Research in Veterinary Science 171 (2024) 105211

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

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

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ARTICLE INFO

Keywords:
Renal damage
Cardiorenal syndrome
Heart failure
Renal biomarkers
MMVD

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.

1. 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

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https://doi.org/10.1016/j.rvsc.2024.105211
Received 3 November 2023; Received in revised form 24 February 2024; Accepted 3 March 2024
Available online 5 March 2024

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.

2. 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).

2.1. 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).

2.2. 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 (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-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.

2.3. 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).

2.4. 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 phased-array 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 (LAV_{Max}) and minimal (LAV_{Min}) 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: $LASV = LAV_{Max} - LAV_{Min}$ (Hollmer et al., 2017). The aortic velocity time integral (VTI_{Ao}) was measured from the subcostal view; the mitral velocity time integral

(VTI_{Mit}), the early diastolic mitral peak velocity (E_{Vmax}), the ratio between E_{Vmax} and the E' wave of the tissue Doppler of the parietal mitral annulus (E/E') were measured from the left parasternal apical four-chamber view. The ratio between the VTI_{Mit}/VTI_{Ao} was then calculated. Tricuspid regurgitation velocity (TR_{Vmax}) 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 TR_{Vmax} 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).

2.5. 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 <2300 pg/ml vs. abnormal uNGAL values ≥2300 pg/ml) and uNGALC results (normal uNGALC values <1400 pg/mg vs. abnormal uNGALC values ≥1400 pg/mg), as previously reported. (Troia et al., 2022) Echocardiographic variables were explored as continuous variables, and uNGAL and uNGALC were expressed dichotomously. The Mann-Whitney *U* test investigated differences between continuous independent, non-normally distributed data. The association between the echocardiographic indexes and increased uNGAL and uNGALC values above the RI was studied using univariate analysis; any significant variable in the univariate analysis was selected as a candidate for the multivariable logistic regression analysis, enter method. Multicollinearity and model goodness-of-fit tests were checked. Multicollinearity between independent variables was investigated using the variance inflation factor (VIF). If the VIF score is 2.5 or higher, it usually suggests significant multicollinearity, considering no correlation between predictors with a VIF = 1, mild between 1 and 2.5, moderate between 2.5 and 5, and potentially high >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 $P < 0.05$ were considered significant for all analyses.

Due to the absence of data in the literature, an a priori analysis (two-tailed test) was made on the first 20 patients included in the study to estimate the sample size. The sample size needed to obtain significant differences between continuous independent non-normally distributed data in normal or abnormal uNGAL groups (Power 1 - $\beta > 0.90$; $\alpha = 0.05$ and $\beta = 0.10$) was 76 dogs, with an effect size of 0.74.

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).

Dogs with increased uNGAL and uNGALC had significantly higher LA/Ao, LASV, LAV_{Max}, LAV_{Min}, LVDDn, TR_{Vmax}, and VTI_{Mit}/VTI_{Ao}. Furthermore, dogs with abnormal uNGALC had higher E_{Vmax}, SF, and VTI_{Mit} (Table 1).

In the univariate analysis, LASV, TR_{Vmax}, LAV_{Max}, LVIDDn, and VTI_{Mit}/VTI_{Ao} 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 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. A moderate correlation between LASV and LAV_{Max} (VIF = 3.3), LVIDDn (VIF = 3.2), and VTI_{Mit}/VTI_{Ao} (VIF = 3.4) was found, whereas a mild correlation between LASV and TR_{Vmax} (VIF = 1.1) was detected.

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). Areas under the ROC curve for TR_{Vmax}, 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

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; E_{Vmax}, E wave peak velocity; LA_{SV}, left atrial stroke volume; LA/Ao, left atrium to aortic root ratio; LAV_{Max}, left atrium maximal volume; LAV_{Min}, left atrium minimal volume; LVIDDn, normalized left ventricular end-diastolic diameter; LVIDSn, normalized left ventricular end-systolic diameter; SF, shortening fraction; TR_{Vmax}, tricuspid regurgitant flow velocity; uNGAL, urinary neutrophil gelatinase-associated lipocalin; uNGALC: NGAL to urinary creatinine ratio; VTI_{Ao}, Aortic velocity-time integral; VTI_{Mit}, Mitral velocity-time integral; VTI_{Mit}/VTI_{Ao}, Mitral to aortic velocity-time integral ratio.
* Significantly different between groups ($P < 0.05$).

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

between sensitivity and specificity for LASV were > 0.76 ml/kg (specificity: 90.62%; sensitivity: 38.64%) and > 1.11 (specificity: 78.95%; sensitivity: 60.53) for uNGAL and uNGALC, respectively; whereas for TR_{Vmax} 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 ($R^2 = 0.045$, F (1,33) = 1.55, $P = 0.222$) for uNGAL and ($R^2 = 0.080$, F (1, 33) = 2.87, P = 0.1) for uNGALC. It was found that diuretic dosage did not influence either uNGAL ($\beta = 0.094$, P = 0.222) or uNGALC ($\beta = 0.122$, P = 0.1) values (Fig. 2).

4. Discussion

This study showed that LASV and TR_{Vmax} 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

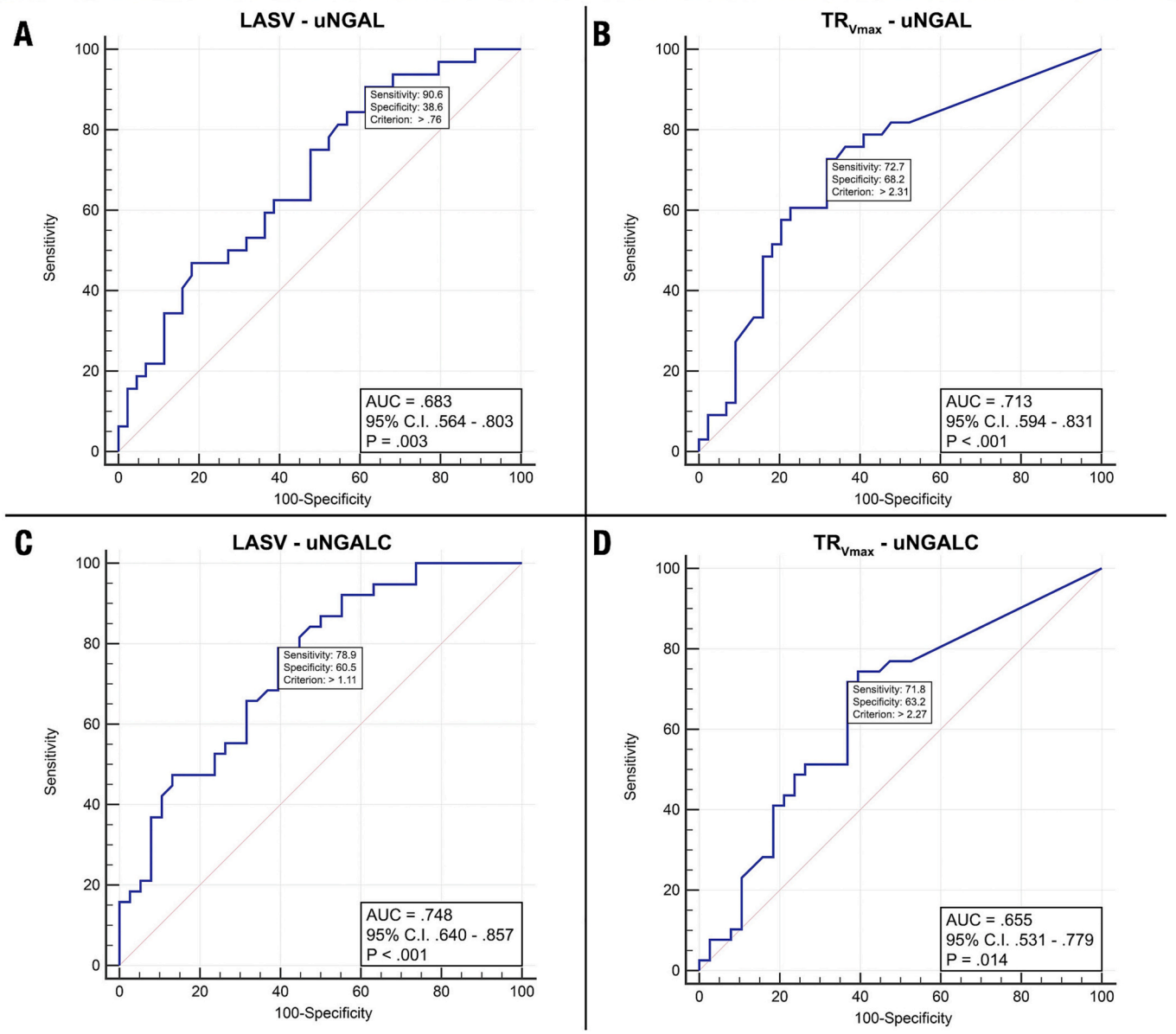

Fig. 1. Receiver operating characteristic curves for LASV and TR_{Vmax} as predictors variables to classify patients with abnormal uNGAL (A, B) and uNGALC (C, D). AUC = Area under the ROC curve.

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, TR_{Vmax}, LAV_{Max}, LVIDDn, and VTI_{Mit}/VTI_{Ao}) were associated with increased uNGAL and uNGALC in our study; however, in the multivariable analysis, only TR_{Vmax} and LASV remained significantly associated. It should be noted that LASV, LAV_{Max}, LVIDDn, and VTI_{Mit}/VTI_{Ao} 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.,

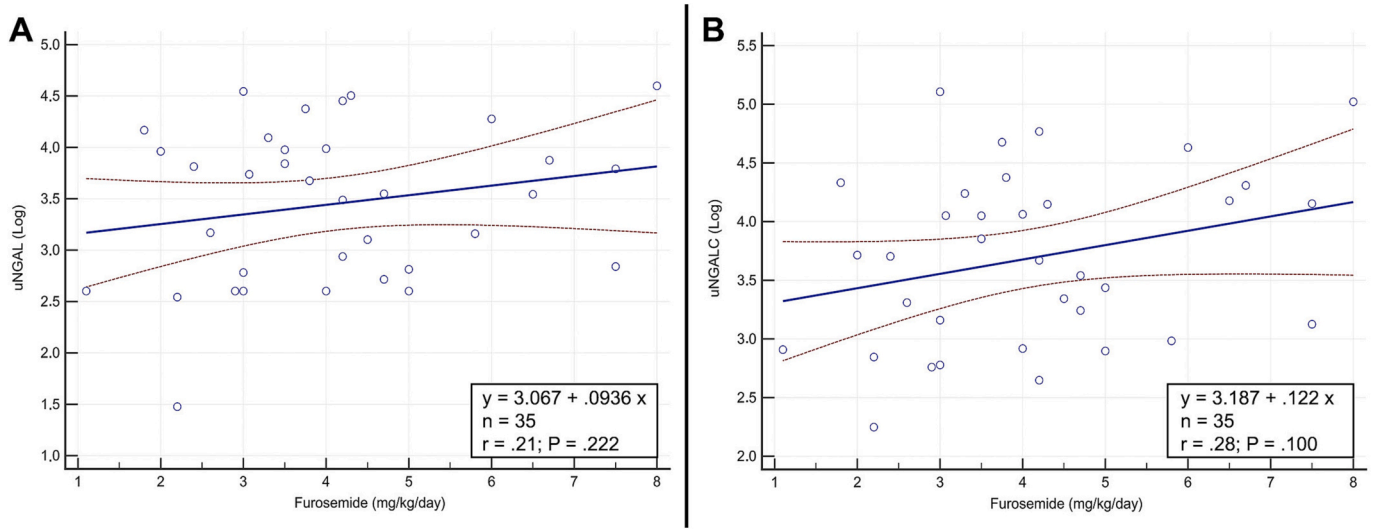

Fig. 2. Relationships between Furosemide daily dosage (mg/kg/day) and uNGAL (A) or uNGALC (B) values.

2009; Husain-Syed et al., 2021; Mullens 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 TR_{Vmax} 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 <2. Conversely, we cannot assume that the presence of PH could be clinically relevant in developing renal damage. However, the results of this study could be partially reconducted to the small number of dogs with PH, classified according to the current guidelines (Reinero et al., 2020).

The primary pathophysiologic mechanism of MMVD in dogs is left-side CHF, which could eventually evolve to type 2 PH and right-sided CHF (Reinero et al., 2020). Moreover, it has been found in humans that elevated intra-abdominal pressure, in the course of ascites, contributes to kidney damage due to the compression of the renal parenchyma (Orvalho and Cowgill, 2017; Pouchelon et al., 2015). Some patients may also develop AF (Franchini et al., 2021b), which could reduce cardiac output. In our population, we had 6 dogs with ascites, and only three of them had concomitant AF, PH, or both; indeed, the association between the presence of abdominal fluid or AF and increased renal biomarkers was not tested due to the low number of dogs affected.

Based on our findings, due to the lack of relationship between uNGAL or uNGALC and VTI_{Ao} or LVIDSn, we might hypothesize that cardiac output is irrelevant in developing tubular damage in our population. This might be related to the nature of MMVD, which is associated with a pure volume overload that rarely evolves into clinically relevant systolic dysfunction (Bonagura and Schober, 2009; Franchini et al., 2021a). However, in the course of MMVD, the detection of impaired systolic function is challenging because the volume overload and the regurgitant fraction could mask it (Bonagura and Schober, 2009). Likewise, a severe regurgitant fraction could affect forward stroke volume even with preserved systolic function; indeed, we found a positive relationship between uNGAL or uNGALC and VTI_{Mit}/VTI_{Ao}, which tends to support this hypothesis. Furthermore, the hemodynamic effect related to the regurgitant fraction and the consequent reduction of the forward stroke volume is partially compensated by the volume overload due to the neurohormonal activation in MMVD dogs (i.e., renin-angiotensin-aldosterone system, natriuretic peptide system, arginine vasopressin system) (Oyama, 2009).

We found no association between daily furosemide dosage and uNGAL and uNGALC. Loop diuretics should not necessarily be considered nephrotoxic agents if properly used; indeed, even if they may negatively affect renal perfusion and glomerular filtration rate, they reduce the congestion with a potentially positive effect on the renal parenchyma (Cruz et al., 2012). At the same time, it is important to underline that the impact of cardiac treatments other than furosemide (e.g., spironolactone, benazepril, pimobendan) on the uNGAL and uNGALC was not evaluated in our study due to the heterogeneous and small treatment subgroups; this constitutes a limitation of the present research.

This study has additional limitations. First, the population has been divided into groups according to normal vs. abnormal uNGAL and uNGALC values. The published reference values refer to a relatively small group of normal ($n = 46$) young dogs and might not be comparable to our study population (Troia et al., 2022). However, even if a sample size of at least 120 subjects is preferred to calculate reference intervals, based on the current guidelines, reference intervals could be reliably calculated despite not being an optimal number (Friedrichs et al., 2012). Moreover, it is known that NGAL concentration may have moderate individual variability (Chen et al., 2023), and the reported cutoff values might not apply to the general population. On the other hand, the reference values considered for this study have been previously reported by the same study group with the same laboratory methods. Moreover, the influence of inflammation on NGAL concentration is well known (Cortellini et al., 2015; Zamagni et al., 2020); however, in our population, there was no difference in C-reactive protein values between dogs with normal or elevated uNGALC (data not shown). Finally, another limitation is the lack of right heart echocardiographic measurement and the small number of patients with right-sided CHF (e.g., ascites) and PH; this limit did not allow us to analyze the role of splanchnic congestion in developing tubular damage.

In conclusion, our study showed an association between uNGAL and uNGALC values and LASV and TR_{Vmax} in dogs with MMVD. These findings suggest that such echocardiographic indexes, mainly indicating tetracameral volume overload, might recognize dogs with MMVD at

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.

CRediT authorship contribution statement

Serena Crosara: Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – original draft, Writing – review & editing. **Francesca Fidanzio:** Investigation, Writing – original draft. **Stefano Oricco:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Francesco Dondi:** Conceptualization, Investigation, Methodology, Supervision, Validation, Writing – review & editing. **Chiara Mazzoldi:** Investigation. **Erika Monari:** Investigation. **Giovanni Romito:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing. **Maria Chiara Sabetti:** Data curation, Investigation. **Roberta Troìa:** Validation, Writing – review & editing. **Cecilia Quintavalla:** Conceptualization, Investigation, Supervision, Validation, Writing – review & editing.

Declaration of competing interest

None.

Acknowledgements

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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