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CICLO XXXVII

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

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Abstract

Cardiorenal syndrome (CRS) describes a reciprocal relationship between the heart and kidneys, where impaired function of one organ—whether acute or chronic—can trigger a similar decline in the other. In dogs with myxomatous mitral valve disease (MMVD), early detection of renal involvement allows for treatment adjustments that benefit both organs. Serum creatinine concentration remains the most common marker for assessing kidney function in these dogs, though it is unfortunately a poor indicator of early kidney damage and cannot differentiate between functional and structural lesions.

This thesis reports the results of a multicenter study conducted at the University of Parma, and the University of Bologna exploring the occurrence and nature of renal damage in dogs with stable MMVD (first study phase) or in congestive heart failure (CHF) (second study phase).

In the first phase, three descriptive studies addressing different aspects of Type 2 CRS were conducted. The objectives were to evaluate in dogs with stable MMVD: 1) the impact of the time frame between oral furosemide administration and sample collection on urinary electrolyte concentration and any potential fluctuations in urinary electrolytes between morning and evening in a group of healthy dogs; 2) the value of urine neutrophil gelatinase-associated lipocalin (uNGAL), a marker of tubular damage, across different American College of Veterinary Internal Medicine (ACVIM) stages and a comparison with healthy dogs; 3) the relationship between uNGAL levels and echocardiographic variables used for staging cardiac disease. The findings from this phase were as follows: 1) urinary electrolyte excretion significantly increases within 6 hours of furosemide administration in MMVD dogs at ACVIM stage C, whereas healthy, untreated dogs showed no significant circadian variations in urinary electrolyte excretion; 2) uNGAL levels were significantly higher in MMVD dogs and increased with higher ACVIM stages, indicating the presence of renal tubular damage even in stable MMVD dogs; 3) two echocardiographic indices left atrium stroke volume (LASV) and tricuspid regurgitation velocity (TRVmax) were associated with elevated uNGALC levels, suggesting these parameters might help identify MMVD dogs at greater risk of kidney damage.

In the second phase of the study, renal responses in Type 1 CRS were evaluated, including dogs with MMVD presented to the intensive care unit in CHF. The aim of the study was to assess the prevalence and characteristics of acute kidney disease (AKI) during a short hospitalization period and evaluate the predictive role of urinary uNGAL for AKI. It was found out that 63%

of dogs with CHF developed AKI within the first 48 hours of admission. However, uNGAL levels were not associated with renal function decline as indicated by creatinine changes.

These studies can contribute to the understanding of cardiorenal syndromes, but future researches should explore reno-protective strategies for managing MMVD in dogs, as these approaches could potentially slow disease progression and mitigate related complications.

Chapter 1

Prelude

This PhD thesis explores some aspects, investigated during the three-year PhD course, of cardiovascular-renal axis disorders in dogs affected by myxomatous mitral valve disease (MMVD) at various stages as defined by the American College of Veterinary Internal Medicine (ACVIM). Specifically, this project explores in-depth the role of urinary chemistry and urine neutrophil gelatinase-associated lipocalin (uNGAL) in identifying and predicting renal tubular damage in dogs with MMVD, both in stable condition and during episode of congestive heart failure (CHF).

This thesis reports the results generated from a multicenter project that began in March 2020 through a collaboration between the University of Parma and the University of Bologna. The study is prospective observational and consists of two phases. The first phase is a descriptive, in-depth examination of kidney disease in dogs with stable MMVD (CRS Type II). This part involves the quantification of serum and urinary chemistry, as well as biomarkers of renal damage (uNGAL) in dogs with MMVD at different ACVIM stages (B1-B2-C-D) (Keene et al., 2019), and the comparison between dogs affected by MMVD and healthy dogs. The second phase provides an in-depth evaluation of AKI in dogs with CHF (acute decompensation in patients at stages C and D) (CRS Type I) and examines the role of uNGAL in predicting AKI.

The present manuscript merges the results of these research activities, some of those already published in peer-reviewed journals or submitted for publication.

First phase (MMVD stable - CRS type II)

Papers already published regarding the first phase studies are presented in Chapters 3, 4, and 5. The first article (**Chapter 3,** Sabetti et al., 2022) was triggered by an observation we made during the sample collection process. Urinary electrolyte excretion has received growing attention as a biomarker for assessing an appropriate diuretic response and for the early recognition of diuretic resistance in human patients and dogs with heart disease (Martens et al., 2019; Adin et al., 2020). Patients on chronic loop diuretic therapy are typically expected to exhibit higher natriuresis compared to healthy, untreated individuals. However, in the urinary samples from MMVD dogs assessed more than six hours after diuretic administration, natriuresis levels were like those in healthy, untreated dogs. Initially, we considered whether

these dogs might have an inherently poor response to diuretics. However, the explanation proved to be simpler, stemming from the pharmacokinetics of loop diuretics. That observation led us to investigate relationship between natriuresis and the timing of diuretic therapy administration. The timing of blood and urine sampling in relation to the time of diuretic administration has a strong influence in urinary electrolyte excretion and therefore needs to be considered and/or standardized when interpreting urinary chemistry as a biomarker of an adequate response to diuretic therapy.

In **Chapter 4**, the second article (Troia et al., 2022) is presented, which describes, for the first time in the literature, the behavior of a urinary tubular damage marker (uNGAL) in dogs with stable heart disease. Findings revealed that in dogs with MMVD, tubular damage is subclinical, occurs at all stages of MMVD even in the absence of azotemia, and worsens with the severity of MMVD. The increase in uNGAL, along with the progression of heart disease, indicates that renal damage during MMVD in dogs may be progressive and potentially contribute to renal fibrosis, renal aging, and chronic kidney disease (CKD) development in more advanced stages of MMVD.

The third study (**Chapter 5**, Crosara et al., 2024) builds on previous research. After demonstrating the progressive increase in uNGAL with worsening heart disease, we aimed to explore whether certain echocardiographic variables were associated with increased uNGAL. Our conclusion suggests that some evaluated blood and urine parameters, such as uNGAL, might help identify dogs with MMVD at higher risk of developing kidney damage. Further studies with larger sample sizes are needed to evaluate the predictive value of these variables in identifying animals at greater risk for renal damage.

Second phase (MMVD CHF – CRS type I)

The first phase, above described, has established the foundation for the second part, which focuses on the predictive role of tubular damage markers in animals with MMVD in CHF. Over the past two years of my PhD, I have concentrated my research on the second part of the project, from data collection to the drafting of the paper. This project will be presented in **Chapter 6** and, as it is the primary focus of my PhD, it will be discussed in greater detail. This prospective observational study aims to assess the occurrence of AKI in dogs with acute CHF and evaluate the role of urinary NGAL for the prediction of AKI during CHF. We included CHF dogs that required emergency in-hospital treatment with IV diuretics (furosemide or torasemide) for at least 24 hours. At admission serum and urine samples were collected within an hour from the

first diuretic administration. Sampling was repeated after 24 and 48 hours. Based on our results, AKI as defined according to the International Renal Interest Society (IRIS) criteria (Segev et al., 2024) occurred in 63% of dogs with CHF within 48 hours from hospitalization. Despite AKI occurrence, no significant changes in uNGAL values were documented during the study period. Furthermore, no correlation was documented between the values of uNGAL and the total dose of furosemide administered during the first 24 hours. This finding indicates that development of mild worsening renal function during CHF is common in dogs but appears to be an expected hemodynamic response to successful decongestion and does not reflect tubular damage. The value of recurrent AKI episodes in more critical scenarios, and its overall impact on long term outcome and diuretic responsiveness, is not known, and should be characterized in further studies.
The results of this study are currently unpublished.

Before going through the specific scientific experiences, is necessary to review the definitions and current literature in the field of cardiovascular-renal axis disorders, MMVD disease, and of urinary chemistry and uNGAL.

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Chapter 2

Scientific Background

Cardiovascular-renal axis disorders

In veterinary medicine, the term "cardiovascular-renal axis disorders (CvRD)" was proposed by Pouchelon et al., 2015 to describe the relationship between the cardiovascular system and kidneys. The syndrome described by CvRD had previously been studied in humans and was defined in 2008 (Ronco et al., 2008) as cardiorenal syndrome (CRS). The working group of Pouchelon et al. (2015) modified the terminology (from CRS to CvRD) "*to emphasize the involvement of both the cardiovascular system and the vasculature, in addition to the heart*" (Pouchelon et al., 2015), thereby highlighting the complexity observed in the clinical manifestations of these disorders in dogs and cats. The CvRD describes how dysfunction of one organ (that can be the cardiovascular system or the kidney or both) leads to injury or function of the other organ. The human CRS classification (Ronco et al., 2018) and the consensus classification for CvRD (Pouchelon et al., 2015) are conceptually similar and overlap in many areas, as both describe the primary insult, sequence of events, and the chronic nature of the process. However, they do not address pathophysiology or therapeutic management. Nevertheless, the term CRS is frequently used in veterinary medicine as well. In this thesis, to align with existing literature, we will often refer to CRS terminology. In dogs and cats, as in human medicine, five types of CvRD are described, depending on whether the primary disorder is cardiac or renal, and whether it is acute or chronic (Table 1).

Table 1
Human and veterinary classification of cardiorenal syndrome (modified from Orvalho and Cowgill, 2017)

Cardiorenal Syndrome (Human)	**CvRD** (Dogs and cats)	**Brief Definition**	**Conditions**
Type 1: Acute cardiorenal syndrome	$CvRD_H$ unstable	Acute impairment of the cardiac function leading to acute kidney injury (AKI)	• Acute heart failure • Cardiogenic shock
Type 2: Chronic cardiorenal syndrome	$CvRD_H$ stable	Chronic cardiovascular disease causing progressive chronic kidney disease (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	$CvRD_K$ stable	Primary CKD that contributes to cardiac dysfunction	• Glomerular disease • Anemia • systemic hypertension
Type 5: Secondary cardiorenal syndrome	$CvRD_O$	Cardiac and renal dysfunction secondary to an acute or chronic systemic condition	• Diabetes mellitus • Sepsis

This thesis focused on exploring unstable $CvRD_H$ (acute CRS, type 1) and stable $CvRD_H$ (chronic CRS, type 2), where CHF can cause AKI, and chronic cardiovascular disease can lead to the progression of CKD, respectively (Orvalho and Cowgill, 2017). In the CRS type 1, AKI (consequent to CHF) can result from reduced renal perfusion, neuroendocrine and sympathetic systems activation, and passive congestion of the kidney (Orvalho and Cowgill, 2017). Furthermore, the congestive state can induce excessive use of diuretics and further kidney injury. Early recognition of AKI remains a challenge due to the lack of biomarkers for early IRIS AKI grades (http://www.iris-kidney.com/guidelines/grading.html) (table 2).

Table 2 IRIS Grading (modified by http://www.iris-kidney.com/guidelines/grading.html)

AKI	**Blood Creatinine**	**Clinical description**
GRADE I	**< 1.6 mg/dl**	**Non-azotemic AKI:** Documented AKI (history, clinical or imaging evidence of AKI) Progressive non-azotaemic increase in creatinine >0.3 within 48 hours Measured oliguria (<1ml/kg/hr) or anuria over six hours
GRADE II	**1.7 -2.5 mg/dl**	**Mild AKI** Documented AKI and static or progressive azotemia

		Progressive azotemic increase in blood creatinine: ≥0.3 mg/dl (within 48 hours), or volume responsiveness Measured oliguria or anuria over six hours
GRADE III	**2.6-5 mg/dl**	**Moderate to severe AKI:** Documented AKI and increasing severities of azotemia and functional renal failure
GRADE IV	**5.1 -10 mg/dl**	
GRADE V	**>10 mg/dl**	

In type 2 CRS, CKD (consequent to cardiovascular disease) can result from a chronic persistently reduced renal perfusion, chronic renal congestion and neurohormonal changes associated with chronic sympathetic stimulation and release of natriuretic peptides (Pouchelon et al., 2015). The prevalence of renal dysfunction in CHF is significant and serves as an independent predictor of outcomes and mortality. Thus, preserving renal function in these patients is of outmost importance (Orvalho and Cowgill, 2017). Since previous studies have confirmed the existence of CRS in dogs with MMVD (Chetboul et al., 2012; Martinelli et al., 2016) for our project we decided to include dogs with MMVD who are stable or in CHF.

Myxomatous Mitral Valve Disease

Classification of heart disease is an important step in characterizing the type of CRS. Chronic MMVD as a result of myxomatous degeneration represents the most common acquired cardiovascular disease in dogs (Keene et al., 2019). Over time, mitral valve regurgitation, depending on its rate of progression, patient age and severity of the condition, leads to left atrial enlargement, compensatory left ventricular remodeling and, potentially, heart failure (Borgarelli et., 2012). The term “heart failure” refers to clinical signs caused by heart dysfunction, and venous pressures increase so severely that fluid accumulates in the lungs or a body cavity (congestive heart failure) (Keene et al., 2019).

The recommended cardiac disease classification is the ACVIM cardiac disease severity classification, which was adapted from the American College of Cardiology/American Heart Association classification system that uses categories A through D. This staging system for MMVD describes 4 stages of heart disease and heart failure (Keene et al., 2019):

- **Stage A:** Dogs who are at risk of developing heart disease but currently do not present with murmurs on auscultation or structural disorders (every Cavalier King Charles Spaniel or other predisposed breed).

- **Stage B:** Dogs with structural heart disease but that have never developed clinical signs caused by heart failure. Dogs with heart disease but have never shown symptoms. This class is further divided into:

  - **B1**: Asymptomatic dogs that do not have radiographic or echocardiographic signs of cardiac remodeling in response to MMVD.

  - **B2**: Asymptomatic dogs that have more advanced mitral valve regurgitation to have caused radiographic signs and echocardiographic signs of left atrial and ventricular enlargement. Medical therapy is recommended to slow the onset of heart failure. For this class, the treatment includes the Pimobendan (positive inotropic and a vasodilator) at dosage of 0.25-0.3 mg/kg PO q12h.

- **Stage C**. Dogs with current or past signs of CHF that required treatment. It distinguishes between dogs with that requiring hospitalization and dogs whose can be managed at home (Table 2).
- **Stage D.** Dogs refractory to treatment, where medical therapy becomes ineffective. Chronic PO furosemide dosages > 8 mg/kg q24h in any dosing regimen to maintain patient comfort in the face of appropriate dosages of pimobendan, an ACEI, and spironolactone indicate disease progression to Stage D (Table 3).

Table 3 Recommendations for treatments (modified by Keene et al., 2019)

	STAGE C	**STAGE D**
HOSPITAL BASED	Furosemide 2 mg/kg IV (or IM) followed by 2 mg/kg IV (or IM) hourly until patient's respiratory signs are substantially improved.	Furosemide 2 mg/kg IV (or IM) followed by 2 mg/kg IV (or IM) hourly until patient's respiratory signs are substantially improved or Torsemide 0.1-0.2 mg/kg q12h-24h.
	Furosemide CRI (0.66-1 mg/kg/hour) for life-threatening pulmonary edema.	Furosemide CRI (0.66-1 mg/kg/hour) for life-threatening pulmonary edema.
	Pimobendan 0.25-0.3 mg/kg PO q12h.	Pimobendan 0.25-0.3 mg/kg PO q8h.
	ACEI (0.5 mg/kg PO q12h)	ACEI (0.5 mg/kg PO q12h)
	Oxygen supplementation, if needed, can be administered.	Oxygen supplementation, if needed, can be administered.

CHRONIC BASED (Home based)	Furosemide (2-4 mg/kg q12h) or Torsemide 0.1-0.3 mg/kg q24h	PO furosemide dosages > -8 mg/kg q24h or torsemide beginning dosage of 0.1-0.2 mg/kg PO up to 0.6 mg/kg, divided q12h if necessary
	Pimobendan, 0.25-0.3 mg/kg PO q12h.	Pimobendan 0.25-0.3 mg/kg PO q8h.
	ACEI (0.5 mg/kg PO q12h).	ACEI (0.5 mg/kg PO q12h).
	Diltiazem (often combined with digoxin) in cases of complicated atrial fibrillation	Diltiazem (often combined with digoxin) in cases of complicated atrial fibrillation
	Spironolactone (2.0 mg/kg PO q12 - 24 h).	Spironolactone (2.0 mg/kg PO q12 - 24 h).

Diuretics are considered essential for removal of CHF fluid and are cornerstone treatments for acute decompensated episodes as well as for the chronic management of MMVD patients. Nonetheless, response to diuretic therapy could be hampered by comorbidities (e.g. renal disease) and progressive sodium retention (Oyama et al., 2022). Significant alterations in sodium balance occur in CHF patients, with failure of adequate natriuresis being one of the major mechanisms behind diuretic resistance (Atkins et al., 2012).

Congestive heart failure and diuretic responsiveness or resistance?

In CHF, characterized by volume retention, which leads to the accumulation of fluid in the interstitial space or body cavities, diuretics like furosemide are prescribed to reduce congestion and ease clinical symptoms, with dosing typically based on the principle of "*maintaining patient comfort*" (Keene et al., 2019). Loop diuretics, such as furosemide and torsemide, inhibit the Na^+-K^+-$2Cl^-$ cotransporter (NKCC) in the ascending limb of the loop of Henle, and urinary excretion of these ions induces water loss (increase urine production) and as consequence resolution of congestion (Felker et al., 2020). Diuretics induce a range of effects on water, Na^+, and clinical signs that can be described as diuretic responsiveness or diuretic resistance (Oyama et al., 2023). Conversely, when natriuresis and diuresis are insufficient to alleviate congestion or achieve euvolemia, diuretic resistance is viewed as a diminished response to diuretics, resulting in inadequate decongestion (Felker et al., 2020). There are various potential causes of diuretic resistance (Oyama et al., 2023). In dogs receiving chronic diuretic treatment, resistance can occur as a result of increased NKCC transporter expression, as well as distal tubular hypertrophy and heightened adenosine activity (ter Maaten et al., 2017). Consequently, distal tubular Na^+ reabsorption can significantly surpass the normally minimal reabsorption that occurs in this part of the nephron. Additionally, repeated parenteral or oral administration of diuretics can lead to resistance due to diuretic braking (Adin et al., 2018). The diuretic braking

describes the abrupt reduction in urine production shortly after diuretic initiation, contributing to the initial loss of diuretic responsiveness (Adin et al., 2018). This occurs because of the activation of the renin-angiotensin-aldosterone system (RAAS) and the sympathetic nervous system, which quickly limit diuresis as the body attempts to compensate for reduced intravascular volume. The increased RAAS activity, which leads to rebound Na^+ and water retention between doses, is known as the post diuretic effect (Cox et al., 2014). This mechanism can lead to a worsening of renal function that contributes to diuretic resistance.

Currently, the assessment of diuretic treatment effectiveness (or diuretic responsiveness) is based on the alleviation of clinical signs, such as tachypnea and dyspnea, or the resolution of radiographic pulmonary edema (Keene et al., 2019). However, several parameters (total urine volume, urinary Na^+ concentrations, urine sodium to urine potassium (uNa^+:uK^+), and fractional excretion of Na^+) have been proposed to quantify the response to diuretics, although they are still being studied in veterinary medicine.

Biomarkers for kidney injury in cardiovascular-renal axis disorders

Recognition of the onset or progressive worsening of kidney function/injury in the contest of CvRDH could provide greater opportunities to facilitate disease surveillance and tailor therapeutic intervention to promote the mutual benefit to both organs (Orvalho and Cowgill, 2017).

Kidney involvement is currently recognized and stratified using functional markers such as urine specific gravity, proteinuria, and serum creatinine. However, these markers may not indicate kidney involvement until it is already well established, potentially missing the early stages of kidney function compromise (Segev et al., 2024). Additionally, a functional marker like serum creatinine may fail to detect active kidney injury resulting from cardiac disease if compensatory adaptation by the remaining kidney mass masks the damage. Only when the active injury exceeds the kidney's repair capacity and compensatory adaptation will signs of kidney involvement become apparent (Orvalho and Cowgill, 2017).

Inside the kidney injury

The factors that triggered and sustained the progression of the kidney injury in CRS are largely unknow, however, mechanisms underlying the relationship between heart failure (HF) and renal dysfunction have been postulated (Fig. 1).

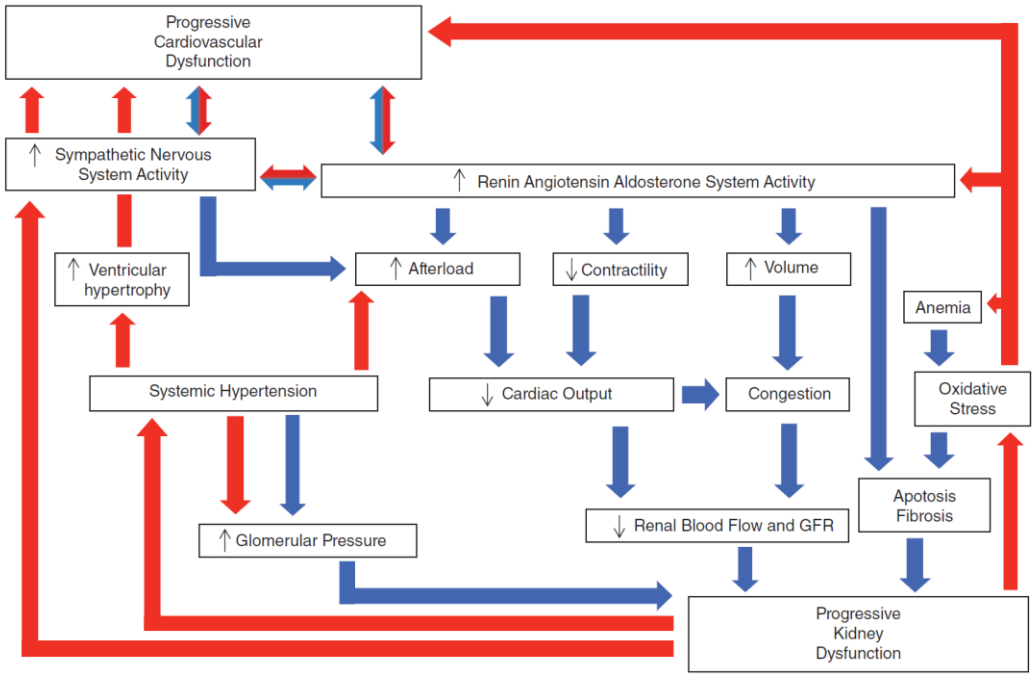

Fig 1. Postulated mechanisms underlying the relationship between HF and renal dysfunction. Blue arrows indicate pathways by which HF may lead to renal failure. Red arrows indicate pathways by which renal failure may lead to HF (Pouchelon et al., 2015).

The injury may occur suddenly and be temporary, or it can gradually worsen, eventually leading to end-stage kidney disease (Pouchelon et al., 2015). Cardiac disease may trigger an acute insult to the kidneys, causing injured tubular cells to undergo necrosis, apoptosis, regeneration, and repair, or remain damaged in a state of cell cycle G2/M arrest. In the latter case, maladaptive signaling pathways may be upregulated, leading to myofibroblast proliferation and fibrosis in the interstitium, which predisposes the kidneys to ongoing damage (Yang et al., 2010). Furthermore, the kidneys are highly sensitive to vascular compromise, hypoperfusion, and relative hypoxia, which can alter their function (Grauer et al., 2005). Tubular epithelial damage resulting from oxidative stress activates vasoconstrictive signals, promoting a vicious cycle of increased ischemia, progressive vascular rarefaction, and stimulation of growth factors that signal interstitial fibrosis and further hypoxia (Grauer et al., 2005). Since the renal tubule is more susceptible to decreased perfusion and hypoxic damage due to CHF, it can sustain injury even while the renal cortex remains intact, thereby preserving the glomerular filtration rate. Additionally, renal blood flow can decrease by 30–40% without noticeably impacting the GFR (van Deursen et al., 2014). Consequently, these factors make tubular markers more sensitive than functional markers (such as creatinine). In this setting an active kidney injury biomarker could expose ongoing or progressive kidney injury subsequent to cardiac dysfunction in

advance of conventional diagnostic methods. Currently, no ideal biomarker exists as a sensitive and specific early indicator of kidney damage resulting from heart disease in CvRD (Pouchelon et al., 2015). However, some of the biomarkers that have been studied so far have shown promising results in dogs.

Fractional excretion tests

Among the new markers of tubular kidney damage, the measurement of excretion fractions (FE) is emerging (Lefebvre et al., 2008). The FE are calculated by assessing the percentage of electrolyte excretion in relation to serum electrolyte concentration, according to the equation reported previously (Brown et al., 2015), as follows:

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

where uX and sX are the concentrations of a specific analyte in urine and serum, respectively, and sCr and uCr are serum and urine creatinine, respectively. FE is reported in percentages.

Urinary electrolytes have been proposed as simple laboratory tools to characterize and prognosticate acute kidney injury (Troia et al., 2018), but the diagnostic and prognostic role of urine chemistry remains largely unknown in dogs with cardiac disease.

However, urinary electrolyte excretion in the context of CRS has been proposed as a method to quantify the patient's response to, or resistance to, diuretics. Indeed, in human, serial assessment of natriuresis in response to loop diuretic administration can predict CHF and provide clinically relevant information: while stable patients tend to have a steady urinary sodium excretion, patients developing progressive decompensation experience a drop in their natriuresis, which can be detected before clinically relevant dyspnea occurs (Biegus et al., 2019). In addition, insufficient natriuresis is associated with poor short and long-term outcomes in CHF. Furthermore, 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. In this regard, the role of urine chemistry has emerged to define diuretic resistance (FE Na^+ <0.2%; uNa^+:uK^+ <1) and as a monitoring tool for prognostication (Biegus et al., 2019; Damman et al., 2020).

In veterinary medicine, analysis of the concentration of urinary electrolytes appears promising, based on preliminary studies (Adin et al., 2020; Loughran et al., 2020). In a study, the authors found that during a 5-h constant rate infusion of furosemide 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 (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). However, in this study was not specifically reported the urine sampling in relation to the time of diuretic administration. A recent study evaluated the correlation between uNa^+ with the frequency of treatment success, defined as successful discontinuation of supplemental O_2 therapy, finding that uNa^+ after IV furosemide was associated with treatment duration, and treatment efficacy (Convey et al., 2024).

Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL) is an acute phase glycoprotein of the lipocalin family produced in the bone marrow during granulocyte maturation. While it was initially identified in activated neutrophils, various other cell types, including renal tubular epithelial cells, can also synthesize NGAL. It is filtered by the glomerulus and almost entirely reabsorbed in the proximal renal tubule through endocytosis (Schmidt-Ott et al., 2007). The NGAL can be measured in plasma, serum or in urine and it is usually referred to as an absolute concentration. In some studies, urinary NGAL (uNGAL) has been normalized to urinary creatinine concentration to reduce variability associated with fluctuations in urine output.

It remains uncertain whether serum or urinary NGAL is more effective in predicting and diagnosing kidney injury. Serum changes may result from systemic responses and are not always directly linked to kidney function, whereas urine samples can be collected noninvasively, contain fewer interfering proteins, and may provide greater specificity for detecting kidney damage (Romejko et al., 2023). Serum NGAL rises after AKI due to impaired tubular secretion into urine, extrarenal production, and/or reduced glomerular filtration. In contrast, elevated uNGAL may indicate increased renal expression, impaired reabsorption, or filtration of plasma NGAL from renal or extrarenal sources (Schley et al., 2015). It has been suggested that urinary biomarkers may be more sensitive to true histological kidney injury, while serum markers may be more reflective of changes in kidney clearance (Schley et al., 2015). In our research project we decided to evaluate the uNGAL for evaluation of kidney tubular injury. Canine-specific ELISA assays have been validated for canine uNGAL (Davis et al., 2021). Furthermore, uNGAL appears robust through freeze-thaw cycles when stored at -80°C (Nabity et al., 2023).

These characteristics make NGAL a strong candidate as a biomarker because of its stability through freezing and thawing, and its reliable measurement across different sample types (plasma, serum, and urine) enhances its practicality and consistency for evaluate renal injury.
Healthy dogs have very low urinary concentration of NGAL. An increase in uNGAL excretion can result from reduced reabsorption due to proximal tubular injury or increase of renal secretion in the thick ascending limb of the loop of Henle because of parenchymal damage (Schmidt-Ott et al., 2007). Furthermore, NGAL is also produced in the distal segments of the nephron, where its synthesis is significantly increased in response to parenchymal kidney damage (Schmidt-Ott et al., 2007). Local and systemic inflammation can increase uNGAL due to its presence within neutrophils; therefore, interpretation should consider the presence of local and systemic inflammation, as even mild pyuria can significantly raise uNGAL levels (Nabity et al., 2023). Currently, uNGAL has been recognized as an early and sensitive marker of renal damage in both human and veterinary medicine (Segev et al., 2013; Monari et al., 2019; Scheemaeker et al., 2020). In the setting of CRS research on NGAL is limited. Studies in CHF dogs have shown that serum NGAL is associated with the development of cardiorenal syndrome (CRS) in acute heart failure cases (Jung et al., 2018) and in dogs with dilated cardiomyopathy (Wrześniewska et al., 2024). Before the studies reported in this thesis, no study had reported uNGAL values in dogs with stable MMVD.

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Chapter 3

MMVD stable - CRS type II

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

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Abstract

Urine chemistry has received growing attention to estimate the diuretic response in dogs with cardiac disease. The aim of the study was to evaluate the impact of time elapsed between the oral furosemide administration and sample collection on urine chemistry in dogs with myxomatous mitral valve disease (MMVD) receiving diuretic therapy in American College of Veterinary Internal Medicine (ACVIM) stage C. Seventy-three dogs with MMVD ACVIM stage C and 106 healthy dogs were prospectively included. Dogs with MMVD were divided, based on the time of sampling, in morning group (MMVD-MG) of one to 6 h and an evening group (MMVD-EG) 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.

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

Keywords: Diuretic, Fractional excretion, Heart disease, Natriuresis Urine chemistry

Introduction

Myxomatous mitral valve disease (MMVD) is a common acquired cardiac disease in dogs and often leads to congestive heart failure (CHF) (1). Furosemide is the most widely used loop diuretic employed to treat this condition (1,2). Despite its extensive use in dogs with naturally-acquired MMVD, the kinetic and diuretic effects of furosemide have mainly been investigated in healthy dogs, dogs with experimentally-induced CHF, and small canine populations affected by various heart diseases (3,4,5,6,7,8). The bioavailability of oral furosemide, which is the preferred route of administration for the treatment of chronic MMVD, is approximately 77% in dogs (9). Furosemide-induced natriuresis is dose-dependent and usually disappears 6 h after oral administration, requiring at least two administrations per day for a sustained diuretic effect (4,7).

In human medicine, diuretic efficacy is assessed based on the relief of CHF-related signs, weight loss, urine output, and urine composition, including natriuresis quantification (10,11). 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 (12,13,14,15). Similarly, monitoring urine electrolytes has recently caught the attention in veterinary medicine (8,16). However, the correct interpretation of urinary electrolytes may be 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 (8,16). To date, little attention has been paid to these possible confounding factors in dogs unlike human medicine (12,13,17).

Therefore, the objectives of this study were (1) 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 American College of Veterinary Internal Medicine (ACVIM) stage C, with medically-controlled CHF, and (2) to explore the possible morning vs. evening fluctuations of urinary electrolytes in a group of healthy, untreated dogs. The hypothesis was that (1) 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 and that (2) no relevant daily fluctuations of urinary electrolytes occur in healthy, untreated dogs.

Materials and methods

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

Study population

Privately owned dogs were enrolled at the cardiology services of both centers. Dogs were eligible for inclusion if they were affected by MMVD at ACVIM stage C, diagnosed, and classified according to the current guidelines (1), and if they were receiving exclusively oral furosemide as a loop diuretic. Further inclusion criteria were the following: (1) oral furosemide had to be administered twice daily at a stable dosage for at least one week prior to enrollment; (2) morning oral administration of furosemide had to occur between 7:00 and 8:00 a.m., at home by the owner; and (3) 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 (1). 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: (1) presence of other acquired or congenital cardiac diseases; (2) presence of one or more concomitant systemic diseases including endocrinopathies, neoplasia, International Renal Interest Society Stage 3 and 4 chronic kidney disease, acute kidney injury, and acute or chronic gastro-enteropathy with malabsorption; (3) acute CHF requiring emergency treatment (e.g. hospitalization with administration of intravenous furosemide); (4) concomitant treatments except of cardiac therapy such as pimobendan, spironolactone, angiotensin converting enzyme inhibitor (ACEI); (5) use of loop diuretics other than furosemide (e.g. torasemide).

Healthy dogs were included as controls for comparative purposes. These dogs were owned by medical staff or students attending the VUH. Dogs were considered healthy in the absence of any clinical signs at the time of sample collection and within the previous two months, and in the absence of clinicopathological abnormalities at complete blood count, serum chemistry profile, and urinalysis. Dogs should not have received any medications within the preceding

two months before inclusion in the study, except for routine preventative 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 (Protocols 747) and conducted with an informed owner consent.

Study groups

The cardiologists of the VUH participating in the study performed the first consultations 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. 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.

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 VUH 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 serum creatinine (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[c].

Urinalysis included urine specific gravity evaluated by a hand refractometer[d], dipstick test[e] read by an automated reader[f] 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 450*g*. 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:

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

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[g].

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 1). 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 A, available in Supplemental Material on-line). This comparison was not possible for MMVD-EG because only one dog did not receive these treatments.

Table 1 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 e maximum value) or mean and 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 (hours)	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			

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.

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 1). 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 FE Na^+ 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 B, available in Supplemental Material on-line). 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 2 and Fig. 1, Fig. 2, Fig. 3.

A further comparison between MMVD-MG and H-MG, and between MMVD-EG and H-EG, respectively, was performed. Urine creatinine and urine-specific gravity were significantly lower in MMVD-MG and MMVD-EG than H-MG and H-EG, respectively (P<0.0001 and 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 P=0.0007)2, [uCa^{++}] to [uCr] (P< 0.0001 and P< 0.0001), and urine phosphate to [uCr] (P=0.0013 and P< 0.0001) ratio. 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 (P=0.0001 and P< 0.0001).

In addition, uNa^+:uCr, uCl^-:uCr, uNa^+:uK^+, 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 2, Fig. 1, Fig. 2, Fig. 3).

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 C available in Supplemental Material on-line).

Table 2 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 emaximum 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)[a]	1.3 ± 0.41[c]	0.330	1.09 ± 0.20	1.12 ± 0.17	0.336
Urea (mg/dL)	17–48	75.2 (33.1–221)[b]	68.7 (34–189)[c]	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[b]	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[b]	107.2 (82.2–111)[c]	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)[b]	1018 (1006–1041)[c]	0.162	1049 ± 13.9	1047 ± 13.9	0.581
UPC (mg/mg)	0–0.5	0.2 (0.1–1.3)[b]	0.15 (0.08–0.61)[c]	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)[b]	78.2 ± 44.76[c]	0.01	293.5 ± 88.4	327 ± 102.6	0.073
[uNa^+] (mEq/L)		71.1 (5.9–192.9)[b]	36.3 (4.6–103.9)[c]	0.021	94.3 (11.7–342.4)	106 (5.8–344.8)	0.547
[uCl^-] (mEq/L)		68.2 (6–148.1)[b]	32.5 (5–114.8)[c]	0.038	184.26 ± 84.95	168.16 ± 85.16	0.332
[uK^+] (mEq/L)		38.9 (7.9–157.1)[b]	41.5 ± 17.71[c]	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)[b]	4.92 (0.87–11.2)[c]	0.903	13.05 ± 0.05	11.1 ± 6.0	0.148
[uP] (mg/dL)		34 (0.2–222)[b]	53.35 (18.4–197.2)[c]	0.070	181.26 ± 70.05	159 ± 81.78	0.135
uNa^+:uK^+		1.67 (0.11–10.21)[b]	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)[b]	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)[b]	0.44 (0.06–2.99)	0.008	0.66 (0.17–2.14)	0.49 (0.057–2.57)	0.132

(*continued on next page*)

Table 2 (*continued*)

Variable	RI	MMVD-MG (n = 47)	MMVD-EG (n = 26)	*p*-value	H-MG (n = 56)	H-EG (n = 50)	P-value
uK^+:uCr	0.00–0.80	0.86 (0.25–5.1)[b]	0.59 (0.18–1.37)[c]	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)[b]	0.056 (0.01–0.34)[c]	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)[b]	0.06 (0.007–0.36)[c]	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[b]	0.89 (0.37–2.3)[c]	0.070	0.59 (0.17–1.36)	0.47 (0.006–1.43)	0.001
FE Na^+:FE K^+		0.05 (0.004–0.24)[b]	0.03 (0.004–0.1)	0.017	0.02 (0.00–0.09)	0.02 (0.00–0.15)	0.260
FE Na^+ (%)	0.00–0.69	1.3 (0.08–10.7)[b]	0.45 (0.06–2.5)	0.008	0.25 (0.03–0.94)	0.25 (0.01–1.55)	0.730
FE Cl^- (%)	0.00–1.09	1.54 (0.08–14.8)[b]	0.53 (0.07–4)	0.015	0.56 (0.15–1.5)	0.48 (0.05–2.28)	0.214
FE K^+ (%)	2.3–23.8	26.4 (8.1–93.8)[b]	17.38 (6.9–49.4)[c]	0.035	11.26 (4.27–45.2)	10.52 ± 4.04	0.240
FE Mg^{++} (%)	0–4	7.1 (1.05–22.5)[b]	4.4 (0.4–20.9)[c]	0.036	2.43 ± 1.1	2.02 ± 1.2	0.105
FE Ca^{++} (%)	0.00–0.33	1.6 (0.1–6.8)[b]	0.7 (0.07–5.2)[c]	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[b]	34.04 ± 18.6[c]	0.835	16.2 (6.07–46.5)	12.6 ± 6.84	0.035

[a] Significantly different with P<0.05 between MMVD-MG vs. H-MG.
[b] Significantly different with P<0.01 between MMVD-MG vs. H-MG.
[c] Significantly different with P<0.01 between MMVG-EG vs. H-EG.

FE Ca: fractional excretion of total calcium; FE Cl: fractional excretion of chloride; FE K: fractional excretion of potassium; FE Mg: fractional excretion of magnesium; FE Na: fractional excretion of sodium; FE Na:FE K: fractional excretion of sodium to fractional excretion of potassium ratio; FE P: 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 further comparison between MMVD-MG and H-MG, and between MMVD-EG and H-EG, respectively, was performed. Urine creatinine and urine-specific gravity were significantly lower in MMVD-MG and MMVD-EG than H-MG and H-EG, respectively (P<0.0001 and 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 P=0.0007)2, [uCa^{++}] to [uCr] (P< 0.0001 and P< 0.0001), and urine phosphate to [uCr] (P=0.0013 and P< 0.0001) ratio. 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 (P=0.0001 and P< 0.0001).

In addition, uNa^+:uCr, uCl^-:uCr, uNa^+:uK^+, 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 2, Fig. 1, Fig. 2, Fig. 3).

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 C available in Supplemental Material on-line).

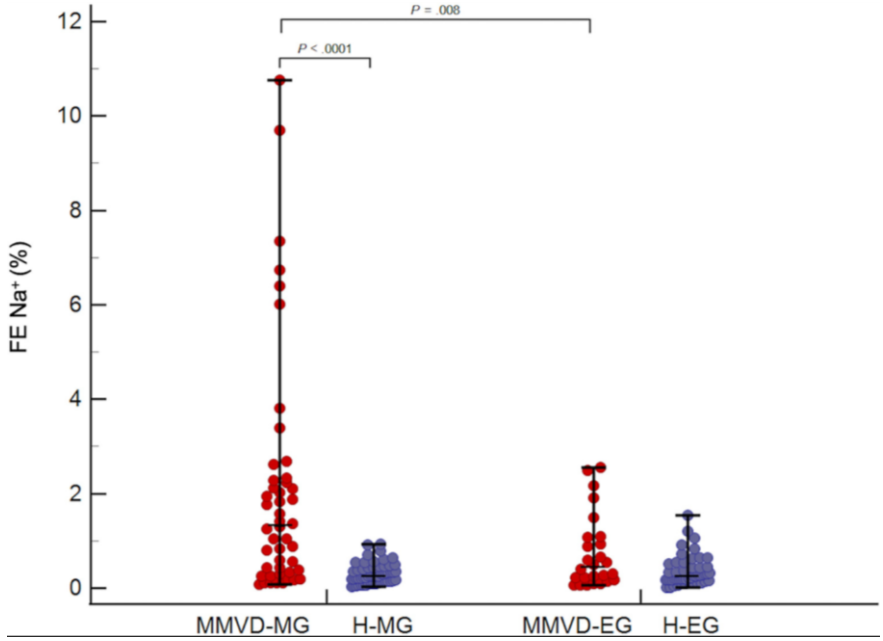

Fig. 1. Dot plot showing results of fractional excretion of sodium in dogs with myxomatous mitral valve disease sampled in the morning (n = 47) and in the evening (n = 26) (red dots) vs. healthy dogs sampled in the morning (n = 56) and in the evening (n = 50) (blue dots). Upright bars represent minimum 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 ($p<0.05$). FE Na^+: 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.

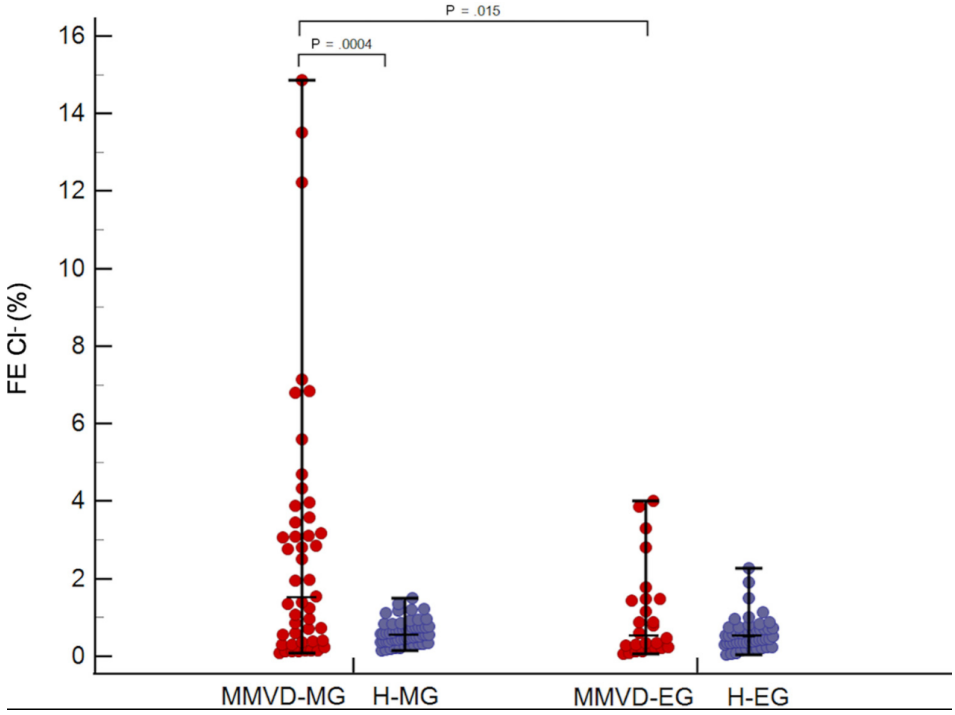

Fig. 2. Dot plot showing results of fractional excretion of chloride in dogs with myxomatous mitral valve disease sampled in the morning (n = 47) and in the evening (n = 26) (red dots) vs. healthy dogs sampled in the morning (n = 56) and in the evening (n = 50) (blue dots). Upright bars represent minimum 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 ($P<0.05$). FE Cl^-: 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.

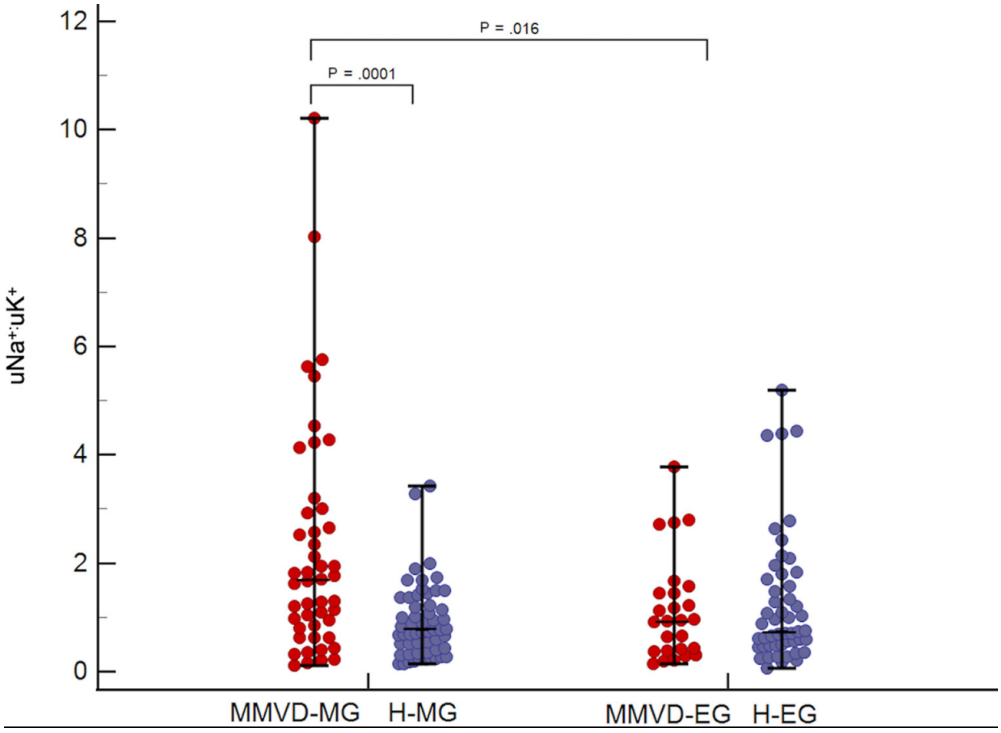

Fig. 3. Dot plot showing results of urine sodium to urine potassium ratio in dogs with myxomatous mitral valve disease sampled in the morning (n = 47) and in the evening n = 26) (red dots) vs. healthy dogs sampled in the morning (n = 56) and in the evening (n = 50) (blue dots). Upright bars represent 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.

Discussion

The present study demonstrates 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 (4,5,7,8). 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 (19,20,21). In humans, a uNa^+:$uK^+ < 1$ is representative of the inadequate urine production and diuretic resistance (21). 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 (22). 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 (23). 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 (23). 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) (8). The median uNa^+:uK^+ measured in cardiopathic ACVIM stage C dogs in another study, was 1.15 (16). 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 (8). 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 (24) or as the failure to reduce the volume of extracellular fluid despite the appropriate use of diuretics (25). Diuretic resistance affects up to 17% of people with heart failure (21) and it is suspected to also

occur in dogs (8,16,23). 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 (21,26). 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 (8,16,23). 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 (23). 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 (16). 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^+] (8). 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 (23). 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

(4,23). 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 (16). 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 (27,28). 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 (29). 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 postdiuretic 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 (30).

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 (31). 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 (32,33,34). 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 (23), 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.

The results of our study 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) (8). 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 (35). 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 (36). 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 (37,38,39) and in some experimental animal models (40,41). 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 (42,43). 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 C, available in Supplemental Material on-line). Lastly, due to the high number of hypothesis tested and comparisons performed, the possibility of cumulative type I error should be acknowledged.

Conclusion

This study demonstrates 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.

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Chapter 4

MMVD stable - CRS type II

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

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Abstract

Dogs with myxomatous mitral valve disease (MMVD) can experience progressive renal tubular damage and dysfunction. The prevalence of renal tubular damage is not known in dogs with stable MMVD. To evaluate renal tubular damage in dogs with stable MMVD by evaluation of urinary neutrophil gelatinase-associated lipocalin (NGAL). Animals: Ninety-eight MMVD dogs grouped according to the American College of Veterinary Internal Medicine (ACVIM) staging (group B1, n = 23; group B2, n = 27; group C + D, n = 48) and 46 healthy dogs. Multicenter prospective observational study. Serum and urine chemistry including NGAL reported as uNGAL concentration (uNGAL) and normalized with urinary creatinine (uNGALC) were compared between MMVD dogs and healthy controls, and among different MMVD ACVIM stages. 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). Renal tubular damage is present in dogs with stable MMVD, as measured by increased uNGAL. This tubular damage is subclinical, occurs in all stages of MMVD even in the absence of azotemia, and increases with the severity of MMVD. Reno-protective approaches to manage MMVD dogs should be explored to slow the progression of renal tubular damage in these patients.

KEYWORDS acute kidney injury, cardiorenal syndrome, heart failure, renal biomarker, tubular damage

Introduction

Myxomatous mitral valve disease (MMVD) is the most common acquired cardiovascular disease in dogs (1). The disease generally has a slow but progressive course, progressing from mitral valve insufficiency to secondary chamber dilatation and, ultimately, congestive heart failure (CHF) (1,2). Dogs with CHF require life-long pharmacological support including diuretic treatment and can develop progressive renal damage and dysfunction (1,3). Indeed, the prevalence of chronic kidney disease (CKD) seems higher in dogs with MMVD compared to the general population of dogs, (4) and azotemia increases with the severity of cardiac disease and diuretic need (5). Renal involvement associated with impaired cardiac function secondary to chronic heart diseases, as in the course of MMVD, is known as cardiorenal syndrome (CRS) type II (3,6,7). When MMVD is severe enough to cause CHF, persistent renal hypoperfusion, chronic congestion of the kidneys and maladaptive neurohormonal changes associated with chronic sympathetic stimulation occur and contribute to progressive renal damage (3,7). More specifically, 2 main mechanisms of renal damage in chronic heart diseases have been proposed: intermittent acute kidney injury (AKI) episodes or sustained kidney injury occurring simultaneously with progressive reduction of functional kidney mass (3). Currently, serum creatinine concentration (sCr) is routinely used to monitor renal function in dogs with MMVD, although it is not a sufficient indicator of early renal damage or worsening organ function and cannot discriminate between functional and structural injury (3). Neutrophil gelatinase-associated lipocalin (NGAL) is a protein belonging to the lipocalin family normally secreted in low amounts by renal tubular epithelial cells and other specific tissues (8,9). Freely circulating NGAL is filtered through the glomeruli and almost completely reabsorbed in the proximal tubules (10). Urinary NGAL concentration is very low under normal physiologic conditions. In active renal tubular injury, NGAL synthesis and secretion are increased and reabsorption is decreased, resulting in increased urinary concentrations of this biomarker (10,11) Therefore, in human and in veterinary medicine, uNGAL represents an early and sensitive biomarker of renal tubular damage (12-14). Specifically, it has been recognized as 1 of the earliest and most strongly induced proteins in both ischemic and nephrotoxic animal models of renal injury, and also has emerged as a promising biomarker for AKI in the clinical setting (12-15). Beyond the kidney, NGAL shows potential as a biomarker in cardiovascular disease (16). Increased concentrations of both serum and uNGAL have been reported in many cardiovascular conditions in people, including both acute and chronic heart diseases leading to CHF (16). To our knowledge, an increase in serum NGAL associated with the development of renal

dysfunction has been documented in dogs with acute (ie, ongoing) CHF.17 However, no data regarding uNGAL are available in dogs with stable compensated MMVD. We aimed to (1) determine whether uNGAL differs between dogs with stable MMVD and healthy controls, and (2) assess uNGAL in dogs with MMVD according to the American College of Veterinary Internal Medicine (ACVIM) staging system. Our hypothesis was that uNGAL increases in dogs with MMVD if compared to healthy control dogs, and that increasing uNGAL is detected with increasing MMVD ACVIM stage.

Material and methods

Ours was a multicentric prospective, observational case-control study performed at 2 Veterinary University Hospitals (VUH; University of Bologna and University of Parma, Italy) between March 2020 and March 2021. The study was approved by the local Scientific Ethical Committee for Animal Testing (Protocol N. 747 of October 13, 2016).

Study population

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 (GR or SC) at both centers. Dogs were eligible for inclusion if affected by MMVD at different ACVIM stages, diagnosed, and classified according to the current guidelines.1 Included dogs were in stable condition (ie, 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 (group B1); asymptomatic dogs with echocardiographic signs of left-sided cardiac remodeling (ie, 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 (group B2); dogs in which at least 1 episode of CHF had occurred were considered to be in stage C; whereas 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 (1). 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). A 12-hour fast had to be enforced before the time of sample collection, but water was always available. Exclusion

criteria were the following: presence of other acquired or congenital cardiac disease, acute CHF requiring emergency treatment (eg, hospitalization and IV administration of furosemide), presence of ≥1 concomitant systemic disease including endocrinopathies, neoplasia, International Renal Interest Society Stage (IRIS) 3 and 4 CKD, acute kidney injury (AKI), acute or chronic gastroenteropathy with malabsorption, evidence of systemic inflammatory disease or sepsis, and administration of nephrotoxic drugs or other concomitant treatments (eg, cortico-steroids, nonsteroidal anti-inflammatory drugs). In contrast, disturbances of cardiac rhythm associated with MMVD did not were not criteria for exclusion. Because urinary tract inflammation can affect uNGAL concentrations, (18) 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. Cardiac treatments routinely used for MMVD, such as diuretics (eg, furosemide, torasemide), pimobendan, angiotensin converting enzyme inhibitors (ACEI; eg, benazepril, enalapril), spironolactone, and antiarrhythmic drugs were allowed. Healthy dogs (n = 46) 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 VUH. Dogs were considered healthy in the absence of any signs of illness on clinical examination and within the previous 2 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.

Clinical and clinicopathological evaluation

Recorded clinical data were signalment, body weight, medical history, physical and echocardiographic examination 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 according to standard procedures and evaluated within 1 hour of collection as previously reported (19). When it was not possible to perform the bio-chemistry analysis within 1 hour, samples were stored at 80C for up to a maximum storage period of 2 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 tests (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 450g. 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 evaluation

Urinary NGAL was measured using a commercial sandwich ELISA according to the manufacturer's instructions (Dog NGAL ELISA kit, BIOPORTO Diagnostics, Hellerup, Denmark) and as previously reported.13 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 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 (20). 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. The concentration of uNGAL in the samples was determined by measuring the absorbance of the solution at 450 nm using an appropriate plate reader (DV990BV4 spectrophotometer, N.T. Laboratory s.r.l. Calenzano, Italy) and calculating from a standard curve using curvefitting 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).

Statistical analysis

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 nonnormal 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).

Results

Baseline characteristics. 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 1. 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 1). 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 ($P = .07$).

Table 1 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					
Number of dog receiving Spironolactone			17/48		
Spironolactone (mg/kg/d)			2 (1.2-5.4)		
Number of dog receiving Pimobendan		18/27	48/48		
Pimobendan (mg/kg/d)		0.5 (0.4-0.7)	0.6 (0.3-1)		
Number of dog receiving Enalapril			2/48		
Enalapril (mg/kg/d)			0.56 (0.32-0.8)		
Number of dog receiving Benazepril			37/48		
Benazepril (mg/kg/d)			0.61 (±0.3)		
Number of dog receiving Furosemide			42/48		
Furosemide (mg/kg/d)			4.5 (±1.9)		
Number of dog receiving Torasemide			6/48		
Torasemide (mg/kg/d)			0.4 (±0.1)		
Number of dog 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.

Clinicopathological data

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 2.

Table 2. 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)	6000-17 000	6485 (5880-7090)	6970 (4290-9470)	8815 (5600-13 700)	.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.

Urinary NGAL

Evaluation in healthy and MMVD dogs 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; Figures 1 and 2). 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 3; Figures 1 and 2).

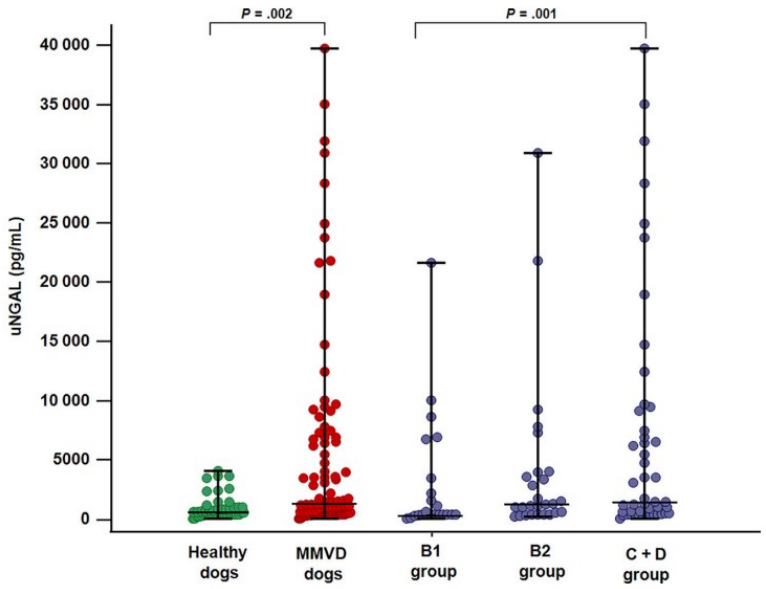

Figure 1. Dot plot showing results of urinary Neutrophil Gelatinase-Associated Lipocalin (uNGAL) comparison between healthy dogs (n = 46) (green dots) and dogs with myxomatous mitral valve disease (MMVD) (n = 98) (red dots), and among dogs with MMVD in different ACVIM stages (blue dots): group B1 (n = 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

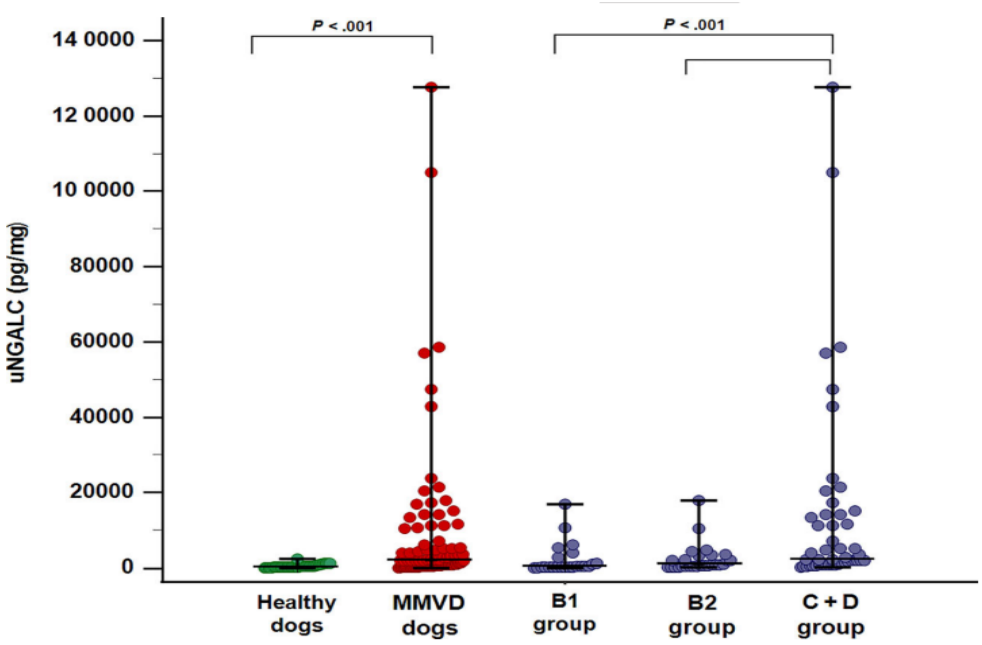

Figure 2 Dot plot showing results of urinary Neutrophil Gelatinase-Associated Lipocalin to urinary creatinine ratio (uNGALC) comparison between healthy dogs (n = 46) (green dots) and dogs with myxomatous mitral valve disease (MMVD) (n = 98) (red dots), and among dogs with MMVD in different ACVIM stages (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

In dogs with MMVD, frequency of abnormal uNGAL results (ie, 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.

Table 3. 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 (70-21 647)[c]	1231 (208-30 916)[d]	1463 (30-39 732)[a,d]	584 (56-4072)[b,c]	.001
uNGALC (pg/mg)	304 (22-16 871)[c,d]	777 (109-17 989)[c,d]	2478 (177-127 639)[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 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.

Discussion

Our study aimed to assess the presence of renal tubular damage in dogs with stable MMVD and to evaluate changes according to the severity of heart disease. The deterioration of renal function linked to chronic cardiac disease by both chronological and causal relationship (so-called CRS type II) has been a matter of growing debate in human medicine, but only scarcely characterized in veterinary medicine (3,5-7,17). 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 (4,5). For the purpose of our study, we enrolled dogs with MMVD, a disease with a high prevalence and a chronic and progressive course, sometimes requiring life-long diuretic treatment (1,2) representing an excellent research model to fill the knowledge gap. In accordance with our initial hypothesis, uNGAL is increased in dogs with MMVD compared to healthy controls and increases with worsening 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,13 but still abnormal with respect to the healthy state. In humans, serum and uNGAL are among the most studied biomarkers of tubular damage in AKI, and increases have been documented extensively in patients with CRS caused by different cardiovascular diseases, including those leading to CHF (16). For example, a previous study showed that renal impairment in patients with CHF with stable disease 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 (21) Interestingly, another study documented that serum NGAL represents a marker of renal injury in people with CHF even when sCr is within the RI (22). In humans, serum and uNGAL also correlate with the clinical severity of CHF and, occasionally, with indices of ventricular structural damage and dysfunction.16,23 In dogs, recent studies determined that serum and uNGAL act as a sensitive and specific biomarkers of AKI and tubular injury, despite being subjected to potential influence during systemic inflammation (13,24,25). 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 7 days of hospitalization compared to those with stable sCr, thus highlighting the potential role of NGAL as an early biomarker of AKI during acute CHF (17). 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 reported previously (3). 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 (3). 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 (7). During MMVD and CRS type II, different scenarios are possible ranging from a chronic decrease in effective circulating volume, with subsequent renal hypoperfusion and ischemia because of decreased cardiac output, as well as chronic renal congestion and venous hypertension causing impaired intrarenal blood flow (7,26). In this regard, the renin-angiotensin-aldosterone system (RAAS) and neurohormonal activation have been associated with fluid overload, venous hypertension, and renal interstitial

edema in humans and in canine experimental models of disease and have been implicated in CKD progression because of ongoing renal interstitial fibrosis and glomerulosclerosis in humans and in animal models of CRS type II (7,26-28). Moreover, episodic cardiac events and repeated occurrence of AKI induce progressive hypoxic and ischemic insults to the kidneys (7,29) These events, overall, can be expressed at any stage of cardiac disease, and become progressively more evident with worsening heart failure (3). All of 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 (30,31). For example, in 1 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 (32). Similar data are lacking in 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 particular aim. 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,20 and uNGAL seems to predict the risk of progressive vs stable CKD in dogs (33). 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 (4). 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 (20,33). 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 CRS type II. 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 (7,34). 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 (16). According to a previous study, higher uNGAL was reported in dogs with inflammatory AKI compared to dogs with noninflammatory AKI (13). Similar to what has been reported in human medicine, mild systemic inflammation has been reported in cardiac diseases of dogs (35-37) 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 (7,38). Moreover, NGAL upregulation has been demonstrated in the myocardial tissue of human patients who died of CHF (7,38). Similarly, in a rat model of postmyocardial infarction heart failure, NGAL/lipocalin-2 gene expression was found to be increased in cardiomyocytes in both normal and failing myocardium (8). Because cardiac remodeling occurs in dogs along with progression of MMVD, (1) 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 disease. More severe 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, (4) or because of frequent renal hypoxic insults associated with worsening heart disease and decompensation (3). Moreover, these dogs could experience progressive kidney damage leading to renal fibrosis and CKD, as previously noted and already discussed (3,4,39). 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 (17). Finally, uNGAL can reflect subclinical injury and could anticipate an increase in sCr. With regard to UPC, most of the MMVD dogs in our study were nonproteinuric or borderline proteinuric according to the IRIS guidelines for CKD (40). Interestingly, such a finding is consistent with previous studies evaluating renal compromise in dogs with MMVD (39,41). Low-grade proteinuria can be associated with tubular damage, and 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. Our study had some limitations. The main limitation 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 (42). 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 with increasing ACVIM stage (data not shown). This finding might be partially expected, because MMVD could progress with aging. 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. Because of the low number of ACVIM stage D dogs, they were incorporated with stage C cases (group C + D) for statistical purposes. This choice might have caused some heterogeneity because of different disease severities and diuretic needs. In addition, no tissue samples to evaluate for the presence

of structural kidney damage consistent with CRS type II were collected in enrolled dogs. 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 (39,41) we did not achieve the sample size recommended by the American Society of Veterinary Clinical Pathology when defining RIs (ie, ≥120 subjects) (43). For this reason, further refinement of RIs should be carried out in the future. In conclusion, our study identified the presence of renal tubular damage 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.

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Chapter 5

MMVD stable - CRS type II

Association between echocardiographic indexes and urinary Neutrophil Gelatinase-Associated Lipocalin (uNGAL) in dogs with myxomatous mitral valve disease.

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Abstract

Neutrophil gelatinase-associated lipocalin (NGAL) is a biomarker of tubular damage, and its elevation has been described in human and canine cardiorenal syndrome. The aim was to evaluate the association between echocardiographic indexes and urine NGAL (uNGAL) and uNGAL normalized to urine creatinine (uNGALC) in dogs with MMVD. This is a multicentric prospective cross-sectional study. A total of 77 dogs with MMVD at different ACVIM stages were included. All dogs underwent echocardiography, serum chemistry, and urinalysis. Echocardiographic data analyzed were shortening fraction (SF), left ventricular diastolic (LVIDDn) and systolic (LVIDSn) diameters normalized for body weight, left atrium to aortic root ratio (LA/Ao), maximal (LAV_{Max}) and minimal (LAV_{Min}) left atrial volumes, LA stroke volume (LASV), early diastolic mitral peak velocity (E_{Vmax}), E_{Vmax} to tissue Doppler E' wave (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}). 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: 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: 10.07-2.10) P = 0.015 for NGALC] remained statistically significant in the multivariable analysis. Based on our results, LASV and TR_{Vmax} are associated with increased uNGAL and uNGALC. These parameters might detect dogs with MMVD at higher risk of developing kidney damage.

Keywords: Cardiorenal syndrome; Heart failure; MMVD; Renal biomarkers; Renal damage.

Introduction

The heart and kidneys are closely related, whereby acute or chronic dysfunction of one organ may lead to acute or chronic dysfunction of the other (Jung et al., 2018; Ronco et al., 2008). Heart failure, whether resulting from systolic or diastolic dysfunction or both, can be associated with renal dysfunction (Andrukonis et al., 2014). In human medicine, progressive chronic kidney disease (CKD) due to primary chronic heart disease is known as cardiorenal syndrome (CRS) type 2 (Ronco et al., 2008; Uduman, 2018). In humans, renal function is strongly and independently associated with prognosis in patients with congestive heart failure (CHF) (Hillege et al., 2006). One study found that CKD prevalence in dogs with myxomatous mitral valve disease (MMVD) was significantly higher compared to healthy dogs and that the American College of Veterinary Internal Medicine (ACVIM) class of cardiac failure (Keene et al., 2019) and the International Renal Interest Society (IRIS) stage were directly related (IRIS, modified 2023; Martinelli et al., 2016). Serum creatinine concentration is currently the most widely used marker for monitoring kidney function in dogs with MMVD. However, it is a late indicator of kidney damage or worsening renal function and is unable to discriminate between functional and structural injury (Orvalho and Cowgill, 2017). As the early recognition of renal involvement would allow therapy modification to promote a mutual benefit for both organs, the use of novel early biomarkers of renal damage has been proposed (Orvalho and Cowgill, 2017). Neutrophil gelatinase-associated lipocalin (NGAL) is a protein belonging to the lipocalin family, expressed by neutrophils and many other cells, including renal tubular epithelial cells, pulmonary cells, and cardiomyocytes (Cruz et al., 2012; Jung et al., 2018). Neutrophil gelatinase-associated lipocalin has emerged as a promising and sensitive biomarker of early acute kidney damage in different settings because it quickly appears in blood and urine in response to tubular renal damage (Shrestha et al., 2011). 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 a few reports in this regard. In one study, urinary NGAL was increased in dogs with stable MMVD compared to healthy dogs; moreover, urinary NGAL was higher in dogs at more advanced ACVIM stages (Troia et al., 2022). Another study, including dogs with acute heart failure, found a strong positive correlation between serum NGAL concentrations and left atrium to aortic root ratio (LA/Ao) (Jung et al., 2018). In the present study, we aimed to evaluate the correlation between selected echocardiographic variables indicative of reduced cardiac output and venous congestion, and urinary NGAL in

dogs with MMVD at different ACVIM stages. We hypothesized that some echocardiographic indexes of disease severity were associated with increased concentration of urinary NGAL in dogs with MMVD, regardless of the ACVIM stage.

Material and methods

This multicentric prospective observational cross-sectional study was performed at two Veterinary Teaching Hospitals of the University of Parma and the University of Bologna between March 2020 and April 2021. The local scientific and ethical committee approved the study for animal testing and animal utilization (Protocol N. 747 of October 13th, 2016); the study was conducted with informed owner consent. The STROBE guidelines were used to ensure the reporting of this observational study (Sargeant et al., 2016; von Elm et al., 2007).

Study population. Privately owned dogs were enrolled at the Cardiology services of both Institutions. Dogs were eligible for inclusion if affected by MMVD at different ACVIM stages, diagnosed, and classified according to current guidelines (Keene et al., 2019). For the inclusion, 12-h fasting had to be guaranteed at the time of sample collection; specific diet restrictions were not recommended, and owners could feed dogs with their preferred diets. Exclusion criteria were the following: 1) presence of other acquired or congenital cardiac disease; 2) acute CHF requiring emergency treatment (e.g., hospitalization with the administration of intravenous diuretic); 3) presence of one or more concomitant systemic diseases including endocrinopathies, neoplasia, CKD IRIS stage 3 and 4, acute kidney injury, clinical evidence of systemic inflammatory disease or sepsis, and symptomatic acute or chronic gastro-enteropathy with malabsorption; 4) presence of pyuria on fresh urine sediment examination (>5 white blood cells per high power field) as well as clinical and laboratory signs of urinary tract infection or inflammation; 5) concomitant treatments with potentially nephrotoxic drugs (e.g., non-steroidal anti-inflammatory drugs, aminoglycosides).

Clinical and clinicopathological data. Recorded clinical data were signalment, body weight, history, and physical examination findings; the current medications and dosage at inclusion were registered; the duration of the treatment before the inclusion was not recorded. On the same day of the echocardiographic exam, blood was collected by standard venipuncture using blood vacuum collection systems; concurrent fresh urine samples were collected by free catch or cystocentesis. All the laboratory analyses were performed at the clinical pathology laboratory of one of the VTHs (University of Bologna). Blood and urine specimens were processed routinely, according to quality standard procedures, and evaluated within one hour of collection.

When it was impossible 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. 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), dipstick test (ComburTest® 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-min centrifugation at 450 ×g. Urine supernatants were immediately analyzed for dipstick examination and then used for chemical analyses or stored. Urine chemistry was determined with the same automated chemistry analyzer used for serum chemistry and included urinary creatinine and total protein and urine protein to creatinine ratio.

Urinary NGAL analysis. According to the manufacturer's instructions, urinary NGAL was measured using a commercial ELISA sandwich (Dog NGAL ELISA kit, BIOPORTO Diagnostics, Hellerup, Denmark) and as previously reported (Monari et al., 2020). Samples of urine supernatant were preserved at − 80 ◦C for a maximum of 2 months before being analyzed. We validated the assay for dogs in our laboratory using a protocol that included testing for linearity and intra-assay variation (< 5%), and the validation results were comparable to the ones previously reported; moreover, the results were consistent with those reported by the manufacturer (Steinbach et al., 2014). Results were reported as urinary NGAL concentration (uNGAL; pg/mL) and uNGAL to urine creatine ratio (uNGALC; pg/mg) (Troia et al., 2022).

Cardiologic evaluation. Transthoracic echocardiographic examination, including M-mode, two-dimensional, and Doppler analysis, was carried out and reviewed by board-certified cardiologists in each dog according to the standard technique (Chetboul and Tissier, 2012; Thomas et al., 1993). Echocardiographic machines (iE33 ultrasound system and Epiq CVx ultrasound system; Philips Healthcare, Monza, Italy) were equipped with phasedarray transducers ranging from 1.6 to 6 MHz. Dogs were gently restrained in lateral recumbency and scanned underneath, from right and left parasternal positions, without sedation. 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 on Doppler examination (Chetboul and Tissier, 2012). From the right parasternal short axis view shortening fraction (SF), left ventricular diastolic (LVIDDn) and systolic (LVIDSn) diameters normalized for body weight, obtained by M-mode images (Cornell et al., 2004), and LA/Ao,

obtained by B-mode images (Hansson et al., 2002), were measured; maximal (LAVMax) and minimal (LAVMin) left atrial volumes were measured with monoplane area-length method from the left parasternal four-chamber view. The left atrial stroke volume (LASV) was calculated according to the formula: LASV = LAVMax – LAVMin (Hollmer et al., 2017). The aortic velocity time integral (VTIAo) was measured from the subcostal view; the mitral velocity time integral (VTIMit), the early diastolic mitral peak velocity (EVmax), the ratio between EVmax and the E' wave of the tissue Doppler of the parietal mitral annulus (E/E') were measured from the left parasternal apical fourchamber view. The ratio between the VTIMit/VTIAo was then calculated. Tricuspid regurgitation velocity (TRVmax) was measured from the left parasternal apical view, angled to optimize the alignment with the tricuspid regurgitant flow. In the absence of right ventricular outflow obstruction, the presence of pulmonary hypertension (PH) was considered when TRVmax velocity was >3.4 m/s (corresponding to a systolic pressure gradient >46 mmHg using the modified Bernoulli equation), with intermediate-high probability (Reinero et al., 2020). All measurements were replicated on three consecutive beats, and the median values were then calculated. At the end of the echocardiographic exam, the presence of ascites was ultrasonographically evaluated. The presence of arrhythmias was based on a 6- or 12-lead surface ECG of least 1-min duration or a good quality 1-lead ECG during the echocardiographic examination. In particular, the diagnosis of atrial fibrillation (AF) was based on the combined presence of the following findings: irregularly irregular cardiac rhythm with narrow QRS complexes, lack of recognizable P waves, and absence of A wave on mitral inflow on Doppler analysis (the latter finding in the case of dogs diagnosed to be affected by AF during echocardiographic examination) (Pedro et al., 2020).

Statistical analysis. 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, PH, 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. Based on their distribution, results were presented as mean ± standard deviation or median and range (minimum-maximum value). Dogs were divided into two groups based on the presence of normal or abnormal (increased) uNGAL (normal uNGAL values 5 (Johnston et al., 2018; Kim, 2019). Significant results from logistic regression analysis were also presented with Receiver Operating Characteristic (ROC) curve 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 values from 0 (a perfectly inaccurate test) to 1 (a perfectly accurate test), with 0.5 suggesting no discrimination, 0.6 to 0.7 poor, 0.71 to 0.8 fair, 0.81 to 0.9 good, and >0.9 outstanding. The Fisher's exact test was used to explore any association between tricuspid regurgitation or PH and increased NGAL; the risk of increased uNGAL or uNGALC in patients with tricuspid regurgitation or PH was explored with the calculation of relative risk (RR). A linear regression model was built to explore any effect of the diuretic dosage on uNGAL and uNGALC results. 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 a non-linear relationship between the independent and the highly skewed dependent variables, a logarithm transformation of the latter was done. Values of P 0.90; $\alpha = 0.05$ and $\beta = 0.10$) was 76 dogs, with an effect size of 0.7 3.

Results

Eighty dogs initially matched the inclusion criteria; however, three were excluded due to the poor echocardiographic image quality. Therefore, 77 dogs with MMVD at different ACVIM stages were included in the study. Forty-three out of 77 were males (55.8%) and 34/77 females (44.2%). Dogs of various breeds were included: 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 and 1 Spinone Italiano. The mean age was 11.7 (± 2.8) years, and the median body weight was 8.9 (2.4–20) kg. The distribution of the population in the different ACVIM stages was: 19/77 dogs (24.7%) at stage ACVIM B1, 22/77 dogs (28.6%) at stage ACVIM B2, 32/77 dogs (41.6%) at stage ACVIM C, and 4/77 dogs (5.2%) at the stage ACVIM D. At the time of inclusion 27 dogs didn't receive any treatment, and 35 dogs were treated with furosemide. Other therapies included pimobendan (50/77 dogs, 64.9%), benazepril (31/77 dogs, 40.3%), spironolactone (10/77 dogs, 130.0%), digoxin (3/77 dogs, 3.9%), enalapril (2/77 dogs, 2.6%), and sildenafil (1/77 dog, 1.3%). Fifty out of 77 dogs (64.9%) had tricuspid regurgitation, 10/77 (130.0%) had PH, and 3/77 dogs (3.9%) had AF; no other arrhythmias were recorded. Six out of 77 dogs (7.8%) had ascites; among these dogs, 1/6 had AF, 1/6 had PH, and 1/6 had both PH and AF. The dogs with increased uNGAL and uNGALC values were 33/77 (43%) and 39/77 (51%), respectively.

The difference in serum creatinine concentration between groups with normal and increased uNGAL (P = 0.333) and uNGALC (P = 0.421) was not significant (Table 1).

Table 1 Data comparison between dogs with myxomatous mitral valve disease (MMVD), grouped according to their normal or elevated uNGAL and uNGALC values. Results are reported as median and range values for every group; the number of dogs (N) and P values are also reported.

Clinicapathologic and echocardiographic variables	uNGAL									uNGALC								
	< 2300 (pg/ml)				≥ 2300 (pg/ml)				P	< 1400 (pg/mg)				≥ 1400 (pg/mg)				P
	Median	Min	Max	N	Median	Min	Max	N		Median	Min	Max	N	Median	Min	Max	N	
Serum creatinine (mg/dl)	1.0	0.6	2.43	44	1.1	0.57	2.76	33	0.333	1.0	0.66	2.1	38	1.1	0.57	2.76	39	0.421
E/E'	9.7	5.8	16.1	31	11.1	7.2	23.3	12	0.110	9.7	5.8	14.9	25	10.6	7.2	23.3	18	0.056
E_{Vmax} (m/s)	1.0	0.52	1.73	44	1.25	0.56	1.8	33	0.077	1.0	0.57	1.73	38	1.26	0.52	1.8	39	**0.033***
LA/Ao	1.7	1.18	3.12	44	2.01	1.03	2.79	33	**0.049***	1.6	1.18	3.12	38	1.9	1.03	2.79	39	**0.041***
LASV (ml/kg)	1.1	0.21	3.55	44	1.72	0.4	5.58	32	**0.007***	0.9	0.21	3.11	38	1.75	0.59	5.58	38	**< 0.001***
LAV_{Max} (ml/kg)	2.5	0.43	10.45	44	3.88	0.67	10.81	33	**0.025***	2.2	0.43	6.78	38	3.99	0.84	10.81	39	**0.002***
LAV_{Min} (ml/kg)	1.0	0.13	6.91	44	1.67	0.25	7.87	32	**0.045***	0.7	0.13	4.6	38	1.68	0.23	7.87	38	**0.010***
LVIDDn ($cm/kg^{0.294}$)	1.9	1.3	2.6	44	2.1	1.5	3	33	**0.027***	1.8	1.3	2.4	38	2.1	1.5	3	39	**0.007***
LVIDSn ($cm/kg^{0.315}$)	1.0	0.6	1.3	44	1.0	0.6	1.5	33	0.265	1.0	0.6	1.3	38	1	0.6	1.5	39	0.389
SF (%)	45.1	22	63.4	44	47	32.4	67	33	0.207	44.2	22	63.4	38	48.2	35	67	39	**0.030***
TR_{Vmax} (m/s)	1.8	0	4.08	44	2.64	0	4.2	33	**0.001***	1.8	0	4.08	38	2.54	0	4.2	39	**0.017***
VTI_{Ao} (cm)	11.0	7	19	44	10	4	17	32	0.112	11.5	7.7	19	38	10	4	19	38	0.051
VTI_{Mit} (cm)	14.0	7	21	43	15	9	23.7	31	0.210	14.0	7	20	37	15	9	23.7	37	**0.012***
VTI_{Mit}/VTI_{Ao}	1.3	0.5	3	43	1.62	0.64	3	30	**0.020***	1.3	0.5	2.42	37	1.65	0.56	3	36	**0.003***

E/E', E to E' waves ratio; EVmax, E wave peak velocity; LASV, left atrial stroke volume; LA/Ao, left atrium to aortic root ratio; LAVMax, left atrium maximal volume; LAVMin, left atrium minimal volume; LVIDDn, normalized left ventricular end-diastolic diameter; LVIDSn, normalized left ventricular end-systolic diameter; SF, shortening fraction; TRVmax, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTIAo, Aortic velocity-time integral; VTIMit, Mitral velocity-time integral; VTIMit/VTIAo, Mitral to aortic velocity-time integral ratio. * Significantly different between groups (P < 0.05).

Dogs with increased uNGAL and uNGALC had significantly higher LA/Ao, LASV, LAVMax, LAVMin, LVDDn, TRVmax, and VTIMit/VTIAo. Furthermore, dogs with abnormal uNGALC had higher EVmax, SF, and VTIMit (Table 1). In the univariate analysis, LASV, TRVmax, LAVMax, LVIDDn, and VTIMit/VTIAo were independent predictors of increased uNGAL and uNGALC (Table 2); 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 TRVmax [(OR: 1.73, 95% CI: 1.20–2.51), P = 0.002 for NGAL, and (OR: 1.50, 95% CI: 10.07–2.10), P = 0.015 for NGALC] remained statistically significant in the multivariable analysis. A moderate correlation between LASV and LAVMax (VIF = 3.3), LVIDDn (VIF = 3.2), and VTIMit/VTIAo (VIF = 3.4) was found, whereas a mild correlation between LASV and TRVmax (VIF = 1.1) was detected.

Table 2 Univariate Logistic Regression Analysis. Strength of the relationship between dependent (uNGAL and uNGALC) and independent variables (echocardiographic indexes).

Echocardiographic parameters	uNGAL ≥2300 (pg/ml)			uNGALC ≥1400 (pg/mg)		
	OR	95% CI	P	OR	95% CI	P
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***

CI, Confidence interval; EVmax, E wave peak velocity; LA/Ao, left atrium to aortic root ratio; LASV, left atrial stroke volume; LAVMax, left atrium maximal volume; LAVMin, left atrium minimal volume; LVIDDn, normalized left ventricular enddiastolic diameter; SF, shortening fraction; TRVmax, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTIMit, Mitral velocity-time integral; VTIMit/ VTIAo, Mitral to aortic velocity-time integral ratio.

Areas under the ROC curve for LASV as a predictor variable to classify patients with abnormal uNGAL and uNGALC 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 (Fig. 1A-B).

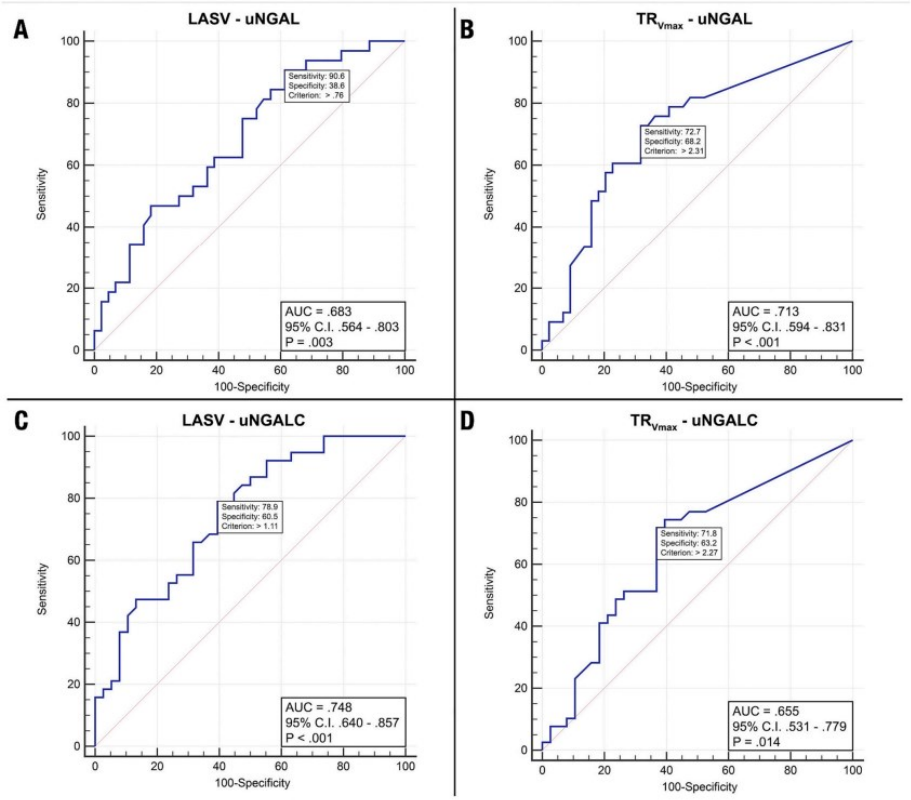

Fig. 1. Receiver operating characteristic curves for LASV and TRVmax as predictors variables to classify patients with abnormal uNGAL (A, B) and uNGALC (C, D). AUC = Area under the ROC curve.

Areas under the ROC curve for TRVmax, as a predictor variable to classify patients with abnormal NGAL values, were 0.71 (95% C.I. 0.59–0.83; P < 0.001) for uNGAL and 0.66 (95% C.I. 0.53–0.78; P = 0.014) for uNGALC (Fig. 1C-D). The best compromises ficity: 90.62%; sensitivity: 38.64%) and > 1.11 (specificity: 78.95%; sensitivity: 60.53) for uNGAL and uNGALC, respectively; whereas for TRVmax 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. There was a statistically significant association between tricuspid regurgitation and abnormal NGAL values (P = 0.009 for uNGAL; P = 0.033 for uNGALC), but the association with PH was not significant (P = 0.311 for uNGAL; P = 0.737 for uNGALC). The RR to have increased uNGAL or uNGALC for dogs with tricuspid regurgitation was 2.43 (95% CI: 1.15 to 5.15; P = 0.02) for uNGAL and 1.80 (95% CI: 10.01 to 3.21; P = 0.047) for uNGALC. In patients with PH, the elevation in risk to have abnormal values was not statistically significant for both uNGAL [RR: 1.49, 95% CI: 0.83 to 2.67 (P = 0.182)] and uNGALC [RR: 1.22, 95% CI: 0.70 to 2.14 (P = 0.491)]. The fitted regression models to test the relationship between daily furosemide dosage and uNGAL, uNGALC values were: uNGAL =

30.0670 + 0.09357 * (mg/kg/day) Furosemide) and uNGALC = 3.1874 + 0.1224 * (mg/kg/day of Furosemide). The overall regressions were not statistically significant (R2 = 0.045, F (1,33) = 1.55, P = 0.222) for uNGAL and (R2 = 0.080, F (1, 33) = 2.87, P = 0.1) for uNGALC. It was found that diuretic dosage did not influence either uNGAL (β = 0.094, P = 0.222) or uNGALC (β = 0.122, P = 0.1) values (Fig. 2).

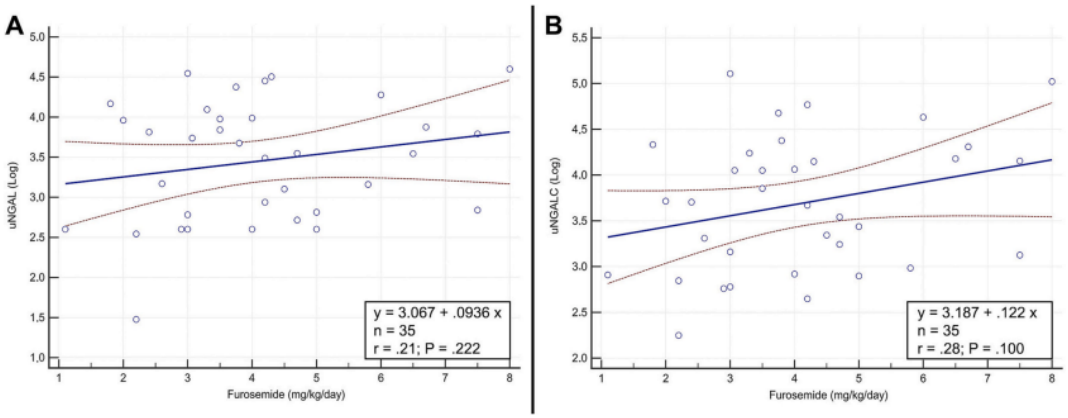

Fig. 2. Relationships between Furosemide daily dosage (mg/kg/day) and uNGAL (A) or uNGALC (B) values.

Discussion

This study showed that LASV and TRVmax are associated with increased uNGAL and uNGALC in dogs affected by MMVD. Moreover, we found evidence that diuretic treatment does not affect uNGAL and uNGALC values. Given the complexity of the pathophysiological interplay between the heart and kidneys, we choose to evaluate the influence of cardiac function on biomarkers of renal tubular damage by analyzing echocardiographic indexes related to systolic function, cardiac output, volume overload, and mitral regurgitant volume (Baron Toaldo et al., 2018; Borgarelli et al., 2008; Franchini et al., 2021a; Hollmer et al., 2017; Oricco et al., 2019; Reinero et al., 2020; Schober et al., 2011; Tribouilloy et al., 1994). Several echocardiographic indexes (LASV, TRVmax, LAVMax, LVIDDn, and VTIMit/VTIAo) were associated with increased uNGAL and uNGALC in our study; however, in the multivariable analysis, only TRVmax and LASV remained significantly associated. It should be noted that LASV, LAVMax, LVIDDn, and VTIMit/VTIAo are all markers of left-side volume overload and are mutually connected; therefore, we hypothesize that LASV was the only significant echocardiographic index at the multivariable analysis due to the multicollinearity detected between these variables. Left atrial stroke volume is an echocardiographic index of atrial function strongly related to the maximum atrial volume and MMVD severity (Hollmer et al.,

2017), and human patients with high atrial volume had a higher risk of renal damage and progression (Paoletti and Zoccali, 2014). On the contrary, a reduction of LASV is associated with a relative increase of minimum atrial volume compared to the maximum, as it happens in AF without atrial volume overload (Fatema et al., 2009). In the population of dogs taken in the study, we had only 3 patients with AF; therefore, the low number probably did not affect this parameter; moreover, they were all ACVIM C and D dogs in which LASV probably could be increased by severe atrial remodeling and not strongly related to the actual atrial function. Generally, we assume that pulmonary edema is the only direct consequence of increased left atrial volume; however, in humans, the left-side volume overload, associated with increased left atrial and ventricular diastolic pressures, also reduces right-sided compliance due to ventricular interdependence and could result in systemic congestion and increased renal venous pressures (Naeije and Badagliacca, 2017). This mechanism could explain the higher uNGAL in dogs with larger left atrial size; however, due to the lack of data, we can't evaluate the effect related to the right-size remodeling. The cardiac output intuitively influences the renal perfusion; however, in humans, venous congestion has been documented to damage kidneys more than hypoperfusion due to receded antegrade systolic flow, a condition known as “congestive nephropathy” (Damman et al.,2009) Indeed, 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. Our study evidenced a relationship between elevated uNGAL and uNGALC and the presence of TR and TRVmax as an index of the right ventriculo-atrial gradient. This result could confirm the role of tricuspid regurgitation and right-size pressures in developing tubular damage due to congestive nephropathy. Unfortunately, the extent of the right-sided impairment and the quantification of the tricuspid regurgitation severity were impossible due to the lack of right-sided cardiac dimension measurements. In the study population, there were 50 dogs with tricuspid regurgitation; 10 of them had PH with a tricuspid regurgitant velocity higher than 3.4 m/s (Reinero et al., 2020). The association between tricuspid regurgitation and renal biomarkers was statistically significant, as well as the relative risk of having abnormal uNGAL and uNGALC for patients with MMVD and tricuspid regurgitation; nevertheless, the clinical relevance (Andrade, 2015) was reached for uNGAL but not for uNGALC because of a RR higher risk of developing renal damage potentially related to renal congestion. Further studies on a larger sample size are needed to confirm these findings and explore the clinical application of NGAL to prevent CRS in dogs.

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Chapter 6

MMVD CHF - CRS type I

Neutrophil gelatinase-associated lipocalin (NGAL) as biomarker of acute kidney injury (AKI) in dogs with congestive heart failure (CHF) due to myxomatous mitral valve disease (MMVD)

UNPUBLHISED

Introduction

The term cardiorenal syndrome (CRS) describes the connection between the cardiovascular and renal systems, whereby the acute or chronic dysfunction of one of these two organs may lead to the acute or chronic dysfunction of the other (Ronco et al., 2008; Pouchelon et al., 2015; Di Lullo et al., 2017; Prastaro et al., 2022).

The CRS type 1, or acute cardio-renal syndrome, refers to the development of acute kidney injury (AKI) in the course of acute congestive heart failure (CHF) (Ronco et al., 2012; Di Lullo et al., 2017). Irrespective of the different hemodynamic profiles that might affect these patients, both the reduction in effective circulation fluid volume or the increase in central venous pressure can lead to a decrease in glomerular filtration rate (GFR) and AKI development (Ronco et al. 2012; Di Lullo et al., 2017; Verma et al. 2022). In addition, non-hemodynamic causes of CRS type 1 include neurohormonal activation, tubular collapse due to increased renal interstitial pressure, chronic inflammation and oxidative damage (Ronco et al., 2012; Di Lullo et al., 2017). In humans, worsening renal function is an important independent prognostic factor for adverse events including cardiovascular mortality, longer length of in-hospital stays and CHF re-hospitalization (Fu et al., 2021). Although such renal impairment is usually regarded as an acute increase in serum creatinine (sCr), a precise definition is lacking. Moreover, sCr has pitfalls for the diagnosis and quantification of CRS type I: its relationship with GFR is non-linear and exponential, especially in the acute setting, and its uplift is considered an insensitive biomarker to detect AKI and estimate the degree of tubular damage (Ronco et al., 2008; Ferrari et al., 2020; Fu et al., 2021; Gembillo et al., 2021). A multitude of AKI biomarkers, including neutrophil gelatinase-associated lipocalin (NGAL), have been investigated in this regard, with controversial results (Ferrari et al., 2020; Fu et al., 2021; Dutta et al., 2023).

Occurrence of renal damage in dogs affected by different cardiac diseases has been described in veterinary literature (Pouchelon et al., 2015; Martinelli et al., 2016; Giorgi et al. 2022; Troia et al., 2022). However, the characterization of this injury has not been well defined, especially in the setting of acute CHF, whereby the increase in sCr could simply be the consequence of appropriate decongestion and less diuretic resistance (Giorgi et al., 2022). Combining sCr changes with biomarkers of tubular damage like NGAL could offer better insights for the understanding AKI in canine CHF. In a recent study including dogs with acute decompensated myxomatous mitral valve disease (MMVD), enrolled patients had higher serum NGAL than healthy ones. Moreover, those developing worsening renal function within 7 days showed higher serum NGAL concentrations upon admission 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). In the current study, we aimed to 1) assess the occurrence and characterize AKI in dogs with MMVD in acute CHF; 2) evaluate the role of urinary NGAL for the prediction of AKI during CHF.

Material and Methods

Study design

This is a multicentric prospective observational study performed at two Veterinary Teaching Hospitals (VTHs) (Veterinary Teaching Hospitals of the University of Parma and of the University of Bologna), between April 2020 and May 2021. The study was approved by the local Scientific Ethical Committee for Animal Testing (Protocol N. 747 of 13/10/2016).

Study population

Privately-owned dogs with a previous or newly diagnosed MMVD according to the current ACVIM guidelines (Keene et al., 2019), were eligible for inclusion in the present study. Specifically, dogs were recruited if presented in acute CHF requiring emergency in-hospital treatment with IV diuretics (furosemide or torasemide) for at least 24 hours.

Diagnosis of CHF was based on the following criteria: clinical signs consistent with CHF (increased respiratory rate and effort, cough, exercise intolerance); echocardiography showing severe left heart disease; radiographic evidence of pulmonary edema including interstitial and alveolar lung patterns; positive clinical response to diuretic treatment. All clinical examinations and cardiac ultrasound examinations were performed or reviewed by a board-certified cardiologist (Giovanni Romito or Serena Crosara) at both centers. Stages C and D dogs with CHF were classified according to published guidelines (Keene et al., 2019).

Dogs with other acquired or congenital cardiac disease, systemic diseases including International Renal Interest Society Stage (IRIS) 3 and 4 CKD, evidence of sepsis, presence of pyuria on fresh urine sediment examination (>5 white blood cells per high power field) or urinary tract infection, and administration of nephrotoxic drugs, were excluded from the study. In contrast, the presence of disturbances of cardiac rhythm associated with MMVD did not preclude exclusion. Healthy dogs (n = 46) and dogs with stable MMVD (n = 98) included in a previous study on urinary NGAL performed by our research group (see Chapter 4), were used for comparative purposes. Enrolled dogs were treated with IV furosemide or torasemide (CRI or bolus, at the discretion of the attending clinicians). Individual clinical assessment and clinical needs guided the treatment with oxygen therapy through nasal cannula or oxygen cage.

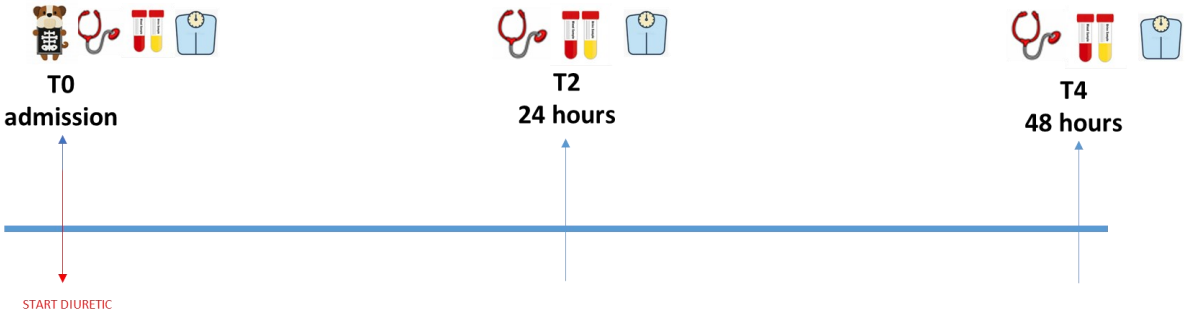
Data collection and analysis

For the purposes of the study, complete clinical and clinicopathological data were collected at the time of hospital admission (T0), and after 24 hours (T1) and 48 hours (T2) of hospital stay. At T0, the following data were collected: signalment, body weight, medical history including comorbidities and prior treatments, physical, echocardiographic and radiographic examination findings. At T1 and T2 we collected: signalment, body weight, therapies administered including the need and duration (hours) of oxygen supplementation, and the type and dose of diuretics administered to treat CHF in the first 24 and 48 hours of hospital stay (mg/kg/day).

When deemed possible based on the animal clinical status, non-invasive blood pressure measurement (SunTech® Vet20TM Veterinary Blood Pressure Monitor, SunTech Medical, Inc., USA) and pulse oximetry (Nellcor SpO2 module for Dash 3000, GE Healthcare, Rahway, NJ), were performed. Duration of hospitalization and final outcome, defined as survival to hospital discharge, death, or euthanasia for disease severity, were additionally recorded.

At T0, blood was collected by standard venipuncture using blood vacuum collection systems; concurrent fresh urine samples were collected by spontaneous voiding or cystocentesis (see Chapter 3 and Chapter 4), within an hour from hospital admission and diuretic administration. Sampling was repeated at T1 and T2 (Fig. 1).

Fig 1. Timeline. At timepoint 0 (admission) we collected: thoracic radiography, history, physical examination, bodyweight, therapies and, serum and urine samples. At each timepoint physical examination, bodyweight, therapies and serum and urine samples.



Blood was collected in plain tubes with gel separators, allowed to clot at room temperature, then centrifuged and divided into two aliquots. One aliquot was analyzed within 60 minutes of collection to assess parameters necessary for managing the hospitalized patient, while the other was stored at −80°C for up to 2 months and used for research purposes (sCr, urea, C-reactive protein [CRP], and serum electrolytes). Urine samples were centrifuged (1000g for 10 minutes) and examined within 1 hour of collection. For uNGAL measurement, aliquots of the urine supernatant were stored at −80°C for up to 2 months until analysis.

The following clinicopathological analyses were performed at the clinical pathology laboratory of one of the VTHs (University of Bologna): serum chemistry including sCr, urea, CRP, and serum electrolytes (AU480, Beckman Coulter, Brea, California, USA); urinalysis including urine specific gravity (USG) evaluated using a hand-held refractometer (American Optical, Buffalo, NY), dipstick tests (Combur-Test® 10 UX, Roche, Switzerland) read by an automated reader (URISYS 1100, Roche, Switzerland) and the evaluation of urine sediment at low (100x) and high (400x) power field; urinary chemistry, including urinary creatinine (uCr), total protein concentrations, urine protein-to-creatinine ratio (UPC), and urinary electrolytes (AU480, Beckman Coulter, Brea, California, USA).

Urinary NGAL evaluation

Urinary NGAL was measured using a commercial sandwich ELISA according to the manufacturer's instructions (Dog NGAL ELISA kit, BIOPORTO Diagnostics, Hellerup, Denmark) and as previously reported (see Chapter 4). Aliquots of the urine supernatant of dogs with CHF enrolled in the study were stored at -80 C for up to 2 months until assayed. The assay was performed as previously described (see Chapter 4). The assay was validated in our laboratory for dogs following a validation protocol including linearity and intra-assay variation, and validation results were similar to those previously reported and consistent with those reported by the manufacturer (Steinbach et al., 2014). Specifically, the regression coefficient's linearity check yielded a calculated R2 of 0.985 ($P < 0.001$) in each run. The intra-assay CV

was determined by calculating the standard deviation (SD) and mean of the two duplicate measurements for each dog, and then dividing the SD by the mean, resulting in a CV of 6.3%. Urine samples from CHF dogs an initial dilution of 1:100 was used, followed by1:300, 1:500 and 1:900 dilutions for samples where analyte concentration could not be determined. The concentration of uNGAL in the samples was determined by measuring the absorbance of the solution at 450 nm using an appropriate plate reader (DV990BV4 spectrophotometer, N.T. Laboratory s.r.l. Calenzano, Italy) and calculated 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).

Definition of AKI and animal grouping

Acute kidney injury was defined as an increase in sCr of ≥0.3 mg/dL within 48 hours from admission and was graded based on IRIS guidelines (http://www.iris-kidney.com/pdf/4_ldc-revised-grading-of-acute-kidney-injury.pdf).

Enrolled dogs were grouped based on the occurrence of AKI 48 hours from CHF diagnosis and hospital admission (CHF AKI vs. CHF no-AKI dogs).

Baseline sCr values were retrospectively obtained from patients' medical records, based on sCr measurements taken within 1 month prior to the occurrence of CHF and their inclusion in the study, only when available. Similarly, creatinine levels were recorded retrospectively at 7 days from T0, only if available. Dogs with CHF were additionally compared with a group of stable MMVD dogs (n=98), and healthy controls (n=46), included in a previous study on CRS published by our study group. Specifically, these were privately-owned dogs affected by MMVD at different ACVIM stages diagnosed and classified according to the current guidelines (Keene et al., 2018), enrolled during cardiological re-checks and with a stable clinical condition, as previously described (see Chapter 4).

Statistical analysis

Data distribution was assessed graphically and using the Shapiro-Wilk test. Data were expressed by means of standard descriptive statistics and presented as mean ± SD or median and range (minimum-maximum) based on normal or non-normal data distribution, respectively. Variables of interest in dogs with CHF were compared among different timepoints using the Wilcoxon test for comparisons between timepoint (T0 vs T1; T1 vs T2; T0 vs T2). The Kruskal-Wallis test with post hoc comparison (Conover test), the Mann Whitney U test and the Student's t-test were used to compare different study groups (healthy dogs vs. stable MMVD vs. CHF

dogs; CHF AKI vs. CHF no-AKI dogs). Categorical variables among groups were assessed using both the Chi-squared test and Fisher's exact test for 2x2 tables. The Spearman's correlation coefficient was used to assess potential correlations between variables. Results were considered significant if $P < 0.05$.

We conducted a power analysis to estimate the necessary sample size for our study. Utilizing prevalence data for the development of AKI from literature (Giorgi et al., 2022), we set the Type I error (α) to 0.05, Type II error (β) to 0.2, and the Null Hypothesis value (%) to 25%. The minimum required sample size obtained was 31 dogs. Statistical analyses were performed using a commercially available statistical software package (MedCalc Statistical Software version 19.5.1; Ostend, Belgium).

Results

Baseline characteristics

The study population consisted of 30 CHF dogs: 25/30 (83%) in stage ACVIM C and 5/30 (17%) in stage ACVIM D. Overall, 19/30 were males (2/19 neutered) and 11/30 were females (3/11 spayed); 18/30 were mixed breed dogs whereas 12/30 were purebred dogs. Median body weight was 7.7 kg (range, 2.7-29.3 kg) and mean age was 12±2.2 years. At the time of enrollment 21/30 dogs received cardiovascular drugs, of these 21/21 (100 %) dogs received pimobendan, 12/21 (57%) dogs received an Angiotensin-Converting Enzyme Inhibitors (ACEI), 7/21 (33%) dogs received spironolactone. Furthermore, 15/21 (71 %) received furosemide (mean dosage 4±1 mg/kg/daily) and 3/21 (2 %) were treated with torasemide (median dosage 0.4 mg/kg/day, range 0.22-0.5). The mean duration of diuretic therapy in these dogs before CHF occurrence was 278±195 days. Digoxin was administered to 3/21 (21%) dogs for the treatment of atrial fibrillation. No other antiarrhythmic drugs were prescribed, since no hemodynamically relevant cardiac rhythm disturbances were found in the study population. Among the dogs included, 12/30 (40%) were at their first CHF episode. At the time of inclusion, CHF dogs presented a mean respiratory rate of 64±21 breaths/min, and a mean systolic blood pressure of 133±11.6 mmHg. No dog was in cardiogenic shock.

In-hospital treatment and outcome

All dogs after admission started IV diuretic therapy. Diuretics were administered both in boluses and in constant rate infusion (CRI) in 21/30 dogs, whereas 9/30 were managed exclusively by IV furosemide boluses. The median IV furosemide dosage administered in CHF

dogs was 11.5 mg/kg/day (range 6-22) during the first 24 hours of hospital stay, and 17.2±2 mg/kg/day after 48 hours. In 5/30 dogs, torasemide was used as a diuretic; in four of the latter dogs, torasemide was associated with a furosemide CRI. During hospitalization, a shift was made from furosemide to torasemide in 2/30 dogs. All but three dogs received oxygen supplementation by means of a nasal cannula (13/27) or oxygen cage (14/27). The mean duration of oxygen therapy was 19±7.7 hours. None of the included dogs was mechanically ventilated. The cardiovascular therapies that the dogs were receiving at time of inclusion were discontinued during hospitalization. Enrolled dogs were hospitalized for a median of 49 hours (range, 24 – 120). Overall, 26/30 dogs (87%) were survivors 48 hours after admission, and 11/30 dogs were discharged between 28 hours and 30 hours of hospital stay because of pulmonary edema resolution. Four dogs (13%) died of cardiac causes during hospitalization, 3/4 after the first 24 hours and 1/4 after 48 hours. At 80 days after discharge 21/30 dogs (70%) were alive, and 9/21 had died with a mean time of 23 days (±33) after the enrollment.

Occurrence of AKI in CHF dogs

Baseline sCr measurements were available in 11/30 dogs (37%), revealing the presence of previous azotemia in 4 dogs. At the time of enrollment (T0), 6/30 dogs (20%) were azotemic (sCr >1.6 mg/dL). Nineteen out of 30 dogs (63%) developed AKI within the first 48 hours of admission. Specifically, kidney injury was grade I (non-azotemic) in 10/19 patients (53%), grade II in 7/19 dogs (37%) and grade III in 2/19 dogs (10%).

Demographic and clinical data of CHF AKI dogs and CHF non-AKI ones were comparable (Table 1). Similarly, the median duration of oxygen therapy and the median dose of furosemide received in the first 24 and 48 hours of hospital stay was similar between CHF AKI dogs and CHF non-AKI ones. No additional difference was detected with regard to duration of hospital stay and outcome between groups (Table 1).

Table 1. Population description of two subgroups, namely dogs that developed AKI during hospitalization (AKI) (n=19) and dogs that did not develop AKI during hospitalization (non-AKI) (n=11).

	CHF AKI (n=19)	CHF non-AKI(n=11)	*P value*
Age (years)	12.1 ± 1.8	11.9 ± 2.7	0.81
Number of dogs receiving PO furosemide at home	10/19	5/11	0.68
Dosage of furosemide (mg/kg/die)	4 (3-6)	4 (2.5-5.8)	0.8
Days of therapy	238 (24-563) (n=5)	224 (26-459) (n=8)	1.00
Number of dogs receiving PO torasemide at home	1/19	2/11	
Dosage of torasemide (mg/kg/die)	0.22	0.45 (0.4-0.5)	
Number of dogs receiving PO pimobendan at home	13/19	8/11	
Dosage of pimobendan (mg/kg/die)	0.6 (0.25-0.8)	0.5 (0.25-0.9)	0.66
Number of dogs receiving PO spirolattone at home	3/19	4/11	
Dosage of spirolattone (mg/kg/die)	2.3 (1.5-3)	2 (1.8-2.5)	0.72
Number of dogs receiving PO ACEi at home	9/19	2/11	
Dosage of ACEi (mg/kg/die)	0.7 (0.3-1.1)	0.47 (0.25-0.7)	0.28
Hours of hospitalization	50 (30-288)	50 (30-120)	0.72
Total dosage of furosemide administered in the first 24 h of hospitalization (mg/kg)	11.1 (6-22)	12 (8-12.4)	0.94
Total dosage of furosemide administered in the first 48 h of hospitalization (mg/kg)	18 (14-21)	18 (16-19)	0.74
Number of dogs alive at 90 days from discharge	15/19	6/11	0.32
Days of survival at 90 days from discharge	4 (2-6)	9 (2-71)	0.4
Albumin (g/dL) (T0)	2.99 ± 0.31	3 ± 0.54	0.92
Cl (mEq/L) (T0)	109.8 ± 4.9	108.8 ± 4.9	0.64
Creatinine (mg/dL) (T0)	0.92 (0.32-2)	1.18 (0.74-2.14)	0.3
CRP (mg/dL) (T0)	3.8 (1-28.9)	3.14 (1.6-20)	0.78
K (mEq/L) (T0)	4.4 (2.9-5)	4.5 (3.4-5.8)	0.34
Na (mEq/L) (T0)	151 (138-156)	149 (147-156)	0.89
Total protein (g/dL) (T0)	6.37 ± 0.7	6.37 ± 0.42	0.99
UPC (T0)	0.28 (0.08-2.6)	0.31 (0.14-2.7)	0.34
Urea (mg/dL) (T0)	59 (28-200)	59.8 (28-125)	0.53
uNGAL (pg/mL) (T0)	2774 (50-42565)	9216 (262-124994)	0.20
uNGALC (pg/mL) (T0)	4243 (94-315514)	25361 (746-1413967)	0.43
uNGAL (pg/mL) at 24h after hospital admission	3779 (400-59685)	805 (27-148765)	0.57
uNGALC (pg/mL) at 24h after hospital admission	6672 (768-65372)	3203 (76.4-1565945)	0.9
uNGAL (pg/mL) at 48h after hospital admission	2987 (29.4-50537)	262 (67.7-51708)	0.23
uNGALC (pg/mL) at 48h after hospital admission	6338 (225-75181)	825 (326-193448)	0.35

Abbreviations: AKI: acute kidney disease; CRP, C-reactive proteins; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC, urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio.

Seven-day sCr values from the time of enrollment were obtained for 21/30 dogs. In the CHF AKI group, 14/19 dogs had available sCr measurements at 7 days. Among these, sCr levels returned to baseline in 10/14 dogs, while 4/14 exhibited persistently elevated levels. In the CHF non-AKI group, sCr measurements were available for 7/11 dogs at 7 days, with sCr remaining stable in 6/7 dogs and increasing (> 0.3 mg/dl) in 1/7 dog.

Clinicopathological data comparison at different time points

Included CHF dogs had significantly higher sCr and urea concentration at T1 and T2 compared to T0 ($P < 0.001$ for both comparisons). No statistical differences were observed for UPC considering all time points evaluated. In addition, dogs had significantly lower serum chloride and potassium concentration at T1 and T2 compared to dogs at T0 ($P < 0.001$ for both comparisons). Complete clinicopathological results are reported in Table 2.

Urinary NGAL evaluation at different time points

The median values for uNGAL and uNGALC measured in CHF dogs are reported in Table 2. Overall, increased uNGAL values above the reference range (previously published, Troia et al., 2022) were detected in 16/30 (53%) dogs at T0, 16/30 (53%) at T1 and 6/15 (40%) dogs at T2. Similarly, increased uNGALC values were documented in 23/30 (77%) dogs at T0, 22/30 (73%) at T1 and 9/15 (60%) cases at T2. No significant differences were documented for either uNGAL nor uNGALC among the considered time points (T0 vs T1, $P = 0.5$, $P = 0.7$, respectively, T0 vs T2, P = 0.7, P = 0.8, respectively, T1 vs T2, P = 0.9 and P = 0.9, respectively).

There was no statistical difference in the correlation between CRP and uNGAL ($P = 0.65$) and uNGALC ($P = 0.51$) at the time of admission. At T1, no correlation was documented between the values of uNGAL and uNGALC and the total dose of furosemide administered during the first 24 hours ($r = 0.34$, $P = 0.09$; $r = 0.35$, $P = 0.08$, respectively). Similarly, at T2, the values of uNGAL and uNGALC were not correlated with the total dose of furosemide administered during the first 48 hours ($r = 0.56$, $P = 0.06$; $r = 0.46$, $P = 0.13$, respectively). No correlation between urinary NGAL and torasemide dosage was performed given the low number of dogs receiving this drug.

Table 2. Clinicopathological variables at time of admission (T0), after 24h (T1) and 48h (T2). Data were reported as mean ± SD or median and range (minimum-maximum value), based on their distribution.

					P value		
	RI	Time Point 0 N=30	Time Point 1 N=30	Time Point 2 N=15	T0 vs T1	T0 vs T2	T1 vs T2
Albumin (g/dL)	2.75-3.85	2.99 ± 0.4	3.2 ± 0.4	3.2 ± 0.2	0.001	0.09	0.33
Chloride (mEq/L)	108.0-118	109.8 ± 4.9	104.3 (96-122)	97.8 (89-122)	0.002	0.004	0.03
Creatinine (mg/dL)	0.75-1.4	1.13 ± 0.4	1.37 (0.88-3.56)	1.4 (0.7- 4.5)	<0.001	0.001	0.6
CRP (mg/dL)	0-1	8.6± 9.1	15.3 ± 12.8	11.9 ± 9.5	0.08	0.7	0.2
Potassium (mEq/L)	3.8-5.0	4.32±0.69	3.9±0.4	3.8± 0.28	0.007	0.17	0.8
Sodium (mEq/L)	143-151	149 (138-156)	148 (140-166)	147±3.9	0.6	0.04	0.11
Total protein (g/dL)	5.6-7.30	6.37 ± 0.6	6.9± 0.7	6.7±0.38	<0.001	0.04	0.3
UPC mg/mg	0-0.5	0.28 (0.08-2.6)	0.34 (0.11-3.6)	0.21 (0.1- 2.9)	0.9	0.27	0.7
Urea (mg/dL)	17-48	59 (28-200)	111±49	110.5±59	<0.001	0.002	0.8
uNGAL (pg/mL)	0-2300	3322 (50-124995)	2897 (27-148765)	1439 (29-51709)	0.5	0.5	0.9
uNGALC (pg/mL)	0-1400	6578 (95-1413968)	4708 (76-1565945)	3601 (225-193448)	0.7	0.8	0.9

Abbreviations: CRP, C-reactive proteins; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC, urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio.

Comparison of urinary NGAL between groups

The values of uNGAL and uNGALC at the time of admission were not significantly different between dogs that developed AKI vs. dogs that did not developed AKI ($P = 0.20$ and $P = 0.42$, respectively). Similarly, no difference between CHF AKI dogs and CHF non-AKI ones was detected for uNGAL and uNGALC values measured at T1 ($P = 0.74$ and $P = 0.47$, respectively) and T2 ($P = 0.89$ and $P = 0.89$, respectively).

In the comparison among CHF dogs (at T0) with healthy dogs and those with stable MMVD of different ACVIM stages, the overall population of dogs with CHF had significantly increased uNGAL (3322 pg/ml; range, 50-124995) and uNGALC (6579 pg/mg; range, 95-1413967) compared to healthy dogs (uNGAL, 584 pg/ml; range, 56-4072; uNGALC, 231 pg/mg; range, 15-2407) and stable MMVD dogs (uNGAL 1204 pg/ml; range, 30-39732; uNGALC 1816 pg/mg; range, 22-127693) ($P = 0.002$, $P < 0.001$, respectively; Figures 2 and 3). When urinary NGAL and NGALC values were compared between CHF dogs and stable MMVD dogs in stage ACVIM C and D only, no differences were detected ($P = 0.8$ and $P < 0.1$, respectively).

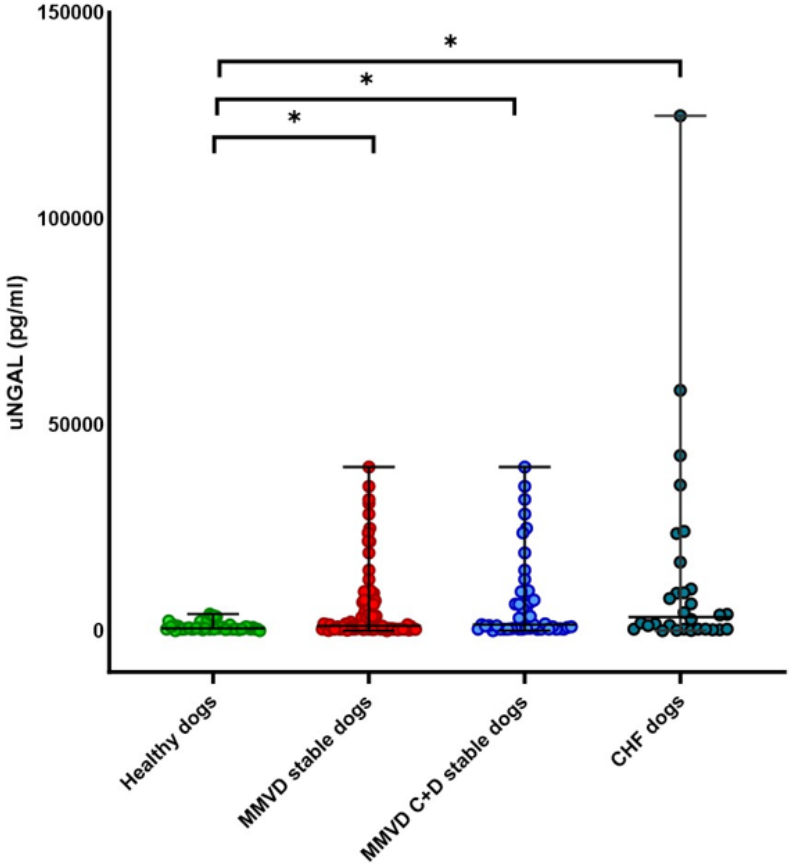


Fig 2. Comparison of uNGAL among CHF dogs with healthy dogs and those with stable MMVD of different ACVIM stages. Abbreviations: MMVD: myxomatous mitral valve disease; CHF: cardiac heart failure. uNGAL urinary neutrophil gelatinase-associated lipocalin; uNGALC, urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio.

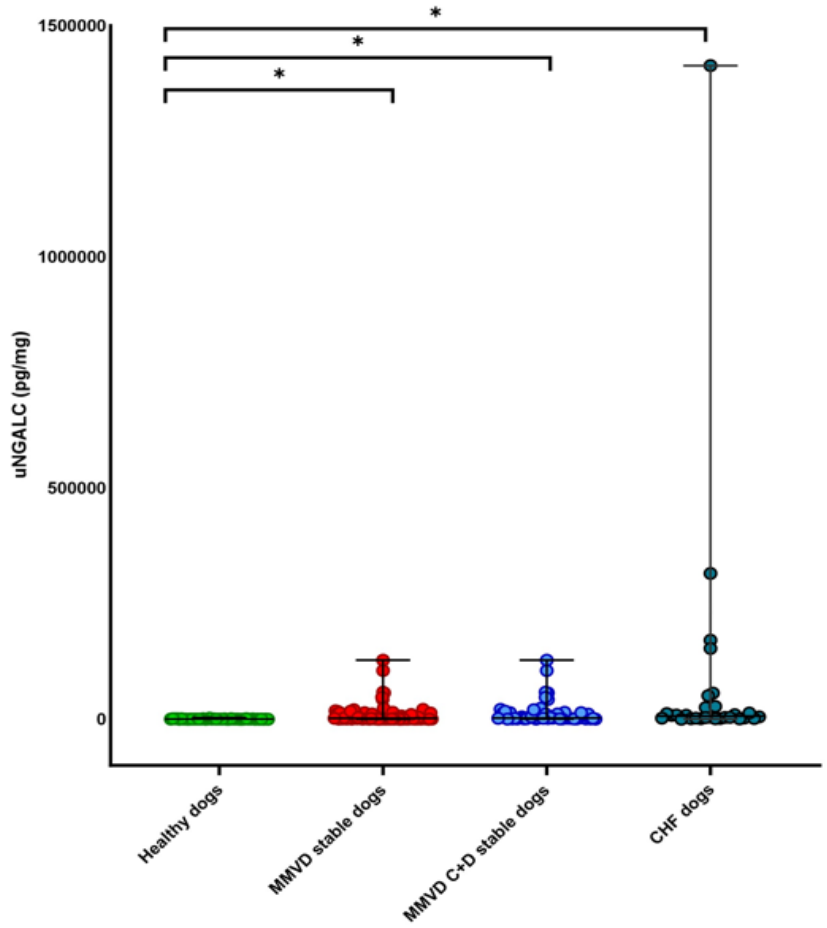


Fig 3. Comparison of uNGALC among CHF dogs with healthy dogs and those with stable MMVD of different ACVIM stages. Abbreviations: MMVD: myxomatous mitral valve disease; CHF: cardiac heart failure. uNGAL urinary neutrophil gelatinase-associated lipocalin; uNGALC, urinary neutrophil gelatinase-associated lipocalin to urinary creatinine ratio.

Discussion

The present study aimed to assess the occurrence of AKI during in-hospital treatment of acute decompensated MMVD in dogs, and to investigate the diagnostic role of urinary NGAL in this clinical setting. In this study, AKI defined according to the IRIS criteria occurred in the 63% of dogs with CHF within 48 hours from hospitalization but was not directly related to diuretic therapy and did not impact short-term outcome.

Despite AKI occurrence, no significant changes in urinary NGAL values were documented during the study period, with comparable concentrations between acute decompensated MMVD dogs with AKI and those without occurrence of renal injury. This finding indicates that development of mild kidney injury in the course of CHF is common in dogs, appears to be mainly functional and does not reflect tubular damage. Acute kidney injury occurring during

CHF has been labelled CRS type 1 and represents a well-known entity in people with a reported incidence between 25 and 40%. The real epidemiology of such worsening renal function is hard to estimate due to inconsistency in definition criteria and timeframes considered for its occurrence (Bagshaw et al. 2010; Cruz et al., 2013; Palazzuoli et al., 2014). Overall, CRS type 1 in people carries a negative prognostic impact, increases healthcare costs, and might predispose to diuretic resistance (Bagshaw et al., 2010; Palazzuoli et al., 2014). On the other hand, changes in sCr induced by diuresis and decongestion may be largely functional or hemodynamic, and thus clinically benign. Indeed, severe deterioration in renal function is not common in people with acute heart failure, as most worsening renal function events represent small to moderate-sized changes in sCr measurement (Ahmad et al., 2018). In this regard, the negative connotation of worsening renal function during CHF has been challenged, being not correlated with biomarkers of tubular damage but representing an expected hemodynamic response to successful decongestion (Ahmad et al., 2018).

The epidemiology of CRS type 1 is less well-characterized in dogs. Prevalence varies among studies because of variable definition of renal insufficiency and heterogeneous cardiac diseases in published clinical trial (Ohad et al., 2010; Besche et al., 2020; Giorgi et al., 2022). In a recent retrospective study evaluating dogs with left-sided CHF treated with parenteral furosemide, AKI was reported in the 48% of cases, was generally in the non-azotemic range and was not related to cumulative in-hospital furosemide dosage or long-term outcome (Giorgi et al., 2022). In the paper by Jung et al. 2018, 11/31 dogs (35%) with degenerative valve disease and CHF developed worsening renal function within 7 days from CHF occurrence. In that study, however, dogs experiencing transient azotemia that resolved after 24 hours of water supplementation or IV fluids were excluded from this analysis (Jung et al., 2018).

In the present study, a higher incidence of in-hospital AKI based on sCr changes within 24-48 hours (63%, 19/30 CHF AKI dogs) was documented. Recording the sCr measurement after 7 days in 14 out of 19 CHF AKI dogs, it was found out that the majority of these animals (10/14) had only a transient worsening renal function, with sCr returning to baseline values within one week from CHF occurrence and hospitalization. Similarly, in the CHF non-AKI group, sCr measurements were available for 7 of 11 dogs at 7 days post T0, and in the majority of these dogs (6/7), creatinine levels remained stable without any significant increase. The pathogenesis of AKI during CHF is complex, with both arterial underfilling and renal venous congestion being involved (Ronco et al., 2012; di Lullo et al., 2017; Fu et al., 2021; Verma et al., 2022). Drugs administered during CHF might additionally contribute to worsening renal function:

diuretics, which are the cornerstone for the treatment of pulmonary edema, may resolve congestion and ameliorate global tissue perfusion and function, but over diuresis could promote kidney hypoperfusion (Cruz et al., 2013; Palazzuoli et al., 2014; Giorgi et al., 2022). Moreover, during CHF, renal perfusion is strictly dependent on neurohormonal activation, which maintains GFR through both afferent and efferent arteriolar vasoconstriction. For this reason, drugs working on the renin-angiotensin-aldosterone system like ACEi, which can be chronically administered in stable heart disease, are generally avoided in the acute decompensated phase of heart failure because of the hemodynamic changes they might induce at the kidney level (Orvalho and Cowgill, 2017; Keene et al., 2019,). Finally, aging or previous AKI episodes contribute to reducing the renal reserve of such patients and to the development of CRS type 1 (Cruz et al., 2013; Martinelli et al. 2016; Orvalho and Cowgill, 2017). In this study setting, all these factors might have contributed to AKI occurrence in CHF dogs. Indeed, most of enrolled dogs (60%) had already experienced previous MMVD decompensation and could have suffered from previous concurrent AKI episodes. All dogs received IV diuretics which could have partially led to AKI; however, the volume of diuretics administered did not differ between CHF AKI and CHF no-AKI dogs, and no events of over diuresis and massive dehydration or hypovolemia were documented. In addition, many patients (57%) received ACEi therapy, which was discontinued at the time of hospital admission but might have limited the kidney ability to sustain perfusion in the timeframe before presentation. Finally, the study population consisted of elderly patients with a median age of 12 years, where the possibility of aging kidneys, reduced renal functional mass, concurrent CKD and sub-clinical tubular damage could all coexist, as previously highlighted (see Chapter 4). Nonetheless, the degree of worsening renal function documented in the study population was mild (mainly AKI IRIS grade I and II), asymptomatic, transient in most cases and did not affect duration of hospital stay or short-term outcome. Among the four dogs experiencing death, none died because of AKI and AKI-related signs. Thus, the pattern of AKI in our CHF population was apparently functional and benign, only reflecting appropriate decongestion. In this regard, the results concerning urinary NGAL in our population of CHF dogs supported the above-mentioned statement. Urinary NGAL was not higher in dogs experiencing AKI, and did not increase in the course of hospitalization. There was, on the contrary, an unexpected (not statistically significant) decrease in the median values of both uNGAL and uNGALC from T0 to T2. These results support the concept that the mild decline in GFR noticed with decongestion in our dogs did not reflect tubular injury but was more probably a clinically benign change in filtration, with no

significant impact on tubular cells. There is only one study in veterinary literature assessing the diagnostic potential of serum NGAL in dogs with degenerative valve disease and CHF (Jung et al. 2018). In that setting, serum NGAL evaluated at the time of hospitalization was able to predict the occurrence of worsening renal function, defined as a rise in sCr (>1.6 mg/dL) within 7 days from decompensation, and acted as an early biomarker of CRS type 1 (Jung et al. 2018). However, dogs developing transient azotemia that resolved after 24 hours of fluid therapy or water supplementation were excluded from the final study population (Jung et al. 2018). For this major reason, and due to additional differences in the case series composition, study setting, and type of biomarker measured (serum vs. urinary NGAL), it is hard to make a comparison with our results.

Neutrophil gelatinase-associated lipocalin assays have been recently proposed to estimate the risk of AKI in human cardiovascular diseases. According to the paper by Palazzuoli and colleagues (2014), admission plasma NGAL was predictive of in-hospital worsening renal function and post discharge outcome in a cohort of people with CHF. On the contrary, discharge NGAL levels were not significantly increased with respect to patients without worsening renal function. In that same study, outcome and development of worsening renal function were strictly related (Palazzuoli et al., 2014). In contrast with Palazzuoli et al., (2014), another study in similar settings, measuring plasma NGAL, revealed no apparent tubular involvement in the course of mild CRS type 1 in people with low-grade CHF (Ferrari et al., 2020). Other studies measured urinary NGAL reported results comparable to ours finding (Ahad et al., 2018; Murray et al., 2020; Natov et al., 2023). In the work conducted by Ahmad and colleagues (2018), baseline and 72 hours biomarkers of tubular injury including urinary NGAL were measured in people with CHF included in a multicenter trial. None of the measured biomarkers was related to the development of worsening renal function, which was transient, mild and had no prognostic implication in that study population (Ahmad et al., 2018).

Based on the available human literature, it seems to be the context of the development of AKI/worsening renal function, rather than its presence, during acute cardiac decompensation to promote true parenchymal damage and affect prognosis. In this regard, it should be noted that our study population was affected by mild forms of cardiac and renal damage, since the mortality rate was low and no patient required non-invasive or invasive ventilation, or inotropic drugs support. In addition, the majority of CHF AKI dogs had IRIS grade I to II kidney injury, a none required renal replacement therapies. Moreover, cases were managed by clinicians experienced in advanced CHF care, making efforts to avoid significant increases in sCr (e.g.,

through frequent laboratory and imaging re-checks, or providing early assisted nutrition to inappetent animals). In settings of less stable patients with higher heart-related complications, higher baseline sCr, need for more aggressive support to resolve congestion and hypoxemia, and less highly trained caregivers deliberately avoiding worsening renal function, the detection of AKI could be associated with the rise of tubular injury biomarkers and preclude survival. Moreover, the long-term effects of these worsening renal function events are not known, and even in the case they are transient and benign the consequences on diuretic responsiveness, CKD incidence and renal functional reserve have yet to be determined.

In the comparison of urinary NGAL values among dogs with CHF (at time point 0), stable MMVD and healthy ones, dogs with CHF had significantly increased uNGAL and uNGALC compared to the others. Thus, the presence of subclinical tubular damage is confirmed in dogs with CHF, similarly with previous results reported in MMVD dogs with stable disease (Troia et al., 2022). However, when only stable MMVD dogs in ACVIM stage C and D were compared with CHF dogs, no difference in urinary NGAL measurements were detected. It could be hypothesized that factors like cardiac disease severity and diuretic therapy play a role in the occurrence of some degree of tubular damage and could make any MMVD dogs with advanced disease at risk for developing more severe kidney disease, regardless the presence of concurrent CHF. Moreover, it should be noted that the increases in urinary NGAL values detected in CHF dogs are significantly lower than those reported in dogs with AKI in the literature (Monari et al., 2020).

There are some limitations to acknowledge when interpreting the results of this study. Since this was a single observational study, it is prone to several forms of bias, limiting the value of our results in different critical care settings (e.g., dogs with more severe forms of CHF requiring invasive mechanical ventilation, or CHF dogs with cardiogenic shock requiring inotropic support), as previously stated. In addition, the lack of a basal sCr measurement for the majority of the enrolled dogs was a major limitation, complicating renal function evaluation at the time of hospital admission. Specifically, six dogs (20% of the whole CHF population) were azotemic at T0, either because of true AKI secondary to renal congestion and hypoperfusion, or because concurrent CKD. Hence, we could only correctly assess the incidence of in-hospital AKI during CHF treatment. Furthermore, the timeframe chosen to investigate AKI occurrence was limited to the hospitalization period of CHF dogs and ranged between 24 and 48 hours. However, since in most cases people experience worsening renal function within the first week following CHF (Bagshaw et al., 2010), a longer period of time for monitoring, up to 7 days, could have been a

better ascertainment time for the diagnosis of CRS type 1. Nevertheless, sCr values retrieved retrospectively at 7 days from hospital admission in non-AKI dogs did not indicate a worsening of renal function in 6/7 of them.

Moreover, additional tests to characterize tubular damage (e.g.: urine protein electrophoresis or even other renal biomarkers) could have been useful to better investigate urinary NGAL origin and correlations in our study. Although the overall management of enrolled dogs was made by the attending clinicians in accordance with a board-certified cardiologist (SC or GR), specific emergency treatments (eg, amount and duration of oxygen supplementation, modalities for oxygen supply, need of non-invasive ventilation) could have been operator-dependent and were based on specific equipment availability at the VTHs involved in the study. Thus, a more detailed approach to characterize the respiratory support of our dogs and the severity of the respiratory compromise was not attempted for the possibility of multiple confounders. Similarly, the degree of hypoxemia was not systematically evaluated to avoid stressful or invasive procedures in affected animals, precluding the assessment of CHF severity at the time of hospital admission. Finally, the majority of enrolled dogs had a mild to moderate increase in serum C-reactive protein concentration, which is an expected finding during CHF in dogs, as previously reported (Reimann et al., 2016). However, we did not perform a methodic screening for additional causes of such inflammatory state if the clinical and laboratory picture of dogs was not suggestive of potential comorbidities. Thus, even in the absence of correlation between urinary NGAL and serum CRP values based on our results, the inflammatory state of enrolled dogs was not regularly characterized.

In conclusion, about two-thirds of dogs with CHF in this study developed in-hospital AKI, documented by increase in serum creatinine, which was mild and transient in most cases and did not affect short-term survival. Urinary NGAL levels were not associated with a decline in renal function defined by creatinine changes and did not predict the transient AKI in CHF dogs. These findings support the concept that, in the majority of acute decompensated MMVD dogs, functional AKI is expected and related to successful decongestion, but does not reflect tubular damage, demonstrating that the benefits of decongestion may outweigh any modest or transient increases in serum creatinine. The value of recurrent AKI episodes in more critical scenarios, and its overall impact on long term outcome and diuretic responsiveness, is not known, and should be characterized in further studies.

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Chapter 7

Conclusion

In this thesis project, we focused on the study of different aspects of cardiorenal syndrome (Type I and Type II) in dogs.

In the first study phase, we reported the importance of defining a timing (between the measurement of natriuresis and the administration of loop diuretics) to more effectively study urinary electrolyte excretion for quantifying the diuretic response in a patient. Indeed, we demonstrated that urinary excretion of urinary electrolytes increases in the first 6 h from diuretic administration in dogs with MMVD stage C. On the other hand, MMVD dogs stage C, evaluated six hours after diuretic administration have the same urinary levels as a healthy dog. Although we were unable to further explore this aspect with subsequent studies, having a quantitative marker of renal response to decongestive therapy could guide clinical decision-making and help define patient prognosis more efficiently.

In subsequent studies we focused on describing the presence or development of renal damage resulting from known heart disease. Patients with MMVD at various stages of disease show an increase in uNGAL excretion, which translates to subclinical renal damage, even in dogs not receiving diuretic treatment. Furthermore, the association found between uNGAL and left atrial stroke volume and tricuspid regurgitation in dogs with MMVD suggests that these echocardiographic indicators may help identify dogs with MMVD at a higher risk of developing renal damage. Although not entirely clarified, the underlying mechanisms may vary. The kidneys of a MMVD dog can experience mild to moderate oxygen depletion and consequent tubular cell damage. Additionally, venous underfilling observed in cardiac patients activates hormonal mechanisms (RAAS), triggering compensatory responses that may overload the kidneys. It remains unclear whether diuretic therapy itself can exacerbate and trigger renal damage.

Focusing on the present thesis study project, it was shown that in MMVD dogs with CHF, renal damage (assessed by increased creatinine) appears to be functional to the decongestive response. Dogs with CHF exhibit the same uNGAL levels as those with stable advanced MMVD, and a short hospitalization (48 hours) with intravenous diuretic therapy does not exacerbate renal damage. Indeed, in this class of patients, uNGAL does not increase during hospitalization, despite the presence of creatinine elevation, and it does not predict the

occurrence of AKI in dogs that develop it. The worsening renal function documented in the study population was mild (IRIS grade I and II), asymptomatic, and transient in most cases, with no significant impact on the duration of hospital stay or short-term outcomes. Thus, the pattern of AKI in our CHF population appears to be functional and benign, reflecting appropriate decongestion rather than severe renal impairment.

Future research should explore reno-protective strategies in the management of dogs with MMVD, as these approaches have the potential to slow disease progression and reduce complications related to cardiorenal syndrome.