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CICLO XXXV

Cardiovascular-renal axis disorders: renal biomarkers in dogs affected by myxomatous mitral valve disease

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Effect of sampling time on urinary electrolytes following oral furosemide administration in dogs with myxomatous mitral valve disease

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Evaluation of urinary neutrophil gelatinase-associated lipocalin to detect renal tubular damage in dogs with stable myxomatous mitral valve disease

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List of abbreviations

[uCa^{++}]	urine calcium
[uCl]	urine chloride
[uCr]	urinary creatinine
[uK^{+}]	urine potassium
[uMg^{++}]	urine magnesium
[uNa^{+}]	urine sodium
ACEI	angiotensin converting enzyme inhibitor
ACVIM	American College of Veterinary Internal Medicine
AKI	acute kidney injury
CHF	congestive heart failure
CKD	chronic kidney disease
CRP	C-reactive protein
CRS	cardiorenal syndrome
CvRD	cardiovascular-renal axis disorders
E/E'	E to E' waves ratio
E_{Vel}	E wave velocity
FE	fractional excretion
FE Na^{+}:FE K^{+}	fractional excretion of sodium to fractional excretion of potassium ratio
H-EG	healthy evening group
H-MG	healthy morning group
IRIS	International Renal Interest Society
LA:Ao	left atrium to aortic root ratio
LA_{Max}	left atrial maximal volume
LA_{Min}	left atrial minimal volume
LASV	left atrial stroke volume
LVIDDn	normalized left ventricular end-diastolic diameter
LVIDSn	normalized left ventricular end-systolic diameter
MMVD	myxomatous mitral valve disease
MMVD-EG	dogs with myxomatous mitral valve disease sampled in the evening
MMVD-MG	dogs with myxomatous mitral valve disease sampled in the morning

NGAL	neutrophil gelatinase-associated lipocalin
RAAS	renin-angiotensin-aldosterone system
RI	reference interval
sCr	serum creatinine concentration
TR_{Vmax}	tricuspid regurgitant flow velocity
uCl:uCr	urine chloride to urine creatinine ratio
uCr	urinary creatinine
uNa^+:uCr	urine sodium to urine creatinine ratio
uNa^+:uK^+	urine sodium to urine potassium ratio
uNGAL	urinary neutrophil gelatinase-associated lipocalin
uNGALC	urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio
UPC	urine protein-to-uCr ratio
USG	urine specific gravity
VTH	Veterinary Teaching Hospital
VTI_{Ao}	aortic velocity-time integral
VTI_{Mit}	mitral velocity-time integral
VTI_{Mit}/VIT_{Ao}	mitral to aortic velocity-time integral ratio

Abstract

The heart and kidneys are closely related whereby acute or chronic dysfunction in one organ may lead to acute or chronic dysfunction in the other organ; cardiovascular- renal axis disorders have been described in dogs with myxomatous mitral valve disease (MMVD). Early recognition of renal involvement would allow adjustment of cardiovascular therapy, however, serum creatinine, that is currently the most widely used marker for monitoring kidney function in dogs, is a late marker and it is not able to detect early renal disfunction. Urine neutrophil gelatinase-associated lipocalin (uNGAL) and uNGAL to urinary creatinine ratio (uNGALC) are novel promising biomarkers of renal damage, proposed in humans and dogs. In addition, urine chemistry has received growing attention to estimate the diuretic response in dogs with cardiac disease.

This doctoral thesis work is aimed to study different aspects of cardiovascular-renal axis disorders in dogs affected by MMVD at different American College of Veterinary Internal Medicine (ACVIM) stages. In particular, the aims of the study are:

- to evaluate renal tubular damage in dogs with stable MMVD by evaluation of uNGAL
- to evaluate the association between echocardiographic indexes and uNGAL or uNGALC, in dogs with MMVD.
- to evaluate the impact of the time elapsed between the oral furosemide administration and the sample collection on urine chemistry in dogs with MMVD stage ACVIM C.

This is a multicentric prospective observational case-control study. Dogs with MMVD at different ACVIM stage were included and underwent physical exam, echocardiographic exam, blood and urine sample for the determination of serum and urine chemistry, including uNGAL and uNGALC. Dogs were monitored over time at established time intervals based on the ACVIM stage.

The values of uNGAL and uNGALC were compared between MMVD dogs and healthy controls, and among different MMVD ACVIM stages. Moreover, echocardiographic data were correlated with uNGAL and uGALC values. The echocardiographic indexes analyzed were shortening fraction (SF), normalized left ventricular diastolic (LVIDDn) and systolic (LVIDSn) diameters, left atrium to aortic root ratio (LA/Ao), maximal (LAV_{Max}) and minimal (LAV_{Min}) left atrial volumes, LA stroke volume (LASV), E wave peak velocity (E_{Vmax}), E/E', aortic (VTI_{Ao}) and mitralic (VTI_{Mit}) velocity time integrals and their ratio (VTI_{Mit}/VTI_{Ao}) and tricuspid regurgitation velocity (TR_{Vmax}).

For the last aim of the study, dogs with MMVD were divided, based on the time of blood and urine sampling, in two groups. The morning group (MMVD-MG) included the dogs in which the sampling

was obtained between 1 to 6 h after the oral furosemide administration, and the evening group (MMVD-EG) included the dogs sampled over 6 h from oral furosemide administration. Analogously, healthy dogs sampled between 9 a.m. and 1 p.m. and between 2 and 7 p.m. were divided in a morning group (H-MG) and an evening group (H-EG), respectively. Urine chemistry, including fractional excretion of electrolytes, was evaluated and compared among groups.

The MMVD dogs had significantly higher uNGAL and uNGALC (1204 pg/mL; range, 30-39 732 and 1816 pg/mg; range, 22-127 693, respectively) compared to healthy dogs (584 pg/mL; range, 56-4072 and 231 pg/mg; range, 15-2407, respectively; P=.002 and P< .0001, respectively). Both uNGAL and uNGALC increased with the increasing ACVIM stage (P=.001 and P< .001, respectively). In the univariate analysis LASV, TR_{Vmax}, LAV_{Max}, LVIDDn and VTI_{Mit}/VTI_{Ao} were independent predictors of increased uNGAL and uNGALC, however only LASV [(OR: 3.26, 95% CI: 1.13–9.38), P= 0.03 for NGAL and (OR: 4.62, 95% CI: 1.44–14.88), P= 0.01 for NGALC] and TR_{Vmax} [(OR: 1.77, 95% CI: 1.16–2.73), P < 0.01 for NGAL and (OR: 1.52, 95% CI: 1.01–2.30), P = 0.05 for NGALC] remained statistically significant in the multivariable analysis. An higher excretion of sodium and chloride and higher urine sodium to urine potassium ratio (uNa^+:uK^+) were detected in MMVD-MG than MMVD-EG (P= 0.021, P= 0.038, and P= 0.016, respectively). Natriuresis, chloriuresis, and uNa^+:uK^+ were higher in MMVD-MG than H-MG, while no differences were found in the comparison between H-MG and H-EG and between MMVD-EG and H-EG.

In conclusion, renal tubular damage is present in dogs with stable MMVD, as measured by increased uNGAL. In our population the tubular damage is subclinical, occurs in all stages of MMVD even in the absence of azotemia, and increases with the severity of MMVD. Based on our results LASV and TR_{Vmax} are associated with increased uNGAL and uNGALC. These parameters might detect patients at higher risk to develop kidney damage in course of MMVD. Reno protective approaches to manage MMVD dogs should be explored to slow the progression of renal tubular damage in these patients.

Urinary electrolyte excretion is significantly increased within 6 h from furosemide administration in dogs with MMVD ACVIM stage C. Time of sampling from furosemide administration significantly affects urine chemistry in MMVD dogs and should be considered in the clinical practice and the research field.

Introduction

Myxomatous mitral valve disease

Myxomatous mitral valve disease (MMVD) is the most common acquired cardiac disease in dogs, accounting for 75% of all cardiac disease (Keene et al., 2019; Franchini et al., 2021; Mead et al., 2022). The prevalence of MMVD varies between breeds, but it is more represented in small breed dogs (90%) older than 8 years of age (Fox, 2012). In particular, Cavalier King Charles spaniels (CKCS) seem to be particularly prone to develop MMVD (Mead et al., 2022).

The disease is approximately 1.5 times more common in males than in females (Keene et al., 2019). Myxomatous mitral valve disease is characterized by slow progressive myxomatous degeneration with subsequent mitral valve regurgitation and left atrial and ventricular dilatation (Borgarelli et al., 2008).

As a result of this degeneration, part of the systolic blood volume of the left ventricle returns to the left atrium (Smith et al., 2015); this condition reduces the cardiac output and increases hydrostatic pressure in the heart. These changes cause the response of multiple neurohormonal systems, whose activation maintains an adequate heart rate, blood pressure and tissue perfusion (Figure 1; Oyama, 2009).

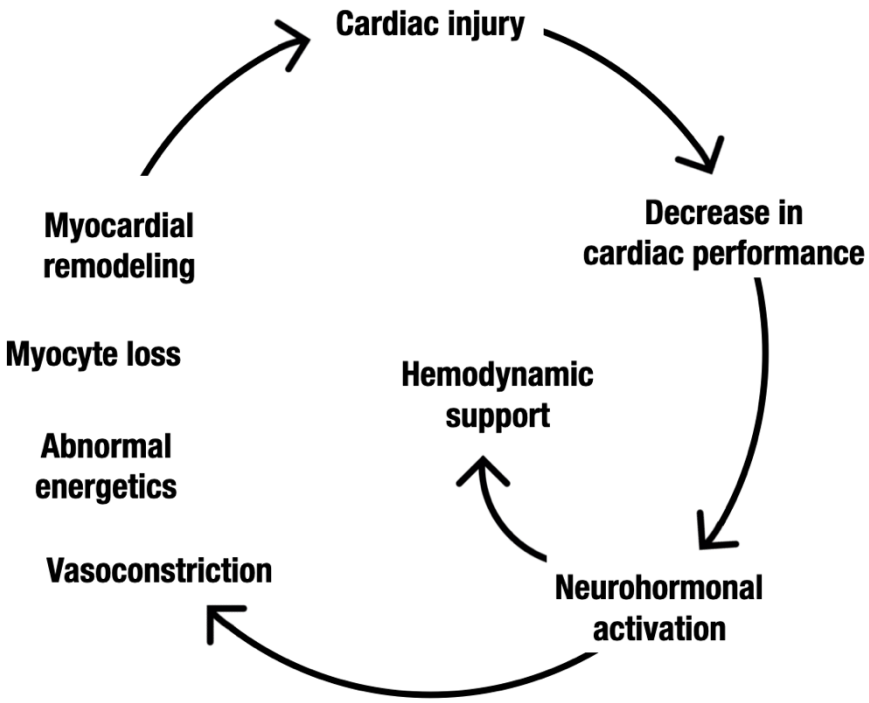

FIGURE 1. Neurohormonal pathophysiology of heart failure. From Oyama, 2009

The compensatory mechanisms activated are: the sympathetic nervous system (SNS), the renin-angiotensin-aldosterone system (RAAS), Atrial Natriuretic Peptide (ANP), Brain Natriuretic Peptide (BNP), the arginine vasopressin (AVP) and endothelin-1 (ET-1) systems (Ettinger et al, 2017).

These systems provide only a temporary hemodynamic support but, in chronic, they are associated with vasoconstriction, abnormal myocardial energetics, myocyte death and myocardial remodeling, which further injure the heart (Figure 1; Oyama, 2009).

The progression of the disease causes a volume overload of the left compartment, leading to left atrial and ventricular remodeling and congestive heart failure (CHF) (Borgarelli et al., 2012).

The diagnostic suspect of MMVD is generally related to the recognition of a systolic heart murmur at the left apical level during auscultation (Keene et al., 2019).

The gold standard for the diagnosis of MMVD is the echocardiographic exam, performed by an experienced operator, to identify the cause of the murmur, the severity of cardiac chamber enlargement, and identify any comorbidities. The diagnostic plan should also include thoracic radiography to assess the hemodynamic relevance of the valve disease and to obtain baseline chest radiographs, even when the patient is asymptomatic for MMVD, and blood pressure measurement to identify or rule out concurrent systemic hypertension and to establish baseline blood pressure.

In order to understand and manage a patient with MMVD, it is necessary to follow the guidelines for the diagnosis and treatment of MMVD outlined by the American College of Veterinary Internal Medicine (ACVIM) (Keene et al., 2019).

Keene et al. proposed this staging system:

- Stage A: dogs at high risk for developing heart disease (every CKCS or other predisposed breed without a heart murmur).
- Stage B: dogs with structural heart disease without clinical signs. In these patients a reevaluation by echocardiography is suggested (or radiography if echocardiography is unavailable) every 6-12 months, depending on the disease severity. Stage B is divided in two sub-classes based on the presence of cardiac remodeling:
    - Stage B1: asymptomatic dogs with no radiographic or echocardiographic evidence of cardiac remodeling.
    - Stage B2: asymptomatic dogs with radiographic and echocardiographic findings of left atrial and ventricular enlargement.

- Stage C: dogs with current or past clinical signs of CHF caused by MMVD that require treatment.
- Stage D: dogs with end-stage MMVD, in which clinical signs of CHF are refractory to standard treatment

There are currently no therapies capable of preventing myxomatous degeneration of the mitral valve, but medical treatment aims to control symptoms, improve quality of life and, therefore, increase survival. The veterinary literature of the last decade aims to understand the most appropriate moment to start the therapy (Boswood et al., 2016; Boswood et al., 2018).

According to ACVIM staging patients in stage A and B1 do not require treatment (Keene et al., 2019).

In stage B2 Pimobendan (positive inotropic and a vasodilator) is recommended at a dosage of 0.25-0.3 mg/kg PO q12h (Boswood et al., 2018; Keene et al., 2019).

Dogs with MMVD stage C and D present clinical signs and a history of tachypnea, cough, exercise intolerance and respiratory distress. Dogs with acute CHF need hospitalization, while patients with chronic CHF can be managed at home (Bagardi et al., 2022).

The cornerstones of therapy for symptomatic patients are (Keene et al., 2019):

- Loop diuretics:
    - In chronic patients: Furosemide 2 mg/kg administered q12h PO or as needed to maintain patient comfort. In alternative torasemide 0.1-0.3 mg/kg q24h (approximately 5% to 10% of the furosemide dosage). Chronic PO furosemide dosages ≥8 mg/kg q24h (or the equipotent torsemide dosage) indicate disease progression to Stage D.
    - In patients who need hospitalization, Furosemide 2 mg/kg IV or IM followed by 2 mg/kg IV or IM hourly until the patient's respiratory signs are improved or constant rate infusion (CRI) at a dosage of 0.66-1 mg/kg/hour after the initial bolus (Adin et al., 2003; Adin et al., 2018).
- Pimobendan at a dosage of 0.25-0.3 mg/kg PO q12h.
- Angiotensin converting enzyme inhibitor (ACEI), eg, enalapril or benazepril, at a dosage of 0.5 mg/kg PO q12h or an equivalent dosage of another ACEI.
- Spironolactone (aldosterone antagonist) at a dosage 2.0 mg/kg PO q12 - 24 h as adjunctive treatment (Bernay et al., 2010)

In cases complicated by atrial fibrillation, digoxin (cardiac glycoside), often in combination with diltiazem (calcium channel blocker), is recommended to control ventricular rate (Keene et al., 2019). For the management of patients with ascites referable to severe pulmonary hypertension, Sildenafil (phosphodiesterase 5 inhibitors) at a dosage of 1-2 mg/kg PO q8 h. Dogs with CHF require life-long pharmacological support and can develop progressive renal damage and dysfunction; the chronic usage of diuretics and RAAS blocking agents can cause drug-induced hypovolemia and hypotension and changes in intra-renal hemodynamics. (Orvalho et al., 2017).

Cardiovascular-renal axis disorders

The heart and kidneys are closely related organs whereby acute or chronic dysfunction in one organ may lead to acute or chronic dysfunction in the other organ (Jung et al., 2018). In human medicine the term cardiorenal syndrome (CRS) has been adopted to describe this pathological interplay (Ronco et al., 2008).

In particular, 5 types of CRS are described, depending on whether the primary disorder is cardiac or renal, acute or chronic (Table 1).

TABLE 1. Human and veterinary classification of cardiorenal syndrome. From Orvalho et al., 2017

Type of Cardiorenal Syndrome (Human classification)	CvRD Consensus	Brief Definition	Conditions
Type 1: Acute cardiorenal syndrome	$CvRD_H$ unstable	Acute impairment of the cardiac function leading to AKI	Acute heart failure Cardiogenic shock
Type 2: Chronic cardiorenal syndrome	$CvRD_H$ stable	Chronic cardiovascular disease causing progressive CKD	Chronic heart failure “Congestive nephropathy”
Type 3: Acute renocardiac syndrome	$CvRD_K$ unstable	Acute primary worsening of kidney function that leads to cardiac dysfunction	AKI Hyperkalemia, uremia
Type 4: Chronic renocardiac syndrome	$CvRD_K$ unstable	Primary CKD that contributes to cardiac dysfunction	Chronic glomerular disease, anemia, systemic hypertension
Type 1: Secondary cardiorenal syndrome	$CvRD_O$ unstable	Cardiac and renal dysfunction secondary to an acute or chronic systemic condition	Diabetes mellitus Sepsis

Abbreviations: CvRD, cardiovascular-renal axis disorders; AKI, acute kidney injury; CKD, chronic kidney disease

Clinical manifestations in dogs and cats can be widely different between individuals and between species; for this reason, in veterinary medicine the term "cardiovascular-renal axis disorders (CvRD)" has been proposed (Pouchelon et al., 2015). In dogs and cats, similarly to human medicine, five types of CvRD are described (Table 1).

Heart failure, whether resulting from systolic or diastolic dysfunction or both, is associated with renal dysfunction (Andrukonis et al.,2014). A progressive chronic kidney disease (CKD) due to primary chronic cardiovascular disease is known as CRS type 2 (Chronic Cardiorenal Syndrome) or or $CvRD_H$ (S: stable disease) (Ronco et al., 2008; Orvalho et al., 2017; Uduman, 2018).

One study found that CKD prevalence in dogs with MMVD was significantly higher compared to a general dog population and that ACVIM class (Keene et al., 2019) and International Renal Interest Society (IRIS) stage (IRIS Staging of CKD, 2019) were directly related (Martinelli et al., 2016), and azotemia increases with the severity of cardiac disease and diuretic need (Nicolle et al., 2007).

In humans, renal function is strongly and independently associated with prognosis in patients with CHF (Hillege et al., 2016), the same information is not available for veterinary patients.

When MMVD is severe enough to cause CHF, persistent renal hypoperfusion, chronic congestion of the kidneys ("congestive nephropathy") and maladaptive neurohormonal changes associated with chronic sympathetic stimulation occur (production of epinephrine, angiotensin, endothelin, and release of natriuretic peptides and nitric oxide) and contribute to progressive renal damage (Orvalho et al., 2017; Cruz et al., 2013).

Early recognition of renal involvement would allow modification of therapy to promote mutual benefit for both organs. Serum creatinine concentration (sCr) is currently the most widely used marker for monitoring kidney function in dogs with MMVD; unfortunately, it is an unreliable indicator of early kidney damage and cannot discriminate between functional and structural lesions (Orvalho et al., 2017).

The research of novel biomarkers of kidney injury and markers specific to either CRS or CvRD is a topic of remarkable interest in both human and veterinary medicine (Pouchelon et al., 2015; Segev et al., 2013; Cobrin et al., Alvelos et al., 2011).

In Table 2 are summarizes traditional and potential novel renal biomarkers.

TABLE 2. Traditional and novel blood and urine tests to assess various kidney functions. From Pouchelon et al., 2015.

Kidney Parameter		Test
Glomerular filtration rate	Traditional blood and urine tests	Serum creatinine Plasma clearance techniques
	Potential novel biomarkers	SDMA
Glomerular perselectivity	Traditional blood and urine tests	Serum albumin UPC Microalbuminuria
	Potential novel biomarkers	Urine immunoglobulin G
Tubular damage and dysfunctions	Traditional blood and urine tests	Serum creatinine Serum electrolytes Serum bicarbonate Urine glucose Urine amino acids USG
	Potential novel biomarkers	Urine NAG Urine RBP Urine GGT Urine cystatin-C Urine KIM-1 Urine NGAL Urine clusterine

Abbreviations: SDMA, Symmetric dimethylarginine; UPC, urine protein to creatinine ratio, USG, urine specific gravity; NAG, N-acetyl B-D-glucosaminidase; RBP, retinol-binding protein; GGT, gamma-glutamyl transpeptidase; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin.

The Consensus Statement of Cardiovascular- renal axis disorders in dogs and cats, in Statement 6, says that "currently, there are no biomarkers specific for $CvRD_H$. Consequences of heart disease or cardiac therapy on the kidneys should be evaluated with traditional tests of kidney function/damage as well as newer biomarkers as developed" (Pouchelon et al., 2015).

Neutrophil gelatinase associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is an acute phase protein belonging to the lipocalin family and it is synthesized in the bone marrow during granulocyte maturation; it is released in small concentrations by many other cells, including renal tubular epithelial cells, pulmonary cells and cardiomyocytes. (Cruz et al., 2012; Helanova et al., 2014; Jung et al., 2018)

Freely circulating NGAL is filtered through the glomerulus and almost completely reabsorbed by endocytosis in the proximal renal tubule (Schmidt-Ott et al., 2007).

The concentration of NGAL in the urine is very low under physiological conditions, in contrast an increase of urinary excretion of NGAL can occurs for a reduced reabsorption due to proximal renal tubular injury or for an increased ex novo NGAL synthesis (Schmidt-Ott et al., 2007).

Renal production of NGAL occurs in the most distal sections of nephron and is markedly increased for a parenchymal damage (Haase et al., 2011). Therefore, in human and in veterinary medicine, NGAL is representing an early and sensitive kidney marker of renal damage (Segev et al., 2013; Monari et al.,2019; Scheemaeker et al.,2020).

Neutrophil gelatinase-associated lipocalin has emerged as a promising and sensitive biomarker of early acute kidney damage in a different range of settings, because it quickly appears in both blood and urine in response to tubular renal damage (Shrestha et al., 2011). Specifically, it has been recognized as one of the earliest and most strongly induced proteins in both ischemic and nephrotoxic animal models of renal injury, it also has emerged as a promising biomarker for acute kidney injury (AKI) in the clinical setting (Shang et al., 2011; Monari et al.,2019; Scheemaeker et al.,2020).

The relationship between NGAL and renal dysfunction in cardiovascular disease has been studied in human medicine and elevated levels of NGAL have been reported in many cardiovascular conditions, including both acute and chronic heart failure (Cruz et al., 2012).

In human medicine, due to its association with kidney injury, inflammation and matrix remodeling, NGAL has been proposed as a marker of prognosis in patients with heart failure (Tawfeek et al., 2016).

In veterinary medicine there are only few reports. In dogs, serum NGAL is associated with the development of CRS in dogs with acute heart failure (Jung et al., 2018) but has not been reported, to our knowledge, the NGAL value in dogs with stable MMVD.

Diuretic resistance

Furosemide is the most widely used loop diuretic employed to treat CHF in dogs with MMVD (ACVIM stage C and D) (Atkins et al., 2012; Keene et al., 2019).

Loop diuretics are potent diuretic agents acting on the Na^+:K^+:$2Cl^-$ cotransporter of the thick ascending loop of Henle; this leads to inhibition of sodium, potassium, and chloride reabsorption resulting in a net loss of water and large amounts of potassium (Atkins et al., 2012).

In animals with CHF, successful diuresis resolves edema or effusion, which improves clinical signs (Oyama et al., 2022).

Furosemide can be administered orally (PO), subcutaneous (CS), intravenous (IV) or intramuscular (IM) (Harada et al., 2015). The bioavailability of furosemide is variable depending on the method of administration; it is approximately 77% in dogs after PO administration (El-Sayed et al., 1981), which is the preferred route of administration for the treatment of chronic MMVD (Keene et al., 2019).

The increased natriuresis after furosemide administration is dose-dependent and usually disappears 6 hours after oral administration, for this reason at least two administrations per day are needed for a sustained diuretic effect (Uechi et al., 2003; Hori et al., 2007; Hori et al., 2010; Harada et al., 2015; Chetboul et al., 2017). In contrast, the peak of torasemide is 2-4 hours after administration and persisted for 12 hours in cats and dogs (Uechi et al., 2003).

The majority of the pharmacodynamic studies published in veterinary literature have been conducted on healthy dogs or dogs with experimentally induced CHF, therefore the results might not be applicable to the clinical setting. (Adin et al., 2003; Uechi et al., 2003; Harada et al., 2015; Adin et al., 2017; Chetboul et al., 2017). Loughran et al. evaluated the diuretic responsiveness after oral administration of furosemide in eight healthy dogs and six dogs with CHF, however the population was small and affected by multiple cardiac disease, therefore there is a possible bias related to the different hemodynamic condition (Loughran et al., 2020).

Urine output is an ideal indicator of diuretic response, but it is difficult to quantify in veterinary patients in the routine. Several metrics, other than urine volume, have been proposed to quantify diuretic responsiveness, including weight loss, resolution of CHF, urinary sodium (uNa) concentrations, and fractional excretion (FE) of sodium (Adin et al., 2018).

In veterinary patients with CHF, the diuretic treatment is mainly guided by the evaluation of the hydration status, the urine production and the clinical and radiographic resolution of congestive signs (Keene et al., 2019). In comparison, relatively little attention is paid to direct measures of the

pharmacologic effect of the drug, eg. assessing sodium excretion after diuretic administration (Oyama et al., 2022).

In contrast, natriuresis is increasingly recognized as the primary determinant of both short and long-term decongestion in human patients with CHF; the evaluation of the natriuresis is essential in order to reach an individualization of CHF treatment and improve outcome. (Singh et al., 2014; Brinkley et al., 2018; Rao et al., 2021).

In humans, urine sodium excretion has received growing attention as a cost-effective and readily available biomarker to estimate an appropriate diuretic response and for early recognition of diuretic resistance in patients with heart failure (Biegus et al., 2019; Damman et al., 2020).

The Heart Failure Association of the European Society of Cardiology recommends uNa as one of the primary metrics of treatment efficacy in patients with acute CHF (Mullens et al., 2019)

These recommendations help to establish a quantitative definition for diuretic responsiveness or resistance (Table 3).

TABLE 3. Proposed definitions related to diuretic therapy. From Oyama et al., 2022

Term	Definition
Diuretic responsiveness	Clinicopathological effects of water and uNa loss and subsequent relief of clinical signs secondary to congestion experienced after administration of diuretic drug(s)
Diuretic resistance	Quantity of water and uNa loss sufficiently less than what typically is expected from appropriate diuretic usage, such that morbidity and mortality are increased as compared to patients with typical responses
Decongestion	Removal of excess fluid and Na from interstitial or intracavitary spaces
Dehydration	Depletion of total body water stores across the intracellular, intravascular and interstitial spaces

Abbreviation: uNa, urinary sodium

The urine electrolytes monitoring has caught the attention in veterinary medicine as well (Adin et al., 2019; Loughran et al., 2020). Focusing on natriuresis opens new potential treatment and monitoring strategies, however, the correct interpretation of urinary electrolytes is challenging without robust background knowledge. For example, possible circadian fluctuations, as well as variations in the timing of diuretic administration and sample collection, might complicate the

interpretation of urinary electrolytes in healthy dogs and dogs with CHF, and inconsistency in the timing of sampling was reported as a possible limit of investigation (Adin et al., 2019; Loughran et al., 2020). To date, little attention has been paid to these possible confounding factors in dogs unlike human medicine (Zazzeron et al., 2016; Biegus et al., 2019; Martens et al., 2019).

In Table 4 are summarized quantitative metrics of diuretic resistance proposed in humans and dogs (Uechi et al., 2003; Voors et al., 2014; Testani et al., 2015; Adin et al., 2018; Mullens et al., 2019; Feola et al., 2021; Oyama et al., 2022).

TABLE 4. Proposed quantitative definitions of diuretic resistance after loop diuretic administration in humans and dogs. From Oyama et al., 2022

Physiologic variable	Criteria
uNa	<50-70 mEq/L in spot urine sample at 2-3 h (human; dog?) <90-100 mEq cumulative uNa excretion over first 6 h (human)
uNa^+:uK^+	uNa^+:uK^+ <1 (human; dog?)
uVol	<100-150 mL first 6 h (human) <1.5 mL/kg/h during first 7 h (dog?)
FENa	<0.2% (human; dog?)
Total Na output	<50-100 mEq over first 6 h (human) <1.0 mEq/kg over first 7 h (dog?)
Weight loss	4-day weight loss <0.38-0.67 kg/40 mg furosemide (human) 5-day weight loss <0.22 kg/40 mg furosemide (human)

Abbreviations: uNa, urine sodium; uNa^+:uK^+, urine sodium to urine potassium ratio; uVol, urine volume.

In addition, interpretation of uNa in acute CHF likely depends on whether or not multiple doses of diuretics previously were given because of phenomena such as diuretic braking (i.e., decreased natriuretic and diuretic response to subsequent doses) (Oyama et al., 2022).

Another possible parameter of diuretic resistance is the serum chloride (Oyama et al., 2022); few studies indicated that hypochloremia was a stronger predictor of poor outcomes than hyponatremia, in people with CHF (Cuthbert et al., 2020; Bellino et al., 2021).

In dogs, hypochloremia is associated with advanced disease and correlates with other markers of diuretic resistance such as urine sodium to urine potassium ratio (uNa^+:uK^+) <1. (Adin et al., 2019; Roche-Catholy et al., 2021).

Despite the encouraging results of a limited number of publications in veterinary species, more studies are needed to define expected responses related to dose, route of administration, timing of sampling, and differences between loop diuretics and other classes of diuretics (Oyama et al., 2022).

Design of the study

This project aims to explore the cardiovascular-renal axis disorders in dog affected by MMVD by studying biomarkers of renal damage. It is a multicentric project started in March 2020 and conducted at the University of Parma and the University of Bologna.

We included stable patients at different MMVD ACVIM stages ($CvRD_H$ stable) (descriptive aim) and hospitalized patients with acute heart failure ($CvRD_H$ unstable) (predictive aim).

Descriptive aim

Evaluation of serum and urinary chemistry and biomarkers of renal damage (uNGAL) in dogs with MMVD in the different ACVIM stages (B1-B2-C-D), with or without medical treatment according to the indications of the ACVIM consensus (Keene BR, 2019); comparison between dogs affected by MMVD and healthy dogs.

Privately-owned dogs were enrolled at the cardiology services of both centers. All clinical examinations and cardiac ultrasound examinations were performed or reviewed by a board-certified cardiologist at both centers. Dogs were eligible for inclusion if affected by MMVD at different ACVIM stages, diagnosed, and classified according to the current guidelines (Keene et al., 2019) included dogs were in stable condition (eg, in the case of dogs in stages C and D, subjects had experienced a previous episode of CHF but were free from clinical and radiographic signs of CHF at the time of enrollment). Dogs were grouped according to ACVIM stage as follows:

- asymptomatic dogs without echocardiographic evidence of left-sided cardiac remodeling were considered to be in stage B1;
- asymptomatic dogs with echocardiographic signs of left-sided cardiac remodeling (eg, left atrial-to-aortic root ratio≥1.6 and body weight normalized left ventricular internal diameter in diastole ≥1.7) were considered to be in stage B2;
- dogs in which at least 1 episode of CHF had occurred were considered to be in stage C;
- dogs experiencing relapses of CHF despite regular administration of more than a total daily dosage of 8 mg/kg of furosemide or an equivalent dosage of torasemide (approximately 10% of the dose of furosemide) along with standard doses of the other medications thought to control the cardiac compromise (eg, pimobendan) were considered to be in stage D (Keene et al., 2019)

A 12-h fasting had to be guaranteed at the time of the sample collection. Water supply was always available. Additional cardiac medications were allowed at standard dosages, according to published guidelines (Keene et al., 2019). For the purpose of this study, specific diet restrictions were not imposed, and owners could feed their dogs their preferred diets.

Exclusion criteria were the following:

- presence of other acquired or congenital cardiac diseases;
- presence of ≥1 concomitant systemic disease including endocrinopathies, neoplasia, IRIS 3 and 4 CKD, AKI, acute or chronic gastroenteropathy with malabsorption, evidence of systemic inflammatory disease or sepsis. In contrast, disturbances of cardiac rhythm associated with MMVD were not criteria for exclusion.
- administration of nephrotoxic drugs or other concomitant treatments (eg, corticosteroids, nonsteroidal anti-inflammatory drugs) except of cardiac therapy such as pimobendan, spironolactone, angiotensin converting enzyme inhibitor (ACEI);
- acute CHF requiring emergency treatment (e.g. hospitalization with administration of intravenous furosemide);
- Because urinary tract inflammation can affect uNGAL concentrations (Vaden et al., 2004) dogs with pyuria on fresh urine sediment examination (>5 white blood cells per high power field) as well as dogs with clinical and laboratory signs of urinary tract infection or inflammation also were excluded.

Healthy dogs were included as controls for comparative purposes and to calculate the reference interval (RI) for uNGAL. These dogs were owned by medical staff or veterinary students attending the Veterinary Teaching Hospital (VTH). Dogs were considered healthy in the absence of any signs of illness on clinical examination and within the previous two months, and in the absence of clinically relevant clinicopathological abnormalities on CBC, serum biochemistry profile, and urinalysis. Dogs had not received any medications within the preceding 2 months before inclusion in the study, except for routine preventive healthcare. At time of sample collection, 12-h fasting had to be guaranteed, while water supply was always assured.

The study was approved by the local Scientific ethical committee for animal testing, animal utilization (Protocol N. 747 of October 13, 2016) and conducted with an informed owner consent.

According with ACVIM guidelines (Keene et al., 2019) we set different time points in different stages of the disease (Figure 2): every 12 months for ACVIM B1 dogs, every 6 months for ACVIM B2, every three months in dogs in ACVIM stage C and D. At each time point we performed:

- clinical evaluation including history, physical examination, weight, and non-invasive blood pressure (NIBP)
- cardiological evaluation
- simultaneous blood and urine sampling

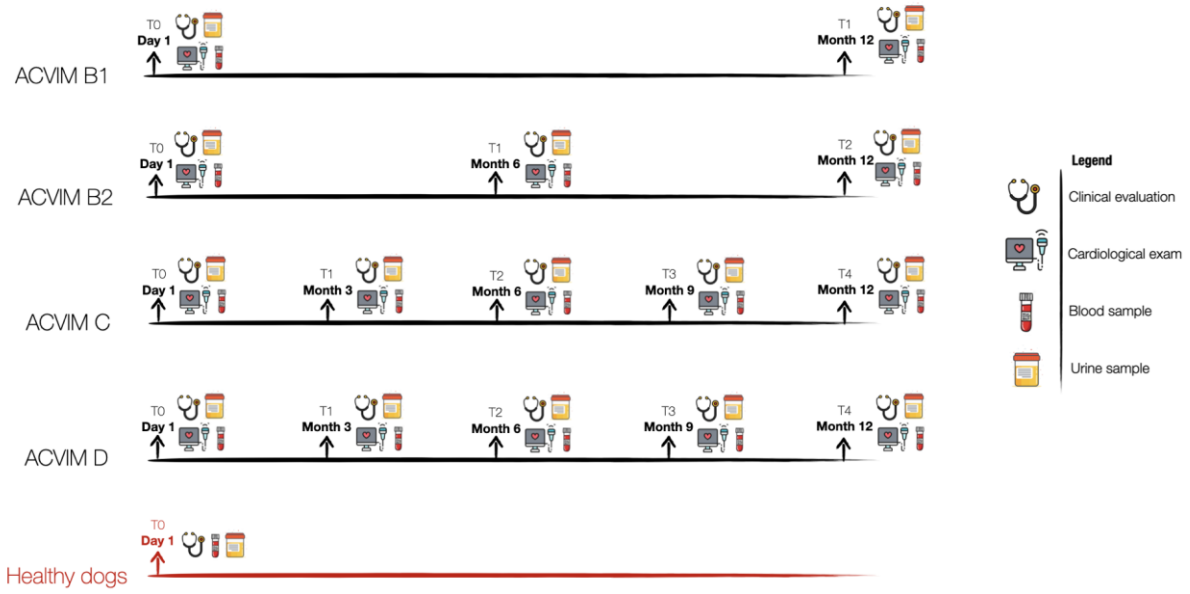

FIGURE 2. Timeline of stable chronic MMVD patients at different stages and control healthy dogs

Predictive aim

Role of urinary chemistry in dogs with CHF (acute decompensation in ACVIM C and D patients) to predict treatment response, clinical signs of edema, duration of hospitalization, short-term (discharge) and long-term (30 days and beyond) prognosis

Dogs were eligible for inclusion if affected by MMVD ACVIM stage C and D with diagnosis of pulmonary edema and treated with intravenous (if hospitalized) or oral diuretic therapy. The exclusion criteria were the same as mentioned above for stable patients with chronic disease. At the time of the diagnosis of CHF were performed: clinical evaluation, weight, thoracic x-rays in two projections, echocardiography (the echocardiographic evaluation was postponed if the

patient was unstable and unable to stay in lateral recumbency), simultaneous blood and urine sampling (Figure 3A and 3B)

If the patient was hospitalized, initial bolus of furosemide (after urine collection) at 2 mg/kg was administered followed by repeated boluses or by CRI 0.6-1 mg/kg/h until respiratory targets are reached. Oxygen therapy was administered if necessary and subsequently recorded; additional cardiac medications were allowed at standard dosages, according to published guidelines (Keene et al., 2019).

During hospitalization clinical assessment and patient weight were performed every 6 hours, simultaneous blood and urine samples were taken 12, 24, 48 hours after the start of the diuretic therapy (Figure 3A).

If the patient was not hospitalized, oral therapy with furosemide was started (dosage chosen by the cardiologist based on the clinical conditions).

Both patients (hospitalized and not) were rechecked seven days after the acute event and subsequently at 1 month, 3 months, 6 months (Figures 3A and 3B). At these time points, clinical examination, weight, echocardiographic evaluation, simultaneous blood and urine sampling were performed.

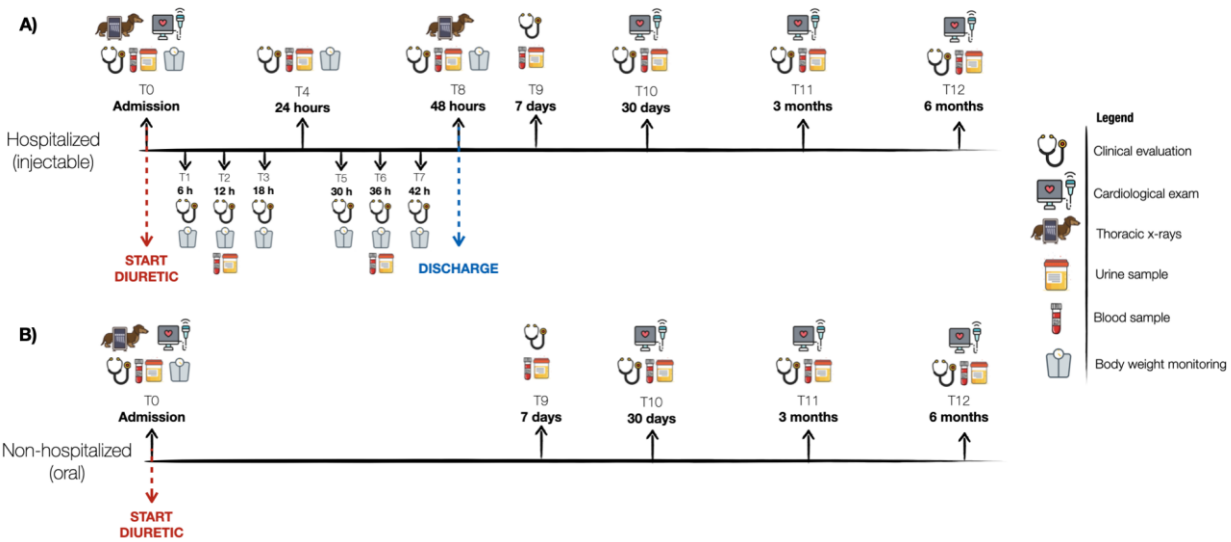

FIGURE 3. A) Timeline of hospitalized patients with congestive heart failure treated with injectable diuretic B) Timeline of non-hospitalized patients with congestive heart failure treated with oral diuretic

Aim of the study

In this thesis work was developed only the descriptive aim ($CvRD_H$ stable) and the data collected at T0 in stable chronic dogs with MMVD at different ACVIM stages (Figure 2) were analyzed.
The work was divided into two projects with the idea of:

- evaluate uNGAL as an early marker of $CvRD_H$ stable (Project 1)
- evaluate urinary electrolytes in stable patients on diuretic therapy as possible markers of diuretic resistance (Project 2)

For the Project 1 we aimed to: determine whether uNGAL and uNGAL differ between dogs with stable MMVD and healthy controls; assess uNGAL and uNGALC in dogs with MMVD according to the ACVIM staging system. Our hypothesis was that uNGAL and uNGALC increase in dogs with MMVD if compared to healthy control dogs, and that increasing uNGAL is detected with increasing MMVD ACVIM stage.
We want also to evaluate the correlation between echocardiographic indexes and uNGAL and uNGALC values.
We hypothesized that the echocardiographic evidence of reduced cardiac output or venous congestion was associated with elevated values of uNGAL and uNGALC in dogs with MMVD, regardless of the ACVIM stage.

The objectives of the Project 2 were: to evaluate the impact of the time frame occurring between the oral furosemide administration and sample collection on the concentration of urinary electrolytes in MMVD dogs with ACVIM stage C, with medically-controlled CHF; to explore the possible morning vs. evening fluctuations of urinary electrolytes in a group of healthy, untreated dogs.
The hypothesis was that: MMVD dogs evaluated within 6 h post-furosemide would have significantly higher urinary sodium, chloride, and other electrolytes if compared to dogs evaluated between six to 12 h post-furosemide; no relevant daily fluctuations of urinary electrolytes occur in healthy, untreated dogs.

Project 1

Neutrophil gelatinase associated lipocalin - NGAL

Materials and methods

This is a multicentric prospective, observational study performed at the Veterinary Teaching Hospital (VTH) of the University of Parma and Bologna between March 2020 and April 2021.

Study population

See Design of the Study (pag 16-18).

Because of the low number of dogs in stage D, dogs in stages C and D were grouped together for statistical purposes (group C+D).

Clinical and clinicopathological data

Recorded clinical data were signalment, body weight, medical history, physical and echocardiographic findings, current medications, and dosage.

Blood was collected by standard venipuncture using blood vacuum collection systems; concurrent fresh urine samples were collected by spontaneous voiding or cystocentesis. Blood and urine specimens were processed on a routine basis, according to quality standard procedures, and evaluated within one hour of collection. When it was not possible to perform the chemistry analysis within one hour, after centrifugation serum and urine supernatant, samples were stored at −80 °C, up to a maximum storage period of two months.

The biochemistry profile included sCr, urea, total protein, albumin, C-reactive protein (CRP), and serum electrolyte (sodium, chloride, potassium, magnesium, calcium, phosphate) concentrations. Serum biochemistry was performed using an automated chemistry analyzer (AU480, Beckman Coulter, Brea, California).

Urinalysis included urine specific gravity (USG) evaluated using a hand-held refractometer (American Optical, Buffalo, NY), dipstick test (Combur-Test 10 UX, Roche, Switzerland) read by an automated reader (URISYS 1100, Roche, Switzerland) and confirmed by visual inspection, microscopic sediment evaluation performed at low power field (100×) and high-power field (400×), and urine chemistry. Urine sediment was obtained after 5-minute centrifugation at 450*g*.

Urine supernatants were immediately analyzed by dipstick examination, and then used for chemical analyses or stored. Urine chemistry was determined using the same automated chemistry analyzer used for serum biochemistry, and included urinary creatinine (uCr), total protein concentrations and urine protein-to-creatinine ratio (UPC).

Urinary NGAL analysis

Urinary NGAL was measured using a commercial sandwich ELISA according to the manufacturer's instructions (Dog NGAL ELISA kit, BIOPORTO Diagnostics, Hellerup, Denmark). Aliquots of the urine supernatant of dogs with MMVD and of control dogs enrolled in the study were stored at −80 C for up to 2 months until assayed. The kit contained microwells plate precoated with mouse monoclonal antibody against dog NGAL, biotinylated anti-canine NGAL monoclonal antibodies, reagents and calibrators.

The assay was validated in our laboratory for dogs following a validation protocol including linearity and intraassay variation, and validation results were similar to those previously reported and consistent with those reported by the manufacturer (Steinbach et at., 2014). A standard curve for uNGAL concentration was made using 8 dilutions of the calibrators included in the KIT (from 0 to 400 pg/ml). Urine samples from healthy dogs were diluted 1:100 whereas for dogs with MMVD an initial dilution of 1:100 was used, followed by 1:300, 1:500, and 1:900 dilutions for samples where analyte concentration could not be determined. Dilutions were made using the sample diluent provided in the kit. One-hundred microliters of diluted sample were placed in the precoated wells and after 60 minutes of incubation, 3 automated washing series were made using the washing solution of the kit. One-hundred microliters of diluted sample were placed in the precoated wells and after 60 minutes of incubation, 3 automated washing series were made using the washing solution of the kit.

Biotinylated Dog-NGAL antibody was added into each microwell and incubated for 60 minutes at room temperature. After incubation another series of washes was carried out. Afterwards was added HRP-Streptavidin (provided in the kit) for a third incubation. After 60 minutes, another series of washes was carried out. A tetramethylbenzidine-based peroxidase substrate was dispensed and incubated for 10 minutes at room temperature in the dark. After this time stop solution was added and have been read within 30 minutes.

The concentration of uNGAL in the samples was determined by measuring the absorbance of the solution at 450nm using an appropriate plate reader (DV990BV4 spectrophotometer, N.T.

Laboratory s.r.l. Calenzano, Italy) and calculating from a standard curve using curve-fitting software (GraphPad Prism software, version 6, San Diego, California). Results were expressed as uNGAL concentrations (pg/mL) and as uNGAL-to-uCr ratio (uNGALC; pg/mg).

Echocardiography

Standard echocardiography and conventional Doppler examination were carried out or reviewed by board-certified cardiologists. Echocardiographic machines (iE33 ultrasound system and Epiq CVx ultrasound system; Philips Healthcare, Monza, Italy) were equipped with phased-array transducers, ranging from 1.6 to 6.0 MHz. Dogs were gently restrained in lateral recumbency and scanned underneath, from right and left parasternal positions. Standard echocardiographic two-dimensional, M-mode, and Doppler images were acquired as per the established standard for veterinary cardiology (Thomas et al., 1993). Myxomatous mitral valve disease was diagnosed based on the typical mitral valve lesions (thickening and prolapse of the mitral valve leaflets) associated with the presence of mitral regurgitation.

The normalized left ventricular end-systolic (LVIDSn) and end-diastolic diameters (LVIDDn) (Cornell et al., 2004), the fractional shortening (SF) and the LA/Ao (Hansson et al., 2002) were measured from the right parasternal short axis views. The maximal (LA_{Max}) and minimal left atrial volumes (LA_{Min}) were measured with monoplane area-length method from the left parasternal four chambers view. The left atrial stroke volume (LASV) was calculated according to the formula: $LASV = LA_{Max} – LA_{Min}$ (Höllmer et al., 2017). The aortic velocity-time integral (VTI_{Ao}) was measured from the subcostal view. The mitral velocity-time integral (VTI_{Mit}), the E wave peak velocity (E_{Vmax}), the ratio between the E wave and the E' wave of the tissue Doppler of the parietal mitral annulus (E/E') were measured from the left parasternal apical four-chamber view. The ratio between the VTI_{Mit}/VTI_{Ao} was then calculated. The velocity of the tricuspid regurgitant flow velocity (TR_{Vmax}) was measured from the left parasternal apical view, angled to optimize the alignment with the tricuspid regurgitant flow.

Statistical analysis

For the evaluation of uNGAL in different ACVIM stages: data distribution was assessed using the Shapiro-Wilk test. Data were expressed by standard descriptive statistics and presented as mean±SD or median and range (minimum-maximum) based on normal or non-normal data distribution. Data obtained in healthy dogs were used to calculate the RI for uNGAL and uNGALC using the Robust method considering a right-sided distribution. The Mann Whitney *U* test and the Student's *t*-test were used to compare dogs with MMVD to healthy control dogs. The Kruskal-Wallis test with post hoc comparison (Conover test) was used to compare continuous variables among dogs with MMVD of different ACVIM stage (group B1 vs B2 vs C+D). Categorical variables were compared among groups using the chi-squared test. Spearman's correlation coefficient was used to assess potential correlations between variables. Results were considered significant if $P<.05$. Statistical analyses were performed using a commercially available statistical software package (MedCalc Statistical Software version 19.5.1; Ostend, Belgium).

For the evaluation of a possible association between echocardiographic indexes and uNGAL: statistical analysis was performed using commercially available software (MedCalc Version 20.114, Ostend, Belgium and G*Power Version 3.1.9.3). Descriptive statistics were used for sex, age, body weight, breed, ACVIM stages, the presence of tricuspid regurgitation, pulmonary hypertension, ascites, and arrhythmias. Data distribution was assessed both graphically and analytically. The Shapiro-Wilk test was used to check if the continuous variables were normally distributed, and results were presented as mean and standard deviation or median and range based on their distribution.

According to normal uNGAL and uNGALC values reported in this thesis (normal uNGAL values < 2300 pg/ml – normal uNGALC values < 1400 pg/mg) the population was divided into two groups for normal and abnormal uNGAL and uNGALC respectively. Echocardiographic variables were explored as continuous variables and uNGAL and uNGALC were expressed dichotomously.

Differences between continuous independent non-normally distributed data were investigated using the non-parametric Mann-Whitney U test.

The association between the echocardiographic indexes and the presence of renal damage were investigated using univariate and multivariable logistic regression analysis. Multicollinearity and model goodness-of-fit tests were checked. Multicollinearity between independent variables was investigated using the variance inflation factor (VIF), considering no correlation between

predictors with a VIF = 1, moderate correlation with VTI between 1 and 5, and potentially severe correlation with VIF > 5. Significant results from logistic regression analysis were also presented with ROC (Receiver Operating Characteristic) analysis to define the ability of some echocardiographic indexes to discriminate between normal and abnormal uNGAL and uNGALC and the best compromise between the true- and false-positive rates was assessed by calculation of the Youden index value; the area under the ROC curve takes value from 0 (a perfectly inaccurate test) to 1 (perfectly accurate test), with 0.5 suggesting no discrimination, 0.6 to 0.7 poor, 0.7 to 0.8 fair, 0.8 to 0.9 good, and more than 0.9 outstanding.

The Fisher's exact test was used to explore any association between the presence of tricuspid regurgitation or pulmonary hypertension and renal damage and the risk of abnormal uNGAL or uNGALC in patients with tricuspid regurgitation or pulmonary hypertension was explored with the calculation of relative risk.

Values of P less than 0.05 were considered significant for all analyses.

To explore any effect of the diuretic dosage upon uNGAL and uNGALC values, a linear regression model was built. The independent variable, furosemide dosage (mg/kg/day), and the dependent ones, uNGAL (pg/ml) and uNGALC (pg/mg), were expressed as continuous variables. Because of the presence of a non-linear relationship between the independent and the highly skewed dependent variables, a logarithm transformation of the latter was done.

Due to the absence of data in the literature, an *a priori* analysis (two-tailed test) was made on the first 20 patients included in the study, to estimate the sample size. The sample size needed to obtain significant differences between continuous independent non-normally distributed data in normal or abnormal uNGAL groups (> 0.90; $\alpha = 0.05$ and $\beta = 0.10$) was 76 dogs, with an effect size of 0.74.

Results

Urinary NGAL in different ACVIM stages

The study population consisted of 98 MMVD dogs: 23/98 (23%) in group B1, 27/98 (28%) in group B2, and 48/98 (49%) in group C+D. In group C+D, 39/48 (81%) dogs were staged in ACVIM C and 9/48 (19%) dogs in ACVIM D. Overall, 43/98 (44%) were females (17/43 spayed) and 55/98 (56%) were males (9/55 castrated); 48/98 (49%) were mixed breed dogs whereas 50/98 (51%) were purebred dogs. Median body weight was 8.9 kg (range, 2.4-31.9) and mean age was 11±2.7 years. Dogs in group B1 were significantly younger compared to those in groups B2 and C+D (P = .003). Body weight was significantly higher in group B1 vs group C+D (P = .03). Demographic data for enrolled dogs and for the different groups are reported in Table 4.

TABLE 4. Demographic data and descriptive statistics of the study population: dogs with myxomatous mitral valve disease (MMVD) in different ACVIM stage (group B1, group B2, group C+D), and healthy dogs

Population	Group B1 (n = 23)	Group B2 (n = 27)	Group C+D (n = 48)	Healthy dogs (n = 46)	*P* value
Age (y)	9.4 (±2.7)[b,c,d]	11.1 (±2.5)[a,d]	11.8 (±2.6)[a,d]	3 (1-8)[a,b,c]	<.001
Weight (kg)	9 (7.2-23.8)[c,d]	9.5 (±4.1)[d]	7.5 (2.9-31.9)[a,d]	27.6 (10-50)[a,b,c]	<.001
Medications					
N. of dogs receiving Spironolactone			17/48		
Spironolactone (mg/kg/d)			2 (1.2-5.4)		
N. of dogs receiving Pimobendan		18/27	48/48		
Pimobendan (mg/kg/d)		0.5 (0.4-0.7)	0.6 (0.3-1)		
N. of dogs receiving Enalapril			2/48		

Enalapril (mg/kg/d)			0.56 (0.32-0.8)		
N. of dogs receiving Benazepril			37/48		
Benazepril (mg/kg/d)			0.61 (±0.3)		
N. of dogs receiving Furosemide			42/48		
Furosemide (mg/kg/d)			4.5 (±1.9)		
N. of dogs receiving Torasemide			6/48		
Torasemide (mg/kg/d)			0.4 (±0.1)		
N. of dogs receiving Digoxin			4/48		
Digoxin (mg/kg/d)			0.007 (0.006-0.008)		

Note: Data are reported as median and range (minimum-maximum value) or mean± SD, based on their distribution.

Abbreviations: Group B1, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B1; group B2, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B2; group C+D, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stages C and D.

[a] Significantly different from group B1.
[b] Significantly different from group B2.
[c] Significantly different from group C+D.
[d] Significantly different from healthy dogs.

At the time of enrollment, no dog in group B1 had received cardiovascular drugs. Pimobendan represented the only cardiovascular drug administrated in dogs in group B2; all dogs in group C+D 48/48 (100%) received pimobendan and diuretics. Additionally, 39/48 (81%) dogs of this group received an ACEI and 17/48 (35%) dogs received spironolactone. Among dogs in group C+D, 4/48 (8%) received digoxin to treat atrial fibrillation (Table 4). No other antiarrhythmic drugs were prescribed because no hemodynamically relevant cardiac rhythm disturbances were found in the study population.

Forty-six healthy dogs were included as controls. The median age was 3 years (range, 1-8) and the median body weight was 27.6 kg (range, 10-50). Sex distribution was as follows: 28/46 (61%) were females (16/28 spayed), and 18/46 (39%) were males (7/18 castrated). Thirteen of 46 (28%) were mixed breed dogs whereas 33/46 (72%) were purebred dogs. Healthy dogs were younger and had a higher body weight compared to MMVD dogs ($P<.001$ and $P<.001$, respectively). No difference in sex distribution was documented between healthy and MMVD dogs ($P = .07$).

Among enrolled dogs, 28/98 (27%) were azotemic (sCr between 1.41 and 2.76 mg/dL) whereas 8/48 (17%) had UPC >0.5. Dogs included in group C+D had significantly higher sCr concentration vs B1 and B2 (overall $P<.001$). The UPC was significantly lower in group B1 dogs compared to dogs in both groups B2 and C+D (overall $P = .002$). Urea concentration was significantly higher in group C+D vs groups B1 and B2 and significantly higher in group B2 vs group B1 (overall $P<.001$). In addition, dogs in group C+D had significantly lower serum chloride concentration compared to dogs in group B1 and B2 (overall $P<.001$). No statistical difference was observed between MMVD dogs in different ACVIM stages for leukocyte count and serum CRP concentration (overall $P = .47$ and $P = .21$, respectively). Complete clinicopathological results are reported in Table 5.

TABLE 5. Data comparison between dogs with myxomatous mitral valve disease (MMVD) grouped according to their ACVIM stage (group B1, group B2, group C+D)

Variable	RI	Group B1 (n = 23)	Group B2 (n = 27)	Group C+D (n = 48)	*P* value
Creatinine (mg/dL)	0.75-1.4	0.97 (±0.2)[c]	0.9 (0.5-2.7)[c]	1.36 (±0.4)[a,b]	<.001
CRP (mg/dL)	0-1	0.99 (0.5-4)	0.93 (0.7-19.7)	1.27 (0.2-6.4)	.21
Leukocytes (/µL)	6000-17000	6485 (5880-7090)	6970 (4290-9470)	8815 (5600-13700)	.47
Urea (mg/dL)	17-48	33 (±8.2)[b,c]	42.1 (16-124)[a,c]	80 (20-296)[a,b]	<.001
Albumin (g/dL)	2.75-3.85	3.2 (±0.19)	3.1 (±0.32)	3.3 (±0.4)	.07
Total protein (g/dL)	5.6-7.30	6.3 (±0.3)[c]	6.3 (±0.6)[c]	6.6 (±0.6)[a,b]	.03
Total calcium (mg/dL)	9.3-11	10.3 (9.2-12.2)[c]	9.9 (±0.65)[c]	10.4 (±0.61)[a,b]	.04
Sodium (mEq/L)	143-151	148 (±1.9)	147 (139-161)	148 (±3)	.47
Potassium (mEq/L)	3.8-5.0	4.5 (±0.2)[b]	4.6 (±0.36)[a,c]	4.3 (±0.48)[b]	.004
Chloride (mEq/L)	108.0-118	111 (±2.68)[c]	111 (±4.3)[c]	106.7 (±3.6)[a,b]	<.001
Magnesium (mg/dL)	1.70-2.35	2.03 (1.36-2.3)	2.05 (±0.2)	2.01 (±0.3)	.65
Phosphate (mg/dL)	2.65-5.40	3.8 (±0.7)	3.9 (±0.9)	3.9 (±1.09)	.62
USG	>1.030	1.030 (±11.6)[c]	1.034 (±12.1)[c]	1.016 (1008-1065)[a,b]	<.001
UPC (mg/mg)	0-0.5	0.11 (0.07-0.8)[b,c]	0.17 (0.07-0.93)[a]	0.22 (0.05-1.36)[a]	.002

Note: Data are reported as mean± SD or median and range (minimum-maximum value), based on their distribution.

Abbreviations: CRP, C-reactive proteins; group B1, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B1; group B2, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B2; group C+D, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stages C and D; RI, reference interval; UPC, urine protein to urine creatinine ratio; USG, urine specific gravity.

[a] Significantly different from group B1.

[b] Significantly different from group B2.

[c] Significantly different from group C+D.

The RI for uNGAL and uNGALC obtained in healthy dogs was 0-2300 pg/mL and 0-1400 pg/mg, respectively. No correlation was identified between either uNGAL or uNGALC and age in healthy dogs ($r = -.02$, $P = .17$; $r = -.01$, $P = .46$, respectively). Similarly, no difference in uNGAL and uNGALC results was documented based on sex distribution ($P = .23$, $P = .07$, respectively).

In the comparison of healthy and MMVD dogs, the overall population of dogs with MMVD had significantly increased uNGAL (1204 pg/mL; range, 30-39 732) and uNGALC (1816 pg/mg; range, 22-127 693) compared to healthy dogs (uNGAL, 584 pg/mL; range, 56-4072; uNGALC, 231 pg/mg; range, 15-2407; $P = .002$, $P < .001$, respectively; Figure 4 and Figure 5).

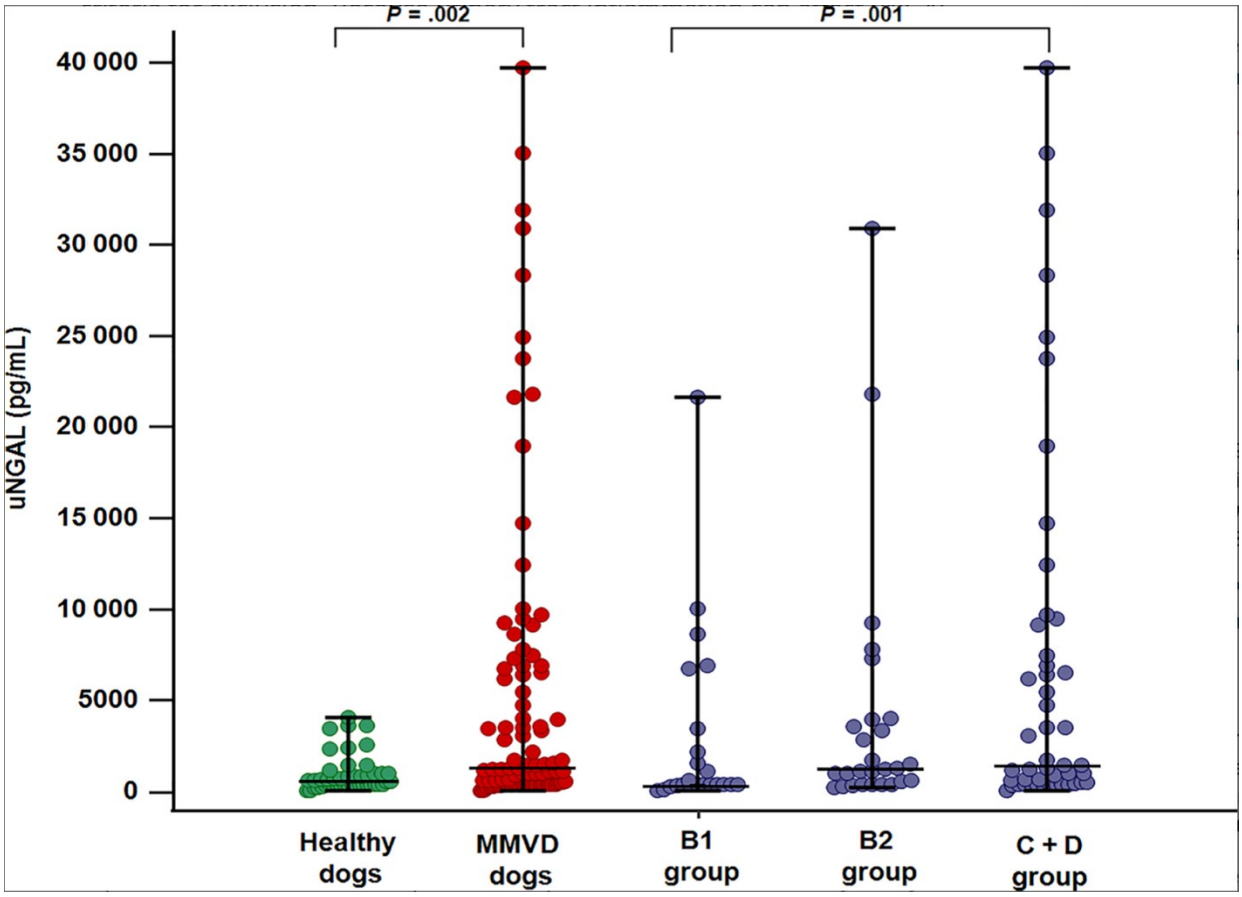

FIGURE 4. Dot plot showing results of urinary Neutrophil Gelatinase-Associated Lipocalin (uNGAL) comparison between healthy dogs (n = 46) (green dots) and dogs with myxomatous mitral valve disease (MMVD) (n = 98) (red dots), and among dogs with MMVD in different ACVIM stages (blue dots): group B1 (n = 23), group B2 (n = 27) and group C+D (n = 48). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent median value. P values are reported for significantly different results ($P < .05$). uNGAL: urinary Neutrophil Gelatinase-Associated Lipocalin; MMVD dogs: dogs with myxomatous mitral valve disease; B1 group: dogs with myxomatous mitral valve disease in ACVIM stage B1; B2 group: dogs with myxomatous mitral valve disease in ACVIM stage B2; C+D group: dogs with myxomatous mitral valve disease in ACVIM stage C and D

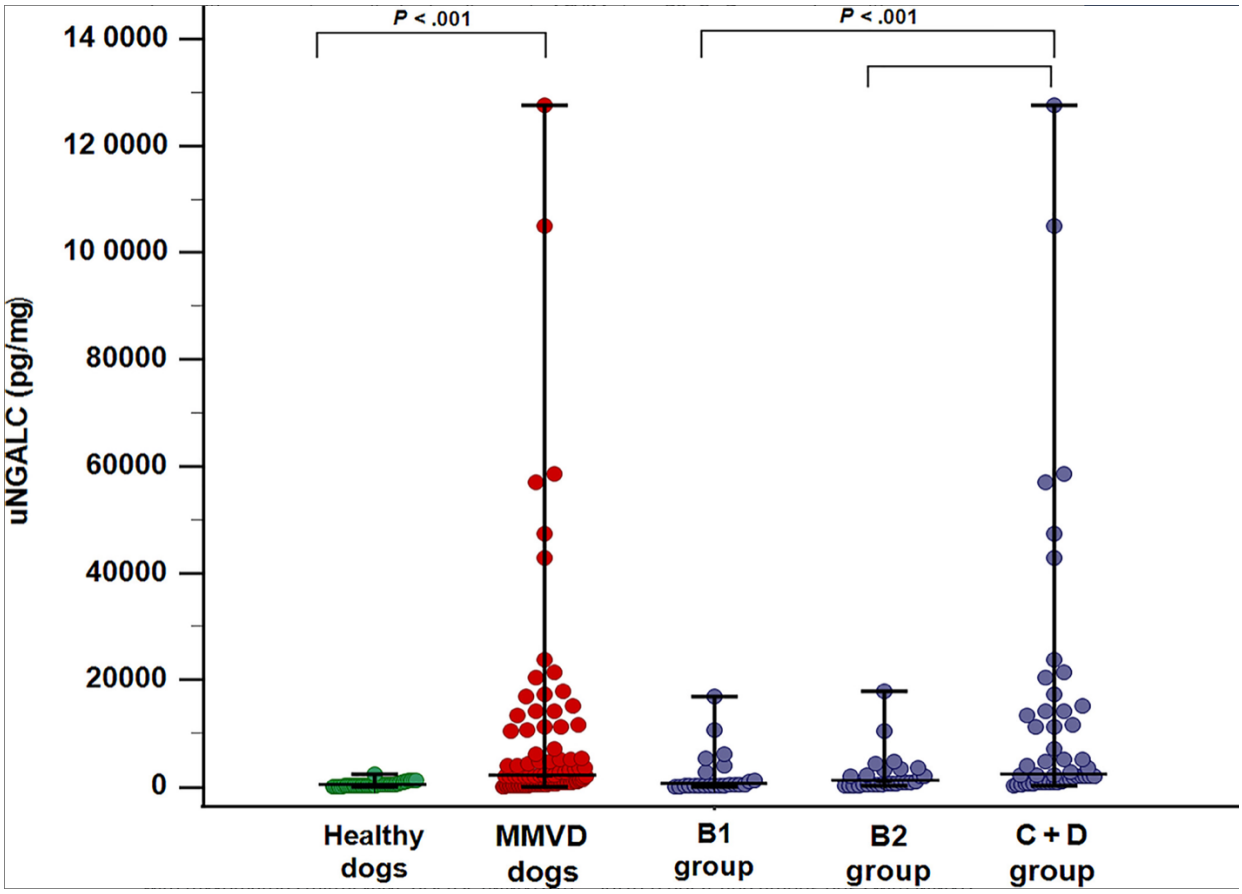

FIGURE 5. Dot plot showing results of urinary Neutrophil Gelatinase-Associated Lipocalin to urinary creatinine ratio (uNGALC) comparison between healthy dogs (n = 46) (green dots) and dogs with myxomatous mitral valve disease (MMVD) (n = 98) (red dots), and among dogs with MMVD in different ACVIM stages (blue dots): group B1 (n = 23), group B2 (n = 27) and group C+D (n = 48). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent median value. *P* values are reported for significantly different results (*P* <.05). uNGALC: urinary Neutrophil Gelatinase-Associated Lipocalin to urinary creatinine ratio; MMVD dogs: dogs with myxomatous mitral valve disease; B1 group: dogs with myxomatous mitral valve disease in ACVIM stage B1; B2 group: dogs with myxomatous mitral valve disease in ACVIM stage B2; C+D group: dogs with myxomatous mitral valve disease in ACVIM stage C and D

When uNGAL values were compared among MMVD dogs in different ACVIM stages, dogs belonging to group C+D had significantly higher uNGAL compared to group B1 and healthy dogs. Furthermore, uNGAL was significantly higher in group B2 compared to healthy controls, whereas no difference was detected in the comparison between group B1 vs B2, between group B2 vs group C+D and between B1 vs healthy dogs (overall *P* = .001). Concerning uNGALC results, all dogs with MMVD had higher values compared to healthy controls, with dogs included in group C+D having significantly higher uNGALC compared to the other MMVD groups (overall *P*<.001; Table 6, Figure 4 and Figure 5).

TABLE 6. uNGAL, uNGALC, and uCr comparison between dogs with myxomatous mitral valve disease (MMVD) grouped according to their ACVIM stage (group B1, group B2, group C+D), and between healthy control dogs

Variable	Group B1 (n = 23)	Group B2 (n = 27)	Group C+D (n = 48)	Healthy dogs (n = 46)	*P* value
uCr (mg/dL)	183 (±80)[c,d]	171 (±62)[c,d]	50 (14-226)[a,b,d]	297 (±129.7)[a,b,c]	<.001
uNGAL (pg/mL)	400 (70-21647)[c]	1231 (208-30916)[d]	1463 (30-39732)[a,d]	584 (56-4072)[b,c]	.001
uNGALC (pg/mg)	304 (22-16871)[c,d]	777 (109-17989)[c,d]	2478 (177-127639)[a,b,d]	231 (15-2407)[a,b,c]	<.001

Note: Data are reported as mean± SD or median and range (minimum-maximum value), based on their distribution.

Abbreviations: uCr, urinary creatinine; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC, urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio; group B1, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B1; group B2, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage B2; group C+D, dogs with myxomatous mitral valve disease in American College of Veterinary Internal Medicine stage C and stage D.

[a] Significantly different from group B1.
[b] Significantly different from group B2.
[c] Significantly different from group C+D.
[d] Significantly different from healthy dogs.

In dogs with MMVD, frequency of abnormal uNGAL results (eg, values above the RI) increased with increasing ACVIM stage: 26% (6/23) in dogs in group B1, 37% (10/27) in dogs in group B2, and 46% (22/48) in dogs in group C+D (*P* = .27). A similar and more significant trend was noted for uNGALC results, because these were above the RI in 26% (6/23) of dogs in group B1, 41% (11/27) of dogs in group B2, and 73% (35/48) of dogs in group C+D (*P* = .0003).

A significant positive correlation was documented for both uNGAL and uNGALC with UPC (*r* = .47, *P*<.001*;* *r* = .54, *P*<.001, respectively) and urea (*r* = .27, *P* = .0071; *r* = .45, *P*<.001, respectively). Serum creatinine and CRP concentrations and leukocyte count were not significantly correlated with uNGAL (*r* = .02, *P* = .08; *r* = .14, *P* = .16; *r* = .14, *P* = .59, respectively) and uNGALC (*r* = .15, *P* = .12*;* *r* = .15, *P* = .14; *r* = −.07, *P* = .78, respectively). Moreover, no correlation was found between uNGAL and uNGALC with daily furosemide dosage (*r* = .005, *P* = .97*;*

r = .09, *P* = .53, respectively). No correlation was performed for torasemide given the low number of dogs receiving this drug.

Association between uNGAL and echocardiographic variables

Eighty dogs matched the inclusion criteria, 3 dogs were excluded due to the poor echocardiographic image quality. Seventy-seven dogs with MMVD, at different ACVIM stages, were included in the study. Forty-three were males (55.8%) and 34 females (44.2%) of different breeds: 36 Mongrel dogs, 18 Cavalier King Charles Spaniels, 7 Poodles, 4 Dachshunds, 3 Pinchers, 2 Chihuahuas, 2 Maltese dogs, 1 Jack Russel terrier, 1 Boston terrier, 1 Schnauzer, 1 Shih-Tzu, 1 Spinone Italiano; the mean age was 11.7 (± 2.8) years and the median weight was 8.9 (2.4 - 20.0; C.I. 95% 7.7-9.5) kg. The distribution of the population in the different ACVIM stages was: 19 dogs (24.7%) ACVIM B1, 22 dogs (28.6%) ACVIM B2, 32 dogs (41.6%) ACVIM C, and 4 dogs (5.2%) ACVIM D.

Fifty dogs (64.9%) had tricuspid regurgitation, 15 of which (19.5%) had pulmonary hypertension and 6 had ascites (7,8%); only 3 dogs (3,9%) had atrial fibrillation.

The dogs with abnormal uNGAL and uNGALC were 33/77 (43%) and 39/77 (51%), respectively.

There was a significant difference between normal and abnormal uNGAL and uNGALC groups for LA/Ao, LASV, LAV_{Max}, LAV_{Min}, LVDDn, TR_{Vmax} and VTI_{Mit}/VTI_{Ao}; furthermore Ao_{Vmax}, SF and VTI_{Mit} were significantly different between normal and abnormal uNGALC (Table 7 and Table 8).

TABLE 7. Data comparison between dogs with myxomatous mitral valve disease (MMVD), grouped according to their normal or elevated uNGAL values. The Mann-Whitney U test results are reported as median and average rank values for every group; U values and P values are also reported.

Echocardiographic parameters	uNGAL					
	< 2300 (pg/ml)		≥ 2300 (pg/ml)		Mann-Whitney U	p < 0.05
	Median	Average Rank	Median	Average Rank		
E/E'	9.7	20.1	11.1	26.9	127.0	0.110
E_{Vmax} (m/s)	1.0	35.1	1.25	44.2	554.0	0.077
LA/Ao	1.7	34.6	2.01	44.8	534.0	0.049*
LASV (ml/kg)	1.1	32.6	1.72	46.6	446.0	0.007*
LAV_{Max} (ml/kg)	2.5	34.0	3.88	45.6	507.0	0.025*
LAV_{Min} (ml/kg)	1.0	34.2	1.67	44.5	531.0	0.045*
LVIDDn (cm/kg$^{0.294}$)	1.9	34.1	2.1	45.5	511.5	0.027*
LVIDSn (cm/kg$^{0.315}$)	1.0	36.6	1.0	42.2	619.0	0.265
SF (%)	45.1	36.2	47	42.7	603.5	0.207
TR_{Vmax} (m/s)	1.8	32.0	2.64	48.4	417.0	0.001*
VTI_{Ao} (cm)	11.0	41.9	10	33.8	554.0	0.112
VTI_{Mit} (cm)	14.0	34.9	15	41.2	552.5	0.210
VTI_{Mit}/VTI_{Ao}	1.3	32.2	1.62	43.9	438.0	0.020*

Abbreviations: E/E', E to E' waves ratio; E_{Vmax}, E wave peak velocity; LA_{SV}, left atrial stroke volume; LA/Ao, left atrium to aortic root ratio; LAV_{Max}, left atrium maximal volume; LAV_{Min}, left atrium minimal volume; LVIDDn, normalized left ventricular end-diastolic diameter; LVIDSn, normalized left ventricular end-systolic diameter; SF, shortening fraction; TR_{Vmax}, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTI_{Ao}, Aortic velocity-time integral; VTI_{Mit}, Mitral velocity-time integral; VTI_{Mit}/VTI_{Ao}, Mitral to aortic velocity-time integral ratio.

* significantly different between groups (P < 0.05)

TABLE 8. Data comparison between dogs with myxomatous mitral valve disease (MMVD), grouped according to their normal or elevated uNGALC values. The Mann-Whitney U test results are reported as median and average rank values for every group; U values and P values are also reported.

Echocardiographic parameters	uNGALC					
	< 1400 (pg/mg)		≥ 1400 (pg/mg)		Mann-Whitney U	p < 0.05
	Median	Average Rank	Median	Average Rank		
E/E'	9.7	18.9	10.6	26.3	147.5	0.056
E_{Vmax} (m/s)	1.0	33.5	1.26	44.4	532.0	0.033*
LA/Ao	1.6	33.7	1.9	44.1	541.0	0.041*
LASV (ml/kg)	0.9	29.1	1.75	47.9	363.5	< 0.001*
LAV_{Max} (ml/kg)	2.2	31.1	3.99	46.7	440.5	0.002*
LAV_{Min} (ml/kg)	0.7	32.0	1.68	45.0	473.5	0.010*
LVIDDn (cm/$kg^{0.294}$)	1.8	32.0	2.1	45.8	476.5	0.007*
LVIDSn (cm/$kg^{0.315}$)	1.0	36.8	1	41.1	657.5	0.389
SF (%)	44.2	33.4	48.2	44.5	527.5	0.030*
TR_{Vmax} (m/s)	1.8	33.0	2.54	44.9	511.5	0.017*
VTI_{Ao} (cm)	11.5	43.4	10	33.6	535.5	0.051
VTI_{Mit} (cm)	14.0	13.3	15	43.7	453.5	0.012*
VTI_{Mit}/VTI_{Ao}	1.3	29.7	1.65	44.5	395.0	0.003*

Abbreviations: E/E', E to E' waves ratio; E_{Vmax}, E wave peak velocity; LA_{SV}, left atrial stroke volume; LA/Ao, left atrium to aortic root ratio; LAV_{Max}, left atrium maximal volume; LAV_{Min}, left atrium minimal volume; LVIDDn, normalized left ventricular end-diastolic diameter; LVIDSn, normalized left ventricular end-systolic diameter; SF, shortening fraction; TR_{Vmax}, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTI_{Ao}, Aortic velocity-time integral; VTI_{Mit}, Mitral velocity-time integral; VTI_{Mit}/VTI_{Ao}, Mitral to aortic velocity-time integral ratio.

* significantly different between groups (P < 0.05)

In the univariate analysis, LASV, TR_{Vmax}, LAV_{Max}, LVIDDn and VTI_{Mit}/VTI_{Ao} were independent predictors of increased uNGAL and uNGALC (Table 9), however only LASV [(OR: 1.96, 95% CI: 1.16 to 3.31), P = 0.01 for NGAL and (OR: 2.79, 95% CI: 1.50 to 5.17), P < 0.001 for NGALC] and TR_{Vmax}

[(OR: 1.73, 95% CI: 1.20–2.51), P = 0.002 for NGAL and (OR: 1.50, 95% CI: 1.07–2.10), P = 0.015 for NGALC] remained statistically significant in the multivariable analysis.A moderate correlation between LASV and LAV_{Max} (VIF = 3.3), LVIDDn (VIF = 3.2) and VTI_{Mit}/VTI_{Ao} (VIF =3.4) was found, whereas no correlation between LASV and TR_{Vmax} (VIF = 1.1) was detected.

TABLE 9. Univariate Logistic Regression Analysis.

Echocardiographic parameters	uNGAL ≥ 2300 (pg/ml)			uNGALC ≥ 1400 (pg/mg)		
	OR	95% CI	P < 0.05	OR	95% CI	P < 0.05
E_{Vmax} (m/s)	3.64	0.91 to 14.55	0.06	4.54	1.12 to 18.37	0.03*
LA/Ao	2.54	0.98 to 6.57	0.055	2.43	0.94 to 6.27	0.07
LASV (ml/kg)	1.96	1.16 to 3.31	0.01*	2.79	1.5 to 5.17	< 0.001*
LAV_{Max} (ml/kg)	1.26	1.03 to 1.55	0.02*	1.41	1.11 to 1.79	0.001*
LAV_{Min} (ml/kg)	1.30	0.98 to 1.74	0.07	1.45	1.06 to 2	0.013**
LVIDDn ($cm/kg^{0.294}$)	5.28	1.24 to 22.46	0.02*	8.31	1.84 to 37.5	0.003*
SF (%)	1.05	0.99 to 1.11	0.14	1.08	1.01 to 1.15	0.015*
TR_{Vmax} (m/s)	1.73	1.2 to 2.51	0.002*	1.50	1.07 to 2.1	0.015*
VTI_{Mit} (cm)	1.10	0.97 to 1.25	0.12	1.19	1.04 to 1.37	0.007*
VTI_{Mit}/VTI_{Ao}	2.80	1.17 to 6.71	0.01*	4.32	1.64 to 11.38	0.001*

Abbreviations: E_{Vmax}, E wave peak velocity; LA/Ao, left atrium to aortic root ratio; LASV, left atrial stroke volume; LAV_{Max}, left atrium maximal volume; LAV_{Min}, left atrium minimal volume; LVIDDn, normalized left ventricular end-diastolic diameter; SF, shortening fraction; TR_{Vmax}, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTI_{Mit}, Mitral velocity-time integral; VTI_{Mit}/VTI_{Ao}, Mitral to aortic velocity-time integral ratio.

Areas under the ROC curve for LASV as a predictor variable to classify patients with abnormal urinary NGAL and NGALC, were 0.68 (95% C.I. 0.56-0.80), P = 0.003, and 0.75 (95% C.I. 0.64-0.86) P < 0.001, respectively (Figure 6A-B). Areas under the ROC curve for TR_{Vmax}, as a predictor variable to classify patients with renal damage, were 0.71 (95% C.I. 0.59-0.83), P < 0.001, for abnormal uNGAL and 0.66 (95% C.I. 0.53-0.78), P = 0.014, for abnormal uNGALC (Figure 6C-D). The best compromises between sensitivity and specificity for LASV were > 0.76 ml/kg (Specificity: 90.62%; Sensitivity: 38.64%) and > 1.11 (Specificity: 78.95%; Sensitivity: 60.53) for uNGAL and uNGALC,

respectively; for TR_{Vmax} the cut-offs were > 2.31 m/s (Specificity: 72.73%; Sensitivity: 68.18%) and > 2.27 m/s (Specificity: 71.79%; Sensitivity: 63.16%) for uNGAL and uNGALC, respectively.

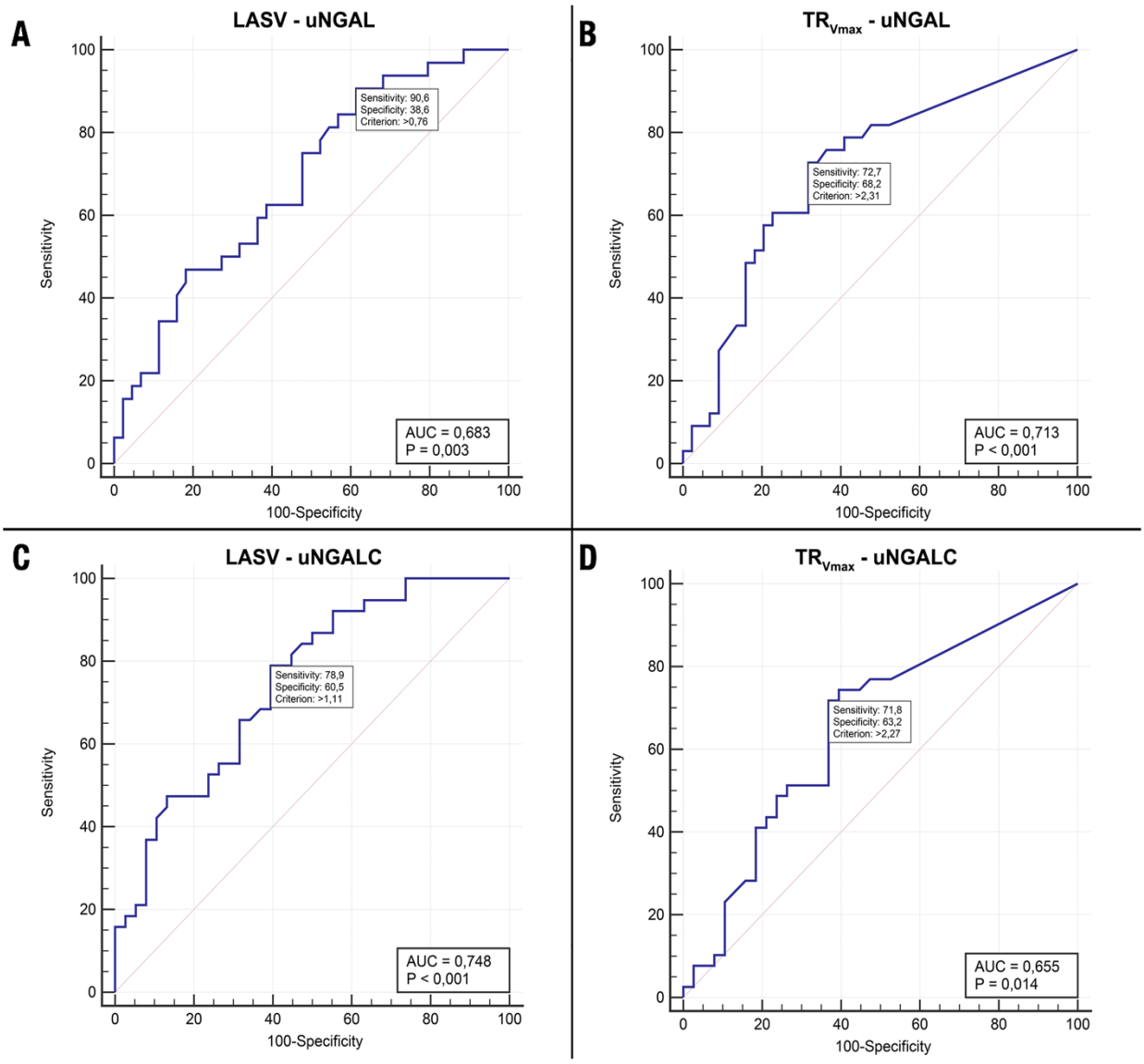

FIGURE 6. Receiver operating characteristic curves of LASV (A-C) and TR_{Vmax} (B-D) with AUC and cutoffs to predict renal damage assessed by abnormal uNGAL and uNGALC in dogs with MMVD. AUC, area under the ROC curve; C.I., Confidence interval; LASV, left atrial stroke volume; MMVD, myxomatous mitral valve disease; TR_{Vmax}, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: uNGAL to urinary creatinine ratio.

There was a statistically significant association between the presence of tricuspid regurgitation and renal damage (P = 0.009 for uNGAL; P = 0.033 for uNGALC), but the association between pulmonary hypertension and renal damage was only significant for uNGAL (P = 0.046 for uNGAL; P = 0.250 for uNGALC). The relative risk to have abnormal uNGAL or uNGALC for dogs with tricuspid regurgitation was statistically significant, with RR: 2.43, 95% CI: 1.15 to 5.15 (P = 0.020) for uNGAL

and RR: 1.80, 95% CI: 1.01 to 3.21 (P = 0.047) for uNGALC. In patients with pulmonary hypertension, the elevation in risk to have abnormal values was statistically significant only for uNGAL [RR: 1.80, 95% CI: 1.11 to 2.91 (P = 0.017)], but not for uNGALC [RR: 1.43, 95% CI: 0.91 to 2.26 (P = 0.119)].

The fitted regression models to test the relationship between daily furosemide dosage and uNGAL, uNGALC values were: uNGAL = 3.0670 + 0.09357 * (mg/kg/day of Furosemide) and uNGALC = 3.1874 + 0.1224 * (mg/kg/day of Furosemide). The overall regressions were not statistically significant (R^2 = 0.045, F (1, 33) = 1.55, P = 0.222) for uNGAL and (R^2 = 0.080, F (1, 33) = 2.87, P = 0.100) for uNGALC. It was found that diuretic dosage didn't influence uNGAL (β = 0.094, P = 0.222) (Figure 7A) and uNGALC (β = 0.122, P = 0.100) (Figure 7B) values.

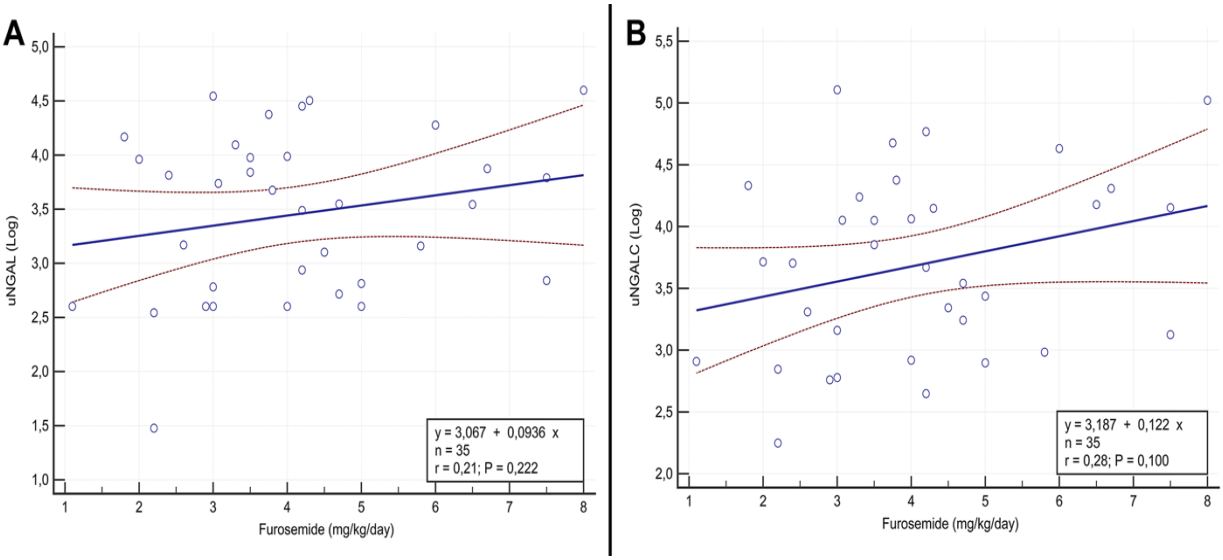

FIGURE 7. Linear regression between Furosemide dosage (mg/kg/day) and uNGAL (A) or uNGALC (B). uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio.

Project 2

Urinary electrolytes

Materials and methods

This was a prospective, observational study performed at the Veterinary Teaching Hospitals (VTH) of two University Institutions (University of Bologna and University of Parma) between March and December 2020.

Study population

See Design of the Study (pag 16-18).

Dogs were eligible for inclusion if they were affected by MMVD at ACVIM stage C, diagnosed, and classified according to the current guidelines (Keene et al., 2019), and if they were receiving exclusively oral furosemide as a loop diuretic.

Further inclusion criteria were the following:

- oral furosemide had to be administered twice daily at a stable dosage for at least one week prior to enrollment;
- morning oral administration of furosemide had to occur between 7:00 and 8:00 a.m., at home by the owner;

Additional exclusion criteria was the use of loop diuretics other than furosemide (e.g. torasemide).

Study groups

The cardiologists of the VTH participating in the study performed the first consultation and routine rechecks between 9:00 a.m. and 7:00 p.m. Such habits were not modified to accomplish the purpose of the study; thus, patient enrollment was conducted in line with daily clinical practice. Dogs with MMVD fulfilling the inclusion criteria were grouped based on the time of sample collection (Figure 8). The MMVD morning group (MMVD-MG) included dogs examined between 9:00 a.m. and 1:00 p.m. (from one to 6 h after furosemide administration); the MMVD evening group (MMVD-EG) included dogs examined between 2:00 p.m. and 7:00 p.m. (over 6 h after furosemide administration). Healthy dogs were also divided into two groups according to the time

of blood and urine sampling: the healthy morning group (H-MG) was sampled between 9:00 a.m. and 1:00 p.m.; the healthy evening group (H-EG) between 2:00 p.m. and 7:00 p.m.

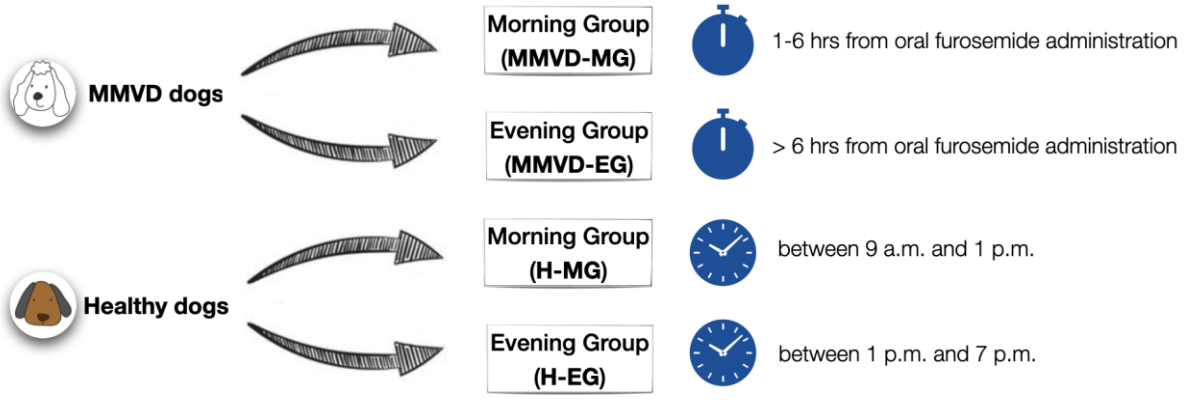

FIGURE 8. Group divisions.
MMVD-MG, dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG, dogs with myxomatous mitral valve disease sampled in the evening; H-MG, healthy dogs evaluated in the morning (healthy morning group); H-EG, healthy dogs evaluated in the evening (healthy evening group).

Clinical and clinicopathological data

Recorded clinical data were signalment, including body weight, medical history, physical and echocardiographic examination findings, current medications and dosage, time elapsed from the morning furosemide administration, duration and dose of furosemide therapy. Blood specimens were collected by standard venipuncture using blood vacuum collection systems; concurrent fresh urine samples were collected by spontaneous voiding or cystocentesis. Blood and urine specimens were processed on a routine basis, according to quality standard procedures, and evaluated at Bologna VTH within 1 h of collection. When it was not possible to perform the chemistry analysis within 1 h, serum and urine samples were stored at 80 C, up to a maximum storage period of two months. The chemistry profile included sCr, urea, total proteins, albumin, and the following serum electrolytes: sodium, chloride, potassium, magnesium, calcium, phosphate. Serum chemistry was determined using an automated chemistry analyzer (AU 480, Olympus/Beckman Coulter, Brea, California, USA). Urinalysis included urine specific gravity evaluated by a hand refractometer (American Optical, Buffalo, NY, USA), dipstick test (Combur-Test 10 UX, Roche, Switzerland) read by an automated reader (URISYS 1100, Roche, Switzerland) and confirmed by visual inspection, microscopic sediment evaluation performed at low power field (100X) and high-power field

(400X), and urine chemistry. Urine sediment was obtained after 5-min centrifugation at 450g. Urine supernatants were immediately analyzed for dipstick examination, and then used for chemical analyses or stored. Urine chemistry was determined using the same automated chemistry analyzer used for serum chemistry, and included urinary creatinine ([uCr]), urine total proteins, urine proteins to [uCr] ratio, and the following urinary electrolyte concentrations: urine sodium ([uNa^+]), chloride ([uCl]), potassium ([uK^+]), magnesium ([uMg^{++}]), calcium ([uCa^{++}]), and phosphate. The [uNa^+] to [uK^+] ratio (uNa^+:uK^+) was calculated. The ratio with [uCr] was calculated for [uNa^+] (uNa^+:uCr), [uCl] (uCl:uCr), and other urinary electrolytes. Fractional excretion (FE) of sodium (FE Na^+), chloride (FE Cl), potassium (FE K^+), magnesium, calcium, and phosphate were also calculated to evaluate electrolytes excretion. Analogous to uNa^+:uK^+, the FE Na^+ to FE K^+ ratio (FE Na^+:FE K^+) was calculated.

Fractional excretion of solute X was calculated according to the equation reported previously [18], as follows:

$$FE\ X = uX\ sCr/uCr\ sX \text{ (based on spot urine sample)}$$

where uX and sX were the concentrations of a specific analyte in urine and serum, respectively, and sCr and [uCr] were serum and urine creatinine, respectively. FE was reported in percentages.

Statistical analysis

Data were expressed by standard descriptive statistics and presented as mean ± standard deviation or median and range (minimum-maximum value) based on their distribution. Normality was assessed graphically and by using the Shapiro-Wilk test. Differences between MMVD-MG and MMVD-EG dogs and among these groups and their healthy counterparts (H-MG and H-EG) were evaluated using an independent sample t-test or Mann-Whitney U test without multiple testing corrections for normally and non-normally distributed data, respectively. Categorical variables were compared between groups using the Fisher exact test. Correlation between variables was assessed using Pearson r or Spearman rank correlation based on data distribution. The results were considered significant when $p< 0.05$. Statistical analyses were performed using an online available statistical software package (MedCalc Statistical Software version 18.10.2; Ostend, Belgium).

Results

Baseline characteristics

During the study period, 73 dogs with MMVD met the inclusion criteria: 29/73 (40%) were females (three neutered) and 44/73 (60%) were males (15 castrated); 42/73 (58%) were mixed breed dogs, 31/73 (42%) were purebred dogs. The median body weight was 7.4 kg (range 2.6-19 kg); the median age was 12 years (range 6-16 years). Overall, 47/73 dogs belonged to MMVD-MG and 26/73 to MMVD-EG. All dogs were treated with furosemide and pimobendan at a median dosage of 4 mg/kg/day (range 1.1-8 mg/kg/day) and 0.5 mg/kg/day (range 0.3-1 mg/kg/day), respectively. Additionally, 63/73 cases received ACEI (enalapril or benazepril) at a median dosage of 0.8 mg/kg/day (range 0.7-0.8 mg/kg/day) and 0.6 mg/kg/day (range 0.20-1.4 mg/kg/day), respectively, and 20/73 received spironolactone at a median dose of 2.07 mg/kg/day (range 1.7-2.8 mg/kg/day). There was no difference between the MMVD-MG and MMVD-EG in terms of treatment dosages (furosemide P = 0.38, pimobendan P = 0.91, ACEI P = 0.29, spironolactone P = 0.84) and number of patients receiving ACEI or spironolactone (P = 0.08, and P = 0.41, respectively) (Table 10).

TABLE 10. Demographic data and descriptive statistics of the study population: dogs with myxomatous mitral valve disease (MMVD) sampled in the morning (MMVD-MG) and in the evening (MMVD-EG) and between healthy control dogs sampled in the morning (H-MG) and in the evening (H-EG). Data were reported as median and range (minimum–maximum value) or mean ± standard deviation (SD), based on their distribution.

Population	MMVD-MG (n =47)	MMVD-EG (n=26)	*P* value	H-MG (n = 56)	H-EG (n=50)	*P* value
Age (years)	12 (6-16)	12.5 (8-16)	0.88	3.9 (1-10)	2.5 (1-7.3)	0.005
Weight (kg)	7.4 (2.6-18.5)	7.5 (3.2-19)	0.6	19 (3-63)	26 (5-78)	0.005
Medications						
Furosemide (mg/kg/day)	4 (1.1-8)	4±1.41	0.38			
Time from morning furosemide administration (h)	4 (2-6)	9 (7-11)	<0.0001			
Pimobendan (mg/kg/day)	0.5 (0.3–0.9)	0.5 (0.3-1)	0.91			

N. of dogs receiving Spironolactone	11/47	9/26	0.41			
Spironolactone (mg/kg/day)	2.1±0.38	2.1 (1.8-2.8)	0.84			
N. of dogs receiving ACEI	38/47	25/26	0.08			
ACEI (mg/kg/day)	0.7 (0.2–1.4)	0.6 (0.2-1.6)	0.29			
N. of dogs receiving Benazepril	37/47	23/26				
Benazepril (mg/kg/day)	0.7 (0.2-1.4)	0.6 (0.2-1.1)	0.21			
N. of dogs receiving Enalapril	1/47	2/26				
Enalapril (mg/kg/day)	0.8	0.75 (0.7-0.8)	0.47			

Abbreviations: ACEI: angiotensin converting enzyme inhibitor; MMVD: myxomatous mitral valve disease; H-EG: healthy dogs evaluated in the evening (healthy evening group); H-MG: healthy dogs evaluated in the morning (healthy morning group); MMVD-MG: dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG: dogs with myxomatous mitral valve disease sampled in the evening.

A further comparison between MMVD-MG receiving ACEI and/or spironolactone vs. MMVD-MG not receiving ACEI and/or spironolactone was performed. No relevant differences were noticed (Table 11). This comparison was not possible for MMVD-EG because only one dog did not receive these treatments.

TABLE 11. Urinary electrolytes comparison between dogs with myxomatous mitral valve disease (MMVD) sampled in the morning (MMVD-MG) receiving RAAS inhibitors (ACEI and/or Spironolactone) and not receiving RAAS inhibitors (ACEI and/or Spironolactone). Data are reported as mean ± standard deviation (SD) or median and range (minimum–maximum value): based on their distribution.

Variable	RI	MMVD-MG Not receiving RAAS inhibitors (n=7)	MMVD-MG Receiving RAAS inhibitors (n=40)	*P* value
[uCr] (mg/dL)		41.8±23	48.8 (9.12-176)	0.5
[uNa] (mEq/L)		66.7±47.8	69.7±46.6	0.8
[uCl] (mEq/L)		66.7±51.5	62 (6-148)	0.9
[uK] (mEq/L)		38.5 ±19.59	38.35 (7.9-157.1)	0.64
[uCa] (mg/dL)		5.5±2.1	6.5(0.7-13.7)	0.6
[uMg] (mg/dL)		3.6±1.36	4.9 (0.85-13.3)	0.09
[uP] (mg/dL)		48.7±26.9	33 (0.2-222)	0.66
uNa:uK		1.08 (0.32-5.7)	1.69 (0.11-10.21)	0.9
uNa:uCr	0.00–1.00	1.68 (0.24-12)	1.47 (0.09-11.1)	0.7
uCl:uCr	0.00–1.25	1.7 (0.16-14.7)	1.43 (0.09-10.98)	0.74
uK:uCr	0.00–0.80	0.85 (0.38-5.1)	0.9 (0.25-3.1)	0.71
uMg:uCr	0.000–0.08	0.08 (0.02-0.54)	0.11 (0.019-0.31)	0.71
uCa:uCr	0.00–0.03	0.2±0.2	0.13 (0.01-0.66)	0.63
uP:uCr	0.00–0.97	1.26±0.36	0.98±0.69	0.3
FENa:FEK		0.03 (0.009-0.16)	0.04 (0.004-0.24)	0.9
FENa (%)	0.00–0.69	1.84 (0.25-10.7)	1.27(0.08-9.7)	0.45
FECl (%)	0.00–1.09	2.5 (0.3-14.87)	1.4(0.08-13.52)	0.65
FEK (%)	2.3–23.8	45.4±30.5	23.64 (8.11-65)	0.11
FEMg (%)	0–4	10.5±8.7	6.7 (1.05-20.3)	0.7
FECa (%)	0.00–0.33	2.8±2.1	1.49 (0.11-6.8)	0.26
FEP (%)	2.22–27.2	46.38±7.6	27.11 (1.19-83.9)	0.025

Abbreviations: ACEI, angiotensin converting enzyme inhibitor; FECa, fractional excretion of total calcium; FECl, fractional excretion of chloride; FEK, fractional excretion of potassium; FEMg, fractional excretion of magnesium; FENa, fractional excretion of sodium; FENa:FEK, fractional excretion of sodium to fractional excretion of potassium ratio; FEP, fractional excretion of phosphate; MMVD-MG, dogs with myxomatous mitral valve disease sampled in the morning; RI: Reference interval; [uCa], urine calcium; uCa:uCr, urine calcium to urine creatinine ratio; [uCl], urine chloride; uCl:uCr, urine chloride to urine creatinine ratio; [uCr], urine creatinine; [uK], urine potassium; uK:uCr, urine potassium to urine creatinine ratio; [uMg], urine magnesium; uMg:uCr, urine magnesium to urine creatinine ratio; [uNa], urine sodium; uNa:uCr, urine sodium to urine creatinine ratio; uNa:uK, urine sodium to urine potassium ratio; [uP], urine phosphate; uP:uCr, urine phosphate to urine creatinine ratio.

One-hundred and six healthy dogs were included as controls. The median age was 3.9 years (range 1-10 years), and the median body weight was 23.9 kg (range 3-78 years). Sex distribution was as follows: 54/106 (51%) were females (27 neutered), and 52/106 (49%) were males (36 castrated). Fifty-five out of 106 (52%) were mixed breed dogs, whereas 51/106 (48%) were purebred dogs. Overall, 56/106 dogs belonged to the H-MG, while 50/106 belonged to H-EG (Table 10). MMVD dogs had a greater median age and a lower median body weight than healthy dogs ($P< 0.0001$ and $P< 0.001$, respectively).

Clinicopathological data

No statistical difference was observed between MMVD-MG and MMVD-EG for sCr, urea, and serum electrolytes. Dogs included in MMVD-MG had significantly lower [uCr] and higher natriuresis, expressed as [uNa^+], uNa^+:uCr, and FENa than the MMVD-EG. Similarly, MMVD-MG dogs had higher chloruresis ([uCl], uCl:uCr, FECl) and calciuresis ([uCa^{++}], [uCa^{++}] to [uCr] ratio, FE Ca^{++}), as well as higher [uK^+] to [uCr] ratio, FEK, [uMg^{++}] to [uCr] ratio and FE of magnesium than MMVD-EG ones, while [uK^+] and [uMg^{++}] were not different in this comparison. Urinary sodium to potassium ratio and FE Na^+:FE K^+ were significantly higher in MMVD-MG than in MMVD-EG dogs. The majority of the urinary variables of interest (uNa^+:uK^+, uNa^+:uCr, uK^+:uCr, FE Na^+:FE K^+, FE Na^+, FE Cl, and FE K^+) resulted significantly negatively correlated with the time from morning furosemide administration expressed in hours. Conversely, no correlation was documented between urinary electrolytes and the duration of furosemide treatment (days) or with the dose of furosemide therapy (mg/kg/day) (Table 12).

TABLE 12. Correlation results between time from morning furosemide administration (hours), time from furosemide treatment date (days), dose of furosemide therapy (mg/kg/day) and urinary electrolytes in 73 dogs with myxomatous mitral valve disease (MMVD). Correlation was assessed using Pearson r or Spearman rank correlation, based on data distribution. Data were reported as correlation coefficient and statistical significance (*P*-value)

Variables	Time from morning furosemide administration (hours)	Time from treatment date (days)	Dose of furosemide therapy (mg/kg/day)
[uCr] (mg/dL)	0.29 (*P*=0.01)	0.110 (*P*=0.35)	-0.23 (*P*=0.049)
[uNa] (mEq/L)	-0.2 (*P*=0.08)	-0.02 (*P*=0.86	-0.199 (*P*=0.09)
[uCl] (mEq/L)	-0.14 (*P*=0.21)	0.02 (*P*=0.8)	-0.164 (*P*=0.16)
[uK] (mEq/L)	0.11 (*P*=0.32)	0.26 (*P*=0.02)	-0.299 (*P*=0.01)
[uCa] (mg/dL)	-0.08 (*P*=0.46)	-0.249 (*P*=0.03)	-0.212 (*P*=0.07)
[uMg] (mg/dL)	0.06 (*P*=0.68)	0.230 (*P*=0.05)	-0.214 (*P*=0.06)
[uP] (mg/dL)	0.17 (*P*=0.13)	-0.04 (*P*=0.7)	-0.16 (*P*=0.15)
uNa:uK	-0.234 (*P*=0.04)	-0.101 (*P*=0.39)	-0.04 (*P*=0.7)
uNa:uCr	-0.27 (*P*=0.01)	-0.023 (*P*=0.84)	-0.04 (*P*=0.72)
uCl:uCr	-0.314 (*P*=0.006)	-0.009 (*P*=0.9)	0.02 (*P*=0.86)
uK:uCr	-0.25 (*P*=0.02)	0.07 (*P*=0.5)	0.06 (*P*=0.6)
uMg:uCr	-0.25 (*P*=0.03)	0.07 (*P*=0.52)	0.03 (*P*=0.79)
uCa:uCr	-0.25 (*P*=0.02)	-0.21 (*P*=0.06)	0.005 (*P*=0.9)
uP:uCr	-0.14 (*P*=0.23)	-0.149 (*P*=0.2)	-0.07 (*P*=0.5)
FENa:FEK	-0.248 (*P*=0.03)	-0.07 (*P*=0.5)	-0.06 (*P*=0.6)
FENa (%)	-0.25 (*P*=0.03)	-0.029 (*P*=0.8)	-0.02 (*P*=0.8)
FECl (%)	-3.05 (*P*=0.008)	0.011 (*P*=0.9)	0.017 (*P*=0.88)
FEK (%)	-0.2 (*P*=0.01)	0.06 (*P*=0.56)	0.04 (*P*=0.7)
FEMg (%)	-0.17 (*P*=0.13)	0.08 (*P*=0.5)	0.07 (*P*=0.5)
FECa (%)	-0.2 (*P*=0.04)	-0.197 (*P*=0.09)	0.03 (*P*=0.8)
FEP (%)	-0.08 (*P*=0.46)	-0.109 (*P*=0.35)	-0.02 (*P*=0.86)

Abbreviations: FECa, fractional excretion of total calcium; FECl, fractional excretion of chloride; FEK: fractional excretion of potassium; FEMg, fractional excretion of magnesium; FENa, fractional excretion of sodium; FENa:FEK, fractional excretion of sodium to fractional excretion of potassium ratio; FEP: fractional excretion of phosphate; [uCa], urine calcium; uCa:uCr, urine calcium to urine creatinine ratio; [uCl], urine chloride; uCl:uCr, urine chloride to urine creatinine ratio; [uCr], urine creatinine; [uK], urine potassium; uK:uCr, urine potassium to urine creatinine ratio; [uMg], urine magnesium; uMg:uCr, urine magnesium to urine creatinine ratio; [uNa], urine sodium; uNa:uCr, urine sodium to urine creatinine ratio; uNa:uK, urine sodium to urine potassium ratio; [uP], urine phosphate; uP:uCr, urine phosphate to urine creatinine ratio.

No significant difference was noted for urinary electrolytes in healthy control dogs between H-MG and H-EG. Complete laboratory data are reported in (Table 13, Figure 9, Figure 10, Figure 11)

TABLE 13. Data comparison between dogs with myxomatous mitral valve disease (MMVD) sampled in the morning (MMVD-MG) and in the evening (MMVD-EG), and between healthy control dogs sampled in the morning (H-MG) and in the evening (H-EG). Data are reported as mean ± standard deviation (SD) or median and range (minimum–maximum value), based on their distribution.

Variable	RI	MMVD-MG (n=47)	MMVD-EG (n=26)	*P* value	H-MG (n=56)	H-EG (n=50)	*P* value
Creatinine (mg/dL)	0.75–1.4	1.2 (0.72–2.45)[aa]	1.3±0.41[bb]	0.330	1.09±0.20	1.12±0.17	0.336
Urea (mg/dL)	17–48	75.2 (33.1–221)[aa]	68.7 (34–189)[bb]	0.454	33.99±8.5	33.3±7.9	0.679
Phosphate (mg/dL)	2.65–5.40	3.7±1.1	3.8±0.87	0.657	4.08±0.72	4.05±0.65	0.773
Albumin (g/dL)	2.75–3.85	3.3±0.4	3.1±0.33	0.03	3.28±0.29	3.27±0.22	0.808
Total protein (g/dL)	5.6–7.30	6.7 (3.9–8)	6.6 (5.1–7.3)	0.254	6.45±0.47	6.41±0.40	0.845
Calcium (mg/dL)	9.3–11	10.56±0.69[aa]	10.35±0.63	0.224	10±0.37	10.1±0.45	0.056
Sodium (mEq/L)	143–151	148.29±3.71[a]	147 (112–153)	0.302	147 (144–152)	147 (139–150)	0.162
Potassium (mEq/L)	3.8–5.0	4.28±0.43[a]	4.31±0.39	0.302	4.4±0.27	4.38±0.29	0.290
Chloride (mEq/L)	108.0–118	117±3.65[aa]	107.2 (82.2–111)[bb]	0.708	113.3±1.9	112.9±2.6	0.418

Mg (mg/dL)	1.70–2.35	1.99±0.35	2.01±0.32	0.781	2.08±0.17	2.01±0.130	0.015
USG	>1030	1014 (1006–1036)[aa]	1018 (1006–1041)[bb]	0.162	1049±13.9	1047±13.9	0.581
UPC (mg/mg)	0–0.5	0.2 (0.1–1.3)[aa]	0.15 (0.08–0.61)[bb]	0.015	0.1 (0.05–0.3)	0.1 (0.049–0.3)	0.192
[uCr] (mg/dL)		48.6 (8.9–176.6)[aa]	78.2±44.76[bb]	0.01	293.5±88.4	327±102.6	0.073
[uNa] (mEq/L)		71.1 (5.9–192.9)[aa]	36.3 (4.6–103.9)[bb]	0.021	94.3 (11.7–342.4)	106 (5.8–344.8)	0.547
[uCl] (mEq/L)		68.2 (6–148.1)[aa]	32.5 (5–114.8)[bb]	0.038	184.26±84.95	168.16±85.16	0.332
[uK] (mEq/L)		38.9 (7.9–157.1)[aa]	41.5±17.71[bb]	0.596	133.9 (31.5–378.6)	124.4 (32.3–256.9)	0.484
[uCa] (mg/dL)		6.27±3.45[a]	4.59±2.61	0.048	3.9 (17–1.3)	3.55 (1–13.4)	0.387
[uMg] (mg/dL)		4.53 (0.85–13.3)[aa]	4.92 (0.87–11.2)[bb]	0.903	13.05±0.05	11.1±6.0	0.148
[uP] (mg/dL)		34 (0.2–222)[aa]	53.35 (18.4–197.2)[bb]	0.070	181.26±70.05	159±81.78	0.135
uNa:uK		1.67 (0.11–10.21)[aa]	0.93 (0.14–3.77)	0.016	0.75 (0.13–3.4)	0.75 (0.07–5.19)	0.299
uNa:uCr	0.00–1.00	1.63 (0.09–12)[aa]	0.50 (0.06–2.82)	0.003	0.35 (0.03–1.78)	0.31 (0.01–2.3)	0.859
uCl:uCr	0.00–1.25	1.51 (0.09–14.7)[aa]	0.44 (0.06–2.99)	0.008	0.66 (0.17–2.14)	0.49 (0.057–2.57)	0.132
uK:uCr	0.00–0.80	0.86 (0.25–5.1)[aa]	0.59 (0.18–1.37)[bb]	0.016	0.48 (0.13–1.5)	0.36 (0.07–1.1)	0.089
uMg:uCr	0.000–0.08	0.11 (0.019–0.54)[aa]	0.056 (0.01–0.34)[bb]	0.006	0.04 (0.006–0.14)	0.035±0.021	0.025
uCa:uCr	0.00–0.03	0.13 (0.01–0.69)[aa]	0.06 (0.007–0.36)[bb]	0.001	0.014 (0.005–0.07)	0.01 (0.003–0.07)	0.119

uP:uCr	0.00–0.97	1.02±0.66[aa]	0.89 (0.37–2.3)[bb]	0.070	0.59 (0.17–1.36)	0.47 (0.006–1.43)	0.001
FENa:FEK		0.05 (0.004–0.24)[aa]	0.03 (0.004–0.1)	0.017	0.02 (0.00–0.09)	0.02 (0.00–0.15)	0.260
FENa (%)	0.00–0.69	1.3 (0.08–10.7)[aa]	0.45 (0.06–2.5)	0.008	0.25 (0.03–0.94)	0.25 (0.01–1.55)	0.730
FECl (%)	0.00–1.09	1.54 (0.08–14.8)[aa]	0.53 (0.07–4)	0.015	0.56 (0.15–1.5)	0.48 (0.05–2.28)	0.214
uK:uCr	0.00–0.80	0.86 (0.25–5.1)[aa]	0.59 (0.18–1.37)[bb]	0.016	0.48 (0.13–1.5)	0.36 (0.07–1.1)	0.089
uMg:uCr	0.000–0.08	0.11 (0.019–0.54)[aa]	0.056 (0.01–0.34)[bb]	0.006	0.04 (0.006–0.14)	0.035±0.021	0.025
FEK (%)	2.3–23.8	26.4 (8.1–93.8)[aa]	17.38 (6.9–49.4)[bb]	0.035	11.26 (4.27–45.2)	10.52±4.04	0.240
FEMg (%)	0–4	7.1 (1.05–22.5)[aa]	4.4 (0.4–20.9)[bb]	0.036	2.43±1.1	2.02±1.2	0.105
FECa (%)	0.00–0.33	1.6 (0.1–6.8)[aa]	0.7 (0.07–5.2)[bb]	0.005	0.17 (0.05–0.64)	0.12 (0.03–0.66)	0.081
FEP (%)	2.22–27.2	35.5±21.1[aa]	34.04±18.6[bb]	0.835	16.2 (6.07–46.5)	12.6 ±6.84	0.035

Abbreviations: FECa, fractional excretion of total calcium; FECl: fractional excretion of chloride; FEK, fractional excretion of potassium; FEMg, fractional excretion of magnesium; FENa, fractional excretion of sodium; FENa:FEK, fractional excretion of sodium to fractional excretion of potassium ratio; FEP, fractional excretion of phosphate; H-EG, healthy dogs evaluated in the evening (healthy evening group); H-MG, healthy dogs evaluated in the morning (healthy morning group); Mg, Magnesium; MMVD-MG, dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG, dogs with myxomatous mitral valve disease sampled in the evening; RI, Reference interval; [uCa], urine calcium; uCa:uCr, urine calcium to urine creatinine ratio; [uCl], urine chloride; uCl:uCr, urine chloride to urine creatinine ratio; [uCr], urine creatinine; [uK], urine potassium; uK:uCr, urine potassium to urine creatinine ratio; uMg, urine magnesium; uMg:uCr, urine magnesium to urine creatinine ratio; [uNa], urine sodium; uNa:uCr, urine sodium to urine creatinine ratio; uNa:uK, urine sodium to urine potassium ratio; [uP], urine phosphate; uP:uCr, urine phosphate to urine creatinine ratio; UPC, urine protein to urine creatinine ratio; USG, urine specific gravity.

[a] Significantly different with $P<0.05$ between MMVD-MG vs. H-MG

[aa] Significantly different with $P<0.01$ between MMVD-MG vs. H-MG

[bb] Significantly different with $P<0.01$ between MMVG-EG vs. H-EG

A further comparison between MMVD-MG and HMG, and between MMVD-EG and H-EG, respectively, was performed. Urine creatinine and urine specific gravity were significantly lower in MMVDMG and MMVD-EG than H-MG and H-EG, respectively (P< 0.0001 for both evaluations). If compared with their healthy counterparts (H-MG and H-EG, respectively), MMVD-MG and MMVD-EG had significantly lower [uNa^+] (P=0.0016 and P< 0.0001), [uCl^+] (P< 0.0001 and P< 0.0001), [uK^+] (P< 0.0001 and P< 0.0001), [uMg^{++}] (P< 0.0001 and P< 0.0001), and urine phosphate (P< 0.0001 and P< 0.0001). Moreover, [uCa^{++}] was higher only in MMVD-MG than H-MG (P = 0.037). Dogs included in MMVD-MG and MMVD-EG, when compared with their healthy counterparts, had a significantly increased [uK^+] to [uCr] (P< 0.0001 and P=0.0007), [uMg^{++}] to [uCr] (P< 0.0001 and P¼0.0007)2, [uCa^{++}] to [uCr] (P< 0.0001 and P< 0.0001), and urine phosphate to [uCr] (P=0.0013 and P< 0.0001) ratio.

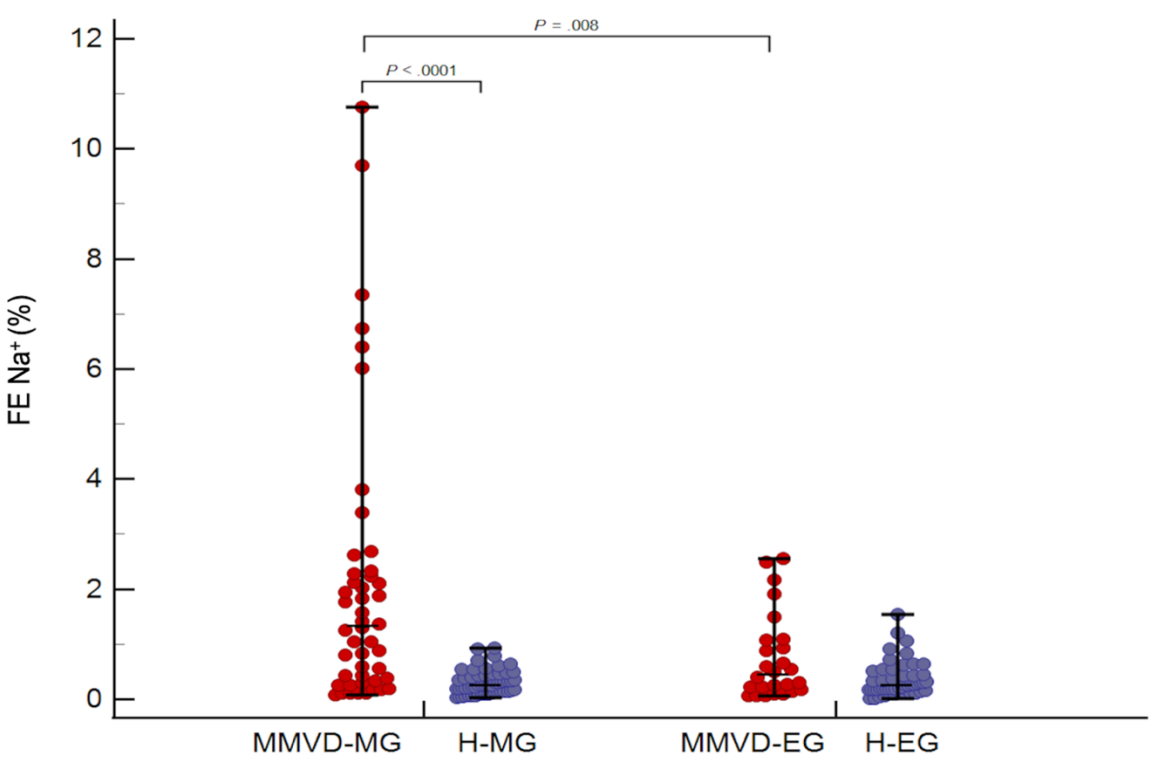

FIGURE 9. Dot plot showing results of fractional excretion of sodium in dogs with myxomatous mitral valve disease sampled in the morning (n ¼ 47) and in the evening (n ¼ 26) (red dots) vs. healthy dogs sampled in the morning (n ¼ 56) and in the evening (n ¼ 50) (blue dots). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent the median value. Each comparison was made using a Mann-Whitney test without correction for multiple testing. p-values are reported for significantly different results (p<0.05). FENa: fractional excretion of sodium; H-EG: healthy dogs evaluated in the evening (healthy evening group); H-MG: healthy dogs evaluated in the morning (healthy morning group); MMVD-MG, dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG, dogs with myxomatous mitral valve disease sampled in the evening.

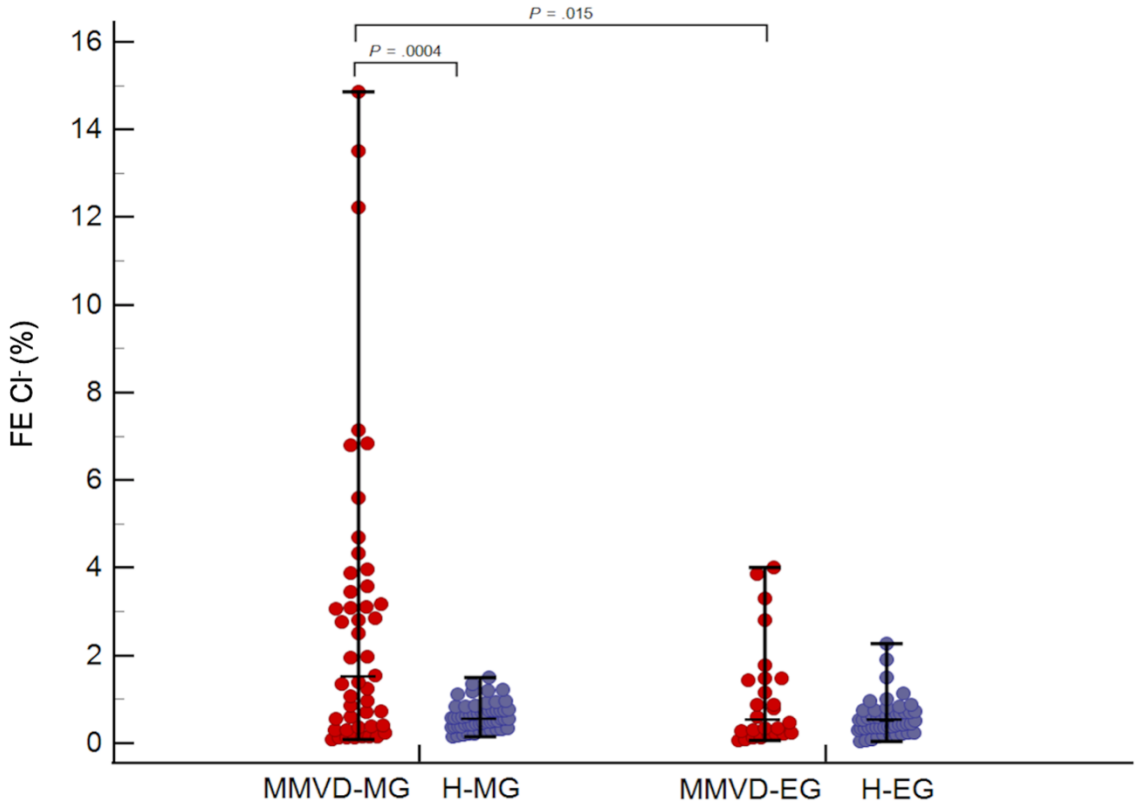

FIGURE 10. Dot plot showing results of fractional excretion of chloride in dogs with myxomatous mitral valve disease sampled in the morning (n = 47) and in the evening (n = 26) (red dots) vs. healthy dogs sampled in the morning (n = 56) and in the evening (n = 50) (blue dots). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent the median value. Each comparison was made using a Mann-Whitney test without correction for multiple testing. P-values are reported for significantly different results (P<0.05). FECl: fractional excretion of chloride; H-EG: healthy dogs evaluated in the evening (healthy evening group); H-MG: healthy dogs evaluated in the morning (healthy morning group); MMVD-MG, dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG, dogs with myxomatous mitral valve disease sampled in the evening.

Similar results were reported for FE K (P< 0.0001 and P< 0.0001), FE of magnesium (P< 0.0001 and P=0.0001), FE of calcium (P< 0.0001 and P< 0.0001), and FE of phosphate (P¼0.0001 and P< 0.0001). In addition, uNa^+:uCr, uCl:uCr, uNa^+:uKþ, FE Na^+, FE Cl, and FE Na^+:FE K^+ were significantly increased only in MMVD-MG if compared with H-MG (P< 0.0001, P=0.0017, P=0.0001, P< 0.0001, P=0.0004, and P=0.0001, respectively) (Table 13, Figure 9, Figure 10, Figure 11).

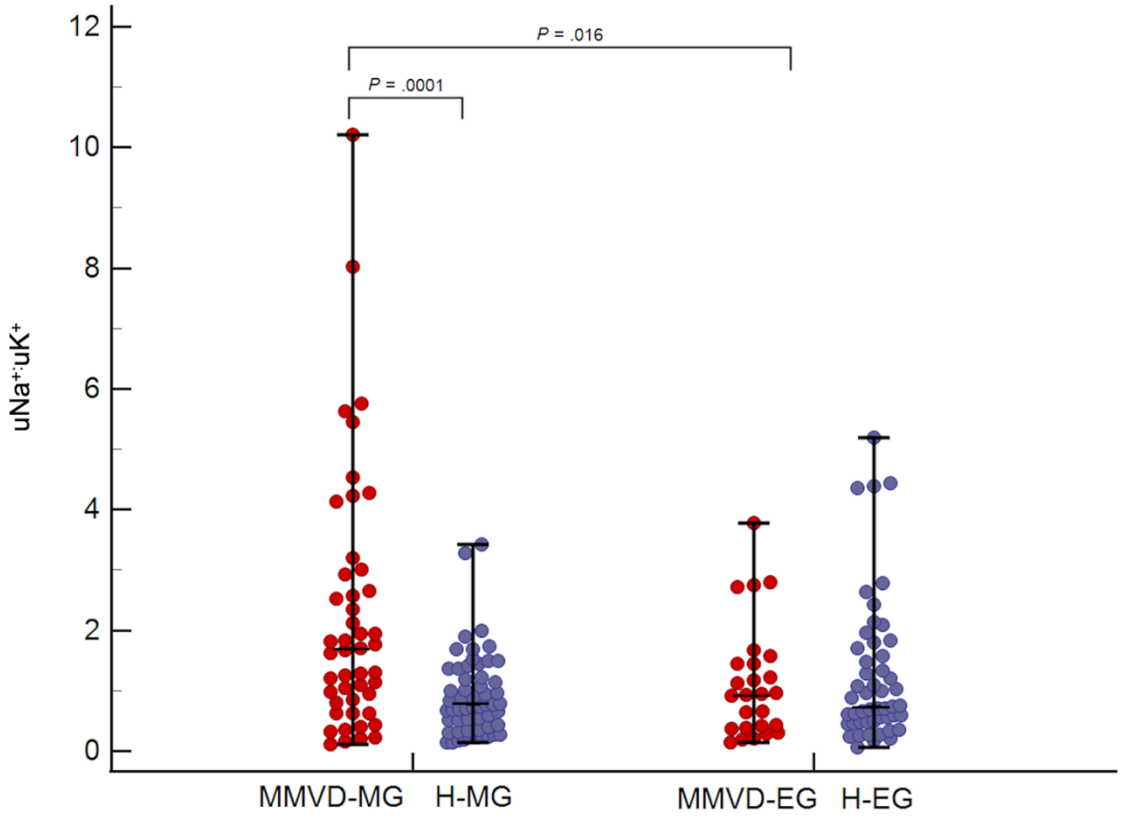

FIGURE 11. Dot plot showing results of urine sodium to urine potassium ratio in dogs with myxomatous mitral valve disease sampled in the morning (n = 47) and in the evening n = 26) (red dots) vs. healthy dogs sampled in the morning (n = 56) and in the evening (n = 50) (blue dots). Upright bars represent minimum and maximum values, while horizontal lines (central bars) represent the median value. Each comparison was made using a Mann–Whitney test without correction for multiple testing. P-values are reported for significantly different results (P<0.05). uNa^+:uK^+: urine sodium to potassium ratio; H-EG: healthy dogs evaluated in the evening (healthy evening group); H-MG: healthy dogs evaluated in the morning (healthy morning group); MMVD-MG: dogs with myxomatous mitral valve disease sampled in the morning; MMVD-EG: dogs with myxomatous mitral valve disease sampled in the evening.

Urinary electrolytes were further compared within the group of healthy dogs grouped by categories based on age and body weight (dogs > 6 years vs. dogs < 6 years; dogs > 20 kg vs. dogs <20 kg). No clinically significant difference was observed (Table 14).

TABLE 14 Urinary electrolytes comparison between Healthy dogs with age < 6 years and > 6 years, and between Healthy dogs with body weight < 20 kg and > 20 kg. Data are reported as mean ± standard deviation (SD) or median range (minimum-maximum value), based on their distribution.

Variable	RI	Healthy dogs <6 years n=85	Healthy dogs >6 years n=21	*P* value	Healthy dogs <20 kg n=44	Healthy dogs >20 kg n=62	*P* value
[uCr] (mg/dL)		312.7±96.9	295.5±95.41	0.46	297.9±79.42	317±106	0.3
[uNa] (mEq/L)		117 (5.8-342)	80 (18-344)	0.18	139.9 (5.8-342.4)	88.5 (9.2-344.8)	0.11
[uCl] (mEq/L)		180±84.81	163±86.64	0.41	196.74±87.58	162.4±80.88	0.04
[uK] (mEq/L)		142.38±62.26	108 (31-378.6)	0.05	135.3 (32-378.6)	127.4±56.14	0.12
[uCa] (mg/dL)		3.4 (1-12)	4.5 (1.4-17)	0.11	5.45 (1.1-17)	3.1 (1-12)	0.01
[uMg] (mg/dL)		12.59 (0.88-30.3)	12.69±5.8	0.4	12.6 ±7.67	11.8±5.8	0.56
[uP] (mg/dL)		173 (2.1-415)	143.9 (49.7-236.7)	0.17	190.9±66.19	156.4±80	0.02
uNa:uK		0.75 (0.07-5.19)	0.74 (0.13-3.43)	0.9	0.816 (0.139-4.36)	0.69 (0.07-5.19)	0.54
uNa:uCr	0.00–1.00	0.35 (0.01-2.3)	0.33 (0.05-0.82)	0.34	0.44 (0.013-2.30)	0.27 (0.02-1.48)	0.052
uCl:uCr	0.00–1.25	0.58 (0.05-2.57)	0.55±0.26	0.48	0.7 (0.05-2.57)	0.48 (0.06-1.79)	0.03
uK:uCr	0.00–0.80	0.46 (0.07-1.5)	0.4±0.18	0.15	0.48 (0.07-1.5)	0.42 (0.08-1.11)	0.02
uMg:uCr	0.000–0.08	0.03 (0.002-014)	0.044±0.01	0.25	0.04 (0.002-0.14)	0.03±0.02	0.64

uCa:uCr	0.00–0.03	0.01 (0.003-0.07)	0.014 (0.005-0.07)	0.04	0.015 (0.003-0.07)	0.011 (0.003-0.04)	0.008
uP:uCr	0.00–0.97	0.54 (0.006-1.43)	0.53±0.23	0.4	0.59 (0.28-1.431)	0.48 (0.006-1.19)	0.0009
FENa:FEK		0.02 (0.002-0.15)	0.02 (0.004-0.09)	0.9	0.02 (0.004-0.132)	0.02 (0.002-0.154)	0.62
FENa (%)	0.00–0.69	0.25 (0.01-1.55)	0.22 (0.05-0.56)	0.30	0.32 (0.01-1.55)	0.210 (0.002-0.15)	0.15
FECl (%)	0.00–1.09	0.55 (0.05-2.28)	0.54 (0.17-1.35)	0.42	0.59 (0.05-2.28)	0.47 (0.06-1.9)	0.18
FEK (%)	2.3–23.8	10.8 (2.23-45.22)	9.95±4.5	0.13	10.7 (2.2-45)	10.82±4.37	0.38
FEMg (%)	0–4	2.15±1.2	2.37±1.06	0.44	2.13±1.2	2.24±1.15	0.57
FECa (%)	0.00–0.33	0.13 (0.03-0.66)	0.2 (0.07-0.64)	0.01	0.17 (0.03-0.66)	0.125 (0.05-0.46)	0.04
FEP (%)	2.22–27.2	15.3 (5.3-25.2)	15.45±6.22	0.9	16.6 (6.8-46.5)	14.2±6.5	0.02

Abbreviations: FECa, fractional excretion of total calcium; FECl, fractional excretion of chloride; FEK, fractional excretion of potassium; FEMg, fractional excretion of magnesium; FENa, fractional excretion of sodium; FENa:FEK, fractional excretion of sodium to fractional excretion of potassium ratio; FEP, fractional excretion of phosphate; RI: reference interval; [uCa], urine calcium; uCa:uCr, urine calcium to urine creatinine ratio; [uCl], urine chloride; uCl:uCr, urine chloride to urine creatinine ratio; [uCr], urine creatinine, [uK], urine potassium; uK:uCr, urine potassium to urine creatinine ratio; [uMg], urine magnesium; uMg:uCr urine magnesium to urine creatinine ratio; [uNa], urine sodium; uNa:uCr, urine sodium to urine creatinine ratio; uNa:uK, urine sodium to urine potassium ratio; [uP], urine phosphate; uP:uCr, urine phosphate to urine creatinine ratio.

Discussion

The Project 1 of this thesis work aimed to assess the presence of renal tubular damage in dogs with stable MMVD, to evaluate changes according to the severity of heart disease and the association between uNGAL and uNGALC and several echocardiographic indexes. The deterioration of renal function linked to chronic cardiac disease by both chronological and causal relationship (so-called CRS type II or $CVRD_H$ stable) has been a matter of growing debate in human medicine, but only scarcely characterized in veterinary medicine (Cruz et al., 2013; Jung et al., 2018; Nicolle et al., 2007; Orvalho et al., 2017; Ronco et al., 2010). In our opinion, such a gap of knowledge can have relevant clinical implications, because renal damage and tubular injury could affect prognosis, influence therapeutic decision-making and impair response to treatment (Martinelli et al., 2016; Nicolle et al., 2007). For the purpose of this thesis work, we enrolled dogs with MMVD, a disease with a high prevalence and a chronic and progressive course, sometimes requiring life-long diuretic treatment (Borgarelli et al. 2012; Keene et al., 2019), representing an excellent research model to fill the knowledge gap. In accordance with our initial hypothesis, we found that uNGAL is increased in dogs with MMVD compared to healthy controls and increases with worsening of the cardiac disease. This finding suggests the presence of subclinical tubular damage in all MMVD stages, occurring in the absence of clinical signs of renal involvement and severe azotemia in the majority of the cases. Indeed, the values of uNGAL documented in enrolled MMVD dogs are far lower compared to those reported in dogs with AKI (Monari et al., 2020), but still abnormal with respect to the healthy state.

In humans, serum and urinary NGAL are among the most studied biomarkers of tubular damage in AKI and their elevation have been documented extensively in patients with CRS caused by different cardiovascular diseases, including those leading to CHF (Cruz et al., 2012). For example, a previous study showed that renal impairment in patients with chronic heart failure is not only characterized by decreased glomerular filtration rate, but also by higher uNGAL results compared to control subjects, indicating the presence of renal tubular damage (Damman et al., 2008). Interestingly, another study documented that serum NGAL represents a marker of renal injury in people with CHF, even when sCr is within the RI (Poniatowski et al., 2009). In dogs, recent studies determined that serum and uNGAL act as a sensitive and specific biomarker of AKI and tubular injury, despite being subjected to potential influence during systemic inflammation (Cortellini et al., 2015; Monari et al., 2020; Zamagni et al., 2020). However, as previously mentioned, the

literature concerning NGAL as a biomarker of CRS is currently scarce in dogs. A recent study identified higher serum NGAL in dogs with acute CHF caused by MMVD compared to healthy dogs. Moreover, higher serum NGAL concentrations were noticed upon admission in dogs with CHF that developed worsening of renal function within 7days of hospitalization compared to those with stable sCr, thus highlighting the potential role of NGAL as an early biomarker of AKI during acute CHF (Jung et al., 2018). To our knowledge, tubular damage in dogs with stable MMVD has never been extensively evaluated. A preliminary evaluation of some biomarkers of kidney damage (clusterin, cystatin B, inosine and NGAL) has been previously reported (Orvalho et al., 2017). In that study, the hypothesized mechanisms contributing to increases in those biomarkers were episodes of intermittent AKI or sustained kidney injury occurring simultaneously with a progressive reduction of functional kidney mass (Orvalho et al., 2017). In that same study, despite being cited, uNGAL results were not reported. Based on our findings, progressive and sustained kidney damage could represent the main mechanism behind CRS type II in MMVD dogs with stable disease, even if the role of episodic AKI cannot be completely ruled out.

The normal function of the cardiorenal axis contributes to normal cardiovascular homeostasis (Cruz et al., 2013). Several mechanisms have been proposed as a cause of renal damage in course of $CvRD_H$ stable or type 2 CRS.

In cardiac patients, a chronic activation of neurohormonal compensatory mechanisms as sympathetic nervous system, RAAS, atrial natriuretic peptide, brain natriuretic peptide, arginine vasopressin and endothelin-1 is reported. These systems provide only temporary hemodynamic support but, in a chronic setting, they are associated with vasoconstriction, abnormal myocardial energetics, myocardiocytes death and myocardial remodeling, which further injure the heart (Oyama, 2009). Renal hypoperfusion, as a cause of neurohormonal activation, is itself a consequence of the latter. These mechanisms negatively affect the normal function of the cardiorenal axis and cardiovascular homeostasis and have been associated with fluid overload, venous hypertension, and renal interstitial edema in humans and in canine experimental models of disease. Moreover, they have been implicated in CKD progression because of ongoing renal interstitial fibrosis and glomerulosclerosis in humans and in animal models of CRS type 2 (Cruz et al., 2013; Deferrari et al., 2021; Kishimoto et al., 1973; Rangaswami et al., 2019).

The renal perfusion is intuitively influenced by the cardiac output, however, in humans, venous congestion was associated with a major effect on renal damage compared to hypoperfusion, a condition well-known as “congestive nephropathy” (Damman et al., 2009; Husain-Syed et al.,

2021; Mullens et al., 2009). The elevated venous pressure could increase renal interstitial pressure and the hydrostatics pressure in the Bowman's capsule, with consequent impairment of both tubular and glomerular function. Moreover, elevated intra-abdominal pressure in course of ascites also plays a role in renal hypoperfusion due to the compression of the renal parenchyma (Orvalho et al., 2017; Pouchelon et al., 2015). Finally, episodic cardiac events and repeated occurrence of AKI induce progressive hypoxic and ischemic insults to the kidneys (Cowgill et al., 2016; Cruz et al., 2013). These events, overall, can be expressed at any stage of cardiac disease, and become progressively more evident with worsening heart failure (Orvalho et al., 2017). All these mechanisms can justify the progressive increase in uNGAL in our study population with increasing ACVIM CHF stages as well as the higher prevalence of abnormal uNGAL in the more advanced stages of MMVD.

In this regard, diuretic treatment might play a role. Indeed, diuretics induce subclinical changes in volume status, which might affect renal and tubular function and integrity. Moreover, because furosemide acts at the tubular level, it theoretically might affect renal excretion of NGAL. However, this effect has not been identified in recent studies of humans (Hamishehkar et al., 2017; Mose et al., 2019;). For example, in one study, although diuretic withdrawal was associated with an increase in some biomarkers of tubular dysfunction, which returned to within normal ranges after diuretic reinstitution, uNGAL concentrations were unaffected by changes in diuretic treatment (Damman et al., 2011). Similar data are lacking in the veterinary literature. In our study, no significant difference in uNGAL results was found between ACVIM stage C and D dogs when considering these stages as separate entities (data not shown), and no correlation was detected between uNGAL and furosemide dosage. In our opinion, a relevant role of diuretic treatment in the excretion of NGAL is unlikely but this hypothesis should be confirmed in additional studies designed with this aim.

To evaluate the influence of cardiac function on renal damage, we analyzed various echocardiographic indexes that are mainly directly or indirectly related to cardiac output (SF, LVIDSn, VTI_{Ao}, VTI_{Mit} /VTI_{Ao}) or venous congestion (LVIDDn, LAV_{Max}, LAV_{Min}, LA/Ao, LASV, VTI_{Mit} /$VTI_{Ao,}$ E_{Vmax}, TR_{Vmax}, VTI_{Mit}, E/E'). These echocardiographic indexes are markers of cardiac output, regurgitation severity, left-side volume, and left- and right-side heart pressure (Borgarelli et al., 2008; Franchini et al., 2021; Höllmer et al., 2017; Shober et al., 2011; Tribouilloy et al., 1994), but an overlap of these effects should be considered.

At the univariate analysis several echocardiographic indexes (LASV, TR_{Vmax}, LAV_{Max}, LVIDDn and VTI_{Mit}/VTI_{Ao}) were associated with increased uNGAL and uNGALC, however, at the multivariable analysis, only TR_{Vmax} and LASV remained significant. It must be noted that LASV, LAV_{Max}, LVIDDn and VTI_{Mit}/VTI_{Ao} are all markers of left-side volume overload and are mutually connected; LASV was the only one that resulted significant at the multivariable analysis likely due to the multicollinearity detected between these variables.

These findings suggest a major effect of venous congestion on the renal damage, while apparently the cardiac output seems not to be relevant. This might be related to the nature of MMVD, which is a condition associated with pure volume overload that normally evolves into systolic dysfunction only at the advanced stages of the disease (Bonagura et al., 2009). Moreover, neurohormonal activation causes an increase in preload, which ensures adequate forward cardiac output, although volume regurgitation, unlike what happens during a sudden onset of mitral regurgitation, in which acute mitral regurgitation affects forward cardiac output and consequently organ perfusion. The presence of arrhythmias could affect the cardiac output; atrial fibrillation is a common arrhythmia in dogs with MMVD and it is hemodynamically relevant (Franchini et al., 2021), however we had only 3 dogs with atrial fibrillation in our population and its effect on renal damage could not be analyzed. The main pathophysiologic mechanism in course of MMVD is left-side CHF that could evolve to type 2 pulmonary hypertension, tricuspid regurgitation, and right-side CHF (Reinero et al., 2020). In our population we had 15 dogs with pulmonary hypertension and 6 dogs with ascites. The small sample size might be the reason why we couldn't try to analyze the association between the presence of clinical signs of right-side CHF (eg. ascites) and the renal damage. However, the left atrial volume and function are markers of MMVD severity and patients with higher values of LASV had a higher risk of renal damage (Baron Toaldo et al., 2018). The left-side volume overload, associated with increased atrial and ventricular diastolic pressures, reduces the right-side compliance due to the ventricular interdependence and could result in systemic congestion (Naeije et al., 2017). The extent of the right-side impairment, as well as the quantification of the tricuspid regurgitation, were not possible due to the lack of the right-side cardiac dimensions' measurements, which is one of the limitations of this study. In the study population there were fifty dogs with tricuspid regurgitation and 15 of them had pulmonary hypertension, with a tricuspid regurgitant velocity higher than 3 m/sec (Reinero et al., 2020). The association between tricuspid regurgitation and renal damage was statistically significant and the relative risk to have abnormal uNGAL and uNGALC for patients with MMVD and tricuspid

regurgitation was significant. Conversely, based on our results and the published literature (Andrade, 2015), we cannot assume that the presence of pulmonary hypertension could be clinically relevant in the development of renal damage. The relative risk to have renal damage for pulmonary hypertension wasn't clinically relevant for uNGAL and uNGALC. However, these results could be partially reconducted to the small number of dogs with pulmonary hypertension, classified according to current guidelines.

In veterinary medicine, the NGAL is a novel biomarker of tubular damage in dogs (Monari et al., 2020). We analyzed both uNGAL and uNGALC because we had an inhomogeneous population in which some patients were treated with diuretics and had diluted urine. This assumes that urine creatinine excretion rate is constant and normalizes the uNGAL value for the urine concentration so the uNGALC should be more accurate than uNGAL in this population (Chen et al., 2023).

We did not find any association between furosemide daily dosage and uNGAL and uNGALC, this could be reconducted to the double effect of loop diuretic on the kidneys: even if on one hand it has a negative effect on the renal perfusion and the glomerular filtration rate, on the other side it reduces the congestion with a positive effect on the kidney parenchyma (Cruz et al., 2012). The effect of other cardiac treatments on the uNGAL and uNGALC was not evaluated because of the small number of patients. However, the use of ACE inhibitors reduces the glomerular filtration rate, but it has no direct effect on renal damage, likely the spironolactone has a positive effect on the cardiorenal axis reducing myocardial fibrosis and volemia. Moreover, a recent study demonstrated that there is no effect of pimobendan on renal clearance in MMVD ACVIM B2 dogs, and we might assume that this drug has no effect on renal damage (Kaplan et al., 2022).

Another possibility for increases in uNGAL in enrolled MMVD dogs could be the presence of preexisting CKD. Previous studies documented higher uNGAL in dogs with CKD compared to healthy dogs (Steinbach et al., 2022), and uNGAL seems to predict the risk of progressive vs stable CKD in dogs (Franchini et al., 2021). Both CKD and chronic valvular disease usually occur in elderly patients, and the estimated prevalence of CKD seems higher in dogs with MMVD than in the general canine population (Martinelli et al., 2016). In a large population of dogs with MMVD included in the LOOK-Mitral registry, the presence of azotemia had a prevalence of 6% (Franchini et al., 2021). In our opinion, the possibility of preexisting CKD influencing uNGAL in the dogs enrolled in our study seems unlikely. Indeed, uNGAL was above the RI in approximately half of the study population of MMVD dogs (uNGAL, 38/98, 39%; uNGALC, 52/98, 53%) and, although not directly comparable, the uNGAL results that we documented seem overall lower compared those

reported in dogs with CKD (Steinbach et al., 2014; Kim et al., 2019). In any case, preexisting renal disease causing the increase in uNGAL would be active progressive renal damage that worsens with increasing ACVIM stage and hence still would fall into type 2 CRS.

Among the potential nonrenal causes of increased NGAL in cardiac disease, chronic inflammation and cardiac remodeling should be mentioned. In humans, chronic inflammation is a hallmark of CHF, and inflammatory mediators have been implicated in cardiovascular disease progression (Cruz et al., 2013; Shirazi et al., 2017). Systemic inflammation could cause an increase in circulating NGAL concentration because NGAL is produced by leukocytes. The role of inflammation in uNGAL excretion, however, is not well defined in humans with heart disease (Cruz et al., 2012). According to a previous study, higher uNGAL was reported in dogs with inflammatory AKI compared to dogs with noninflammatory AKI (Monari et al., 2020). Similar to what has been reported in human medicine, mild systemic inflammation has been reported in cardiac diseases of dogs (Ljungvall et al., 2010; Reimann et al., 2016; Rush et al., 2006), and may contribute, at least in part, to increased NGAL. In our study population, however, the leukocyte count was normal, serum CRP concentrations were only mildly increased, and these results were similar in MMVD dogs of different ACVIM stages. In addition, no correlation was detected between both leukocyte count and serum CRP with uNGAL, making an effect of systemic inflammation on our results unlikely.

In human patients with cardiac diseases, NGAL also plays a direct role in the pathogenesis of cardiovascular remodeling, as well as in atherosclerotic plaque instability (Cruz et al., 2013; Helanova et al., 2014). Moreover, NGAL upregulation has been demonstrated in the myocardial tissue of human patients who died of CHF (Cruz et al., 2013; Helanova et al., 2014). Similarly, in a rat model of post myocardial infarction heart failure, NGAL/lipocalin-2 gene expression was found to be increased in cardiomyocytes in both normal and failing myocardium (Yndestad et al., 2009). Because cardiac remodeling occurs in dogs along with progression of MMVD (Keene et al., 2019), cardiac expression of NGAL also may be hypothesized in this species but has not been documented in dogs so far. Based on our study design, the contribution of cardiac remodeling to NGAL results in our population is hard to evaluate but remains a possibility that should be clarified in future studies.

Besides higher uNGAL, dogs with ACVIM stage C+D MMVD also had higher median concentrations of urea, sCr and higher UPC compared to MMVD dogs with less severe ACVIM stage. A grater impairment of both glomerular and tubular function seems to occur in the more advanced stages of MMVD in dogs, likely because of more severe renal hypoperfusion associated with vigorous

diuretic treatment (Martinelli et al., 2016), or because of frequent renal hypoxic insults associated with worsening heart disease and decompensation (Orvalho et al., 2017). Moreover, these dogs could experience progressive kidney damage leading to renal fibrosis and CKD, as previously reported and already discussed (Martinelli et al., 2016; Orvalho et al., 2017; Szczepankiewicz et al., 2019). Interestingly, no correlation was found between uNGAL and sCr in our study population. This result, however, is expected, because NGAL indicates renal tubular damage whereas sCr is a surrogate of renal function impairment. Specifically, in the context of heart failure, a combined approach measuring both sCr and uNGAL might be preferred to correctly assess renal damage and dysfunction. As an example, iatrogenic prerenal azotemia caused by overzealous diuretic treatment might not necessarily cause parenchymal damage, whereas small decreases in renal function might result in tubular hypoxia and subsequent tubular damage despite relatively normal glomerular filtration rate and sCr (Jung et al., 2018). Finally, uNGAL can reflect subclinical injury and could anticipate an increase in sCr. Regarding UPC, most of the MMVD dogs in our study were nonproteinuric or borderline proteinuric according to the IRIS guidelines for CKD (IRIS Staging of CKD, 2019). Interestingly, such a finding is consistent with previous studies evaluating renal impairment in dogs with MMVD (Szczepankiewicz et al., 2019; Valente et al., 2021). Low-grade proteinuria can be associated with tubular damage and it is consistent with the mild positive correlation observed in our study between uNGAL and UPC. Nonetheless, better characterization of proteinuria in dogs with MMVD using qualitative methods would be needed to confirm its tubular origin.

In the Project 2 of this thesis work we demonstrate a strong influence of the time frame occurring from oral furosemide administration to sample collection on the concentration of urinary electrolytes in dogs with stable MMVD ACVIM stage C. Previous studies in healthy dogs and dogs with cardiac disease observed a peak diuretic effect after 2 h and a return to baseline after 6 h from oral administration of furosemide (Harada et al., 2015; Hori et al., 2007; Loughran et al., 2020; Uechi et al., 2003). Thus, we hypothesized that natriuresis would be higher within the first 6 h after oral furosemide administration, and we divided MMVD dogs into two groups according to the time elapsed from diuretic therapy to sample collection. As expected, we found that cardiopathic dogs sampled in the morning (MMVD-MG) had significantly higher sodium and chloride urinary excretion (when evaluated as concentrations, normalized with [uCr] or FE) than those evaluated in the evening (MMVD-EG). Hence, the time elapsed from furosemide

administration to sample collection should be viewed as a prerequisite to allow proper urine chemistry interpretation in this setting.

The uNa^+:uK^+ is a short-term biomarker of renin–angiotensin–aldosterone system activation in many species (Brandish et al., 2008; Eudy et al., 2021; Doering et al., 2017). In humans, a uNa^+:uK^+ < 1 is representative of the inadequate urine production and diuretic resistance (Doering et al., 2017). In patients who develop resistance, more sodium is reabsorbed at the distal tubular level; decreased sodium excretion and stable potassium excretion result in a decreased ratio than non-resistant patients (Collins et al., 2019). A cut-off for uNa^+:uK^+ to identify an inadequate diuretic response has not been established in dogs. A study in healthy dogs showed that urinary volume is closely related to the uNa^+:uK^+, which seems a useful indicator of urine production after diuretic administration (Adin et al., 2018). In that study, the uNa^+:uK^+ increased in the first hour of constant rate infusion of furosemide and then progressively decreased in parallel to a reduction in urinary output (Adin et al., 2018). In a prospective trial including a small population of dogs with CHF (n = 6) receiving furosemide, the uNa^+:uK^+ resulted significantly correlated with urine volume; lower uNa^+:uK^+ was detected in dogs with reduced diuretic response (median values 1.49) (Loughran et al., 2020). The median uNa^+:uK^+ measured in cardiopathic ACVIM stage C dogs in another study, was 1.15 (Adin et al., 2019). In our study, uNa^+:uK^+ was significantly higher in MMVD-MG than MMVD-EG with a median value of 1.67 vs. 0.93, respectively. Although urine output was not quantified in the current study, it might be assumed that the lower uNa^+:uK^+ noticed in MMVD-EG dogs and its negative correlation with the time elapsed from furosemide administration, reflected reduced sodium excretion potentially linked to reduced urine production >6 h since furosemide administration, similarly to previous observations (Loughran et al., 2020). Overall, our data indicate that beyond 6 h, FE Na^+ and uNa^+:uK^+ in MMVD dogs are not significantly different than healthy dogs, suggesting that the pharmacodynamic effect of furosemide has worn off. Hence, sampling of urine beyond 6 h as a mean to determine diuretic response is not clinically useful. Thus, as part of urinary chemistry, the uNa^+:uK^+ is also affected by the timing of diuretic administration, which should therefore be considered for the correct interpretation of these data.

As mentioned above, the chronic use of furosemide could lead to resistance development. Diuretic resistance has not been unanimously defined in humans, although it has been described as the failure to achieve relief from signs of CHF despite a full dose of diuretic (Collins et al., 2019) or as the failure to reduce the volume of extracellular fluid despite the appropriate use of diuretics

(Shah et al., 2017). Diuretic resistance affects up to 17% of people with heart failure (Doering et al., 2017) and it is suspected to also occur in dogs (Adin et al., 2018; Adin et al., 2019; Loughran et al., 2020). It is crucial to recognize this condition, in order to adjust treatment and formulate a prognosis. In humans, several biochemical markers and specific cut-offs have been proposed to diagnose diuretic resistance, including FE Na^+ < 0.2% and spot [uNa^+] < 50 mEq/L (Doering et al., 2017; ter Maaten et al., 2016). Patients' responses to diuretic therapy have been related to pre-treatment FE Na^+, with low values being associated with a blunted natriuretic response after furosemide administration, both in acute and chronic settings. In veterinary medicine, no markers of diuretic resistance have been conclusively established; however, analysis of the concentration of urinary electrolytes and their FE appears promising, based on preliminary studies (Adin et al., 2018; Adin et al., 2019; Loughran et al., 2020). In a previous study, Adin et al. found that during a 5-h constant rate infusion of furosemide (3.3 mg/kg diluted with 5% dextrose in water to a final concentration of 2.2 mg/mL) in six healthy dogs, the [uNa^+] (and the uNa^+:uK^+) increased in the first hour and then progressively decreased in parallel to a reduction in urinary output, suggesting an early braking phenomenon (Adin et al., 2018). A subsequent study from the same group evaluated urinary electrolyte concentrations in a large cohort of cardiopathic dogs, demonstrating that both the FE Na^+ and FE Cl^- were significantly higher in ACVIM stages C and D dogs receiving diuretics than dogs with preclinical disease (Adin et al., 2019). In that study, samples were likely collected four to 6 h after furosemide administration; however, the time elapsed between diuretic intake and sample collection was not specifically reported in the study methods. In a prospective trial investigating formulas to predict cumulative urine sodium and volume in eight healthy dogs and six dogs with CHF receiving oral furosemide, dogs fulfilling the criteria of low diuretic response showed lower [uNa^+] and uNa^+:uK^+ compared with dogs with an adequate diuretic response. Indeed, in that study dogs with net fluid gain excreted less [uNa^+] (Loughran et al., 2020). Our results further support the importance of considering the time elapsed from diuretic administration when assessing urinary sodium, chloride, and even uNa^+:uK^+ excretion in dogs receiving furosemide, in order to obtain reliable and meaningful data, correctly assess tubular function, and evaluate the expected patient response to diuretic therapy. This forms the basis for future studies on markers of diuretic resistance in dogs.

Kaliuresis (reported as both FE K^+ and [uK^+] to [uCr] ratio but not as [uK^+]) was higher in MMVD-MG than MMVD-EG. This result is likely related to the diuretic action. In fact, furosemide, by blocking the $Na^+ + K^+ + 2Cl^-$ channel at the level of Henle's loop, also blocks potassium

reabsorption resulting in increased urinary excretion (Adin et al., 2018). An increased kaliuresis in dogs receiving furosemide has already been described in healthy dogs and in dogs with iatrogenic mitral regurgitation receiving intravenous or oral diuretic therapy, respectively (Uechi et al., 2003). Moreover, a recent investigation conducted in dogs with heart disease (MMVD or dilated cardiomyopathy) reported an increased FE K^+ in patients with furosemide therapy vs. patients without furosemide therapy (Adin et al., 2019). Data regarding kaliuresis in the above-mentioned study are similar to our results; however, due to differences in study setting, case composition, and intervals between furosemide administration and sampling, a more detailed comparison is difficult to make.

Myxomatous mitral valve diseased dogs sampled in the morning also had greater renal excretion of calcium and magnesium than MMVD-EG ones, while no difference in phosphate excretion was noted. In old experimental studies conducted on healthy dogs treated with intravenous furosemide, the urinary excretion of calcium and magnesium (reported as concentrations or as FE) was already found to be increased (Duarte et al., 1968; White et al., 1981). By decreasing the transepithelial voltage along the thin ascending loop of Henle's loop, furosemide is reported to decrease the absorption of calcium and magnesium at the tubular level in humans; thus, increasing the urinary excretion of these electrolytes (Alexander et al., 2017). There is insufficient literature to establish a link between furosemide and phosphate excretion according to the available human literature. To the best of the authors' knowledge, there are no studies correlating the response to furosemide with urinary excretion of calcium, phosphate, and magnesium in dogs with heart disease. Our results might provide a basis for further studies aimed at a better understanding of the role of furosemide, MMVD, or even renal dysfunction in the excretion of these electrolytes in dogs. Significant negative correlations were observed between most of the measured urinary electrolytes and the hours elapsed from the morning furosemide administration in the enrolled dogs. Although weak, such correlations further corroborate the main finding of our study in regard to the impact of the time from diuretic therapy and urine collection and analysis. On the contrary, neither the dose nor the duration of furosemide therapy was correlated with urinary analytes results. These findings could be partially supported by the basic pharmacokinetic principles of furosemide and its characteristic sigmoidal dose–response curve (threshold dose to start diuretic effect followed by ceiling or plateau effect). Moreover, the post diuretic rebound effects, namely the ‘early’ and ‘late braking phenomenon’, could help to explain, at least in part, the lack of correlation between urinary electrolytes results and treatment

duration (Regolisti et al.,2016). In the comparison of MMVD and healthy dogs, higher urinary electrolyte excretion (FE Na^+, uNa^+:uCr, FE Cl^-, uCl^-:uCr, FE Na^+:FE K^+, uNa^+:uK^+) was detected in MMVD-MG than H-MG, while no difference was documented between H-EG and MMVD-EG. Hence, the tubular reabsorption capacity of sodium and chloride is comparable between healthy dogs and dogs with stable MMVD ACVIM stage C, when analyzed more than 6 h after the last diuretic administration. Although predictable based on the known pharmacokinetic of furosemide, such finding is important to be documented in general clinical practice, raising questions regarding the possibility of rebound sodium and water retention and renin–angiotensin–aldosterone system activation in response to transient volume depletion once furosemide effect is ended, with additional implications regarding the optimal dosing interval for this drug. Circadian fluctuations of urinary electrolytes have previously been observed in humans and animals (Lefebvre et al., 2008). In healthy dogs, a marked day/night variation in urinary excretion of sodium has been reported, with several studies describing an afternoon peak of sodium excretion four to 8 h after meal ingestion, followed by a progressive decrease during evening and night (Boemke et al., 1995; Gordon et al., 1985; Mochel et al., 2013). Despite the aforementioned studies, no information is currently available concerning circadian changes of urinary electrolytes in fasted dogs. To rule out the possible effect of such physiologic fluctuations, we included a group of healthy dogs, fasting for at least 12 h, sampled in the morning or in the evening. Based on our results, the control group experienced no difference in urinary sodium, chloride, and potassium excretion based on the time of sampling, suggesting that in healthy dogs no significant morning vs. evening variation in urinary electrolytes is expected. Therefore, it is possible to claim that the difference in electrolyte excretion in MMVD-MG vs. MMVD-EG dogs depends mainly, if not exclusively, on the time elapsed from furosemide administration.

Of note, [uNa^+], [uCl^-], and [uK^+] were significantly decreased in diseased dogs if compared to the healthy ones. Although as first unexpected, these results are not surprising since the reduction of urine concentration after furosemide treatment significantly affects urine chemistry (Adin et al., 2018), with a potential dilutional effect on urinary analytes. A dilutional effect was also confirmed by the significant decrease in urine specific gravity and [uCr] noticed in diseased dogs of both groups. Hence, the sole evaluation of the concentration of urinary electrolytes could lead to misinterpretation of the diuretic response. For these reasons, normalization with [uCr] ratio or even better, the evaluation of the FE of the substance of interest is recommended in this type of patient.

Limitations

Our study had several limitations. The main limitation of Project 1 was that dogs with MMVD and control dogs were not matched for age and body weight. Specifically, healthy control dogs were younger than dogs with MMVD. Urinary NGAL in people is linearly related to aging. Such variations are mild, potentially related to loss of renal mass and tubulointerstitial fibrosis, but enough to consider the establishment of age-related reference values in the healthy population (Pennemans et al., 2013). Indeed, both cardiovascular and renal disease are considered age-related diseases in people, making it difficult to evaluate the effect of age on a specific biomarker separately from age-associated comorbidities. Whether such age-associated effects on urinary biomarkers are clinically relevant remains a matter of debate in humans. To the best of our knowledge, no similar data are available in dogs. Although an age effect was not identified in healthy dogs enrolled in our study, the possible impact of aging on uNGAL results should be acknowledged in MMVD dogs. In the included MMVD dogs, age increased at increasing ACVIM stage (data not shown). This finding might is expected, because MMVD progresses with aging (Borgarelli et al., 2012). Aging also could be a predisposing factor for progressive functional renal loss and CKD, which could be an additional reason for the obtained NGAL results, as previously discussed. Nonetheless, it seems unlikely that aging alone would be a determining factor behind the increase in uNGAL identified in our study population. However, because of the difficulty in overcoming this limitation when evaluating diseased animals, additional studies specifically addressing the age effect on canine uNGAL in healthy dogs are needed. Another limitation is that due to the low number of ACVIM stage D dogs, they were enrolled in the same group with ACVIM stage C cases (group C+D) for statistical purposes. This choice might have caused some heterogeneity because of different disease severities and diuretic needs. Moreover, additional tests to characterize tubular damage (eg, urine protein electrophoresis, other renal biomarkers) and to assess venous congestion (eg, abdominal ultrasound) could have been useful to better explore uNGAL origin and correlations in our study. Although dogs with signs of urinary tract infection or pyuria were excluded from our study, urine cultures were not systematically performed, which could be considered a minor limitation. Lastly, although the number of control dogs we enrolled is substantially higher than in previous studies in dogs that evaluated renal impairment in MMVD (Szczepankiewicz et al., 2019; Valente et al., 2021), we did not achieve the sample size recommended by the American Society of Veterinary Clinical Pathology when defining RIs (eg, ≥120 subjects) (Friedrichs et al., 2019). For this reason, further refinement of RIs should be carried out in the future.

In order to evaluate a possible association between echocardiographic variables and uNGAL we classified the population in groups according to normal uNGAL and uNGALC values described in this thesis work. It is known that NGAL has a moderate individual variability (Chen et al., 2023) and the cutoff values reported might not be applicable to the general population. On the other hand, the used cutoff values have been reported by our group with the same laboratory methods.

The results of Project 2 should be read in the context of certain limitations. First of all, the time windows of sample collections remained relatively wide (samples could be collected at a range of times between one and 6 h for MMVD-MG or over 6 h for MMVD-EG dogs, after furosemide administration). Possibly, such temporal variability could have led to an increased variability of urinary electrolyte excretion. Moreover, although MMVD and healthy dogs were sampled in the morning or in the evening based on the time of the appointment randomly arranged with the owner, the possibility of systematic sampling generating systematic differences between groups should be considered. However, it should be considered that our study was intentionally designed to be an observational investigation aimed to be as representative as possible of the clinical setting and schedules of many small animal clinical practices. For the same reason, and more importantly, urine volume quantification was not performed. This is a limitation of the study since the interpretation of urinary electrolytes should be linked with the amount of urine volume excreted in a certain amount of time and not only with the time elapsed from furosemide administration. Indeed, urinary volume and urinary sodium are not interchangeable measures and might not change in parallel (e.g. a patient under diuretics could excrete a significant amount of water with low sodium content and thus experience incomplete decongestion) (Loughran et al., 2020). Treatments and dosages were not standardized since they could vary based on the needs of the patients and the clinician's judgment. Nevertheless, there was no significant difference in terms of therapeutic regimens between the two groups of diseased dogs. Dietary sodium intake was not standardized, and this could have affected furosemide responsiveness and natriuresis in our populations. In a recent study involving healthy dogs that were fed with normal, low, or ultralow sodium diets, even if there was a significant difference between the three groups, the authors did not specify whether the difference was assessed in fasting and fed patients. Furthermore, the samples collected shortly after a meal had the greatest fluctuation in FE Na^+, while in fasted dogs FE Na^+ was more commonly less than 1% despite individual variations still being detectable (Lobetti, 2020). The postprandial peak in urinary sodium excretion occurs after four to 6 h and

approximately 60% of the sodium fed is excreted within 8 h after food intake (Reinhardt et al., 1996). Although the time between feeding, diuretic administration, and sampling was inconsistent in enrolled dogs, such inconsistency involved both healthy and MMVD dogs, and the potential derived bias has been similarly distributed between groups. More importantly, at the time of sampling, enrolled dogs had all been fasted for at least 12 h. We can therefore assume that although the lack of dietary standardization in this context remains an important limitation, its impact has been minimized. In addition, MMVD and healthy dogs were not matched for weight and age, especially due to the intrinsic nature of MMVD that commonly affects old, small-sized dogs. Aging, as well as obesity, have been documented as affecting renal hemodynamic and sodium handling in humans (Epstein,1996; Esposito et al., 2010; Hall, 2003) and in some experimental animal models (Friedman et al., 1957; Vargas et al., 1997). Nevertheless, studies considering variations of electrolyte excretion in dogs of different ages and weights are lacking. There are only few studies available, describing FE distribution in puppies up to six-months-old or comparing natriuresis between puppies and mature dogs (Lane et al., 2000; Laroute et al., 2005). For these reasons, the real impact of such physiological differences in the study groups could not be clearly estimated. Nonetheless, in order to partially overcome this limitation, we made further comparisons in urinary electrolytes (dogs > 6 years vs. dogs < 6 years; dogs > 20 kg vs. dogs < 20 kg) within the group of healthy dogs; no clinically significant difference was observed (Table 15). Lastly, due to the high number of hypotheses tested and comparisons performed, the possibility of cumulative type I error should be acknowledged.

Conclusions

In conclusion, this thesis work identified the presence of renal tubular damage, evidenced by uNGAL and uNGALC, in dogs with stable MMVD. This tubular damage was subclinical, and evident even in the initial stages of the disease in dogs not receiving diuretic treatment. This finding emphasizes that MMVD dogs experience functional kidney impairment beyond that related to hemodynamic changes associated with cardiac disease and diuretics. Increasing uNGAL along with the worsening of heart disease indicates that renal damage during MMVD in dogs might be progressive and potentially involved in renal fibrosis, renal aging, and CKD development in more advanced MMVD stages. The role of reno-protective approaches in the management of dogs with MMVD should be explored in the future because they can potentially slow progression and decrease complications of CRS.

We identify an association between uNGAL and uNGALC values and left atrial stroke volume and tricuspid regurgitation in dogs with MMVD. These findings suggest that these echocardiographic indexes might detect dogs with MMVD at higher risk to develop renal damage; this might be the expression of congestive nephropathy.

Moreover, we also demonstrated that urinary excretion of sodium, potassium, and chloride, as well as additional urinary electrolytes, is increased within 6 h from furosemide administration in dogs with stable MMVD ACVIM stage C, compared to dogs sampled beyond 6 h and healthy controls. Since no relevant morning vs. evening variations of urinary electrolytes were found in this study in healthy dogs, the difference in electrolyte excretion between the two time periods assessed in MMVD dogs treated with furosemide depends mainly, if not exclusively, on the time elapsed from furosemide administration to patient evaluation. The results of this study add new information concerning urinary electrolyte handling in dogs with MMVD receiving diuretic therapy, suggesting that urine chemistry interpretation is strongly affected by the timing of blood and urine sampling. This could have important implications in both clinical and research fields and could offer new perspectives in the identification of diuretic resistance in such patients. Further studies with a larger sample size are needed to confirm these findings.

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