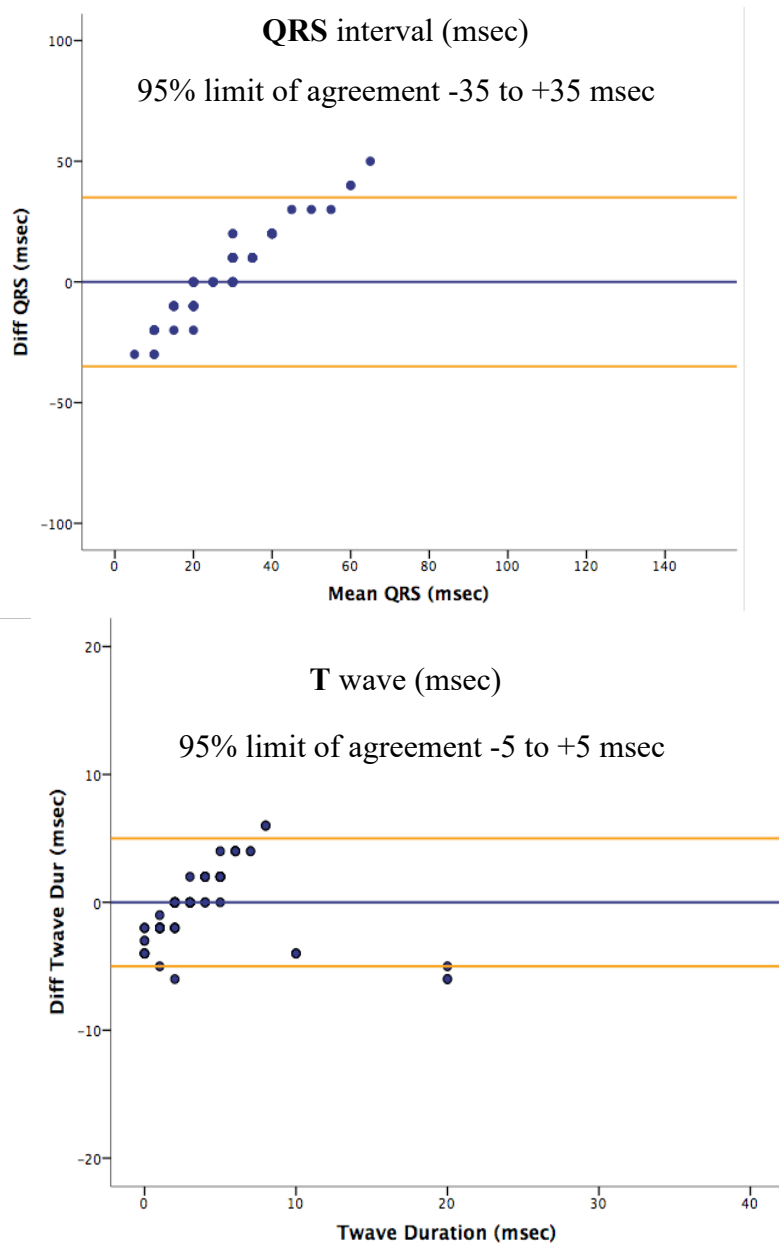


Fig. 3 Limits of agreement (Bland–Altman) plot showing differences between PQ interval, QRS interval and T wave duration manually measured on standard 6 leads-ECG and D-Heart Smartphone ECG device tracings.

Discussion

The aim of the study was to determine the reliability of a new recording method for ECG in dogs. This is the first study in veterinary medicine that compared two multiple lead ECG with different recording systems, one of which based on a portable device for a smartphone.

The first result to point out is that the smartphone ECG was easy to use and that 93% of the trace were successfully recorded and suitable for interpretation, in line both with human and veterinary results from other studies on smartphone systems [Vezzosi, 2016, Maurizi 2017]. Most of the tracings that were not available for interpretation were actually not recorded in the cloud storage. This allows us to affirm that, also if the storage system is very operator friendly, the learning curve should not be underestimated.

The level of concordance between the two recording methods was excellent. In fact, for every rhythm identified, the concordance between D-Heart ECG readings and standard 6-lead ECG was very high according to the Cohen's kappa. Every arrhythmia identified with the traditional method can also be recognized with the D-Heart® technology and there was a 100% concordance for rhythm (sinus rhythm and atrial fibrillation) waves polarity and ST segment description. Identification of the P waves plays a fundamental role in the correct diagnosis of this type of arrhythmia. At the time the ECG were taken, it was not possible to change the amplitude of the waves but, despite that, P waves remains clearly visible in both the tracing, not leading to any misdiagnosis.

The primary objective of the study was to determine the concordance between D-Heart Vet and standard ambulatory veterinary ECG as compared with a score of growing ECG abnormalities. Agreement between the two techniques was excellent (95,19%) with kw test showing a concordance of 0,912 ($p=0,03$). It's important to underline that severe abnormalities were correctly addressed with both ECG recording devices but indeed, for subtle alterations, the standard ambulatory ECG seemed more accurate. In fact, the percentage of normal ECGs was 78% vs 83%, and mildly abnormal 18% vs 12%. However, a possible explanation lies in the fact that the ECGs were not recorded simultaneously but sequentially, therefore slight differences in the HR values could be present, affecting the scoring. HR tends to rise if animals are manipulated, and to normalize during recumbency, also if physical restraint is applied. Moreover, impossibility in adjusting waves' voltage could have contributed to the loss of information regarding subtle abnormalities, like wandering pacemaker, pretty common in canine patients.

Finally, in dogs in which R waves were very tall, impossibility of adjustments in voltage led to overlapping between D1 and D2, preventing correct measurements of this segments.

Comparison of the PR, QRS and T waves duration with Bland Altman method showed a good accuracy for D Heart vet technology. Less concordance was obtained for QRS duration, for which the confidence interval was slightly wider. In our opinion, smartphone recording methods should not be used to assess the waves in substitution of standard ECG for the diagnosis of mild alteration and/or cardiac chamber enlargement. Indeed, it's application can find large utility for first opinion practice and in all the facilities in which a cardiologist may not be always be present, thanks to the possibility to easily share the tracing.

Few limitations were present in our study. First, despite a reasonably large cohort of canine patients, it was composed mainly by patients with low cardiovascular risk. Because of that, the number of dogs with arrhythmias was relatively small. The explanation of the scarce prevalence of arrhythmias in our sample derives from the origin of the enrolled patients, mainly student-owned healthy dogs or referred for routinely screening prior to anaesthesia, with low prevalence of ECG alterations. The minority of cases were dogs presenting heart conditions. Nevertheless, this was a pilot study pointed on the determination of the level of concordance between the D-Heart Vet and the standard 6-lead ECG tracings, not specifically focused on the identification of rhythm alterations. A larger number of arrhythmias might have needed to validate more extensively smartphone ECG on pathologic tracings. Another limitation might be the underestimation of isolated arrhythmic events by Smartphone ECG since the ECGs were not simultaneously recorded. However, every isolated arrhythmic event was evidenced in both recording systems tracings. Moreover, the smartphone ECG tracing allowed diagnosis of all the arrhythmias detected by standard 6-leads ECG included in our study.

This is the first study in veterinary medicine that compared, in a rigorous and systematic way, two recording ECGs methods with such different characteristics. Moreover, for the first time, it is here stated that a smartphone based, multiple lead device can be used with the same reliability of current traditional recording technologies.

D-Heart is indeed the world's first ECG device that is as reliable as hospital ECGs, but usable anywhere, in any condition and by anyone, even with no medical background. This may be less important than in the human since a spontaneous evidence of acute myocardial infarction is not described in dog as frequently as in humans. However, it is certainly true that d-heart opens the gate to new scenarios. The owners of animals suffering from heart disease already diagnosed, can easily share the ECG information with the veterinarian cardiologist in real-time manner. Even if they live far from referral centres, and with less stress for the patients. The possibility to share ECGs tracing with specialists is a very powerful aspect of technology innovation, because sharing makes the decision process faster, powerful and more accurate. Veterinary physicians that are not fully trained in cardiology can benefit of the possibility to have an opinion leader one click away. This can effectively must be considered one of the most important results of the age of communication we're living.

Evaluation of aldosterone concentration through urinary aldosterone:creatinine ratio in urine of healthy dogs and dogs affected by preclinical myxomatous mitral valve disease: a pilot study

The importance of being Aldosterone

Aldosterone is a mineralocorticoid hormone produced mainly by the glomerular area of the adrenal cortex. The major stimulus to its production is given by the interaction of angiotensin II with the angiotensin receptor 1 (AT1); other important stimuli are high levels of potassium and Adreno Cortico Tropic Hormone (ACTH). Mineralocorticoid receptors are located in kidneys, heart, brain and vascular system. Aldosterone main effect is to promote sodium reabsorption and renal elimination of potassium, thus favouring increase in blood volume and pressure. Further effects have been attributed to this hormone over the years, such as sympathetic activation, fibrosis and production of oxygen free radicals [Ettinger, 2016].

Aldosterone is one of the main actors in the heart and kidney crosstalk and, because of its main role in determining volume overload in patients with impaired heart function, it's always been a potential target of pharmacological treatment. Historically, Aldosterone was thought to be produced only by its main pathway, the RAAS and, because of that, the main class of drugs developed were directed at the inhibition of this way of production (ACE inhibitors). Late studies, conducted on humans, mice and dogs, have highlighted a variety of different mechanism of production, both tissutal and intracellular, for the synthesis of angiotensin II and aldosterone. The components of these systems, despite being located at different levels, are mostly overlapping [Fyhrquist, 2008; Paul, 2006; Kumar, 2007; Fleming, 2006].

Tissue RAAS has been found in many organs, including kidneys, adrenal glands, heart, vascular system and brain. Its effects affect the growth, proliferation and cell metabolism of the respective organ and contribute, under physiological conditions, to maintain tissue homeostasis [Fyhrquist, 2008; Paul, 2006; Fleming, 2006]. A study from Barlucchi et al. reported the existence of a local RAAS with aldosterone production at the level of ventricular myocytes in dogs [Barlucchi, 2001].

The production of aldosterone in cardiac tissue has also been demonstrated in both healthy dogs and dogs with dilated cardiomyopathy [Palomar, 2017], as well as increased ACE tissue activity following experimentally induced mitral insufficiency in healthy dogs [Fujii, 2007]. The progression of CKD in patients with overactivation of the RAAS, like in HF, is being attributed to aldosterone through the promotion of renal fibrosis and glomerulosclerosis [Cruza, 2013]. Onozato and colleague studied a murine model of HF and postulated that increases in aldosterone levels have detrimental effect on renal function because of the increased oxidative stress and TGF- β overproduction. In fact, untreated animals ended up developing proteinuria, azotemia and glomerulosclerosis. On the other hand, mice treated with ACE inhibitors showed lower azotemia and proteinuria [Ozonato,2007].

Another class of drugs that can be used to reduce the effect of Aldosterone are the Aldosterone Receptors Blockers (ARB), whose best-known molecule is spironolactone. Spironolactone is an antagonist of mineralocorticoid receptors, through which aldosterone has its main effects. In 1999, the human RALES study showed that the addition of this drug to standard cardiology therapy improves clinical conditions and reduces the risk of death [Pitt, 1999]; beneficial effects were also documented in patients with concomitant heart and renal failure, despite the negative effects of spironolactone on the kidneys, mainly due to hyperkalemia [Gu, 2016]. More recently, the same results were obtained in dogs with HF caused by MMVD [Bernay, 2010]. In 2017 a study by Hezzell et al. reported favorable results for the use of spironolactone also in dogs with MMVD in preclinical phase [Hezzell, 2017]. The beneficial effects of spironolactone demonstrate the pathological role of aldosterone, exerted through interaction with mineralocorticoid receptors [Funder, 2009].

ACE inhibitors and ARBs are then beneficial in the treatment of cardiovascular and renal disorders, but frequently CKD patients are not given this therapy due to the concern on the effect on kidney function. As reported by Orvalho and colleagues in 2017, “a better understanding of the relative risk of using these and other drugs may be very important in patients with CRS” [Orvalho, 2017].

In literature, little is known about the normal values of aldosterone in healthy dogs and dogs with a natural occurring history of MMVD. Aldosterone is normally secreted in a pulsatile way, making measurement on serum poorly correlated to the actual aldosterone concentration for each patient [Tolagen, 1978; Steele, 2002].

The measurement of urinary aldosterone levels these changes since the hormone present in urine, stored in the bladder, represents an average of the secretion of aldosterone that occurred in the previous hours [Gardner, 2007]. The measurement of aldosterone in urine produced and collected in 24 hours is considered the gold standard. However, the procedure is a long, expensive and poorly feasible in animals. A study by Gardner et al. from 2007 reported that the urinary aldosterone:creatinine ratio on a single urine sample is comparable to the urinary excretion of aldosterone in 24 hours and is therefore an accurate parameter to evaluate the production of the hormone; furthermore, only one urine sample obtained for spontaneous micturition is considered sufficient [Gardner, 2007]. This has been validated both in physiological conditions and in conditions in which the RAAS is pharmacologically influenced or by variations in the salt in diet [Gardner, 2007].

So, in order to identify possible positive effects and risk related to the use of this drugs in patients affected by MMVD, the evaluation of aldosterone in healthy and sick dogs could be a turning point.

Materials and methods

The study was performed in two subsequent steps.

In the first part of the study, the urine of 4 subjects were retrospectively selected for the evaluation of the ELISA kit performances. Two patients were selected among a healthy pool, based on clinical examination and complete blood workout, one among urine of patients classified as CKD IRIS stage 4 (proteinuric, no systemic hypertension), one was a dog with MMVD stage C.

Urinary aldosterone determination was performed on these 4 urinary supernatants, previously stored at -18° for a period of time comprised among 3 weeks and 3 months.

The concentration of urinary aldosterone was determined by a competitive ELISA kit. The determinability limit of the kit is 4.7 pg/mL and the linear calibration line up to 250 pg/mL. The cross reactivity is reported in Table 17.

ELEMENT	%XR
Deoxycorticosterone	0,30%
Progesterone	0,20%
Corticosterone	0,19%
Cortisol	≤0,001%
DHT	≤0,001%
Estradiol	≤0,001%
Testosterone	≤0,001%

Table 17

The kit is based on a solid phase anti-IgG donkey anti-sheep antibody, a polyclonal anti-aldosterone sheep antibody, alkaline phosphatase labeled aldosterone (AP) and p-nitrophenyl phosphate (pNpp), used as a substrate for detection. For the standard curve, 7 scale dilutions 1: 2 of the standard aldosterone were performed, with a range of concentrations from 250 pg/mL to 3.9 pg/mL.

Aldosterone is mostly present in urine as metabolites, which cannot be directly recognized by anti-aldosterone antibodies [Syme, 2007]. For this reason, each urine sample was subjected to acid hydrolysis. Briefly, a part of urine was transferred into a special tube and was diluted with 2 parts of 0.2N HCl. The sample was then incubated overnight at room temperature, covered and protected from light. Before being processed, the urine samples were diluted 1:30 in Dilution Buffer thus obtaining a final 1/90 dilution.

The results were calculated through the following steps:

1. B value was obtained by the average of the absorbances (ABS) of each standard minus the average of the blank values (Non Specific Bound, NBS)

2. B value was then transformed in percentage with respect to the value with the maximum reading (Maximum binding well, B₀), and expressed as B / B₀%.
3. The values obtained were placed on a graph, in which on the Y axis were reported the values of B/B₀ (in percentage) and on the x axis the values of the Log₁₀ of the standard aldosterone concentrations.
4. The standard line was then obtained by performing linear regression of the points in the graph.
5. The average of the ABS obtained on each sample was converted to B / B₀ (%) and included in the equation of the standard line for calculating the aldosterone concentration.

Parallelism, spike and recovery test and reproducibility test were performed. For parallelism, the curve obtained with the standard aldosterone was compared with the curves obtained from scalar dilutions of the urine of the 4 subjects. The parallelism of the obtained lines was evaluated with a statistical test of the PRISM 6 program (GraphPad Software, San Diego, CA). For spike and Recovery test, 1 nanogram of standard aldosterone was added to a urine sample in triplicate. The sample was tested in ELISA and the amount of aldosterone recovered in the sample was calculated.

For reproducibility test, the urine of the 4 dogs were tested in different times and plates and the coefficient of variability calculated.

The second part was a prospective study including private owned dogs recruited at the Cardiology unit of the Department of Veterinary Medicine (DIMEVET) of the University of Milan in the period between November 2017 and July 2018. For every dog, informed consent signed by the owners was obtained.

The population was composed by both healthy dogs and dogs with stage B1 MMVD.

For every dog, signalment and anamnesis were collected. Complete clinical examination (including measurement of systemic blood pressure using the Doppler method), complete echocardiographic examination, complete blood tests (including CBC and biochemical examinations) and urine examination with evaluation of urinary to protein ratio (UPC) were performed. The CBC and biochemical examinations were obtained only for 82% of the subjects; for the rest of the subjects, the owners did not consent to collect blood sample and did not have previous exams available.

Blood tests carried out up to 6 months before or after urine collection, also performed at external laboratories, were accepted.

The subjects were classified as healthy based on clinical history, clinical examination and the results of the tests performed. The presence of a very mild mitral regurgitation evidenced by colour Doppler, in the absence of heart murmur or any other echocardiographic alteration, was tolerated and defined as trivial. None of the dogs was assuming any cardioactive drug.

Subjects with a diagnosis of MMVD were classified as stage B1 according to the ACVIM classification guidelines. The presence of any other cardiac disease was a reason for exclusion.

Two patients affected by chronic renal failure (IRIS 3) and persistent proteinuria were included. Among the subjects belonging to this subgroup, the patient affected by proteinuria was taking ACE inhibitors.

Free catch Urine were collected directly from the owners or, if cystocentesis was needed, by a trained operator.

The samples were immediately refrigerated at 4 ° C and centrifuged within 8 hours at 1250 rpm for 5 minutes; the supernatant was then immediately frozen at -20 ° C until the determination of urinary aldosterone. For each urine sample between 3 and 9 mL, of supernatant were collected.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics for MAC OS software, version 25.

Continuous variables are expressed as mean \pm standard deviation or median and interquartile range (IQR), when appropriate, and categorical variables as proportions. Normality of distribution was evaluated using Shapiro-Wilk test.

To identify the differences between the two groups, student's t test or Mann-Whitney test were used, respectively, normally or non-normally distributed variables.

The simple linear correlation between two variables was evaluated by calculating the Pearson correlation index.

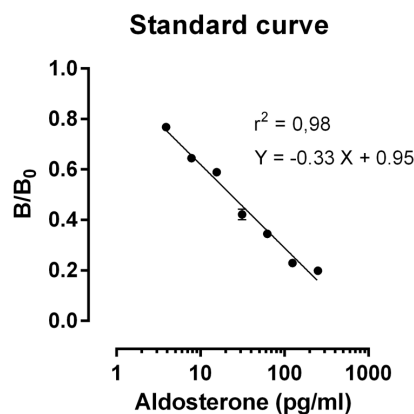
A multiple linear regression was used to identify a correlation between the UAldo: C parameter and the following variables: MF sex (Male or Female), presence / absence of mitral pathology (GROUP), Urea, protein ratio: urinary creatinine (PU / CU), left atrium ratio: aortic root (Asx/Ao), E/A wave ratio (E/A), left ventricular allometric value in diastole and in systole (AlloVSd, AlloVSs).

A value of $p < 0.05$ was considered significant.

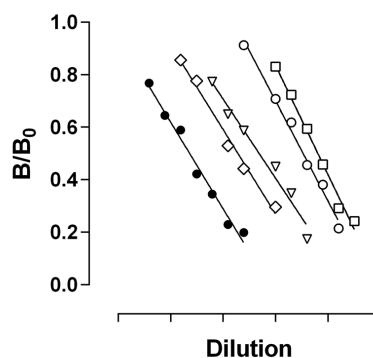
Results

The performances of the ELISA kit were evaluated through the production of a standard curve, parallelism test and spike and recovery test.

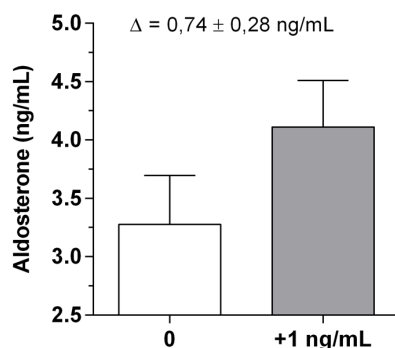
- a. Standard curve: the standard line was linear between 3.9 pg / mL and 250 pg / mL, with $r^2 \geq 0.98$.



- b. Parallelism test: the curves obtained with scaled dilutions of hydrolysed urine were parallel to each other and to the standard line, demonstrating the accuracy of the ELISA kit in the determination of aldosterone in the dog urine.



- c. Spike and recovery test: recovery was evaluated by adding 1 ng/mL of aldosterone in dog's urine. The non-added urine was used as reference white. The average recovery was 84%.



- d. Reproducibility: the intra-assay coefficient of variation was between 12.2 and 17.5%

Aldosterone's concentrations are reported in table 18.

Aldosterone (ng/mL)	
Dog 1	2.593 ± 0.890 (CV 14.9%)
Dog 2	2.737 ± 0.39 (CV 14.3%)
Dog 3	3.620 ± 0.44 (CV 12.2%)
Dog 4	2.399 ± 0.42 (CV 17.5%)

Table 18

The repeatability (intra-assay precision) was between 4.5-6.6% and the reproducibility (inter-assay precision) between 10.8-16.3%.

For the second part of the study, a total of 37 subjects were included, 17 classified as healthy and 20 as affected by stage B1 MMVD. A total of 37 urine samples were collected.

The mean age of dogs enrolled in group healthy was 8,3 years ($\pm 3,28$ SD), and the mean body weight was 22,3 kg ($\pm 12,2$ SD). Most of the dogs included were neutered females (n=9; 53%), followed by intact males (n=4; 23%), neutered males (n=3; 18%) and intact females (n=1; 6%). The most represented breeds were Mongrels (n=5; 29%), Golden retriever (n=3; 17%) and Cavalier King Charles (n=2; 12%). Breeds with less than two dogs were grouped and listed as others (42%).

The mean age of the MMVD group was 12 years ($\pm 2,34$ SD), and the median body weight was 9 kg (IQR 7,13-19,20). The majority of the dogs included were, equally, neutered females (n=8; 40%) and intact males (n=8; 40%), followed by neutered males (n=4, 20%). The most represented breed were Mongrels (n=6; 30%), followed by Dachshunds (n=3, 15%) and Cavalier King Charles (n=2; 10%). All the dogs were classified as ACVIM B1, as it was an inclusion criterion.

Serum creatinine (sCr; nv < 1,4 mg/dL) and urea (nv 20 - 60 mg/dL) were evaluated on 31 of the 37 dogs (84%), 15/17 (88%) of the healthy dogs and 16/20 (80%) of the MMVD dogs. All the subjects belonging to the healthy group presented normal sCr and urea values. Only one subject (6%) in the MMVD had sCr exceeding the normal value, as the dog was diagnosed with CKD IRIS stage 3. In the MMVD group, 3/16 (19%) dogs had urea values exceeding the reference interval. In the healthy group, USG, and UPC were in the reference interval for all the subjects, while in the MMVD group 5 dogs presented UPC > 0,5. Systemic blood pressure was evaluated in 34/37 dogs (94%); in the remaining dogs, the animal wasn't complaining due to an elevated anxious state. Mean value of systemic blood pressure was 151 \pm 27 SD. One dog in the healthy group and 1 dog in the MMVD presented blood pressure exceeding the normal value reported in the consensus.

For each sample, urinary aldosterone and creatinine were determined, respectively through ELISA and standard colorimetric technique. UAldo: C ratio was then calculated. Data are reported in table 19.

	U.Aldo (ng/mL)	U.Crea (mg/dL)	UAldo:C (μg/g)
Healthy 1	0,29	261,4	0,11
H 2	8,33	283,8	2,93
H 3	0,28	53,0	0,52
H 4	2,83	396,8	0,71
H 5	3,15	195,0	1,62
H 6	2,97	210,8	1,41
H 7	0,66	98,4	0,67
H 8	0,97	208,0	0,47
H 9	4,04	302,4	1,34
H 10	0,88	58,7	1,50
H 11	2,06	194,2	1,06
H 12	5,12	111,0	4,61

H 13	2,02	317,6	0,63
H 14	1,59	230,0	0,69
H 15	3,32	80,8	4,11
H 16	8,73	408,4	2,14
H 17	1,57	99,4	1,58
MMVD 1	1,27	132,8	0,96
M 2	0,45	63,6	0,71
M 3	0,66	117,9	0,56
M 4	2,34	340,0	0,69
M 5	0,54	139,0	0,39
M 6	7,01	216,6	3,23
M 7	1,05	141,0	0,75
M 8	1,94	62,5	3,10
M 9	0,65	134,4	0,48
M 10	1,71	42,8	3,99
M 11	0,24	145,8	0,16
M 12	0,55	87,5	0,63
M 13	0,82	148,0	0,55
M 14	0,71	39,3	1,80
M 15	0,65	90,3	0,72
M 16	2,77	96,5	2,87
M 17	1,11	104,6	1,06
M 18	1,39	126,0	1,11
M 19	2,27	131,8	1,72
M 20	2,86	147,0	1,94

Table 19

On a total of 37 subjects, 18 (49%) present UAldo: C values < 1.0 µg/g, 7 (39%) in the healthy group and 11 (61%) in the MMVD group. Nineteen (51%) had UAldo: C values > 1.0 µg / g, 10 (53%) in the healthy group and 9 (47%) in the MMVD group.

The healthy and the MMVD group were tested for the presence of statistically significance differences. The variable for which differences were found and relatives P are reported in table 20.

	Healthy	P	MMVD
Age	8,35±3,28	<0,001	12±2,34
U sCr	206,45±112,32	0,01	125,37±65,84
LVs	24,17±7,21	0,047	19,35±7,02

Table 20

Due to the absence of statistically significant difference with respect to the parameter UAldo:C between healthy and MMVD group, all 37 dogs were reunited and classified according to age; aim was to verify if the relationship UAldo:C was then affected. Six over 37 dogs (16%) were ≤ 6 years, 31/37 (84%) were > 6 years. No statistically significant difference with respect to the UAldo:C parameter between the two groups was evidenced.

Secondly, the presence of a linear relation was evaluated in the whole population for UAldo:C with selected variables. Results are reported in table 21.

	Pearson's coefficient	P Value
Age vs UC	-0,568	<0,001
Age vs UAldo	-0,364	0,027
Sex vs UAldo	0,398	0,015
Sex vs UAldo:C	0,381	0,02
USG vs UAldo	0,468	0,004
UC vs UAldo	0,538	0,001
UAldo vs UAldo:C	0,606	0,001
UAldo vs AlloLVs	-0,346	0,036
UAldo vs FS (%)	0,327	0,048
UAldo:C vs FS (%)	0,429	0,008

Table 21

Multiple linear regression was performed, considering as predictive variables sex (MF), presence/absence MMVD, Urea, UPC, LA/Ao, E/A, normalized LVIDd (LVIDDN), normalized LVIDs (LVIDSN). Analysis was performed on healthy and MMVD groups separately and on all the 37 subjects.

In the healthy group, LVIDDN and LVIDSN showed a statistically significant association with Ualdo:C (p-value=0.039). The portion of total variability explained by the regression line corresponds to 88% (R²=0.88). In the MMVD group, none of the variable presented a statistically significant association with Ualdo: C. In the whole population, only LVIDSN presented a statistically significant association (p value = 0.032) with Ualdo:C. The portion of total variability explained by the regression line corresponds to 49% (R² = 0.49).

Discussion

The population of dogs affected by MMVD enrolled in this study presented the typical characteristics reported in literature. In fact, the dogs were mainly medium age and body weight, mongrels or pure small breed dogs [Borgarelli, 2012].

The healthy group consists of dogs with a mean age of about 8 years, medium to large size, female. The most represented breeds were Mongrels, Golden Retriever and Cavalier King Charles Spaniel. Between the two groups, there was a statistically significant difference in age. This is consistent with what reported in literature, were subjects with MMVD are elderly [Borgarelli, 2012].

MMVD was directly related to age and inversely related to weight: as age increases and weight decreases, the probability of finding the pathology in a subject increase. This result also agrees with literature [Borgarelli, 2012]. A statistically significant difference was also found with respect to the diameter of the left ventricle in systole (VSs). However, we do not believe that this difference has a clinical value, since the respective allometric value is not different. The allometric equation makes it possible to normalize the echocardiographic measurements performed in M-mode, indexing them to body weight.

In the MMVD group, two dogs presented blood urea over the reference interval. They were, respectively, the dog affected by CKD stage IRIS 3 and the dog with persistent proteinuria. Azotemia can be related by the presence of a renal impairment more than to a prerenal azotemia, like it can be seen in advanced congestive heart failure. A reduction in functioning renal mass causes a decrease in GFR with subsequent retention of uremic catabolites [IRIS].

Only 2 out of 37 dogs presented systemic blood pressure values over the reference interval, with a value around 190 mmHg; both presented with a consistent anxious state at the time of measurement. The data can likely be related to the "white coat effect", that can increase, even dramatically, blood pressure [Brown, 2007].

Currently, there are no studies in literature in which urinary aldosterone has been assessed in dog using the ELISA method; in all the published works, RIA method was used [Ames, 2015; Ames, 2016; Ames, 2017].

The ELISA kit used in this study was found to be sensitive, accurate and reproducible for the determination of aldosterone in dog urine after acid hydrolysis.

The possibility of determining urinary aldosterone via ELISA technique would be an advantage for clinical research, as it is quicker, cheaper, does not require specialized technical figures or radiation protection measures and, unlike the RIA, can be performed by most of laboratories. Consequently, there can be a benefit in terms of time and costs, allowing to expand the number of samples analyzed and to make research in this field accessible to research laboratories that, for different reasons, do not have access to radioimmunoassay techniques. Moreover, RIA produce special waste that require special treatment, with considerable costs and a high environmental impact. Facilitate research in this field using kits that guarantee reliable results would allow us to obtain a higher number of data, a very important objective in veterinary medicine, where most of the studies are unfortunately characterized by the inclusion of small number of subjects.

The present study did not find any statistically significant difference in the UAldo: C ratio among the healthy and the MMVD group, nor any correlation with the presence/absence of MMVD.

The ACVIM class B1 represents the earliest stage of the disease [Atkins, 2009]. It can be hypothesized that the progression of the pathology and the consequent hemodynamic alterations may not, in this phase, trigger the RAAS and therefore increase the levels of aldosterone.

Furthermore, as for the healthy subjects, also in the MMVD group (with the exception of the patient treated with ACEI for proteinuria) dogs weren't assuming any cardio-active therapy, as in the guidelines [Atkins, 2009]. The administration of some drugs would represent a stimulus for the RAAS, with increase in aldosterone levels [Ames, 2016].

The median and mean values obtained were respectively 1.34 $\mu\text{g/g}$ (IQR 0.65-1.88) in healthy dogs, 1.37 $\mu\text{g/g}$ (\pm 1.11 SD) in MMVD dogs and 1.06 $\mu\text{g/g}$ (IQR 0.63-1.87) considering the whole sample. Literature report a maximum value of UAldo:C ratio in healthy subjects of 1.0 $\mu\text{g/g}$. This data was presented in an abstract and was obtained from 55 healthy subjects, subdivided in two groups according to age (respectively <6 and > 5 years). The mean values of UAldo: C were respectively 0.40 $\mu\text{g} / \text{g}$ (\pm 0.21DS) for the first group and 0.49 $\mu\text{g} / \text{g}$ (\pm 0.27DS) for the second group [Ames, 2015].

The same authors carried out a series of studies aimed at evaluating the onset of aldosterone breakthrough (ABT) in healthy dogs with pharmacologically activated RAAS, and, on a total of 50 healthy subjects, only 9 (18%) presented a baseline value of UAldo: C higher than 1.0 $\mu\text{g/g}$. In the present study, more than half of the dogs ($n = 19$, 51%) showed values of UAldo:C ratio higher than 1 $\mu\text{g/g}$, 10 (53%) in the healthy group and 9 (47%) in the MMVD group [Ames, 2017].

Mean values of UAldo:C ratio and the percentage of subjects with UAldo: C > 1 $\mu\text{g/g}$ of the present study are superior even to those proposed by Ames and colleagues in their study on subjects with ACVIM B2 and C MMVD [Ames, 2017]. Considering the pathophysiology of the production of aldosterone in cardiovascular diseases, we would have expected the opposite. In fact, the progression of the disease leads to a reduction of cardiac output and progressive activation of compensatory mechanisms, including the RAAS. In advanced stages, the RAAS is chronically activated, leading to higher aldosterone levels than in the early stages. In addition, in more advanced MMVD, drugs, such as ACE inhibitors and antagonists of the aldosterone receptor, are usually prescribed, which should

on one hand decrease the production of aldosterone and, on the other, as in the case of spironolactone, increase its urinary excretion.

However, in comparing the results of our study with those reported in literature, we must consider the fact that the available data are preliminary and not well established.

Furthermore, the method used for the determination of urinary aldosterone and the population on which the study was carried out are different. Although different, there are two studies that identify a good correlation between RIA and ELISA in the determination of aldosterone, one in humans and one in mouse urine [Hanquez, 1987; Al-Dujaili, 2009], one of which reported a result inferior of about 20-35% for the ELISA method [Al-Dujaili, 2009]. It is possible that, in our study, a percentage of variability due to the method was present, even if apparently not in the same direction.

The two techniques have a similar, competitive procedure, but if in the RIA aldosterone is labeled with a radioactive isotope, in the ELISA the labeling is enzymatic. Moreover, differences can be present in antibodies, hydrolysis methods and metabolite of aldosterone determined.

These considerations could explain, in part, the difference between the values of UAldo:C ratio of the present work and those of literature. Furthermore, the form in which aldosterone occurs in the urine, that can influence the amount of hormone detected, differs from species to species: the knowledge, in dog, about the topic are scarce [Syme, 2007].

The present study showed that the kit we use is able to effectively detect aldosterone in dog urine. It cannot be excluded that the reference values of UAldo: C must be revised based on the lab method. Moreover, the production of aldosterone may be influenced by factors varying from subject to subject, such as diet [Lovern, 2001], obesity [Ames, 2015] and genetic [Meurs, 2015]: different populations may have different basal levels of aldosterone. In fact, Ames et colleagues suggest determining a specific threshold of UAldo:C ratio, derived from a cohort of healthy subjects homogeneous for the characteristics of the population one wants to study [Ames, 2016].

In our case, groups were homogeneous for the number of subjects included, but there was a significant difference in terms of age, although only subjects with more than 5 years were included. There were no differences in terms of sex and weight.

The number of Mongrels and Cavalier King Charles Spaniel was similar in both groups. Great homogeneity for environment, diet and lifestyle was achieved.

In the study by Ames and colleagues, there was no significant difference in UAldo:C between the two groups divided for age (<6 years and > 5 years) and the authors therefore believe that this parameter does not vary significantly with age [Ames, 2016]. Our data were in line with this finding. The 37 subjects selected for the presence study were further subdivided according to age (< or > 6 years) and no statistically significant difference in terms of UAldo:C was found.

Considering therefore these data, it can be hypothesized that, for the population of the present study, it is necessary to determine a different cut-off from 1.0 $\mu\text{g/g}$ since, if this value was considered, it would mean that more than half of our healthy subjects would have aldosterone levels over the reference interval. We observed that the majority of subjects in both groups (76% for healthy and 80% for patients) had UAldo:C values <2 $\mu\text{g/g}$. We questioned whether this could represent a possible threshold value in the population of this study. Although aware that, according to our statistical analysis, a larger sample would be necessary to determine a value of UAldo:C threshold in our population, the present preliminary observation seems to point in this direction.

Multiple linear regression showed that, in the healthy group, UAldo:C presents a direct linear association with LVIDDN and inverse with LVIDSN: increases of LVIDDN increases UAldo:C; decreases of LVIDSN increases UAldo: C. In the MMVD group, none of the variables was predictive for UAldo:C. We would have expected no correlation between these parameters in the healthy group and a positive correlation, both with LVIDDN and with LVIDSN in the MMVD group. In fact, with the progression of MMVD, which could probably determine an increase in UAldo:C, there is a progressive increase in the diameters of the left ventricle both in diastole and in systole, due to left ventricular eccentric hypertrophy and associated volume overload. However, it must also be considered that in stage B1 of the mitral pathology, this type of remodeling is absent. We therefore believe that the data are not numerically sufficient to draw conclusions and it is necessary to verify these correlations again by expanding the number of subjects.

General discussion and conclusions

This thesis was aimed to find new diagnostic and prognostic approaches to cardiorenal syndrome in the canine and feline patients. Cardiorenal syndrome is still a highly discussed field in veterinary medicine, since prevalence reported by literature are quite different between veterinary and human medicine. One of the differences that can be called into question is that the median age of the population of subjects treated in the veterinary practice is very different from the median age of a human patient; however, it is certainly true that, even in the feline and canine population, diseases related to aging are recognized. In our opinion, early, fast and reliable diagnosis could have a pivotal role in the management of dogs and cats affected by heart and kidney conditions, as the pet average life grows longer in the countries of the first world. Drug management is particularly important because, in veterinary medicine, medical management of cardiovascular and renal conditions is still the leading therapy (no replacing therapy as transplant can be considered up to now a valid option). The study on aldosterone was driven from this perspective, since drugs that can modulate and reduce the effect of RAAS are still currently used in the treatment of cardiovascular diseases. In the comparison between healthy dogs and dogs with MMVD (B1), no statistically significant difference in urinary aldosterone:creatinine ratio was found. So, stage B1 MMVD does not seem to lead to an increase in aldosterone levels with respect to healthy subjects. The result is in line with Kwart and colleague, authors of the only prospective placebo controlled blinded study on stage B1 dogs on the effect of those drugs and concluding that no positive nor negative effect of the treatment was seen at this stage of MMVD [Kwart, 2002].

Moreover, guarantee a good quality of life to a living subject that is considered a full-fledged part of families must be a priority, in particular considering the impact that quality of life of a pet can have on the compliance of owners in deciding how to treat the animal and how far to go with selected treatments. This can be strengthened through the study of comorbidities.

In human medicine, iron deficiency has been strongly correlated with exercise intolerance and worsen quality of life and prognosis.

In this thesis, for the first time, serum iron concentration in dogs with MMVD was evaluated and resulted to be predictive, for dogs, to be included in a higher ACVIM class. It is true that, in our study, quality of life was not systematically evaluated, but it is also true that, for higher ACVIM classes, in which quality of life can be scarce, the lower concentrations of serum iron were detected.

When it comes to CKD, quality of life usually coincides with palliative cares, often administered at home by the owners themselves. The rapid and reliable evaluation of cardiac rhythm can be of great importance in patients with late stage CKD, patients often managed by the owners themselves on the instructions of the attending veterinarian, for which fast and easy solutions are essential. From this perspective, and with the aim to provide clinicians a multiple lead ECG less expensive but equally reliable, our study on the D-heart technology was performed and proved that this technology, even if less expensive compared to traditional equipment, is equally reliable. Moreover, it has a very strong impact in determining the importance of telecardiology and the need for veterinarians with a specialization to create a network.

Dogs affected by MMVD are usually old dogs, at higher risk for the development of cardiorenal syndrome type 2, due to the reduced performances of the kidney function that can already be present; SDMA proved not to be influenced by the presence of MMVD at any stage of the pathology and can thus be considered a reliable biomarker in these dogs. In our opinion, the real importance of this marker is not so much in the detection of early renal impairment in symptomatic patients, but to permit, through serial blood samples, that can be obtained quickly and effectively, the monitoring of glomerular filtration rate in patients subjected to changes due to improper functioning of the heart pump. Although a normality threshold is reported in literature, in our opinion the strength of this marker lies in the serial evaluation for the single patient: in fact, more than evaluating when this parameter rises above the threshold, in the patient affected by MMVD it would be important to monitor it over time, in order to have an idea of the progress and fluctuations of the GFR over time. This would allow to have, in an economic and technically easy way, the control over the development of renal impairment before azotaemia and to have a direct feedback on the use of diuretic therapy.

Our study on cats affected by HCM was a preliminary study designed to investigate this marker in a specie in which it has never been deepened; feline macro-CK1 may have a different structure compared with other species. In fact, CK-MM represents the main fraction in the electrophoretic separation of CK isoenzymes and macroenzymes in cats, followed by macro-CK1 and macro-CK2. The work that led to the production of this thesis was articulated on several fronts; cardiorenal syndrome is in fact a faceted topic and the curiosity have driven us into different aspects, with a lowest common denominator: the research for diagnostic methods that allow, once formulated a diagnosis of cardiovascular or renal disease, to have a more complete picture of the syndrome, in order to allow a better therapeutic management and a higher quality of life of the treated subjects. Specifically, the main conclusions of this thesis are that:

- Iron deficiency can be considered a common comorbidity in dogs with MMVD, recognized in almost the 20% of dogs and with dogs presenting low serum iron concentrations with a 6.3 higher risk of being included in a higher ACVIM class.
- Symmetric dimethylarginine (SDMA) should be periodically tested in dogs affected by heart failure due to MMVD with normal serum creatinine at any stage of the heart disease, together with urianalysis, in order to detect decreases in glomerular filtration rate that cannot be precociously detected by serum creatinine.
- A possible role of CK-MB in the evaluation of feline cardiomyopathy, particularly preclinical form and in association with other conditions, as in cardiorenal syndrome type 4, emerges from this study. Cat can effectively be considered a suitable animal model for progression in clinical research.
- D-Heart proved effective and accurate recording of ECG in the canine patient. It represents an excellent option for fast evaluation of cardiac rhythm in different clinical settings.
- The selected ELISA kit for the determination of aldosterone's levels in dog's urine demonstrated its ability to effectively detect urinary aldosterone, and can therefore constitute a simpler, safer and more economical alternative to the RIA method. The mean values of UAldo:C in the present study were greater than those reported in literature, even compared to

more advanced MMVD subjects (ACVIM class B2 and C). Although this represents a pilot study, these results point us towards a reevaluation of the normality threshold set by the literature (1.0 µg/g), which does not seem to be valid in the population of the present study.

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