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PhD Thesis

AN EXPANSION ON DIAGNOSTIC AND PROGNOSTIC MARKERS IN DOGS WITH ACUTE AND CHRONIC HEART FAILURE

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INDEX

LIST OF ABBREVIATIONS

ABT - aldosterone breakthrough

ACE - angiotensin converting enzyme

ACE-I - angiotensin converting enzyme inhibitor

ACVIM - American college of veterinary internal medicine

A-II - angiotensin II

ANP - atrial natriuretic peptide

ARB - angiotensin receptor blocker

AT1 - angiotensin receptor 1

AT2 - angiotensin receptor 2

BCS - body condition score

BNP - Brain natriuretic peptide

BW - body weight

CHF - congestive heart failure

CKCS - cavalier king Charles spaniel

CNP - C-type natriuretic peptide

Cr - creatinine

DCM - dilated cardiomyopathy

E/A - E peak velocity-to-A peak velocity ratio

ELISA - enzyme-linked immunosorbent assay

GFR - glomerular filtration rate

HC - haemoconcentration

HF - Heart failure

HR - hazard ratio

IQR - interquartile range

IRF - improving renal function

JRT - Jack Russell terrier

LA/Ao - left atrium-to-aortic ratio

LVEDD - left ventricular end diastolic diameter

LVESD - left ventricular end systolic diameter

MMVD - myxomatous mitral valve disease

MR - mineralocorticoid receptor

MRA - mineralocorticoid receptor antagonist

MR-proANP - midregional pro-A-type natriuretic peptide

NGAL - neutrophil gelatinase-associated lipocalin

NP - natriuretic peptide

NPR - natriuretic peptide receptor

NT-proANP - N-terminal pro-A-type natriuretic peptide

NT-proBNP - N-terminal pro-B-type natriuretic peptide

NT-proCNP - N-terminal pro-C-type natriuretic peptide

PH - pulmonary hypertension

PIIINP - serum procollagen type III N-terminal peptide

PRA - plasma renin activity

RAAS - Renin angiotensin aldosterone system

RI - reference interval

SAP - systolic arterial pressure

SD - standard deviation

SNS - sympathetic nervous system

ST2 - interleukin-1 receptor-like 1 protein

UAldo - urinary aldosterone

UAldo:C - urinary aldosterone-to-creatinine ratio

UC - urinary creatinine

UP - urinary protein

UP/UC - urinary protein-to-creatinine ratio

USG - urine specific gravity

WRF - worsening renal function

PREMISE AND PROJECT AIMS

In the last decades, human and veterinary literature has increasingly highlighted the importance of heart failure (HF) as a complex clinical syndrome[1]. By its definition, HF is a condition in which the heart is unable to maintain sufficient organ perfusion to meet the body's needs[2]. Consequent hemodynamic derangements turn into increased sympathetic nervous system activity and neurohormonal dysregulation, which progressively lead to inflammation, oxidative stress and multi-organ damage and dysfunction[1,3-5].

Neurohormonal dysregulation is likely the hallmark of the HF syndrome. In recent years, veterinary cardiology has particularly focused on the renin-angiotensin-aldosterone system (RAAS) from both a diagnostic and therapeutic perspective[3]. Blocking RAAS activity is a cornerstone of HF therapy, but its trend during the course of the disease is difficult to predict[3,6,7]. Effective diagnostic tools to monitor RAAS activity could help optimize management and therapeutic decisions in dogs with heart diseases.

Natriuretic peptides act as a counterregulatory pathway of RAAS[8,9]. Brain natriuretic peptide (BNP) and its inactive fragment N-terminal pro-B-type natriuretic peptide (NT-proBNP) represent the recommended diagnostic and prognostic markers in acute and chronic heart failure guidelines in people[10,11]. In dogs with cardiac diseases, several studies have highlighted the utility of NT-proBNP measurement in stratifying the risk of morbidity and mortality and in guiding the therapeutic strategy[8]. Current reference intervals are non-breed specific, and a significant interbreed variability has been reported[12-14]. Thus, breed-specific reference ranges might improve sensitivity and specificity of this parameter.

Hemodynamic abnormalities, neurohormonal dysregulation and consequent systemic inflammation and oxidative stress turn HF into a multi-organ syndrome[2,4,5]. The cardiorenal axis is probably the most studied interaction between heart and peripheral organs[15]. However, worsening renal function (WRF) during acute HF still represents a diagnostic and prognostic dilemma which usually influences the therapeutic

approach[16]. Recent human literature highlighted how achieving an adequate decongestion is more prognostically important than avoiding WRF, which is often transient and not related to a true kidney injury[16-19]. Even electrolyte disorders appeared to affect outcome in people[20,21]. Identifying new potential predictors of survival in dogs with acute HF might help to develop an in-hospital therapeutic strategy characterized by specific goals.

The aim of the present project is to achieve a better understanding of the diagnostic and prognostic significance of different markers related to the primary aspects of the HF syndrome: neurohormonal dysregulation and multi-organ damage. In particular, the first aim is to assess aldosterone concentration in healthy dogs and dogs with different stages of myxomatous mitral valve disease (MMVD) and investigate potential factors that affect this parameter. The second aim is to assess breed-specific reference intervals for NT-proBNP in Chihuahuas, an increasingly widespread breed whose most common causes of death are heart diseases and low respiratory tract disorders[22,23]. The last aim is to evaluate the prognostic significance of haemoconcentration (a marker of decongestion), WRF and electrolyte disorders in dogs hospitalized for congestive heart failure (CHF).

SCIENTIFIC BACKGROUND

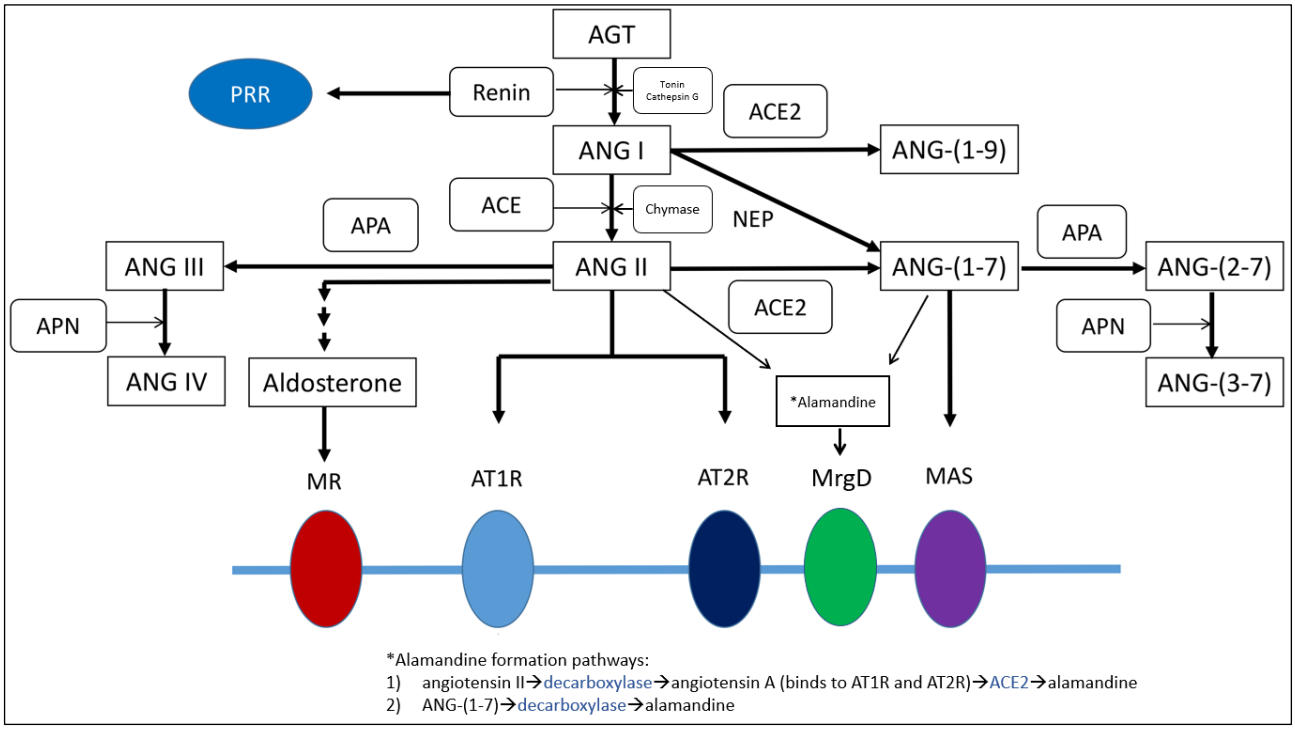
Renin-angiotensin-aldosterone system

Overview on physiology and cardiac pathophysiology

The physiologic function of the renin-angiotensin-aldosterone system is to preserve blood volume and pressure, thus ensuring an adequate perfusion to vital organs. Hypovolemia, hypotension, hyponatremia and sympathetic activity are the major triggers of this system[1,3]. Once renin is released into the circulation, it cleaves angiotensinogen (constitutively released by the liver) into angiotensin-I, which is physiologically inactive[1,3]. Angiotensin-converting enzyme (ACE), released from endothelium, converts angiotensin-I into angiotensin-II (A-II), which acts through two receptors. Angiotensin-type 1 receptor (AT1) activation leads to sodium retention, vasoconstriction, cellular growth, increased sympathetic activity, increased thirst and desire of salt, release of aldosterone from the adrenal zona glomerulosa and vasopressin from posterior pituitary gland[1,3,23,25]. Activation of angiotensin-type 2 receptor (AT2) counterbalances the effects of AT1s, leading to vasodilation and inhibiting cellular growth and proliferation[1,3,24]. Aldosterone, whose secretion is primarily regulated by A-II and potassium levels, acts via the mineralocorticoid receptors (MR) inducing sodium and water reabsorption and potassium excretion[1,3,24]. Angiotensin-type 1 receptors are located in several tissues, such as kidney, heart, vascular smooth muscle cells, brain, adrenal glands and lung[25-27]. Angiotensin-type 2 receptors are primarily located in brain and adrenal glands, while they are expressed at lower levels in other districts; however, their expression generally declines after birth[1,3,26]. Mineralocorticoid receptors are expressed in both epithelial (e.g., kidney) and non-epithelial (e.g., myocardium, vascular smooth muscle cells) tissues. Expression and activity of AT1s, AT2s and MRs can vary under pathological conditions, such as heart failure[28-31].

In the last decades, the classical pathway of RAAS has been revisited and several new components have joined the system (Figure 1)[32]. For example, mast cells chymases have been found to mediate the conversion of angiotensin I to A-II, thus forming an ACE-independent pathway[33-36]. Great interest has arisen around the ACE2-angiotensin 1-7-mas axis, which acts as a counter-regulatory pathway of the classical RAAS and mediates cardiovascular protection[37]. Recently, alamandine has been proposed as a novel cardioprotective RAAS component, derived from A-II and angiotensin 1-7[37,38].

Figure 1. Expansion of the renin-angiotensin-aldosterone system



AGT, angiotensinogen; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ANG, angiotensin; ANG I, angiotensin I; ANG II, angiotensin II; ANG III, ANG-(2–8); ANG IV, ANG-(3–8); APA, aminopeptidase A; APN, aminopeptidase N; AT1R, angiotensin II type 1 receptor; AT2R, angiotensin II receptor; MAS, angiotensin-(1–7) receptor MAS; MR, mineralocorticoid receptor; NEP, neutral endopeptidase; PEP, prolyl endopeptidase; PRR, (Pro)renin- receptor; MrgD, Mas-related G-protein-coupled receptor, member D. From Hsu CN, Tain YL (2021), modified (Creative Commons CC BY license: https://creativecommons.org/licenses/by/4.0/legalcode). The figure has been modified to add "Tonin, Cathepsin G", "Chymase", "Alamandine" and "MrgD" boxes, and Alamandine formation pathways explanation; effects derived from receptor activation have been removed from the original figure.

This complexity has been further expanded by the introduction of the concept of tissue RAAS, a local system with paracrine, autocrine and intracrine effects[39]. Tissue RAAS has been found in several organs, including heart, kidney and vessels, and its components are likely both captured from the circulation and synthesized locally[39-41]. The circulating and the local form represent two complimentary and integrated systems, which exert a physiological function in normal conditions and a pathological role in course of several diseases[3,39,42,43]. Existence of tissue RAAS is also relevant from a pharmacological perspective, since the affinity of RAAS blockers might be different for circulating and tissue components[44,45].

Dysregulation of RAAS represents a cornerstone of the HF syndrome[1,3]. The inadequate cardiac output (i.e., renal hypoperfusion and increased adrenergic nervous system activity) activates the neurohormonal cascade, which has an important compensatory and beneficial effect in the early stages of the disease. However, this overactivity is maladaptive over the long-term. A chronic exposure to high concentrations of A-II and aldosterone leads to several detrimental cardiovascular effects (Figure 2)[3], such as vascular and myocardial hypertrophy, fibrosis and inflammation, other than volume and pressure overload[3,46-53]. Taken together, these effects contribute to cardiovascular remodelling and progression of the HF syndrome[1,3]. Several studies have demonstrated the beneficial effects of ACE-inhibitors (ACE-I), angiotensin receptor blockers (ARB), neprilysin inhibitor and mineralocorticoid receptor antagonists (MRA) on morbidity and mortality in people with cardiovascular diseases[51,52,54-59]. Indeed, they represent a cornerstone of human HF therapy[10,11]. In dogs, multiple studies have showed the positive impact of ACE-I and MRAs on survival, disease progression and clinical signs in patients with congestive HF secondary to MMVD[6,60-63], and both drugs are included in the recommended chronic HF therapy[7]. In contrast, the use of RAAS blockers in dogs with asymptomatic MMVD is still subject of debate[7,64-68]. Potential beneficial effects of ARBs (alone or associated with neprilysin inhibitor) in dogs with cardiac diseases have been suggested by few experimental studies[69-71] and three recent preliminary trials[72-74].

However, current evidence is not sufficient to recommend their use in the clinical practice.

Figure 2. Harmful cardiovascular and renal effects of aldosterone and angiotensin II

Adverse effect	Direct effects of angiotensin II	Direct effects of aldosterone
Myocardial remodeling: fibrosis, hypertrophy, necrosis, apoptosis	Yes	Yes
Vascular remodeling: hypertrophy, fibrosis	Yes	Yes
Increase ROS	Yes	Yes
Pro-inflammatory (cytokines, ROS)	Yes	Yes
Arrhythmogenic	Yes	Yes
Vascular endothelial dysfunction (ET-1, vasopressin, acetylcholine-mediated vasodilatory dysfunctin)	Yes	Yes
Systemic hypertension	Yes	Yes
Glomerular damage	Yes	Yes
Glomerular dysfunction: proteinuria	Yes	Yes
Increased intraglomerular pressure	+++ (vasoconstriction)	+ (fluid retention & SNS)
Tubulointerstitial injury	Yes	Yes
Baroreceptor dysfunction→HR increase	Maybe	Yes
Increased SNS tone	Yes	Yes
Inotropy	Yes	No
Direct HR increase	Yes	No
Salt appetite	Yes	Yes
Increased thirst	Yes	No
Na^+ and H_2O retention, congestion	Yes	Yes
K^+ wasting	No	Yes

ET-1, endothelin 1; HR, heart rate; SNS, sympathetic nervous system activation; ROS, reactive oxygen species. From Ames MK, Atkins CE, Pitt B (2019) without changes (Creative Commons CC BY-NC license: https://creativecommons.org/licenses/by-nc/4.0/legalcode).

Focus on aldosterone in heart failure

In the last decades, aldosterone has acquired a dominant role in the pathophysiology and management of the HF syndrome. As reported in Figure 1, the adverse effects of aldosterone are not limited to the volume/pressure overload due to sodium and water reabsorption[3]. Excessive aldosterone concentrations exert a true cardiac and vascular "toxic" effect, inducing organ damage and dysfunction[3,52]. These harmful effects are strongly potentiated by an inappropriate aldosterone-sodium balance (i.e., high

aldosterone and high/normal sodium levels), which is likely to occur in the presence of HF and normal/high-sodium diet[3,52,75,76]. Moreover, aldosterone might also exacerbate the deleterious cardiovascular effects of A-II. In vitro studies have showed that aldosterone upregulates expression and activity of AT1s and ACE in vascular smooth muscle cells and cardiomyocytes respectively[77-80].

The central role of aldosterone in HF syndrome has been further consolidated by the discovery of the aldosterone breakthrough (ABT) phenomenon. Aldosterone breakthrough is defined as a condition in which aldosterone levels rise up to or above pre-treatment levels despite ACE-Is/ARBs therapy[81]. Several mechanisms have been hypothesized to explain this phenomenon: insufficient ACE-Is/ARBs dosage[82,83], ACE-independent formation of A-II (e.g., chymases)[33-36], overwhelming RAAS activity because of therapy and disease progression (e.g, diuretic, vasodilators, salt restriction, renin positive feedback, sympathetic activity, renal hypoperfusion)[84-90], stimulation of aldosterone secretion through AT2s[91,92], inadequate suppression of tissue RAAS[87], secretion of aldosterone by RAAS-independent paracrine regulatory factors at the level of adrenal cortex (e.g., endothelin 1, serotonin, leptin, catecholamines)[93,94], decreased neurohormones metabolism and clearance because of organs hypoperfusion[90,95], and increased potassium concentrations[82]. Aldosterone breakthrough has been reported to occur in 10-53% of human patients subjected to ACE-Is/ARBs treatment for various diseases[81,88,96]. In dogs, several studies have reported the occurrence of ABT in 40-56% of healthy research subjects with pharmacologically induced RAAS activation[97,98]. More recently, ABT has been found in 32% of dogs with chronic HF secondary to MMVD[90] and 34-59% of dogs with proteinuric chronic kidney disease[99]. The prevalence of this phenomenon is likely influenced by several factors, such as use of different cut-offs to define its presence, underlying disease, comorbidities, and type of therapy and its duration[81]. Interestingly, after treatment with ACE-I (i.e., enalapril), dogs with MMVD and positive for ACE gene polymorphism showed higher serum aldosterone concentrations and greater incidence of ABT compared to polymorphism negative dogs[100]. This result suggests

that even the genetic profile might be involved in the mechanisms behind the ABT, and this factor, in canine species, could also be related to the breed. Considering the detrimental effects of excessive aldosterone concentrations, it's clear that ABT might contribute to HF syndrome progression and worse prognosis[101,102]. Indeed, higher aldosterone levels have been associated with an increased risk of HF re-hospitalizations, cardiovascular mortality and all-cause mortality in people with cardiovascular diseases[103-110]. These results, in association with the concept of ABT and the existence of aldosterone alternative pathways, strongly support the need of MRAs in addition to the standard RAAS blocker therapy (i.e., ACE-Is, ARBs).

Assessment of aldosterone in dogs with heart diseases

As stated before, a sequential RAAS blockade is recommended in dogs with chronic HF[7] because of neurohormonal overactivity[1,111-114]. In contrast, RAAS activation in the asymptomatic phase of heart diseases (i.e., MMVD, dilated cardiomyopathy) is not clearly established[1,53,113-122], as well as the benefits of a RAAS blocking therapy[64-66,68,123]. This ambiguity is also related to the heterogeneity of asymptomatic patients, which range from those with a very mild disease to those near to the onset of congestive HF. However, the trend of RAAS activity could change even over the course of chronic HF due to the ABT phenomenon[90]. In light of the above considerations, assessment of neurohormonal activity might help to optimize the monitoring and management of patients with heart diseases.

A comprehensive evaluation of different RAAS components would be the best way to estimate RAAS activity; the recent development of the RAAS equilibrium analysis (RAS-Fingerprint™; Attoquant Diagnostics GmbH, Vienna, Austria), performed by liquid chromatography-mass spectrometry/mass spectroscopy, opened the door to this possibility. However, this technique remains not very accessible because of the high costs and, currently, is not very suitable for the use in routine clinical practice. Among several RAAS components, aldosterone has the advantage of being the terminal hormone of the cascade. Thus, its assessment takes into account the alternative

pathways of RAAS (e.g., ACE and A-II independent pathways), estimates the efficacy of ACE-I/ARBs therapy and may identify the presence of ABT. Moreover, as mentioned above, aldosterone has been showed to have a prognostic value in people with cardiovascular diseases and to be a predictor of HF[103-110]. In previous studies performed in dogs with heart diseases, aldosterone was measured on plasma[111-113, 115, 116, 118, 119, 122]. However, circulating aldosterone might be subjected to continuous variations because of the pulsatile secretion of this hormone and its sensitivity to stress and posture[124-126]. Thus, it might not be an accurate marker of RAAS activity. Measurement of 24-h urinary aldosterone blunts these fluctuations because it reflects the total amount of aldosterone secreted during the day; however, this method is not feasible in the clinical practice. In 2007, Gardner et al. showed that the urinary aldosterone-to-creatinine ratio (UAldo:C), derived from a spot urine sample, was comparable to the 24-h urinary aldosterone excretion in dogs[124]. In a subsequent study, this parameter mirrored the increase in angiotensin peptides measured with RAAS equilibrium analysis in healthy dogs treated with diuretics[127]. Thus, UAldo:C is economically affordable, can be easily obtained by non-invasive methods (i.e., free catch urine sample) and likely reflects the overall RAAS activity. When this project was designed, data about this parameter were poorly consolidated since only 2 studies investigated it in a clinical setting in dogs with MMVD, and only one of these had a healthy control group[53,90]. Further investigations were required to get a better comprehension of UAldo:C in healthy dogs and in dogs with heart diseases and understand its applicability in the diagnostic routine of these patients.

Natriuretic peptides

The Family

The family of natriuretic peptides (NP) consists of a series of hormones involved in the regulation of fluid homeostasis[9]. In mammals, three members of this family have been found: atrial natriuretic peptide (ANP), BNP and C-type natriuretic peptide (CNP)[9]. Dendroaspis natriuretic peptide (DNP) has only been isolated from the venom of the Green Mamba (*Dendroaspis angusticeps*)[128], while ventricular natriuretic peptide (VNP) is probably only present in fishes[9].

Atrial natriuretic peptide is primarily expressed in both cardiac atria and, in smaller amount, in the ventricles and brain. It is stored in granules and released following atrial myocardial stretch secondary to increased intravascular volume and/or pressure[9,129,130]. However, components of the neurohormonal system, such as endothelin, arginine, vasopressin and catecholamines, can directly stimulate ANP secretion[131]. Moreover, the small production of ANP in the ventricles can be enhanced during pathological conditions (e.g., volume overload, hypertrophy)[9,132]. Urodilatin is the kidney version of ANP; it is produced in the renal distal tubules and has mainly a local action[9,133,134].

Brain natriuretic peptide is primarily localized in the ventricles, while only a small amount is present in the brain and in the atria[129,131,135]. Unlike ANP, BNP is not stored in granules and is rapidly produced on demand following myocardial stretch and hypoxia[9,129,136-138]. As ANP, BNP production is also stimulated by other factors, such as A-II and adrenergic agonists[9,139].

Production of ANP and BNP starts from a pre-pro hormone (pre-proANP and pre-proBNP respectively). Cleavage of these pre-pro hormones results in pro-ANP and pro-BNP. Subsequently, the pro-hormones are cleaved by serine proteases (corin and furin respectively) in the biologically active mature ANP and BNP and their inactive fragments N-terminal proANP (NT-proANP) and NT-proBNP[129]. The mature hormone and its fragment are released into the circulation. Atrial natriuretic peptide has a half-life of 2-3 minutes, while that of BNP is around 20 minutes[140,141]. The inactive fragments NT-proANP and NT-proBNP have a much longer half-life, which is about

60-120 minutes[141]. Recently, new immunoassays that detect the mid-region of NT-proANP (MR-proANP) have been developed, since this part is easily detectable and less prone to further fragmentations[142].

C-type natriuretic peptide is primarily expressed in the vascular endothelial cells, central nervous system, kidney, chondrocytes and pituitary gland; secondarily, it can be found also in the heart and brain[9,129,131,143]. It acts as a paracrine and autocrine factor[9,144,145]. Indeed, CNP is particularly expressed in tissues, while its plasma concentration is low[9,129]. Stimuli for CNP release are not clearly established, although its expression seems to be enhanced by volume overload[146]. Similarly to ANP and BNP, production of CNP starts from pre-proCNP, followed by proCNP, which is then cleaved by furin into the biologically active mature CNP (CNP-53) and the inactive fragment NT-proCNP[129]. In some tissues, CNP-53 is cleaved in another biologically active peptide, the CNP-22[129]. The half-life of CNP-22 is approximately 2-3 minutes[147]; that of CNP-53 is supposed to be a bit longer, but it has not been reported in humans[148]. The half-life of NT-proCNP is estimated to be 40 minutes[148].

Two other "minor" peptides are guanylin and uroguanylin, which are localized in the gastrointestinal mucosa[131,149].

In mammals, NPs exert their action through two natriuretic peptide receptors (NPR) on cell surface: NPR-A (lungs, brain, heart, adrenal glands, kidneys, terminal ileum) and NPR-B (lungs, brain, skin, kidneys, adrenal glands, uterus, ovaries)[9]. They are expressed also in blood vessels, with NPR-B particularly abundant in veins compared to arteries[9,150]. Atrial natriuretic peptide and BNP have high affinity for NPR-A[129,151], while NPR-B has high selectivity for CNP[152].

In contrast, natriuretic peptide receptor type C (NPR-C) is responsible of the clearance of NPs and it is localized in several tissues, such as lungs, brain, kidneys, heart, adrenal glands, mesentery, adipose tissue, veins and aorta[9,153]. Its affinity is higher for ANP and CNP compared to BNP[129,151]. Neprylisin is a metalloprotease which contribute, along with NPR-C, to circulating NPs degradation via hydrolysis[9,154]. Neprylisin is primarily expressed in kidneys, but it is found also in other tissues such as brain and lungs[9,155]. It

has good affinity for ANP and CNP, while BNP is more resistant[129]. Inactive fragments of NPs are instead eliminated through organs such as kidneys[9].

Physiological action

Atrial natriuretic peptide induces vasorelaxation by acting on vascular smooth muscle cells and increasing permeability of vascular endothelium[129,156-158]. Moreover, ANP stimulates vagal afferents and blunts the sympathetic tone in the peripheral vasculature[131,158,159]. Atrial natriuretic peptide exerts cardiovascular protective effects suppressing vascular fibrosis and proliferation of vascular smooth muscle cells, as well as inhibiting myocardial fibrosis and hypertrophy[129,156,158].

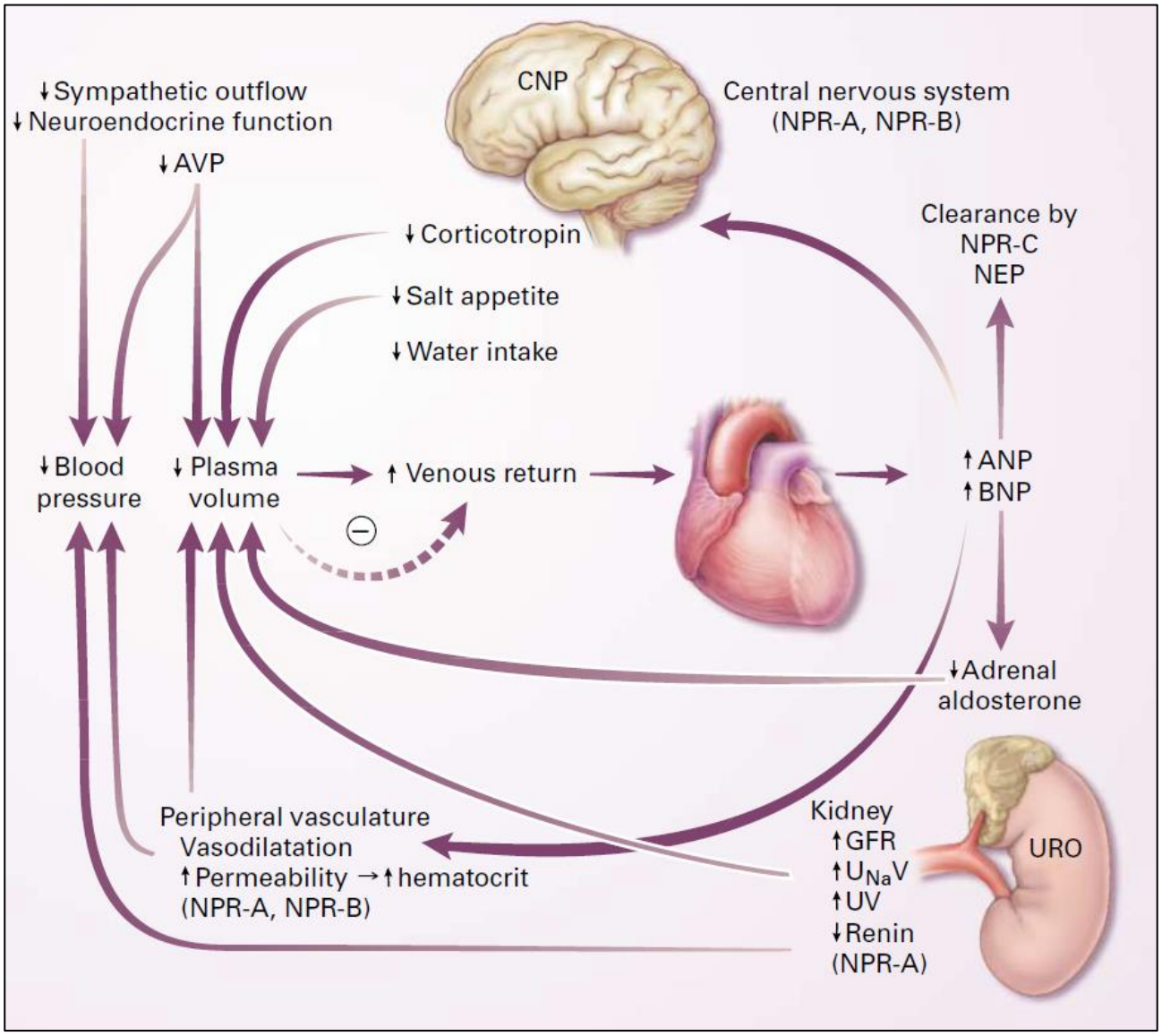
In the kidney, ANP increases glomerular filtration rate (GFR) inducing afferent arteriolar dilation and efferent arteriolar constriction[160]. Moreover, ANP exerts important natriuretic and diuretic effects through direct tubular actions and RAAS suppression. This peptide inhibits Na^+ and H^+ exchanger in the proximal tubule, Na^+-Cl^- cotransporter in the distal tubule and Na^+ in the collecting duct[129,158]. Moreover, it antagonizes arginine vasopressin contrasting water reabsorption in the collecting duct[161]. Atrial natriuretic peptide counterbalances the effect of RAAS through the reduction of renin secretion from the macula densa and the inhibition of water and sodium reabsorption induced by A-II in the proximal tubules[158,162-164]. This hormone also inhibits aldosterone secretion induced by different stimuli in the adrenal zona glomerulosa[158,165]. Urodilatin, the local kidney version of ANP, exerts the same action on RAAS components[9]. Atrial natriuretic peptide is present also in the lungs, where it induces bronchodilation and contrasts pulmonary hypertension[158].

Cardiovascular and renal effects of BNP are very similar to those of ANP. These two peptides are found also in the brain, where their local actions reinforce the effects in the periphery[131]. Figure 3 offers an overview on the cascade of ANP and BNP effects starting from the increased venous return to the heart[131].

Instead, CNP has some different effects, such as simulation of long bone growth e reduction of pulmonary hypertension and fibrosis[9,129,158,166,167]. However, even CNP has a vasodilatory action, an antiproliferative effect on vascular smooth muscle cells, an

antigrowth effect in glia, blunts sympathetic nervous system activity and regulates cardiac hypertrophy and remodelling[9,129,131,158,168-170].

Figure 3. Physiologic effects of natriuretic peptides released from the heart when venous return is increased.



URO denotes urodilatin; NEP neutral endopeptidase; CNP C-type natriuretic peptide; NPR-A, NPR-B, and NPR-C natriuretic peptide receptors A, B, and C, respectively; AVP arginine vasopressin; ANP and BNP atrial and brain natriuretic peptides, respectively; GFR glomerular filtration rate; U Na V urinary sodium excretion; UV urinary volume; and BP blood pressure. Reproduced with permission from Levin ER, Gardner DG, Samson WK (1998) without changes (Copyright Massachusetts Medical Society: https://www.nejm.org/about-nejm/permissions, reuse of content within a thesis or dissertation).

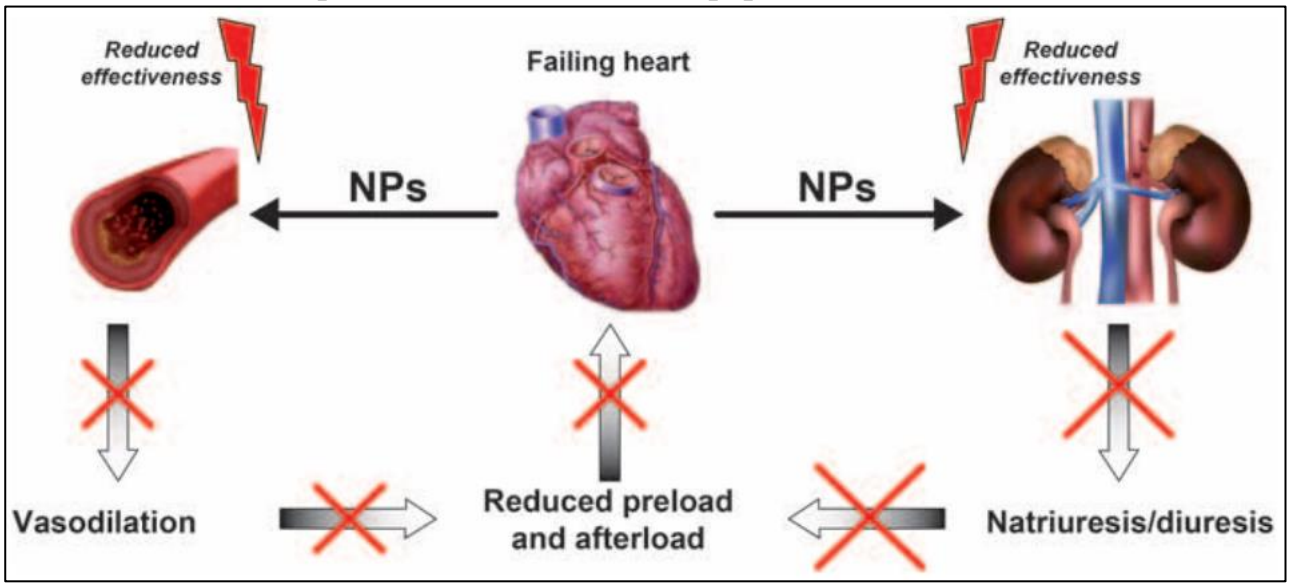
Pathophysiology in heart failure

Heart failure is generally characterized by volume/pressure overload, reduced cardiac output and RAAS overactivity. For these reasons, NPs system is progressively activated under such conditions[158]. As a consequence, circulating ANP and BNP concentrations are elevated during HF and positively correlated with the severity of the disease, with a greater increase of BNP compared to ANP in the presence of congestion and in the more advanced states[129,158,171]. Elevation of circulating CNP is instead weak, probably because it is more concentrated in tissues[129]. Elevated NPs concentrations serve as a counter-regulatory mechanism to sympathetic nervous system, RAAS and vasopressin overactivity and, consequently, to volume/pressure overload and cardiac remodelling[158]. Several experimental studies in dogs have shown that the elimination of NPs or the blockade of NPRs induced a rise in sympathetic nervous system (SNS) and RAAS activity, as well as an increase in sodium and water retention[131,158,171], thus aggravating renal and cardiac function. On the other hand, intravenous administration of NPs in people and dogs showed an improvement of clinical status, cardiac output, diuresis and pulmonary and systemic pressure[158,171-174]. However, as HF worsens, these elevated NPs concentrations are insufficient to contrast the deleterious effects of SNS and RAAS overactivity[158,171,175,176]. Indeed, people and experimental animals with overt HF still show significant sodium and water retention because of reduced renal and vascular effectiveness to these peptides[171,177-181]. Several mechanisms have been proposed to explain this condition. A decreased availability of biologically active NPs may occur because of delayed conversion of proBNP to mature BNP (reduced levels of corin), glycosylation of proBNP (more resistant to proteases), increased levels of dipeptidyl peptidase IV (producing less active forms of BNP), increased neprilysin activity and upregulation of NPR-C[177]. Decreased response by target organs has been also associated with a reduced expression, desensitization and inhibited intracellular signalling (by phosphodiesterases) of NPRs in course of HF[177]. Lastly, NPs actions can be counter-regulated by the overactivity of RAAS (e.g. reduced renal blood flow, A-II-induced reduction in NPRs activity and expression, stimulation of neprilysin activity

by angiotensin I)[171,177,182-184], SNS (e.g. reduced renal blood flow, further increase in renin secretion, downregulation of NPRs)[171,177] and endothelin-1 (e.g. reduced NPRs activity)[177,185].

It's clear that the HF syndrome is a condition characterized by a profound neurohormonal dysregulation, with excessive SNS and RAAS activity and blunted response to NPs.

Figure 4. Reduced responsiveness to natriuretic peptides in chronic heart failure.



NPs, natriuretic peptides. From Diez J (2016), without modifications (Creative Commons CC BY NC-ND license: https://creativecommons.org/licenses/by-nc-nd/4.0/legalcode).

Application in veterinary medicine (dogs)

Brain natriuretic peptide and NT-proBNP have been historically the most studied members of NPs family because of their greater stability and easier assessment; moreover, these peptides concentration tend to be higher in advanced congestive HF compared to ANP and seems to perform better in terms of sensitivity and specificity[9,129,186]. Indeed, these two hormones are recommended diagnostic and prognostic tools in the current American and European heart failure guidelines[10,11]. However, despite promising results and the presence of ongoing clinical trials, they are

not included in the recommended heart failure therapy in humans because of the short bioavailability, no evidence of long-term effects on mortality and hospitalization and possible side effects (e.g., hypotension)[177,187]. On the other hand, RAAS blockers associated with neprilysin inhibitors are first-line treatments in people[10,11]. In particular, neprilysin degrades several vasoactive peptides, such as natriuretic peptides, bradykinin and adrenomedullin[59,188-190]. Its inhibition leads to increased concentrations of these substances, thus helping to counteract RAAS and SNS overactivity[59,191,192]. Even in dogs with congestive HF, inhibitors of RAAS are recommended drugs[7], while the use of neprilysin inhibitor in association with ARBs is still in an experimental stage[73,74].

Several studies have showed the potential utility of BNP and, especially, NT-proBNP as diagnostic and prognostic markers in dogs with cardiac diseases. Brain natriuretic peptide and NT-proBNP have been found to be significantly higher in blood of dogs with occult and overt dilated cardiomyopathy (DCM) compared to controls[193-196], as well as in healthy Doberman Pinschers which subsequently developed the disease within 1.5 years[194]. N-terminal pro-B-type natriuretic peptide was significantly higher in Doberman Pinschers with symptomatic DCM compared to asymptomatic patients[194]. Moreover, concentration of this peptide resulted an independent predictor of all-cause mortality in Doberman Pinschers with the occult form[195]. Nevertheless, this test was usually characterized by low sensitivity and/or specificity in detecting dogs with various forms of this disease, especially in the absence of echocardiographic abnormalities[193-195]. However, the association of plasma NT-proBNP concentration with 24-hours Holter showed good performance in detecting occult DCM[195]. Studies in dogs with MMVD showed more relevant results. N-terminal pro-B-type natriuretic peptide concentration resulted significantly higher in dogs with both asymptomatic and symptomatic MMVD compared to healthy ones[197-199]. Moreover, plasma concentration of this hormone was significantly associated with echocardiographic and radiographic measures of cardiac size, as well as significantly correlated with disease severity[199-201]. N-terminal pro-B-type natriuretic peptide was turned out to be an important predictor

of all-cause and cardiac mortality in dogs with MMVD[197,198,200-203], and a 100 pmol/L increase in this parameter increased the hazard by 7%[202]; Reynolds CA et al. (2012) also showed that it was able to independently estimate risk of first-onset of congestive HF[204]. Lastly, radiographic evidence of congestive HF was unlikely and cardiac survival was longer in dogs with lower plasma NT-proBNP concentration that underwent diuretic treatment[8,205]. Overall, these results suggest that NT-proBNP can be a useful tool for the screening of dogs with suspected cardiac diseases, as well as to stratify the risk of morbidity and mortality, monitor the disease progression and, potentially, guide the therapeutic strategy[8]. Another important indication for NT-proBNP testing is the diagnostic screening of dogs with respiratory signs of unknown causes[8]. Indeed, several studies have showed its ability in differentiating dogs with cardiac and non-cardiac causes of respiratory signs[206-210].

Potential limitations of the use of these peptides are related to daily and weekly biological variability, in terms of both within-subject and between-subjects variations[8,211-213]. Moreover, impairment of renal function and body mass index can affect BNP and NT-proBNP levels[8,9]. Even sex differences have been reported for NT-proBNP in dogs and people[201,214-218].

Currently, a second-generation enzyme-linked immunosorbent assay (ELISA) is available for NT-proBNP measurement in dogs (Cardiopet® proBNP test, IDEXX Laboratories, Westbrook, ME), along with non-breed specific interpretive criteria[219]. However, breed represent another potential confounding factor in the canine species. Indeed, a significant interbreed variability has been reported in a previous study[12]. Subsequently, two studies have reported high values of NT-proBNP in healthy Labrador Retrievers and Greyhounds, respectively[13,14], suggesting that the proposed generic canine reference intervals might not be appropriate for all breeds. Breed-specific reference intervals might improve the diagnostic accuracy of this parameter.

A multi-organ syndrome

An overview

Heart failure is characterized by pathologic hemodynamic derangements which can lead to both a "backward failure" (i.e. elevated cardiac filling pressures) and a "forward failure" (i.e. reduced cardiac output)[2]. Consequent hypoperfusion, congestion, neurohormonal and metabolic dysregulations, oxidative stress and inflammation are inevitably responsible of multiple organs damage and dysfunction[2,4,220-222]. These multi-organ alterations in turn contribute to the progression of cardiac disease, establishing a persistent and deleterious vicious circle.

Heart

The heart itself is affected by these pathological perturbations, which lead to loss of cardiomyocytes and alterations of the residual viable myocardium (e.g., fibrosis, hypertrophy, alterations of extracellular matrix, abnormal energy handling)[221,223,224]. Myocardial injury is a primary consequence of HF and contributes to progressive ventricular stiffness, remodelling and impaired diastolic and systolic function, thus resulting in a vicious circle[221,223,224]. Cardiac troponins T and I (cTn-T and cTn-I) represent the primary direct marker of myocardial injury, as they are intracellular proteins which are released into circulation when cardiomyocytes death occurs[224]. Elevated serum levels of cardiac troponins have been associated with increased mortality and morbidity in people with both chronic and acute HF[225-228]. Their correlation with a poorer prognosis has been also reported in dogs with various heart diseases[203,224,229-231]. Serum procollagen type III N-terminal peptide (PIIINP), galectin-3 and interleukin-1 receptor-like 1 protein (ST2) are involved, under pathological conditions, in extracellular matrix turnover, fibroblast proliferation and inflammation[221,232,233]. Thus, they are considered as marker of myocardial fibrosis in people and have a prognostic value in patients with HF[221,233-236]. In dogs with heart diseases, encouraging results have been reported for galectin-3, while ST2 and PIIINP appear less promising biomarkers[53,237-240].

Lung

Lungs are directly connected to the heart and inevitably suffer from a failing myocardium. Forward failure, that is reduced cardiac output, mainly compromises ventilation efficiency, increasing ventilation-perfusion mismatch and physiologic dead space and reducing respiratory muscle strength and tidal volume[2]. Inefficient ventilation has been showed to increase resistance and decrease compliance of pulmonary vessels[241]. However, elevated left-sided cardiac filling pressure (i.e., backward failure) are the primary responsible of lung injury in patients with HF. Pulmonary venous congestion leads to increased pulmonary venous and capillary pressure and culminate in alveolar oedema and/or pleural effusion[2,5]. Consequent pulmonary alterations can be reversible in the short-term[242]. However, repeated and sustained elevations in pulmonary capillary pressure are responsible for mechanical, inflammatory and oxidative injuries, which progressively lead to endothelial dysfunction, vasoconstriction, myofibroblasts proliferation and thickening and fibrosis of interstitium and alveolar-capillary barrier[2,5,221,243]. These pathological changes result in impaired gas diffusion (i.e., arterial hypoxia)[2,243-245] and pulmonary venous remodelling (i.e., postcapillary pulmonary hypertension [PH])[2]. Progressively, even the arterial compartment is involved and combined postcapillary and precapillary PH occur[2]. This exacerbates right ventricular dysfunction, ventricular interdependence and abnormal right ventricular to pulmonary arterial coupling, thus further worsening lung function[2,242,246]. Increase in right-sided cardiac filling pressure (i.e., central venous pressure) also impairs lymphatic drainage and favour pleural effusion[2]. Even the bronchial system can suffer from these hemodynamic and ventilatory abnormalities, and cardiac asthma is a recognized entity in the HF syndrome[247]. Presence of pulmonary hypertension has been associated with increased mortality and morbidity in people with chronic HF[248,249]. Right-heart catheterisation represents the gold standard to diagnose PH, but it is an invasive technique[250]. Several echocardiographic parameters represent a valid non-invasive alternative, and they are routinely used to indirectly estimate PH in veterinary medicine[251,252]. Peak tricuspid regurgitation

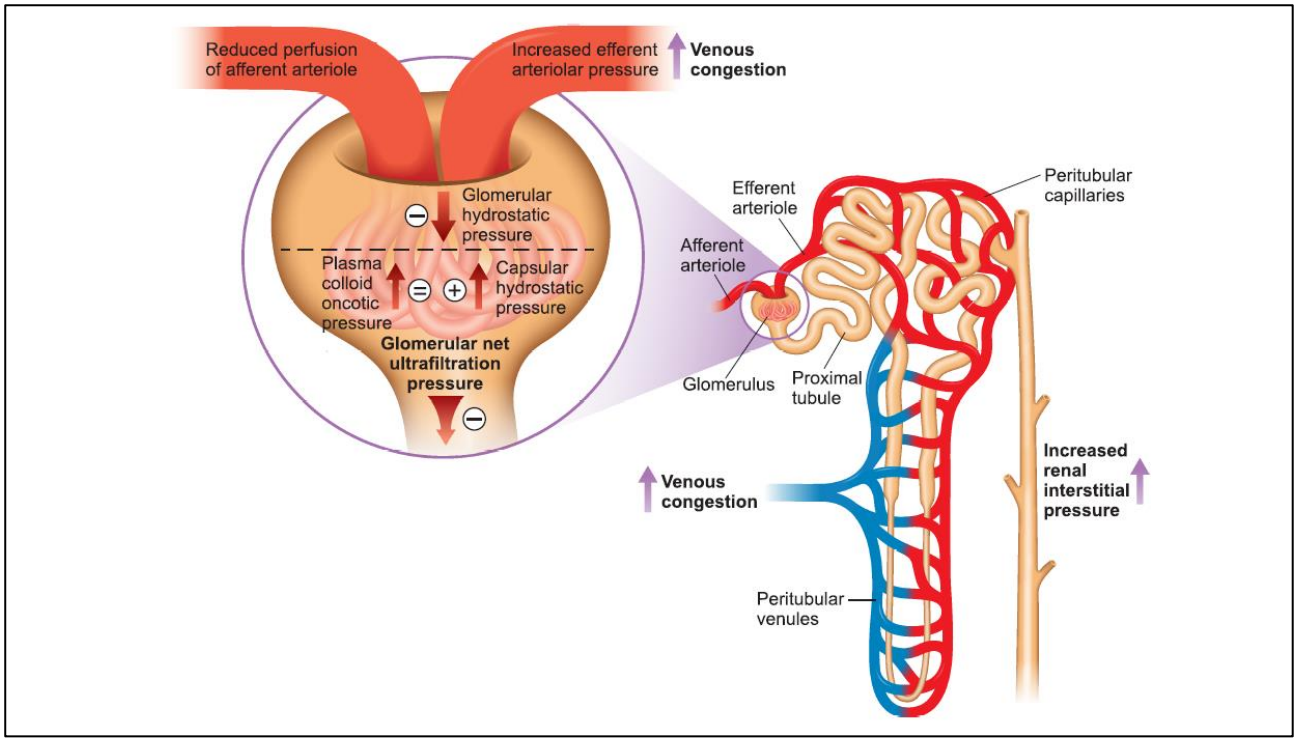
gradient is the main echocardiographic marker of PH and it has been associated with a worse outcome in dogs with pre-clinical and clinical MMVD[252].

Kidney

The crosstalk between the heart and kidney is the most studied interaction of the HF syndrome. Veterinary classification of the cardiorenal syndrome has been recently adapted to human classification[253], and 5 phenotypes have been proposed based on the primary failing organ and disease acuity (e.g. acute/chronic HF, acute/chronic kidney disease, failure of both organs due to independent systemic disease)[254,255]. The following discussion will focus on the role of HF as primary cause. Cardiac output is the primary regulator of renal perfusion. Generally, in the presence of reduced cardiac output, kidney has an excellent capacity to adjust renal blood flow and maintain an adequate intraglomerular pressure (and hence glomerular filtration rate) through the autoregulation mechanism and the early compensatory RAAS activation[2,253,254]. However, prolonged (e.g. chronic HF) or severe (e.g. acute HF) hypoperfusion/hypotension states can overwhelm renal autoregulation capacity[2,254,256]. Concomitant increased sympathetic nervous system activity, extreme RAAS overactivity (see Figure 2), inflammation and oxidative stress strongly contribute to such dysregulation[2,253,254,256]. Overall, this renal hemodynamic instability can lead to reduction of functional nephrons, glomerular surface and permeability and intraglomerular hydrostatic pressure, resulting in kidney injuries and decreased renal function[2,256-261]. However, it's now well established that systemic venous congestion (secondary to backward failure) has a central role in the cardiorenal syndrome, and it is probably the primary driving force of worsening renal function in patients with HF, especially during decompensation episodes (Figure5)[2,5,16,253,262,263]. This phenomenon is called “congestive nephropathy”. A marked increase in central venous pressure is directly transmitted to renal veins, resulting in renal venous hypertension[2,254,256,264]. As the kidney has a tight capsule, such congestion lead to increased renal interstitial hydrostatic pressure; consequently, hydrostatic pressure of the tubular system increases as well, directly opposing filtration forces[2,254,256,264]. Indeed, the concomitant increase

in efferent arteriolar pressure and intratubular pressure decreases the pressure gradient between glomerular capillaries and proximal tubule, as well as between renal arterial and venous compartment[2,254,256,264]. A decrease in afferent arteriolar perfusion, which frequently occurs during HF, further exacerbates these gradients. This renal hemodynamic derangement results in reduced renal blood flow, decreased glomerular filtration rate and impairment of tubular secretion and reabsorption[2,254,256,264]. Even in this case, sympathetic nervous system and RAAS overactivity sustain this pathological condition[264]. While renal autoregulation promptly responds to low cardiac output, it is less clear if and how these intrinsic autoregulatory forces are able to adapt to venous congestion[2]. Even elevated intra-abdominal pressures (e.g. ascites) can contribute to congestive nephropathy[254].

Figure 5. Visual explanation of congestive nephropathy



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Overall, renal function in patients with HF is determined by a complex interaction among three main components: extracellular fluid volume, sympathetic nervous

system and RAAS[42]. Moreover, several drugs routinely used in the management of HF (e.g. diuretics, ACE-I, vasodilators) and other concomitant diseases (e.g., systemic hypertension, endocrine/metabolic disorders) can further alter renal hemodynamic[254-256]. The hemodynamic profile of patients with acute HF can be extremely complex and the association of hypoperfusion and congestion represents the worst scenario for renal function; achieving a good balance between an adequate perfusion and decongestion probably remains the best approach, although challenging. However, whenever possible, obtaining an adequate decongestion seems to be preferable compared to worry about preserving cardiac index and preventing excessive dehydration; certainly, severe hypotensive episodes must be always avoided[265]. Serum creatinine still represents the most used biomarker to estimate glomerular filtration rate in patients with HF and, along with serum urea, they are the only recommended renal biomarkers in human and canine HF guidelines[10,11,16]. Although less diffused, cystatin C seems to be more precise in estimating glomerular filtration rate compared to serum creatinine[16,266]. However, these estimations are validated in stable patients and may not reflect well true changes in glomerular filtration rate during dynamic hemodynamic conditions, such as acute HF[16,267]. Moreover, these parameters usually fail in detecting kidney injuries at early stages[267]. Assessing electrolyte disorders, diuresis, natriuresis and tubular function would help to get a better understanding of global renal function during acute HF[16]. Serum and urinary neutrophil gelatinase-associated lipocalin (NGAL) is the primary marker of tubular injury[16,253]. An increase in this parameter predicted the occurrence of WRF in people with acute HF[268], although controversial results have been reported in other studies[269,270]. In dogs with stable MMVD, urinary NGAL increased with disease severity[271], but it has not been evaluated during the acute symptomatic phase. Even urinary electrolytes can be reflective of tubular function, as well as indicative of diuretic responsiveness[16,272]. See next paragraph for further diagnostic and prognostic information on WRF in acute HF.

Other organs: liver, intestine, skeletal muscle and brain

Cardio-hepatic syndrome represents another well recognized entity during HF[273]. Similarly to WRF, liver dysfunction during HF comes from a combination of hypoperfusion and venous congestion, where the latter seems to have predominant role even in this case. Liver injuries secondary to decreased cardiac output (also called "hypoxic hepatitis") are more frequent during severe and acute decompensation episodes (e.g., cardiogenic shock, severe hypoxemia) and are characterized by acute centrilobular hepatocellular damage and necrosis[273-276]. On such occasion, increases in serum alanine and aspartate aminotransferases activity are the main laboratory findings[273,275]. However, liver has an excellent capacity to extract oxygen from circulation and it is particularly resistant to hypoperfusion alone[277]. Thus, concomitant venous congestion is likely determinant for the onset of hypoxic lesions, increasing the susceptibility of the liver to reduced cardiac output[273,274,278]. However, venous congestion is also a direct cause of liver injuries, especially during chronic HF. This hemodynamic alteration leads to elevated hydrostatic pressure in liver sinusoids, enlargement of liver cells and consequent compression of bile canaliculi and ducts[273]. Laboratory abnormalities secondary to venous congestion are typical of cholestasis, such as increase in alkaline phosphatase, gamma-glutamyl transpeptidase and hypoalbuminemia[273,274]. Bilirubin elevations occurs with both hypoperfusion and congestion[273]. Increase in liver enzymes and bilirubin can be reversible if HF is well controlled and cardiac function is restored[273,277]. Lastly, liver dysfunction can alter drugs metabolism, as well as some drugs commonly used in HF can have a hepatotoxic effect[274]. In dogs with acute HF, elevated liver enzymes were one of the most common abnormalities[279]. In people with HF, several liver laboratory parameters have been showed to have a prognostic value[273,274].

Venous congestion and low cardiac output are also responsible of decreased flow in splanchnic circulation, leading to intestinal oedema and hypoperfusion[4,280]. The consequent increase in gut permeability favours endotoxins and bacterial translocation into systemic circulation, which in turn contribute to the overall pro-inflammatory and

oxidative state[4,5,280-283]. Altered intestinal environment also modify the microbiota, which further promote metabolic abnormalities and disruption of gut barrier[284,285]. Dysbiosis, oedema and thickening of gastrointestinal wall decrease nutrients absorption and, along with inflammation, contribute to cardiac cachexia[280,282,286]. Protein-losing enteropathy can be another consequence[280,287]. Gastrointestinal dysfunction also leads to alteration in the pharmacokinetics and pharmacodynamic of drugs and can complicate the pharmacological treatment of HF[280,288].

Skeletal muscle is seriously compromised in patients with HF. Hypoperfusion, congestion, RAAS overactivity, inflammation, oxidative stress, altered systemic metabolism and endothelial dysfunction lead to major structural and functional changes of skeletal muscle, such as mitochondrial dysfunction, decrease of type 1 fibers and impaired energy metabolism (e.g., reduced oxidative capacity, increased glycolysis)[4,289,290]. These abnormalities result in sarcopenia, that is muscle atrophy and weakness. Muscle wasting is the primary responsible of exercise intolerance and it is a major component of cardiac cachexia, a condition driven by an anabolic/catabolic imbalance and associated with a devastating prognosis in people and dogs with HF[291-297].

Lastly, brain is particularly sensitive to hypoperfusion[5]. During HF, global and regional atrophy, vascular brain injuries and increased blood-brain barrier permeability can occur[4]. Hypoxemia is likely the primary determinant of cerebral dysfunction in patients with HF, but also inflammation, oxidative stress and neurohormonal dysregulation play an important role[4,5]. In humans, cognitive dysfunction, depression and delirium can be consequence of these brain injuries[5,297].

The dilemma of worsening renal function in acute heart failure

Worsening renal function is usually defined as an increase in serum creatinine ≥ 0.3 mg/dL and it is one of the most common complications in people and dogs hospitalized for acute HF[298-302]. As explained above, the hemodynamic instability secondary to HF

is a direct responsible of impaired renal function/WRF in these patients. Moreover, it is well known that diuretics and RAAS blockers, the cornerstone of HF therapy, contribute to this complication[16,254,255]. Worsening renal function has been historically associated with worse outcome in people hospitalized for acute HF[299,303-307]. As a consequence, clinicians are used to reduce diuretic doses and/or stop RAAS blockers when an increase in serum creatinine occurs, at the expense of inadequate decongestion. However, this approach has been questioned in recent years. Worsening renal function alone was not an independent determinant of outcome in people with acute HF and had a negative prognostic value only in patients with residual congestion[18,308,309]. Moreover, haemoconcentration and aggressive diuresis were associated with improved survival despite an increased risk of in-hospital WRF[17,19,310-314]. Interestingly, even improving renal function (IRF) has been paradoxically correlated with a higher risk of mortality and HF re-hospitalization[315-317]. However, a recent study has shown that IRF is not the true cause of worse outcome, but it is instead reflective of a more severe acute decompensation; these patients have more vulnerable kidneys which experience WRF prior to admission, that is then identified as IRF during hospitalization[318]. Indeed, IRF patients had worse admission parameters and the benefit of decongestion overwhelmed the negative impact of IRF on mortality[316,318]. Wettersten et al. (2021) also speculated that the occurrence of IRF might be misinterpreted as a general improvement of organ perfusion, inducing clinicians to a less aggressive therapeutic (i.e., diuretics, RAAS blockers) approach[318]. Lastly, lowering right-cardiac filling pressures resulted more prognostically relevant than preserving cardiac index[319]. Overall, recent human literature highlights that achieving adequate decongestion should be the driving goal of acute HF therapy, regardless of WRF or IRF occurrence. In dogs with acute HF, a previous study did not find any association between serum urea and creatinine (at admission or discharge) and survival[301]. More recently, WRF was found to be a common abnormality in dogs hospitalized for left-sided congestive HF, but it did not impact on survival[300]. Thus,

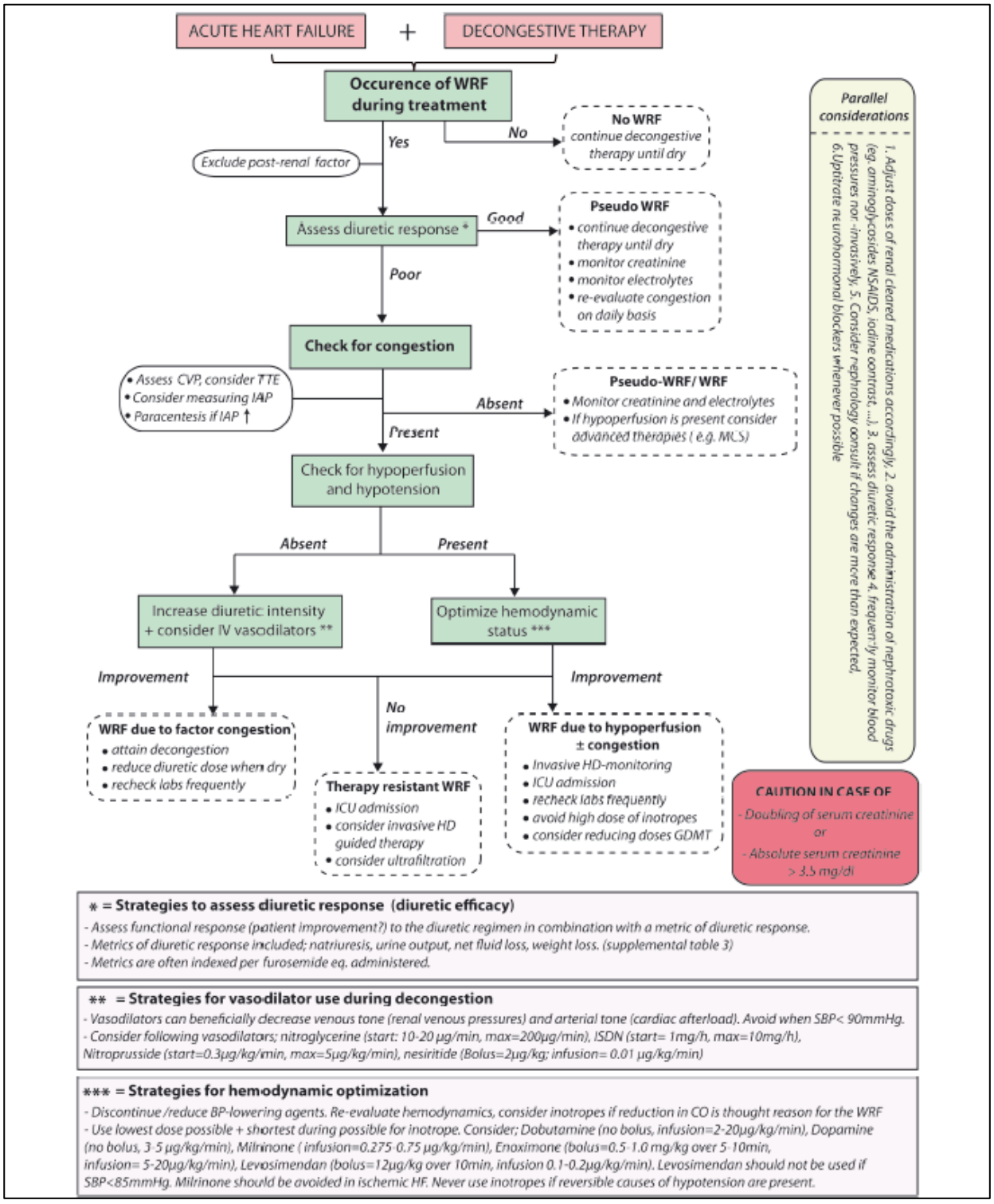
results in canine species seem to follow the same direction of human literature, although further investigations in this field are needed.

Hemodynamic alterations secondary to HF are likely responsible for the increase in serum creatinine prior to or early during hospitalization[16]. In most patients, occurrence of WRF later during hospitalization (i.e., after treatment) might just be reflective of an appropriate renal response to therapy and changes in intraglomerular hemodynamic, rather than a true kidney injury[5,16,320]. Indeed, people that experience adequate diuretic response and decongestion frequently develop WRF during hospitalization but have the best prognosis at the same time[16,17,19,310-314]. Moreover, WRF in patients receiving aggressive diuresis was not associated with tubular injury, supporting the hypothesis that a small to moderate decrease in renal function in this setting diverts from a true acute kidney injury[320]. Ahmad et al. (2018) suggested that "it is the context by which WRF develops, rather than simply its presence, that is the principal determinant of adverse outcomes"[320]. If the overall clinical status of a patient improves or remains stable and adequate diuretic response and decongestion are achieved, the occurrence of WRF (called "pseudo-WRF") may be acceptable, it does not impact on outcome and should not trigger therapy discontinuation[16,321,322]. In contrast, if diuretic response is poor and/or the clinical status does not improve or deteriorates in the face of WRF, multiple causes should be investigated. Firstly, diuretic response and the status of congestion/perfusion must be checked and adequately optimized. Possible reversible causes such as increased intrabdominal pressure (e.g., ascites) or urinary tract obstructions must be considered[16]. Then, other extracardiac causes of true WRF can be supposed, such as use of nephrotoxic drugs (e.g., non-steroidal anti-inflammatory drugs), primary kidney failure (e.g., glomerulonephritis) and various systemic comorbidities (e.g., infections, blood loss, uncontrolled endocrine/metabolic diseases)[16,322]. Regardless of the underlying scenario, extreme increases in serum creatinine, especially if associated with electrolytes and acid-base disorders, must be always interpreted cautiously because they could be indicative of true WRF[51]. Moreover, monitoring of renal function is always recommended up to and after

discharge, and home therapy must be adjusted during follow-up, aiming to a good cardio-renal balance[16,322].

However, the clinical picture of a patient can be extremely complex and dynamic, and it does not always re-enter under a single category. Thus, distinguishing between pseudo and true WRF and establishing a cause-effect relationship remain a challenge in the clinical practice. For this reason, renal function should be assessed through a more holistic approach compared to the sole estimation of the glomerular filtration rate[16]. The assessment of the diuretic response is becoming a recommended practice in people with acute HF, and this topic has been recently arisen also in dogs[16,272,323-325]. Diuretic response can be quantified through different metrics, such as net fluid loss, weight changes, urine volume and urinary sodium[16,272]. Spot urinary sodium and hourly urine output assessed during the first hours of treatment seem to be the most useful methods to estimate the diuretic efficacy[16,272,323-325], but also haemoconcentration has been used as a surrogate of intravascular volume depletion[310]. Several urinary biomarkers can add further information on glomerular function and integrity (e.g., urinary creatinine, albuminuria), tubular function (e.g., urinary electrolytes) and tubular injury (e.g., NGAL, N-acetyl-β-D-glucosaminidase, kidney injury molecule 1, sediment analysis)[16,325-327]. Even serum electrolytes imbalances can be prognostically important in people[20,328-332], while controversial results have been obtained in dogs[231,333,334]. Figure 6 proposes a schematic approach to WRF during treatment of acute HF.

Figure 6. Approach to WRF during treatment of acute HF.



BP, blood pressure; CO, cardiac output; CVP, central venous pressure; GDMT, guideline-directed medical therapy; HD, haemodynamic; HF, heart failure; IAP, intra-abdominal pressure; ICU, intensive care unit; ISDN, isosorbide dinitrate; IV, intravenous; MCS, mechanical circulatory support; NSAIDS, non-steroidal anti-inflammatory drugs; SBP, systolic blood pressure; TTE, transthoracic echocardiography. From Mullens W, Damman K, Testani JM et al (2020) without changes (with permission from John Wiley and Sons).

RESEARCH ACTIVITIES

STUDY 1: Factors affecting the urinary aldosterone-to-creatinine ratio in healthy dogs and dogs with naturally occurring myxomatous mitral valve disease

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Phd student activity: participation in the conception and design of the study, acquisition of clinical data and drafting the manuscript.

Abstract

Background Chronic RAAS activation in course of heart diseases contributes to cardiac remodeling and heart failure. Myxomatous mitral valve disease is characterized by different stages of severity and trend of RAAS activity during the course of the disease is still uncertain. Urinary aldosterone-to-creatinine ratio has been proven to reflect RAAS activation in dogs and might be a useful marker in monitoring therapy and disease progression, but data about this parameter need to be expanded. The objective of this study was to evaluate the UAldo:C in healthy dogs and dogs with naturally occurring MMVD, and to investigate the relationships between this parameter and clinical, echocardiographic and laboratory variables.

Methods Dogs enrolled in this prospective cross-sectional study were recruited among private owned dogs referred for cardiological check-up. All subjects underwent complete physical examination, systemic pressure measurement, echocardiography, urinalysis, serum urea and creatinine analysis. Dogs with MMVD were classified

according to ACVIM guidelines. Urinary aldosterone was measured on left-over samples with an enzyme-linked-immunosorbent-assay, previously validated in dogs.

Results The study population consisted of 149 dogs: 49 healthy and 100 MMVD dogs (45 stage B1, 13 stage B2 and 42 stage C). Urinary aldosterone-to-creatinine ratio was not significantly different among healthy and MMVD dogs of any stages. Breed, sex and age showed a significant impact on UAldo:C. In particular, Chihuahua and Cavalier King Charles spaniel showed significantly higher UAldo:C than other breeds, as well as intact females than other genders. In stage C dogs, UAldo:C appeared to be increased by spironolactone and was positively associated with furosemide dose (P = 0.024). Aldosterone breakthrough appeared to occur in 36% (8/22) of stage C dogs not receiving spironolactone. A significant positive association between UAldo:C and left atrium-to-aortic root ratio (LA/Ao) was found.

Conclusions Individual factors such as breed, sex and age appeared to influence UAldo:C, and therapy seemed to add further variability. In the light of these results, comparing the UAldo:C of a single patient with a population-based reference value might lead to wrong interpretations and an individual monitoring should be considered. The prevalence of ABT in the present study (36%) was in line with those previously reported. However, due to the high individual variability of UAldo:C found in the study, even this result should be re-evaluated in the setting of an individual longitudinal approach. The positive association between UAldo:C and LA/Ao supports the mutual relationship between RAAS and cardiac remodeling.

Introduction

Renin-angiotensin-aldosterone system represents an important compensatory mechanism of heart failure. However, a chronic activation is maladaptive and contributes to the development of cardiovascular remodelling and congestive pattern because of the harmful cardiovascular and renal effects of A-II and aldosterone [1,3,46,49,335-338]. Indeed, higher aldosterone levels have been associated with cardiac

remodelling and worse outcome in humans with heart diseases[104,105,109,337,339-343], and urinary aldosterone (UAldo) concentration have appeared to be associated with greater ventricular remodelling and a worse prognosis in dogs with MMVD[53,344]. Moreover, the beneficial effects of angiotensin-converting enzyme inhibitors (ACEI) and spironolactone in patients with CHF have been showed in both species, indirectly proving the negative impact of chronic RAAS stimulation [3,55,60,63,345].

The MMVD is the most common acquired cardiovascular disease in dogs. Due to its chronic and progressive nature, MMVD is characterized by different stages of severity, ranging from a pre-clinical phase (stage B1 and B2, American College of Veterinary Internal Medicine [ACVIM] classification) to the onset of CHF and related clinical signs (stage C and D) [7]. The trend of RAAS activity during the course of the disease is still uncertain. While it is fairly established that RAAS is overstimulated after the onset of CHF secondary to various heart diseases [1,111-113], there are conflicting data about the neurohormonal activation during the asymptomatic phase [1,53,68,114-122]. Accordingly, whereas the administration of RAAS blockers (eg, ACEI, spironolactone) is recommended in stage C and D, their use in pre-clinical MMVD is still subject of debate [3,7]. Moreover, the ABT phenomenon suggests the possibility of RAAS overexpression even after the beginning of ACEI therapy [81,90].

Therefore, the assessment of RAAS activity in course of MMVD could help optimize the follow-up and the therapeutic management of the patient. Urinary aldosterone to creatinine ratio seems to be a very useful parameter for the monitoring of RAAS activity in the clinical practice. It has been proven to reflect RAAS activation and to be comparable to 24h urinary aldosterone excretion, which, unlike serum/plasma aldosterone, is not affected by the pulsatile variations of aldosterone secretion. Secondly, it can be easily determined from a single “free-catch” urine sample, thus avoiding blood sampling and reducing the impact of stress of in-hospital visit [90,97,124]. Moreover, compared to other RAAS components, aldosterone has the advantage of being the last effector of the cascade; thus, its assessment also takes into account the alternative pathways of RAAS, such as the ACE or A-II independent ones [3]. However,

to our knowledge, data about UAldo:C in healthy and MMVD populations are still not enough consolidated and should be expanded before introducing it in the diagnostic routine. The first aim of this study was to evaluate the UAldo:C in healthy dogs and dogs with naturally occurring MMVD in stage B1, B2 and C, in order to assess their RAAS activity. A second aim of the study was to investigate the relationships between UAldo:C and certain clinical, echocardiographic and laboratory variables.

Materials and Methods

Animals and study timeline

This prospective cross-sectional study was conducted in accordance with the guidelines of the Animal Care and Use Committee of the University of Milan (approval number 2/2016) and with informed consent of the owners. All procedures to which patients have been subjected were part of their routine health screening; blood and urine analysis were performed on leftover samples. Private owned dogs were recruited among those referred to the cardiology service of the Veterinary Teaching Hospital - University of Milan, between November 2017 and December 2019.

Inclusion and exclusion criteria

To be enrolled in the study, dogs had to be either healthy or affected by MMVD stage B1, B2 or C (ACVIM classification)[7]. No age, sex or breed restrictions were applied.
All included dogs underwent indirect blood pressure measurement, complete physical examination, echocardiography, standard urinalysis and UREA, SCr and UAldo evaluations.
A dog was considered healthy if the medical history and the results of the aforementioned procedures did not reveal any alterations. Patients that were diagnosed with MMVD by echocardiography, were classified in stage B1, stage B2 or stage C according to the criteria of the ACVIM guidelines[7]. Dogs with any cardiovascular disease other than MMVD were excluded. Subjects with clinically relevant diseases (ie, metabolic, endocrine or neoplastic) were excluded. Hypertension and chronic

kidney disease were reason of exclusion only in healthy, stage B1 and stage B2 dogs. The administration of non-cardiovascular drugs with known effects on RAAS (i.e., corticosteroids) was not accepted. The administration of cardiovascular drugs was allowed for stage B2 (pimobendan) and stage C (standard therapy: furosemide, ACEI, pimobendane; ± spironolactone) dogs.

Systolic arterial pressure measurement

Each dog was allowed to acclimate to the room for 5-10 minutes and SAP measurement was the first procedure to be performed based on previously published guidelines[346]. Dogs were gently restrained in ventral or lateral recumbency and SAP was measured by a Doppler sphygmomanometry method on the left thoracic limb of each dog, with a cuff size approximately 40% of the limb circumference. Blood pressure results were obtained by discarding the first measurement and averaging the following 5 consecutive ones.

The SAP measurement included in the analysis was the one recorded during the day of urine collection, in order to have temporal agreement between systemic pressure and aldosterone levels. Subjects with SAP >160 mmHg have been re-evaluated at subsequent examinations and true hypertension was excluded in all dogs, except for 2 stage C dogs in which hypertension was confirmed.

Echocardiography

The echocardiographic examination was performed by two experienced echocardiographers using an ultrasonographic unit (Esaote MyLab50 Gold Cardiovascular ultrasound scan) equipped with two different multifrequency phased array probes. All echocardiographic scans were carried out on conscious dogs in right and left lateral recumbency, in accordance with published standards[7,347].

All measurements were taken from at least three consecutive cardiac cycles, and the mean was recorded. The following measurements were taken from the right parasternal short-axis view: LA/Ao measured in 2D-mode using the Hansson's method[348], and LVEDD and LVESD measured in 2D-guided M-mode with the leading edge to inner edge method at the level of the papillary muscle. Normalized left ventricular end-

diastolic diameter and LVESDn were obtained using the allometric equation, as previously described[349]. Transmitral flow [E peak velocity, A peak velocity, E/A] was measured using continuous-wave Doppler from the left four chamber apical view.

Sample collection, storage and analysis

All urine samples were collected by spontaneous micturition and were immediately refrigerated. Within 8 hours, standard urinalysis was performed by dipstick chemistry test and refractometer (for USG evaluation); all samples were then immediately centrifuged at 1250 rpm for 5 minutes and supernatant was stored at -20°C. Supernatant underwent urinary protein (UP) and urinary creatinine (UC) evaluation by Pyrogallol Red Method and UP/UC was calculated (values < 0.5 were considered normal[350]). Samples were then submitted for determination of UAldo.

Blood samples were carried out by venipuncture at least 8 hours after meal and collected into serum gel tubes. Serum urea and SCr was determined by Urease-GLDH Method and Modified Jaffe's Method respectively (internal laboratory reference value: 20-60 mg/dL for UREA and <1.5 mg/dL for SCr).

Measurement of urinary aldosterone

Urinary aldosterone was determined by a commercially available species-independent ELISA kit (Enzo Life Sciences Aldosterone ELISA kit, Enzo Life Sciences Inc., Farmingdale, NY, USA).

Before analysis, urine samples were hydrolysed to extract aldosterone metabolites using a 3-fold dilution with 0.2 N HCl and incubation in the dark at room temperature for 24 hours. After the hydrolysis, the samples were diluted 30-fold in assay buffer (final dilution: 1/90) and processed immediately. The concentration of UAldo was determined following the manufacturer's recommendations. Cross-reactivity to various steroid hormones was: 11-deoxycorticosterone 0.30%; progesterone 0.20%; corticosterone 0.19%; cortisol, dihydrotestosterone, estradiol and testosterone < 0.001%.

As the kit was developed for measuring aldosterone in human and rat samples, the accuracy of the kit for measuring aldosterone in dog urine was evaluated by recovery and parallelism studies.

In the recovery study, a pooled urine of low endogenous aldosterone was prepared by thoroughly mixing urine samples from three dogs, that was then hydrolysed as previously described. Aldosterone solution was prepared by dissolving 1 mg aldosterone (SIGMA-Aldrich, Schnelldorf, Germany) in 1 ml of 100% ethanol which was further diluted to 100 ng/mL with assay buffer. Known amounts of aldosterone (10-50-100 pg/mL) were added into the urine pooled sample and the total urine aldosterone (including endogenous aldosterone) was measured using the kit. The amount of aldosterone recovered was then calculated by subtracting the spiked dose from the value obtained for the non-spiked urine samples and the overall recovery was summarized in linear regression analysis between the measured and the added concentrations.

In the parallelism study, the slope of the standard aldosterone curve was compared with the slope of the curves obtained assaying four urine samples taken from different dogs and serially diluted in assay buffer (1/30 – 1/960).

Furthermore, verification of performance for precision was tested to establish that the laboratory's performance was consistent with the manufacturer's claims.

Precision was determined by replicate determinations of aldosterone in four urine samples with different aldosterone concentrations. Intra-assay precision was determined by evaluating each sample five times within the same run of the assay on three separate occasions. Inter-assay precision was determined by evaluating each sample in three assays on separate days. The results are reported as coefficient of variation (CV%).

Parallelism and recoveries calculations were performed with statistical methods included in the GraphPad PRISM 8.0 software package (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 26.

Distribution of variables was tested for normality using the Shapiro-Wilk test at the $\alpha = 0.05$ level. Normally distributed data were presented as mean ± standard deviation and compared by the two-sided Student's t-test and non-normally distributed data were presented as median and IQR and compared by the median test; categorical data were presented as frequencies and compared by the Chi-square test.

Multiple comparisons were performed by ANOVA or median test as appropriate. Post hoc tests were performed when appropriate and Bonferroni adjusted P values were reported for significant findings.

Correlation was tested by the Pearson rho correlation coefficient, with the following interpretation: ≤ 0.3 weak correlation, > 0.3 and ≤ 0.7 moderate correlation, > 0.7 strong correlation.

Multiple linear regression was performed, and the backward method was used. An R square value greater than 0.1 (at least a weak correlation) at the 0.05 significance level was considered suitable. Regression was performed in the entire sample, in the group of healthy and stage B1 dogs and in stage C group, while it was not performed in stage B2 group because of low number of subjects.

A p-value of 0.05 was taken as statistical significance.

Results

Animals

The study population consisted of 149 dogs of which 49 were healthy, 45 were stage B1, 13 were stage B2 and 42 were stage C. Demographic data for all of the dogs included and for the different groups are reported in Table 1.

Age was significantly lower in healthy dogs compared to stage B1, B2 and C dogs (P values <0.001). Body weight (BW) was significantly higher in healthy dogs compared to stage C dogs (P values <0.05). No significant differences were detected in age and

BW between any other group pairs. There were no significant differences by sex among the four groups. Neuter status distribution was significantly different for healthy dogs compared to stage B1 and stage C dogs (P values <0.05). Breed distribution was significantly different for healthy dogs compared to stage B1, B2 (P values <0.05) and C dogs (P value ≤ 0.001), as well as between stage B1 and stage C dogs (P value <0.05). No significant differences were detected in neuter status and breed distributions between any other group pairs.

At the time of enrolment, 9/13 stage B2 dogs were already receiving pimobendan. All 42 stage C dogs were already receiving standard therapy (furosemide, ACEI, pimobendan) and 20 out of these were also treated with spironolactone. For stage C dogs, the median (interquartile range [IQR]) dosages of furosemide and ACEI were 2.93 (2-5) and 0.58 (0.39-0.82) mg/kg/day, respectively; the median (IQR) durations of furosemide and ACEI administration were 307.5 (46-579.75) and 344 (47-959) days, respectively. The median (IQR) dosage and duration of spironolactone administration (20/42 stage C) were 2.75 (2.23-2.97) mg/kg/day and 248 (70-488.5) days, respectively. Nine stage B2 and all stage C were receiving pimobendan at a standard dose of 0.25-0.30 mg/kg q12h.

Table 1. Demographic data in all dogs, healthy dogs and dogs with different stages of MMVD

	All dogs	Healthy	Stage B1	Stage B2	Stage C
Number	149	49	45	13	42
Age (years)	9 (6-12.25)	6 (3-7.50) †‡§	9.99 ± 3.37	9 (8-12)	11.71 ± 2.88
Weight (kg)	9.40 (6.18-17.25)	16 (7.33-27.90) §	9.40 (7.45-18.15)	8.6 (3.08-11.75)	7 (4.95-10.25)
Sex (F/M)	75/74	30/19	20/25	6/7	19/23
Neuter status (IF/NF/IM/NM)	23/52/52/22	16/14/13/6 †§	3/17/16/9	1/5/6/1	3/16/17/6
Breed		†‡§	§		
CKCS	27	6	8	6	7
CHH	18	7	4	3	4
JRT	8	3	4	0	1
Other breeds <15 kg	53	6	16	3	28
Other breeds ≥15 kg	43	27	13	1	2

CKCS: Cavalier King Charles Spaniel, CHH: Chihuahua, JRT: Jack Russell Terrier, F: Females, M: Males, IF: Intact Females, NF: Neutered Females, IM: Intact Males, NM: Neutered Males. Other breeds <15 kg**:** 22 crossbreed (1 healthy, 8 stage B1, 2 stage B2, 11 stage C), 6 Dachshund (2 stage B1, 4 stage C), 5 Miniature Pinscher (1 stage B2, 4 stage C), 4 Toy Poodle (2 healthy, 2 stage B1), 4 Maltese (4 stage C), 3 Pug (2 healthy, 1 stage B1), 2 Shih-tzu (1 stage B1, 1 stage C), 1 Bichon Frisé (1 healthy), 1 Chinese Crested Dog (1 stage C), 1 English Cocker Spaniel (1 stage C), 1 Épagneul Breton (1 stage B2), 1 Fox Terrier (1 stage C), 1 Bruxelles Griffon (1 stage B1), 1 Pekingese (1 stage C), 1 Yorkshire Terrier (1 stage B1). Other breeds ≥15 kg: 15 crossbreed (6 healthy, 7 stage B1, 1 stage B2, 1 stage C), 4 American Staffordshire Terrier (4 healthy), 4 Golden Retriever (4 healthy), 3 Pointer (2 healthy, 1 stage B1), 2 German Shepherd (1 healthy, 1 stage B1), 2 Staffordshire Bull Terrier (2 healthy), 1 Great Dane (1 healthy), 1 Standard Poodle (1 healthy), 1 Border Collie (1 stage B1), 1 Boxer (1 stage B1), 1 Cane Corso (1 stage B1), 1 Drahthaar (1 stage C), 1 Labrador Retriever (1 healthy), 1 Belgian Shepherd (1 healthy), 1 Rhodesian Ridgeback (1 healthy), 1 Standard Schnauzer (1 healthy), 1 English Setter (1 stage B1), 1 English Springer Spaniel (1 healthy), 1 Italian Hound (1 healthy).

Data are reported as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables.

†values significantly differ ($p<0.05$) from stage B1; ‡values significantly differ ($p<0.05$) from stage B2; §values significantly differ ($p<0.05$) from stage C.

Echocardiographic parameters

Echocardiographic parameters and systolic arterial pressure (SAP) for all of the dogs included and for the different groups are shown in Table 2.

There were no significant differences in any echocardiographic parameters between healthy and B1 dogs.

Left atrium-to-aortic root ratio was higher in stage B2 and C dogs compared to healthy and stage B1 dogs (P values <0.001), as well as in stage C dogs compared to stage B2 dogs (P value <0.05).

Stage B2 and C dogs had higher normalized left ventricular end-diastolic diameter (LVEDDn), compared to healthy (P values <0.001) and stage B1 dogs (P values ≤0.05). Stage C dogs had higher normalized left ventricular end-systolic diameter (LVESDn), compared to healthy and B1 dogs (P values <0.001), while was not significantly different for stage B2 dogs compared to healthy and stage B1 dogs. Normalized left ventricular end diastolic diameter and LVESDn were not statistically different between stage C and B2 dogs.

E peak velocity was higher in stage B2 and C dogs compared to healthy (P values <0.001) and stage B1 dogs (P values <0.05), as well as in stage C dogs compared to stage B2 dogs (P value <0.001). A peak velocity was higher in stage B2 and C dogs compared to healthy dogs (P value ≤0.001) and in stage C dogs compared to stage B1 dogs (P value <0.05), while was not significantly different for stage B2 dogs compared to stage B1 and stage C dogs. E peak velocity-to-A peak velocity ratio (E/A) was higher in stage C dogs compared to healthy, stage B1 (P values <0.001) and stage B2 dogs (P value <0.05), while it was not significantly different for stage B2 dogs compared to healthy and stage B1 dogs.

There were no significant differences in SAP among the four groups.

Table 2. Echocardiographic parameters and systolic arterial pressure in all dogs, healthy dogs and dogs with different stages of MMVD

	All dogs	Healthy	Stage B1	Stage B2	Stage C
Number	149	49	45	13	42
LA/Ao	1.42 (1.17-1.89)	1.20 (1.08-1.39) ‡§	1.26 (1.14-1.43) ‡§	1.79 ± 0.22 §	2.31 (1.84-2.69)
LVEDDn	1.55 (1.35-1.86)	1.42 ± 0.18 ‡§	1.39 (1.27-1.63) ‡§	1.83 ± 0.22	2.00 ± 0.35
LVESDn	0.91 (0.78-1.05)	0.89 ± 0.15 §	0.86 ± 0.18 §	0.96 ± 0.14	1.04 (0.95-1.14)
E peak velocity (m/s)	0.83 (0.65-1.13)	0.67 ± 0.16 ‡§	0.75 ± 0.17 ‡§	0.97 ± 0.20 §	1.32 ± 0.32
A peak velocity (m/s)	0.70 (0.55-0.83)	0.59 ± 0.13 ‡§	0.68 (0.54-0.82) §	0.76 (0.70-0.86)	0.81 (0.70-0.97)
E/A	1.23 (1.00-1.51)	1.17 ± 0.27 §	1.12 ± 0.30 §	1.20 ± 0.26 §	1.66 ± 0.55
SAP	142.94 ± 19.73	145 (140-150)	141.76 ± 20.62	155 (127.50-170)	140.67 ± 20.96

LA/Ao: Left atrium-to-aortic root Ratio; E/A: E peak velocity-to-A peak velocity ratio; LVEDDn: Normalized left ventricular end diastolic diameter; LVESDn: Normalized left ventricular end systolic diameter; SAP: Systolic arterial pressure.

Data are reported as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables.

†values significantly differ ($p<0.05$) from stage B1; ‡values significantly differ ($p<0.05$) from stage B2; §values significantly differ ($p<0.05$) from stage C.

Standard laboratory parameters

Standard laboratory parameters are reported in Table 3. Serum urea was higher in stage C dogs compared to healthy dogs (P value <0.05), while no significant differences were detected between any other group pairs. Serum creatinine (SCr) was not significantly different among the four groups.

Urine specific gravity (USG) was lower in stage C dogs compared to healthy and stage B1 (P values <0.001). Urinary protein-to-creatinine ratio (UP/UC) was significantly higher in stage B2 and C dogs compared to healthy dogs (P values <0.05). No significant differences were detected in USG and UP/UC between any other group pairs.

Table 3. Laboratory parameters in all dogs, healthy dogs and dogs with different stages of MMVD

	All dogs	Healthy	Stage B1	Stage B2	Stage C
Number	149	49	45	13	42
UREA (mg/dL)	39 (31-49.28)	33 (27.93-40-50) §	35.16 ± 10.60	58 ± 25.85	49 (38.68-68.04)
SCr (mg/dL)	0.92 (0.80-1.10)	0.91 ± 0.20	0.89 ± 0.18	1.04 ± 0.32	1 (0.80-1.18)
USG	1036 (1020.50-1052)	1046.98 ± 18.41 §	1042.07 ± 16.16 §	1039.69 ± 20.50	1019 (1011.50-1028.50)
UP/UC	0.12 (0.04-0.27)	0.07 (0.03-0.12) ‡§	0.15 (0.06-0.24)	0.20 (0.10-0.41)	0.25 (0.35-0.69)
UAldo:C (µg/g)	1.86 (0.88-3.77)	1.75 (0.83-4.02)	1.75 (0.73-3.13)	1.95 (0.85-4.65)	2.03 (1.16-4.85)

UREA: serum urea; SCr: serum creatinine; USG: Urine specific gravity; UP/UC: urinary protein-to-creatinine ratio; UAldo:C: Urinary aldosterone-to-creatine ratio.

Data are reported as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables.

†values significantly differ ($p<0.05$) from stage B1; ‡values significantly differ ($p<0.05$) from stage B2; §values significantly differ ($p<0.05$) from stage C.

Urinary aldosterone assay validation

The enzyme-linked immunosorbent assay kit (Enzo Life Sciences Aldosterone ELISA kit, Enzo Life Sciences Inc., Farmingdale, NY, USA) resulted appropriate for the measurement of aldosterone in dog urine after acid hydrolysis.

The ELISA standard curve in a semi-log plot was linear between 250 and 3.9 pg/mL. Recoveries of added aldosterone were satisfactory, ranging between 93.0% and 113.1% (mean 101.0% ±8.1). Furthermore, slope of the regression was not different from unit (slope 0.97±0.05; y-intercept 4.70±5.42; r^2=0.99).

Dilutional parallelism was also demonstrated. Dilutions of dog urine samples were compared with the dose-response curve for standard aldosterone. There was no significant difference (P>0.1) between the slopes, after log transformation of the dilutions (P>0.1).

The intra- and inter-assay %CV were calculated by measuring the urine aldosterone in four dogs. Aldosterone concentrations ranged between 1.22 and 13.51 ng/mL. The intra-assay %CV ranged between 8.2% and 16.6% and the inter-assay %CV between 14.2% and 21.3%.

Urinary aldosterone-to-creatinine ratio (UAldo:C)

There were no significant differences in UAldo:C among healthy, stage B1, stage B2 and stage C dogs (Table 3). Pearson's correlation and multiple linear regression analysis results are shown in Table 4 and Table 5 respectively.

For the evaluation of correlations between UAldo:C and other variables, healthy and stage B1 dogs were grouped together (H+B1 group), since they did not differ in therapy, SAP, echocardiographic measures and laboratory parameters. Comparison of UAldo:C values among breed and sex categories were performed only in H+B1 group in order to avoid any possible influence of therapy and MMVD severity. Chihuahua, Cavalier King Charles spaniel (CKCS) and Jack Russell terrier (JRT) were chosen as comparator breeds because they were the most common pure breeds in this group (as well as in the entire study population). Other breeds (see Table 1) were divided into two groups according to BW (other breeds <15kg vs other breeds ≥15 kg). Chihuahua

was chosen as the comparator breed for Pearson's and multiple linear regression analysis since showed the highest median value of UAldo:C in H+B1 group (Figure 1). For the linear regression analysis, the following baseline variables were considered in all analysed groups (total population, H+B1, stage C): age, BW, sex (female=1, male=0), breed (Chihuahua=1, any other breeds=0), LA/Ao, LVEDDn, E peak velocity, serum urea, UP/UC. In H+B1 group, the variable STAGE (Healthy=0, stage B1=1) was also included. In total population, the following variables were included in addition to the baseline ones: furosemide (YES=1, NO=0), ACEI (YES=1, NO=0), pimobendan (YES=1, NO=0) and spironolactone (YES=1, NO=0). In stage C dogs, only spironolactone (YES=1, NO=0) were considered, since all subjects in this stage were receiving furosemide, ACEI and pimobendan. In this group, even dosage and duration of both furosemide and ACEI were added to the regression analysis.

Table 4. Pearson's correlation between UAldo:C and other variables

	All dogs		Healthy + Stage B1		Stage B2		Stage C	
	Pearson's coefficient	P value	Pearson's coefficient	P value	Pearson's coefficient	P value	Pearson's coefficient	P value
UAldo:C-Sex	0.167	0.042*	0.247	0.016*	0.193	0.528	0.012	0.942
UAldo:C-Breed	0.346	<0.001*	0.480	<0.001*	0.257	0.396	0.109	0.494
UAldo:C-Age	-0.233	0.004*	-0.365	<0.001*	0.016	0.958	-0.181	0.252
UAldo:C-BW	-0.242	0.003*	-0.283	0.006*	-0.181	0.554	-0.125	0.429
UAldo:C-USG	0.274	0.001*	0.510	<0.001*	0.449	0.124	-0.049	0.756
UAldo:C-UP/UC	0.069	0.405	0.165	0.113	-0.040	0.896	0.048	0.762
UAldo:C-UREA	0.124	0.203	0.164	0.199	0.722	0.043*	0.024	0.888
UAldo:C-SCr	0.103	0.291	0.047	0.717	0.557	0.151	0.127	0.459
UAldo:C-LA/Ao	0.154	0.061	0.056	0.590	0.624	0.023*	0.230	0.148
UAldo:C-LVEDDn	0.077	0.354	-0.092	0.377	-0.255	0.401	0.262	0.094
UAldo:C-LVESDn	0.096	0.246	-0.083	0.426	0.098	0.750	0.282	0.070
UAldo:C-E peak	0.035	0.681	-0.040	0.717	-0.259	0.417	0.095	0.550
UAldo:C-SAP	0.039	0.680	0.193	0.105	0.338	0.282	-0.342	0.065

Sex: female=1, male=0; Breed: Chihuahua=1, any other breeds=0; BW: body weight; USG: urinary specific gravity; UP/UC: urinary protein-to-creatinine ratio; Sur: serum urea; SCr: serum creatinine; LA/Ao: left atrium-to-aortic root ratio; LVEDDn: normalized left ventricular end diastolic diameter; LVESDn: normalized left ventricular end systolic diameter; SAP: systolic arterial pressure.

*= significant (P value <0.05).

Table 5. Multiple regression final models for UAldo:C dependent variables in total population, Healthy + Stage B1 group and stage C group

	Goodness of fit	Variables in the model		
	R squared		**Coefficient**	**Significance**
Total population (a)	0.263	(constant)	-0.148	0.881
		LA/Ao	1.204	0.013*
		Sex (female vs male)	1.838	0.003*
		Chihuahua (YES vs NO)	3.615	<0.001*
Healthy + Stage B1 (b)	0.444	(constant)	-2.537	0.101
		Sex (female vs male)	1.958	0,014*
		UP/UC	4.903	0,052
		Chihuahua (YES vs NO)	4.323	<0.001*
		UREA mg/dL	0.089	0.018*
Stage C (c)	0.145	(constant)	2.395	0.003
		Furosemide dose (mg/kg/day)	0.336	0.024

LA/Ao: left atrium-to-aortic root ratio; sex: female=1, male=0; Chihuahua: YES=1, NO=0; UP/UC: urinary protein-to-creatinine ratio; UREA: serum urea.

(a) Backward method step 0 predictors: body weight (kg), age (years), sex (female=1 vs male=0), Chihuahua (YES=1 vs NO=0), UREA mg/dL, UP/UC, LA/Ao, LVEDDn, E peak velocity m/s, spironolactone (YES=1 vs NO=0), pimobendan (YES=1 vs NO=0), furosemide (YES=1 vs NO=0), ACEI (YES=1 vs NO=0).

(b) Backward method step 0 predictors: body weight (kg), age (years), sex (female=1 vs male=0), Chihuahua (YES=1 vs NO=0), UREA mg/dL, UP/UC, LA/Ao, LVEDDn, E peak velocity m/s.

(c) Backward method step 0 predictors: body weight (kg), age (years), sex (female=1 vs male=0), Chihuahua (YES=1 vs NO=0), UREA mg/dL, UP/UC, LA/Ao, LVEDDn, E peak velocity m/s, spironolactone (YES=1 vs NO=0), furosemide dose (mg/kg/day), furosemide duration (days), ACEI dose (mg/kg/day), ACEI duration (days).

*= significant (P value <0.05).

Healthy and stage B1 dogs

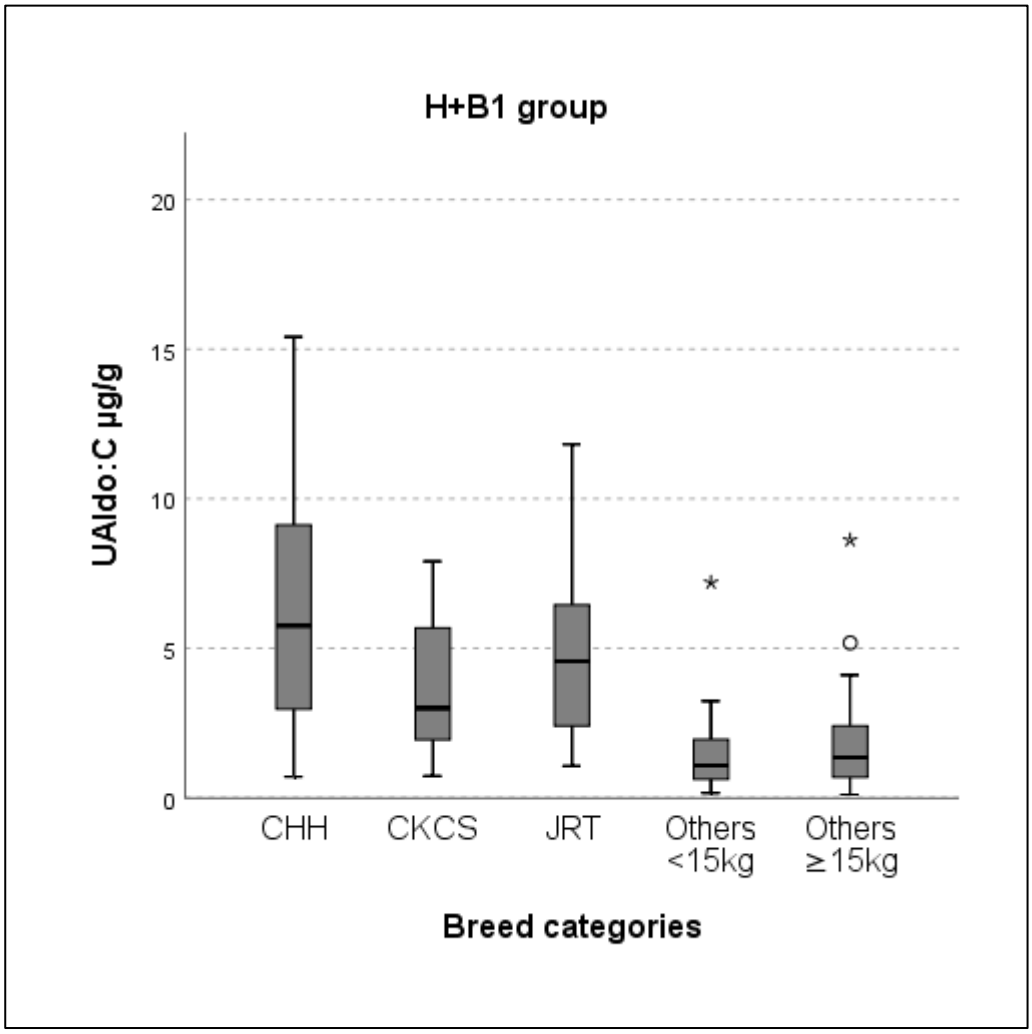
Urinary aldosterone-to-creatinine ratio showed a positive weak correlation with sex (female=1, male=0), a positive moderate correlation with breed (Chihuahua=1, any other breeds=0) and USG, a negative weak correlation with BW and a negative moderate correlation with age.

Chihuahua and CKCS showed a significantly (P values <0.05) higher UAldo:C than other breeds <15kg and other breeds ≥15kg (Figure 1). Jack Russell terrier had numerically, but not statistically, higher UAldo:C compared to CKCS, other breeds <15kg and other breeds ≥15kg.

Females showed higher UAldo:C than males (median 2.51 interquartile range [IQR] 1.06-3.98 µg/g vs median 1.33 IQR 0.64-2.29 µg/g; P value <0.05); intact females showed a significantly (P values <0.05) higher UAldo:C than other neuter status (neutered females, intact males [P values <0.05] and neutered males [P value ≤0.001]) (Figure 2).

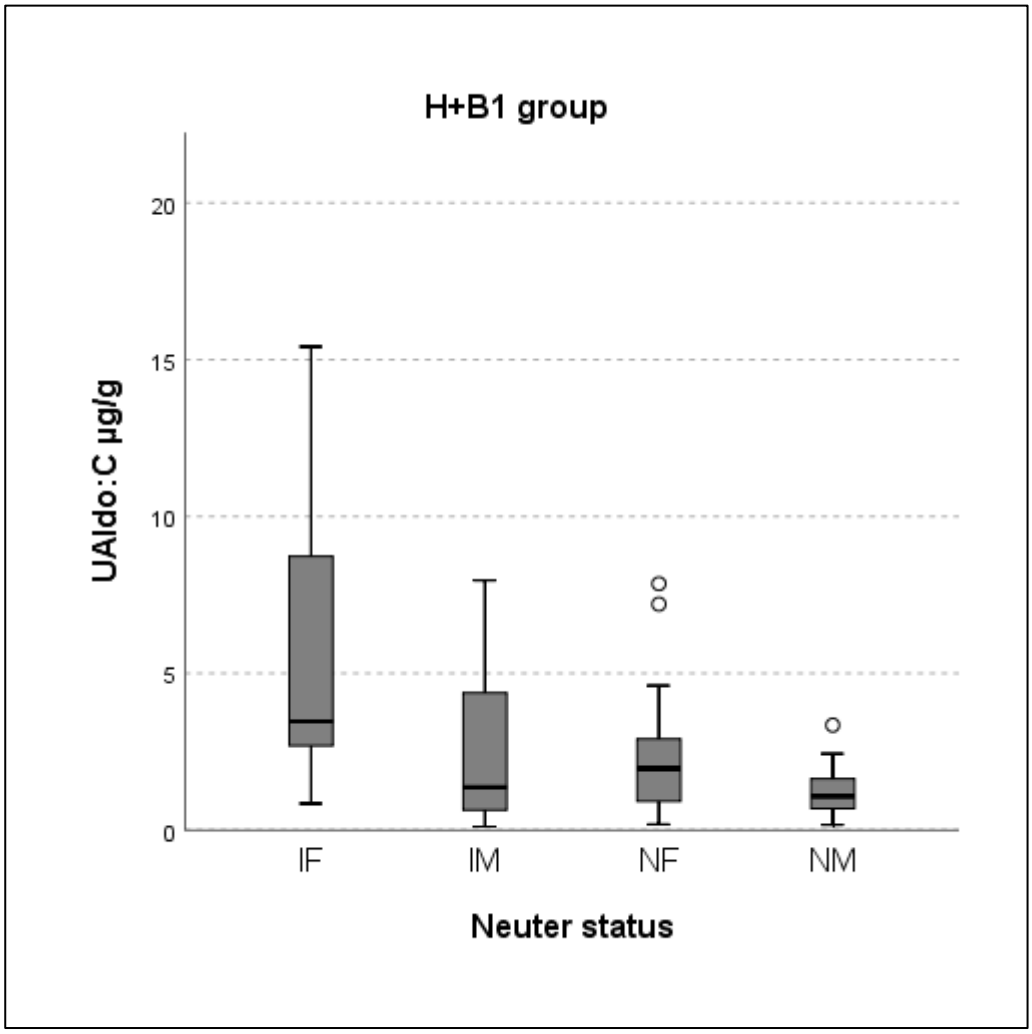
In the multiple linear regression analyses, UAldo:C was positively associated with sex (female=1, male=0), breed (Chihuahua=1, any other breeds=0) and UREA.

Figure 7. Comparison of UAldo:C among breed categories in H+B1 group



CHH: Chihuahua; CKCS: Cavalier King Charles spaniel; JRT: Jack Russell terrier; Others <15kg: other breeds with a body weight <15kg (see Table 1); Others ≥15kg: other breeds with a body weight ≥15kg (see Table 1). Chihuahua (median 5.75 IQR 2.91-9.40) and CKCS (median 3.00 IQR 1.90-5.70) showed significantly higher UAldo:C than others <15kg (median 1.08 IQR 0.61-2.02) and others ≥15kg (median 1.35 IQR 0.69-2.42) (P values <0.05). There were no significant differences between JRT (median 4.57 IQR 2.07-7.85) and any other breed categories, as well as between others <15kg and others ≥15kg.

Figure 8. Comparison of UAldo:C among neuter status in H+B1 group.



IF: intact females; IM: intact males; NF: neutered females; NM: neutered males. Intact females (median 3.47 IQR 2.64-8.83) showed significantly higher UAldo:C than IM (median 1.36 IQR 0.61-4.47), NF (median 1.96 IQR 0.87-2.93) (P values <0.05) and NM (median 1.08 IQR 0.67-1.75) (P value ≤0.01). No significant differences were detected in UAldo:C between any other group pairs.

Stage B2 dogs

In stage B2 dogs, UAldo:C showed a moderate positive correlation with LA/Ao and positive strong correlation with UREA.

Stage C dogs

In stage C dogs, Pearson's correlation did not show significant results for UAldo:C. In the multiple linear regression analysis, UAldo:C was positively associated with furosemide dose (mg/kg/day).

Within the stage C group, there was significant difference in UAldo:C between dogs treated with spironolactone (n=20; 2.50 IQR 1.71-5.76 µg/g) and those not treated (n=22; median 1.37 IQR 0.84-3.05 µg/g; P value<0.05). These two groups were separately compared with healthy, stage B1 and stage B2 dogs, but no significant differences in UAldo:C among groups were found in both cases.

Using the median UAldo:C value of healthy dogs as cut-off (1.75 µg/g), ABT occurred in 36% (8/22) of stage C dogs not receiving spironolactone.

Total population

In the entire study population, UAldo:C showed a positive weak correlation with sex (female=1, male=0) and USG, a positive moderate correlation with breed (Chihuahua=1, any other breeds=0) and a negative weak correlation with age and BW. In the multiple linear regression analysis UAldo:C was positively associated with sex (female=1, male=0), breed (Chihuahua=1, any other breeds=0) and LA/Ao.

Discussion

Urinary aldosterone-to-creatinine ratio was not significantly different among healthy and MMVD dogs of different stages, similarly to what reported by two recent studies[53,121]. Several factors may have influenced this result.

The RAAS activity during the asymptomatic phase is one of the most debated topics of MMVD pathophysiology since several studies have showed conflicting data[53,68,114-121]. Different assays, substrates (plasma, serum, urine, tissues), RAAS components and MMVD aetiology (naturally occurring vs experimentally induced) may have contributed to the lack of univocal results. However, these conflicting data probably also reflect the heterogeneity of the pre-clinical MMVD population: the severity of the disease and the consequent hemodynamic alterations could differ widely between patients that has just developed mitral insufficiency and those near to the onset of CHF. The recent results of the DELAY study seem to support this hypothesis, since UAldo:C was found to be significantly higher in advanced stage B2 (42 months) compared to early one (day 0), showing a progressive increase over time[68]. Definitely, the number of stage B2 dogs was very low in the present study and this may have decreased the statistical power for detecting significance in differences between this group and the other ones for UAldo:C. For the same reason, the impact of pimobendan on this parameter was not evaluated. In previous studies, short-term administration of pimobendan in healthy dogs had no effect on UAldo:C[86,351,352]. Even in the setting of a long-term treatment, pimobendan was not significant in the multivariable model for UAldo:C in dogs with MMVD. However, data are limited to one study[53] and further investigations about chronic administration are warranted.

Stage C dogs were expected to show an overstimulation of RAAS compared to healthy and pre-clinical MMVD dogs[1,111], but the complexity of therapy and its influence on RAAS makes difficult to interpret the result. All stage C dogs were receiving drugs with opposite effects on RAAS (ACEI vs diuretic)[3,53,84,86,351,352] and 20 dogs were also treated with spironolactone, which prevents aldosterone's binding to mineralcorticoid receptor, leading to an increase in blood and urine aldosterone concentrations[88,90,121,335].

As expected, dose of furosemide was positively associated with UAldo:C in the regression analysis and stage C dogs receiving spironolactone showed a significantly higher UAldo:C than those not treated.

Moreover, individual factors affecting UAldo:C were found in the present study, adding further variability to this parameter.

Cavalier King Charles spaniel and Chihuahua showed a significantly higher UAldo:C than other breeds <15kg and other breeds ≥15kg. In particular, Chihuahua showed the highest median UAldo:C value among breeds in H+B1 group, and in the multiple linear regression analyses this breed was positively associated with this parameter. Breed differences in RAAS activity were already reported by Pedersen et al. in 1995, which found higher plasma renin activity and aldosterone in CKCS and Poodles compared to other breeds[353]. More recently, Hezzell et al. have found higher UAldo:C in CKCS compared to other breeds[53], while, to our knowledge, there are no reports about Chihuahua. The influence of breed on RAAS activity could be related to differences in genes encoding for RAAS components. Polymorphism of ACE gene has been found in several breeds, including CKCS and Chihuahua, but it has been suggested that it could be more common in certain breeds[100,354,355]. Little is known about the effect of this polymorphism on RAAS components in dogs, but, as previously reported in humans[356], a recent study has found higher aldosterone levels and ABT incidence in MMVD dogs with ACE polymorphism after enalapril treatment and in presence of adequate ACE-A-II suppression, suggesting that this genotype could be involved in the upregulation of alternative pathways for aldosterone secretion[100]. Furthermore, polymorphism of genes encoding for angiotensinogen, A-II type 1 receptor, aldosterone synthase and chymase have been reported in humans and may also be present in dogs[357-360].

The negative association between UAldo:C and BW highlighted by Pearson's correlation is difficult to interpret because this parameter is strictly related to breed. Parameters such as body condition score (BCS) were not recorded, but we excluded clinically relevant underweight or overweight at physical examination.

In the multiple linear regression analyses, sex (female=1, male=0) was positively associated with UAldo:C. In H+B1 group, females showed a significantly higher UAldo:C than males and, distinguishing between neutered and intact, UAldo:C was significantly higher in intact females compared to other neuter status. To our knowledge, this is the first study that has reported an association between sex and aldosterone levels in dogs. Aldosterone levels have been showed to rise during the luteal phase of menstrual cycle in women and a relationship with progesterone concentration was found[361-363]. Progesterone showed an antimineralcorticoid effect in human, rats and guinea pigs, binding to mineralocorticoid receptor and preventing aldosterone interaction. Thus, the physiological increase in progesterone concentration during the luteal phase likely lead to an increase in serum and urinary aldosterone levels because of the receptor occupation and the compensatory activation of RAAS[361-367]. However, it has been also reported that progesterone is able to both directly stimulate the aldosterone production from zona glomerulosa and increase adrenal sensitivity to A-II[362]. Data about estrogens are instead controversial since they have been reported to induce both an increase and decrease in RAAS components[362,363,367-370]. Overall, sex hormones fluctuations during menstrual/estrous cycle seem to have a significant impact on RAAS components and may have been responsible for sex differences in aldosterone levels in the present study. Thus, gender, neutering status and phase of estrous cycle should be taken into account when evaluating aldosterone levels in dogs. Sex hormones and history of estrus were not evaluated in the current study. Moreover, the interaction between gender and RAAS could be even more complex; indeed, a relationship between sex and polymorphisms of RAAS genes has been reported in people[371-374]. Thus, further investigations are needed to better elucidate the association between sex and RAAS in dogs.

Lastly, UAldo:C showed a negative weak and moderate correlation with age, although this association was lost in the linear regression analyses, suggesting that other factors (eg, breed and sex) had greater impact on this parameter. However, similar result was obtained by one previous study in dogs[53]. In people and animal models, age-related

changes in RAAS were reported by several studies, which found a decline in plasma renin activity (PRA) and aldosterone production with advancing age; moreover, these changes were more pronounced under stimulatory conditions, such as sodium restriction or ACTH stimulation[375-380]. With aging, normal function of many physiological systems progressively decline. Mechanisms underlying the decrease in PRA still need to be clarified, but it may be related to morphological and functional alterations of aging kidney and consequent reduction in renin activation and synthesis[375,379,381,382]. The decline in aldosterone levels in older people could directly depend on lower PRA, but it has been also associated to a progressive decrease of aldosterone synthase expression[383]. Overall, older age appears to be associated with a lower physiological aldosterone secretion and with a reduced ability to respond to physiological stimuli of RAAS. For these reasons, age might contribute to the individual variability of aldosterone levels.

On the basis of the aforementioned results, a population-based reference value of UAldo:C might not be representative of the neurohormonal activity of the single patient and could lead to wrong interpretations. An individual monitoring of this parameter would likely be more accurate since it would take into account the impact of therapy and the influence of individual characteristics (ie, breed, sex and age). To our knowledge, individual monitoring of UAldo:C in MMVD dogs has been only performed in the recent DELAY study[68], which included only stage B2 dogs and did not aim to investigate the prognostic role of this parameter. Although the results are promising, showing an increase in UAldo:C as the disease progresses, further longitudinal studies are needed. This approach would help clarify the real diagnostic and prognostic value of UAldo:C in MMVD dogs, which might be misrepresented by mean/median values comparisons, and would help better define the ABT phenomenon.

Aldosterone breakthrough occurs when aldosterone levels rise up to or above pre-treatment levels despite ACEI/angiotensin receptor blockers administration[81,90]. In the present study, ABT was investigated in stage C dogs not receiving spironolactone using the median UAldo:C value of healthy dogs as cut-off. According to these criteria, ABT

occurred in 36% (8/22) of dogs. This percentage fit well with those previously reported both in humans and dogs[81,90]. However, this study showed that individual factors can affect UAldo:C. Thus, the use of median values might be misleading even for ABT definition. An individual monitoring before and after ACEI administration would be more accurate even for the identification of this phenomenon.

Our median normal UAldo:C value was remarkably higher than those of previous studies. Considering the aforementioned results, it is likely that breed and gender differences played an important role. In the present study, CKCS, Chihuahua, and JRT represented, together, 33% of breeds in healthy group. Females were the prevalent gender (61%), and most of them were sexually intact. In previous studies, the recruited healthy dogs were beagles or hounds (except for few crossbred) and most of them were males[85,86,97,351,352,384-386]. Further studies will help confirm breed and gender inconsistencies about UAldo:C.

In the present study, LA/Ao showed a positive correlation with UAldo:C in the linear regression analyses in total population. The progression of MMVD is mediated in part by the RAAS. Impairment of cardiac function leads to RAAS activation; on the other hand, persistent high aldosterone levels contribute to cardiac remodelling through several harmful cardiovascular effects[1,3,46,49,335-337]. In humans, the association between aldosterone and left ventricular remodelling has been shown[337,339-341] in recent studies, aldosterone levels have also been associated with left atrial structural and functional remodelling in patients with hypertension and primary aldosteronism[342,343]. In dogs with MMVD, UAldo:C has been positively associated with echocardiographic indicators of left ventricular remodelling[53]. In the DELAY study, treatment with spironolactone and ACEI in stage B2 dogs led to a reduction of LA/Ao and LVEDDn, confirming the negative effects of aldosterone; moreover, a progressive increase of both LA/Ao and UAldo:C during the study period (42 months) was observed in the placebo group[68]. The statistically significant association between UAldo:C and LA/Ao found in the present study support the mutual relationship between RAAS and cardiac remodelling. Left atrium dilation is probably the most important marker of MMVD

progression and LA/Ao is strongly associated with time to the onset of CHF or cardiac death[387,388]. On the basis of our result, MMVD dogs with higher UAldo:C are expected to show a more severe LA dilation, suggesting a possible role of aldosterone as a marker of disease progression and negative prognostic factor. In people affected by heart diseases, higher aldosterone concentrations have been associated with development of CHF and increased cardiovascular mortality[104,105,109], while the evidence of an influence on survival is minimal in dogs[344]. Thus, further studies in veterinary medicine are needed to explore the effect of aldosterone on outcome in patients with heart diseases.

It's well established that aldosterone also contributes to renal damage through multiple mechanisms, such as renal hemodynamic alterations, fibrosis and oxidative stress, and has been associated with a decline in estimated glomerular filtration rate (eGFR) in humans[1,46,338,389]. Preliminary findings in support of a relationship between aldosterone and renal function have been found in the present study: in the linear regression analyses UAldo:C was associated with serum urea. However, this association is difficult to explain as a result of aldosterone-induced renal damage since all healthy and stage B1 dogs had normal serum urea and creatinine. Veterinary medicine lacks specific studies about the pathological role of aldosterone on renal function and these preliminary findings warrant further investigations, especially in patients affected by kidney diseases.

The positive association between UAldo:C and USG observed was likely related to the sodium reabsorption and water retention induced by aldosterone.

The present study has several limitations. The first one is the low number of stage B2 dogs, which may have influenced the results obtained within this group and may have decreased the statistical power for detecting significance in differences among groups. Secondly, dietary sodium intake, time of feeding and time of urine collection were not controlled. Sodium-restriction has been associated with an increase in aldosterone levels and aldosterone has daily fluctuations, especially in relation to meals[3,84]; thus, these aspects may have influenced UAldo:C. Thirdly, this study focused on UAldo:C,

but other RAAS components also may be clinically relevant in MMVD dogs[114]. However, little is still known about their diagnostic and prognostic utility, as well as their individual variability, and further studies are needed, especially in a longitudinal setting. Definitely, a comprehensive assessment of different RAAS components should be always preferable, whenever possible, to the evaluation of a single factor in order to better characterized RAAS activity and its relation with certain variables. Lastly, aldosterone was not evaluated on other substrates, such as plasma and serum. At the current knowledge, UAldo:C seems to be the most accurate method to assess aldosterone levels in dogs[90,97,124]. However, further investigations about the comparison of aldosterone measurements on different substrates would be of interest. In conclusion, urinary aldosterone-to-creatinine ratio was not significantly different among healthy, stage B1, stage B2 and stage C dogs. This parameter appeared to be influenced by individual factors, such as breed, sex and age, and therapy probably added further variability. This means that the use of median values of UAldo:C to interpret the RAAS activity of a single patient or of a specific MMVD stage might be misleading. An individual monitoring of this parameter may be more appropriate and would help clarify its real diagnostic and prognostic value in dogs affected by MMVD. Aldosterone breakthrough showed a prevalence of 36% in stage C dogs not receiving spironolactone and this percentage is in line with those previously reported[90]. However, due to the high individual variability of UAldo:C found in the present study, even these results should be re-evaluated in the setting of an individual longitudinal approach. Lastly, UAldo:C was positively associated with LA/Ao, sustaining the mutual relationship between RAAS and cardiac remodelling and suggesting a possible role of UAldo:C as marker of MMVD progression.

STUDY 2: Aldosterone-progesterone relationship in sexually intact Chihuahua bitches

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Phd student activity: conception and design of the study, acquisition of clinical data, performing statistical analysis and drafting the manuscript.

Abstract

Background Aldosterone represents an important target of heart failure therapy and may be a valuable indicator of the renin-angiotensin-aldosterone system activity. However, its assessment might be challenging because of the effect of individual factors. In a recent study, intact female dogs showed the highest value of UAldo:C compared to other sex categories. In humans and rodents, an influence of progesterone has been reported by several studies. To our knowledge, the relationship between aldosterone and progesterone has not yet been investigated in dogs. The aim of this prospective study was to investigate this relationship in sexually intact Chihuahua females, measuring both hormones twice in the same bitch, that is in anoestrus when progesterone concentrations are baseline and in dioestrus when they are high.

Methods Dogs enrolled in this prospective study were recruited among breeding dogs referred for cardiological and reproductive screening/monitoring. All dogs underwent physical examination, systemic blood pressure measurement, vaginal cytology, complete blood count, biochemistry profile, urinalysis, echocardiography and hormones measurement during anestrus. In mid-diestrus, urinalysis, vaginal cytology and hormones assessment were repeated. Aldosterone and NT-proBNP (IDEXX

Cardiopet® proBNP) were measured by ELISA kits; progesterone was measured by a MINI-VIDAS® analyzer (bioMérieux). Hormones were assessed on left-over samples.
Results The study population consisted of 14 sexually intact Chihuahua bitches. Serum progesterone (34.06 (21.17-44.90) vs 0.19 [0.13-0.38] ng/ml; P<0.001) and urinary aldosterone (9886.98 ± 5735.22 vs 5005.72 ± 2127.73 pg/ml; P=0.01) were significantly higher in dioestrus compared to anoestrous. Urinary aldosterone-to-creatinine ratio was higher in dioestrus compared to anoestrus (4.16 [3.17-6.80] vs 3.39 ± 1.64 µg/g), but it did not reach the statistical significance (P=0.056). Serum progesterone showed a moderate positive correlation with urinary aldosterone (ρ=0.638, P<0.001) and UAldo:C (ρ=0.516, P=0.005).
Conclusions The results of the present study suggest the existence of a progesterone-aldosterone relationship in canine species, indicating that sex and phase of reproductive cycle should be taken into account when interpreting aldosterone concentrations. Further studies are needed to confirm these results on a larger canine population and to identify the underlying mechanisms in this species.

Introduction

Chronic RAAS activation promotes and perpetuates the CHF syndrome[1,3]. Prolonged and excessive aldosterone secretion leads to several harmful cardiovascular effects (eg, volume and pressure overload, inflammation, fibrosis), and aldosterone has become an important target of CHF therapy[3,49,53,63,340]. The assessment of RAAS activity in patients with heart diseases could help optimize their monitoring and therapeutic management. Aldosterone represents the terminal hormone of the cascade, and its assessment takes into account the RAAS alternative pathways and the aldosterone breakthrough phenomenon[3,90,100]. Thus, it holds potential as a valuable indicator of a patient's overall RAAS activity. However, the interpretation of this marker might be challenging. In a recent study in dogs, aldosterone levels appeared to be affected by individual factors. In particular, Chihuahuas and sexually intact females showed the

highest values of UAldo:C among breed and sex categories respectively[390]. The influence of breed might be related to polymorphisms of genes encoding for RAAS components, as previously suggested[100,355]. Regarding gender, RAAS activity has been suggested to be affected by sexual hormones. Aldosterone levels have been showed to rise during the luteal phase of menstrual cycle in women and progesterone has been found to be a high affinity antagonist for human MRs, preventing aldosterone interaction. The rise in progesterone concentration during the luteal phase leads to an increase in serum and urinary aldosterone levels because of the receptor occupation and the compensatory activation of RAAS[361,362,391]. However, progesterone might contribute to the rise of aldosterone concentration even through RAAS-independent mechanisms, such as directly stimulating aldosterone production from zona glomerulosa[362,366]. In dogs, an increase in RAAS activity during pregnancy has been suggested[392,393], but, to our knowledge, the relationship between progesterone and aldosterone levels has not yet been investigated in this species. The aim of this prospective study was to investigate the relationship between progesterone and aldosterone in sexually intact Chihuahua bitches, measuring these hormones in anoestrus and dioestrus (ie, low and high progesterone state respectively).

Materials and methods

Animals and study timeline

This prospective study was conducted in accordance with guidelines of the Animal Welfare Organisation of the Università degli Studi di Milano (approval number 2/ 2016) and with informed consent of the owners. Subjects enrolled in this study were recruited among private owned breeding dogs referred to the Veterinary Teaching Hospital – University of Milan (January-December 2021) for cardiological and reproductive screening/monitoring. All procedures to which patients have been subjected were part of their screening/monitoring program. Adjunctive analyses on

serum/plasma were performed on leftover samples, while urine analyses were performed on free-catch samples.

Inclusion and exclusion criteria

To be enrolled in the study, dogs had to meet the following criteria: Chihuahua breed, intact female and older than 12 months. All dogs had to be either healthy or affected by MMVD ACVIM stage B1[7]. Subjects with any other known disease or subjected to any pharmacological treatment at the time of examinations were excluded.

All dogs included in the study had to undergo two examinations as part of their reproductive monitoring program: the first one during anoestrus (i.e., at basal levels of progesterone) and the second one during following dioestrus (i.e., at high levels of progesterone). During anoestrus, each dog underwent indirect blood pressure measurement, complete physical examination, echocardiography, vaginal cytology, blood sampling (for CBC, biochemistry profile and serum progesterone), collection of a free-catch urine sample (for urinalysis and urinary aldosterone) and assessment of plasma NT-proBNP. In dioestrus, the following procedures were repeated: vaginal cytology, blood sampling (for serum progesterone), collection of a free-catch urine sample (for urinalysis and urinary aldosterone) and assessment of plasma NT-proBNP. Diet information was also collected. A dog was considered healthy if the medical history and the results of the aforementioned procedures did not reveal any alterations. Patients diagnosed with MMVD by echocardiography were classified as stage B1 following the criteria of the ACVIM guidelines[7].

Systolic arterial pressure and echocardiography

Non-invasive arterial pressure measurements were obtained and interpreted based on published guidelines[346], using a veterinary high definition oscillatory device (VET-HDO ®- MONITOR, S + B medVET GmbH, Babenhausen, Germany).

The echocardiographic examination was performed by two experienced echocardiographers using an ultrasonographic unit (Esaote MyLabOmega) equipped with two different multifrequency phased array probes (1-9 and 2-5 Mhz respectively).

All echocardiographic scans were carried out on conscious dogs in right and left lateral recumbency, in accordance with published standards[347].

Sample collection, storage and analysis

Blood sampling was performed by venepuncture on fasted dogs (i.e., at least 6-8 hours after meal), and blood was immediately collected into EDTA and serum gel tubes.

Blood collected into EDTA tubes was used for CBC. Samples were then immediately centrifuged at 3750 rpm for 5 minutes and plasma was stored at -80°C. Refrigerated plasma samples were sent at IDEXX laboratory (Korwestheim, Germany) for plasma NT-proBNP measurement by a second-generation ELISA (Cardiopet® proBNP test, IDEXX Laboratories, Westbrook, ME).

Blood collected into serum gel tubes was left to clot at room temperature for 30 minutes, and then it was centrifugated at 3750 rpm for 5 minutes. Serum was used for biochemical analysis (urea, creatinine, glucose, total protein, albumin, alanine aminotransferase, alkaline phosphatase). Residual serum was stored at -80°C and then submitted for the determination of progesterone.

Urine samples were collected by spontaneous micturition. Standard urinalysis was performed by dipstick chemistry test and refractometer; all samples were then immediately centrifuged at 1250 rpm for 5 minutes. Supernatant underwent urinary protein and urinary creatinine assessment by Pyrogallol Red Method and UP/UC was calculated (values <0.5 were considered normal[350]). Residual supernatant was stored at -80°C and then submitted for the determination of urinary aldosterone.

Urinary aldosterone concentrations were determined by a commercially available species-independent ELISA kit (Enzo Life Sciences Aldosterone ELISA kit, Enzo Life Sciences Inc., Farmingdale, NY, USA), following the manufacturer's recommendations. The ELISA kit was previously validated for the measurement of aldosterone in dog urine after acid hydrolysis[390].

Serum progesterone concentrations were determined by an Enzyme-linked Fluorescent Assay method (Enzyme-linked Fluorescent Assay) (MiniVidas automated analyser, bioMérieux, Marcy-l'Étoile, France) validated in dogs[394].

Statistical analysis

Statistical analysis was performed with commercially available statistical software (IBM SPSS® Statistics 27). Distribution of variables was tested for normality using the Kolmogorov-Smirnov test. Normally distributed variables were presented as mean ± standard deviation (SD) and compared by paired-sample t-test; non-normally distributed variables were presented as median and IQR and compared by paired-sample Wilcoxon signed-rank test. To compare a normally distributed variable with a non-normally distributed one, paired-sample Wilcoxon signed-rank test was used. Correlation was tested by the Pearson correlation coefficient (ρ) with the following interpretations: ≤ 0.03 weak correlation, > 0.3 and ≤ 0.7 moderate correlation, > 0.7 strong correlation. A P value <0.05 was considered statistically significant.

A constant value corresponding to half of the limit of detection (LOD) was assigned to patients with serum progesterone and plasma NT-proBNP below the LOD (0.25 ng/ml and 250 pmol/l respectively).

Results

Animals

The study population consisted of 14 sexually intact Chihuahua females. The mean age was 3.71 ± 1.55 years and the mean body weight was 2.82 ± 0.41 kg; 6 out of 14 dogs had a BCS of 4/9 and 8 out of 14 dogs had a BCS of 5/9. All dogs were fed a normal-sodium diet (0.34% [OASY One Animal Protein Adult/Small Mini Salmon; OASY, Republic of San Marino] and 0.4% sodium [Royal Canin Mini Adult; Royal Canin, Aimargues, France]). The mean systolic arterial pressure was 135.14 ± 16.92 mmHg. Complete blood count (CBC), biochemical profile and urinalysis were unremarkable in all dogs. Cardiac auscultation was unremarkable in 11/14 dogs; 2 dogs presented a I/VI grade left apical systolic murmur and 1 dog an II/VI grade left apical systolic murmur. Echocardiography was unremarkable in 11 dogs (ACVIM stage A), while 3 dogs were diagnosed with MMVD ACVIM stage B1[7]. Dogs in dioestrus were

evaluated between 23 and 48 days after the onset of serosanguinous vulvar discharge. Dogs in anoestrus were evaluated between 99 and 302 days after the last oestrus and before the subsequent proestrus. Phases of reproductive cycle (anoestrus and dioestrus) were confirmed by vaginal cytology in all dogs.

Hormone measurements

Serum progesterone and urinary aldosterone were significantly higher in dioestrus compared to anoestrous. Urinary aldosterone-to-creatinine ratio was higher in dioestrus compared to anoestrus, but it did not reach the statistical significance. N-Terminal pro-B-type natriuretic peptide was not significantly different between anoestrous and dioestrus (Table 6).

Table 6. Hormones measurements in anoestrus and dioestrus

	Anoestrus	**Dioestrus**	**P value**
Serum progesterone (ng/ml)	0.19 (0.13-0.38)	33.39 ± 14.87	<0.001*
Urinary aldosterone (pg/ml)	5005.72 ± 2127.73	9886.98 ± 5735.22	0.010*
UAldo:C (µg/dl)	3.39 ± 1.64	4.16 (3.17-6.80)	0.056
NT-proBNP (pmol/l)	332.86 ± 163.50	394.50 (125-643.75)	0.139

Ualdo:C urinary aldosterone-to-creatinine ratio, NT-proBNP N-Terminal pro-B-type natriuretic peptide.
Normally distributed variables are presented as mean ± standard deviation. Non-normally distributed variables are presented as median and interquartile range (IQR).
*Significant difference (P value <0.05) between anoestrus and dioestrus

In total sample (anoestrus + dioestrus), serum progesterone showed a positive moderate correlation with urinary aldosterone (ρ=0.638, P<0.001) and Ualdo:C (ρ=0.516, P=0.005). N-Terminal pro-B-type natriuretic peptide was not significantly different between anoestrus and dioestrus and no significant correlations were found between NT-proBNP and serum progesterone, urinary aldosterone and Ualdo:C.

Urinalysis

Urinary protein-to-creatinine ratio was significantly higher in anoestrus compared to dioestrus. Urinary creatinine was significantly higher in dioestrus compared to anoestrus. No significant differences in USG and UP were found between the two phases of oestrus cycle (Table 7).

Table 7. Comparison of urinary parameters between anoestrus and dioestrus

	Anoestrus	Dioestrus	P value
Urinary creatinine (mg/dl)	150.94 ± 26.95	194.54 ± 57.39	0.008*
Urinary protein (mg/dl)	35.64 ± 9.29	33.29 ± 12.05	0.488
UP/UC	0.24 ± 0.063	0.17 ± 0.042	0.003*
USG	1060 (1048.75-1062)	1059.07 ± 10.93	0.327

UP/UC urinary protein-to-creatinine ratio, USG urine specific gravity.
Normally distributed variables are presented as mean ± standard deviation. Non-normally distributed variables are presented as median and interquartile range (IQR).
*Significant difference (P value <0.05) between anoestrus and dioestrus

Discussion

The results of the present study suggest the existence of a progesterone-aldosterone relationship in canine species.

As expected, progesterone significantly increased in dioestrus compared to anestrus[395]. Urinary aldosterone showed a significant parallel increase and UAldo:C raised in dioestrus with a P value close to significance (0.056). Moreover, urinary aldosterone and UAldo:C showed a significant positive correlation with progesterone in total population.

In women, the luteal phase of the menstrual cycle is characterized by increased levels of progesterone and oestradiol, and a rise in aldosterone concentration during this period has been reported by several studies[361,362,396-398]. Progesterone represents a high-affinity antagonist of MRs in humans and rats, acting as a competitive inhibitor of aldosterone and inducing natriuresis[364,366,391,396,399-401]. Because of these properties, high levels of progesterone likely lead to an increase in aldosterone concentrations because of the receptor occupation and the compensatory activation of the RAAS[362,397,398,401,402]. However, even a RAAS-independent mechanism has been suggested. Szmuilowicz et al. (2006) reported an increase in aldosterone levels during the luteal phase in women without a concurrent increase in plasma renin activity and angiotensin-II[362]. In the same study, they found that the incubation of rat zona glomerulosa cells with progesterone

caused a significant increase in aldosterone production compared to the incubation with vehicle alone[362]. A similar result has been obtained with human adrenocortical cells[403]. These findings suggest that progesterone directly stimulate adrenal aldosterone production. Progesterone receptors has been found in the adrenal capsular cells of female mice[404] and in human adrenocortical cells[403], and it has been hypothesized that this hormone might influence aldosterone secretion through its own receptors[362]. A second hypothesis is that increased levels of progesterone correspond to increased levels of substrate for 21-hydroxylase in the adrenal cells, since progesterone represent a precursor in the aldosterone biosynthetic pathway[362,405]. A similar mechanism has been already hypothesized for the positive relationship between progesterone and cortisol reported in peripartum bitches and naturally cycling women[406,407]. However, the role of progesterone as a direct stimulator of aldosterone secretion remains subject of debate, since conflicting results have been reported in vitro[408].

Interestingly, progesterone has different properties in other species. In cartilaginous and ray-finned fish, in which aldosterone is not synthetized, progesterone represents a physiological activator of MRs[391]. Mineralocorticoid receptors preceded aldosterone by millions of years, and they evolved from an ancestral protein in common with glucocorticoid, progesterone and androgen receptors. Aldosterone appeared for the first time in lungfish, which represent a transition from aquatic to terrestrial life[391,409]. The role of progesterone as MR activator was lost in amphibians, alligators, rodents and humans[391]. Unexpectedly, progesterone activates MR in chicken and the reason remains unknown at this time, since the receptor has molecular characteristics similar to that of humans[391,410]. Currently, a mutant human MR represents the only other case of MR activated by progesterone in terrestrial vertebrates[411]. In dogs, previous studies suggested an increase in RAAS activity in relation to pregnancy/oestrous cycle[392,393,397], but, to our knowledge, this is the first study that investigated the relationship between progesterone and aldosterone in canine species. Such as humans and rodents, dogs belong to mammals class and, from an evolution perspective, it could be presumed that similar mechanisms regulate the interactions among MR,

progesterone and aldosterone. However, Johnson et al. found that natriuresis did not occur after progesterone infusion in healthy dogs[412], raising doubts about the role of progesterone as MR antagonist in this species. The underlying mechanisms are still to be determined and further investigations are warranted in dogs, especially in the molecular and cellular field.

Szmuilowicz et al. (2006) showed a direct correlation between aldosterone and progesterone among women receiving a high-sodium diet, but no differences in aldosterone levels between follicular and luteal phase were found among women in a low-sodium regimen[362]. Although these results confirm that progesterone is responsible of aldosterone increase, they also indicate that natremia remains a primary modulator of RAAS activity and it likely overwhelms the role of progesterone in case of sodium restriction[362]. In the present study, all dogs included were fed two type of normal-sodium commercial diet throughout the reproductive cycle and their sodium content was superimposable. Thus, it can be excluded that sodium intake influenced the difference in aldosterone levels between anoestrus and dioestrus in the present study.

In a previous study, Chihuahua showed significantly higher UAldo:C compared to other breeds[390]. Polymorphism of angiotensin converting enzyme has been reported in dogs, with wide prevalence variability among breeds[413]. This gene variant has been associated with higher aldosterone levels and aldosterone breakthrough incidence in dogs[100]. In the present study, Chihuahua breed was chosen as inclusion criterion in order to eliminate the breed-related variability of aldosterone. It cannot be excluded that also the relationship between aldosterone and sexual hormones may be breed-related, and further investigation on multiple breeds are warranted.

Urinary aldosterone-to-creatinine ratio was higher in dioestrus compared to anoestrus, but it was not statistically different (P=0.056) between the two phases. However, the following aspects should be taken into consideration: a small sample size (i.e., higher probability of type-II error) and the fact that urinary creatinine was significantly higher in dioestrus compared to anoestrus, in face of a superimposable USG. Urinary creatinine is one of the most-used correction factors to adjust several urinary

biomarkers for dilution in spot urine samples[124]. The rationale is that UC excretion is constant[414]. However, creatinine clearance and UC excretion might be influenced by physiological changes[415]. For example, these parameters appeared significantly higher in mid-luteal phase (high oestrogen and high progesterone state) compared to pre-ovulatory phase (high oestrogen and low progesterone state) in women[416]. Variations of urinary creatinine, creatinine clearance and glomerular filtration rate have been reported also over the course of pregnancy[415,417,418]. Thus, it cannot be excluded that changes in UC excretion between anoestrus and dioestrus may have affected the UAldo:C results in the present study. To our knowledge, there are no reports about the variability of UC excretion over the reproductive cycle in dogs and further investigations are warranted to confirm this hypothesis.

N-Terminal pro-B-type natriuretic peptide was not significantly different between anoestrus and dioestrus and did not show any correlation with progesterone. Previous studies in dogs reported significantly higher NT-proBNP in intact females than intact males[201,214], in agreement with studies in people, where women showed significantly higher NT-proBNP than men[215-217,419,420]. No differences were found between intact and neutered female dogs, as well as between intact and neutered males[201]. In women, NT-proBNP was found to be significantly higher in luteal and follicular phase than in midcycle phase[218]. In humans, the mechanisms behind the influence of gender on NPs are still subject of debate. Initially, the difference between genders was attributed to oestrogens (and, in part, to progesterone), which could have both a direct and indirect stimulatory effect on NPs[216,218,421,422]. More recently, the role of gender has been primarily related to testosterone, which has been found to be inversely correlated with NPs likely because of a suppressive effect on their release[218,419,423]. However, controversial results have been reported for both oestrogens and testosterone. It is likely that both hormones contribute to the regulation and the determination of NPs levels through the sum of their effects[218]. Even the role of progesterone is not clear because of conflicting results[218,421,424-426]. In dogs, no differences were found between intact and neutered female[214], but, to our knowledge, there are no reports about NPs

levels in different phase of reproductive cycle and their correlation with sex hormones. Based on the results of the present study, NT-proBNP seems not to be affected by progesterone and not to significantly vary between anoestrus and dioestrus. However, oestrogens and testosterone were not measured, and the other phases of reproductive cycle were not evaluated. Thus, further investigations are warranted in dogs to better define the variation of NPs through the entire cycle and their relationship with sex hormones.

The present study has several limitations. First of all, the small sample size limits the strength and the accuracy of the results; contributors encourage further studies on a large canine population, and even in other breeds, to verify their replicability. Secondly, oestrogens were not evaluated and a possible influence of these hormones on aldosterone could have been missed. However, oestrogens levels in dioestrus are low[395] and their potential effect on RAAS should be limited during this phase. Thirdly, serum sodium and potassium levels can affect RAAS activity[384], and they were not assessed in the present study. Fourthly, assessment of other RAAS components and urinary sodium would help obtain a better understanding of the aldosterone-progesterone relationships. Lastly, although the time of urine collection was restricted between 10 am and 2 pm, an influence of urinary aldosterone and UC circadian variations on the values obtained in the present study cannot be excluded[427,428].

In conclusion, these results suggest the existence of a progesterone-aldosterone relationship in canine species, indicating that gender and phase of oestrous cycle should be taken into account for a correct interpretation of aldosterone levels in dogs. Mechanisms underlying this correlation in dogs might be similar to those reported for humans and rodents, that are the MRs antagonism by progesterone with the consequent compensatory RAAS activation, and a direct stimulation of aldosterone production by progesterone as well. Further studies are needed both to confirm these results on a larger canine population and to identify the mechanisms behind this relationship in this species.

STUDY 3: Plasma N-terminal pro-B-type natriuretic peptide and urinary aldosterone-to-creatinine ratio in healthy Chihuahuas

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Phd student activity: conception and design of the study, acquisition of clinical data, performing statistical analysis and drafting the manuscript.

Abstract

Introduction Chihuahua represents an increasingly widespread breed predisposed to cardiac disease. N-terminal pro-B-type natriuretic peptide might be a useful point of care biomarker for dogs suspected of heart diseases but breed differences have been reported. The renin-angiotensin-aldosterone system is a cornerstone of heart failure syndrome. Urinary aldosterone-to-creatinine ratio appeared to be a good indicator of RAAS activity in dogs, but Chihuahuas showed significantly higher UAldo:C compared to other breeds. The objective of this study was to assess preliminary breed-specific reference intervals for NT-proBNP and UAldo:C in healthy Chihuahuas, evaluate sex differences in these parameters and confirm the impact of Chihuahua breed on UAldo:C.

Methods Chihuahuas were recruited among private-owned dogs referred for cardiological screening. Dogs had to be healthy and >12 months. The following procedures were performed: collection of medical history, blood pressure measurement, physical examination, echocardiography, complete blood count, biochemistry profile, urinalysis, plasma NT-proBNP and urinary aldosterone assessment. Urinary aldosterone-to-creatinine ratio was compared between intact male

Chihuahuas and intact males derived from a healthy multiple-breed population of a previous study.

Results Forty-three healthy Chihuahuas were enrolled. Median NT-proBNP was 347 (125-515) pmol/L, and median UAldo:C was 2.59 (1.57-4.61) µg/g. The NT-proBNP reference interval was 125 (90% CI 125-125) – 2121.4 (90% CI 941.6-2248) pmol/L and inter-individual coefficient of variation was 87%. The UAldo:C reference interval was 0.6 (90% CI 0.5-0.9) – 16.8 (90% CI 10.9-27.4) µg/g. No sex differences in NT-proBNP and UAldo:C were found. Intact male Chihuahuas showed significantly higher UAldo:C (1.81 [1.51-3.15] µg/g) compared to intact males of other breeds (0.68 [0.54-1.62] µg/g; P=0.019).

Conclusion Median value, inter-individual coefficient of variation and reference interval of NT-proBNP were in line with those reported in other small-size breeds. In contrast to previous studies, no sex differences in NT-proBNP were detected. Impact of Chihuahua breed on UAldo:C was confirmed by the present study.

Introduction

Natriuretic peptides counterbalance the effects of the RAAS, inducing vasodilation, diuresis and natriuresis, and providing antifibrotic and antihypertrophic effects[8,9]. In human and veterinary medicine, brain natriuretic peptide and its inactive fragment NT-proBNP are the most studied NPs in patients with cardiovascular diseases[8,9,129]. Compared to other NPs, they are easier to assess because of longer half-life and greater stability[9,141]. Brain natriuretic peptide is primarily released by ventricles after myocardial stretch and hypoxia, but also under other stimuli such as angiotensin-II and adrenergic agonists[8,9]. Brain natriuretic peptide and NT-proBNP are recommended diagnostic and prognostic tools in acute and chronic heart failure in humans[10,11]. In dogs, several studies have been carried out. These peptides were found to be higher in dogs with overt and occult dilated cardiomyopathy compared to controls[193-196]. N-terminal pro-B-type natriuretic peptide was independently associated with survival in

dogs with the occult form, although its screening utility was limited because of low sensitivity when used alone[195]. Relevant results have been reported in dogs with MMVD. N-terminal pro-B-type natriuretic peptide resulted significantly higher in dogs with both symptomatic and asymptomatic MMVD compared to healthy subjects, and it was found to increase with increasing disease severity[197-200,203]. Moreover, NT-proBNP was predictive of all-cause and cardiac mortality and it was an independent risk factor for the first onset of congestive heart failure; lower levels of this peptide after treatment were associated with longer cardiac survival[197,200,202,203-205]. These results suggest that NPs, in addition to standard diagnostic tests (e.g., echocardiography, thorax radiography), might be of great aid in stratifying the risk of morbidity and mortality and in guiding the therapeutic strategy in dogs with MMVD[8]. Furthermore, several studies have showed the usefulness of brain natriuretic peptide and NT-proBNP in differentiating between dogs with cardiac and non-cardiac causes of respiratory signs[206-210]. Non-breed specific cut-offs of NT-proBNP have been proposed by the manufacturer of the currently available test (Cardiopet® proBNP test, IDEXX Laboratories, Westbrook, ME) for the screening of dogs suspected of heart diseases[219]. However, a significant interbreed variability has been reported and the proposed cut-offs might not be appropriate for some breeds, as already highlighted for Labrador Retrievers and Greyhounds[12-14]. Even sex differences have been reported for this parameter[201,214].

Chihuahua represents an increasingly widespread breed, which is predisposed to MMVD; most common causes of death in this breed are hearth diseases and low respiratory tract disorders[22,23]. Thus, NT-proBNP and a related breed-specific reference interval (RI) might be particularly useful in this breed.

In a recent study, Chihuahuas also showed significantly higher UAldo:C compared to other breeds[390]. However, the number of Chihuahuas included was low and other factors, such as sex, appeared to simultaneously affect aldosterone levels. Although markers of RAAS activity are still far from entering the diagnostic routine of patients

with MMVD, a precise monitoring and therapeutic modulation of this system, titrated on the single patient, might improve the disease management.

The first aim of the present study was to determine a breed-specific RI for plasma NT-proBNP in healthy Chihuahuas, using the currently available second-generation ELISA (Cardiopet® proBNP test, IDEXX Laboratories, Westbrook, ME), and evaluate sex differences in this parameter in a single-breed population. The second aim was to assess UAldo:C on a larger population of healthy Chihuahuas and: 1) obtain a preliminary breed-specific RI, 2) evaluate sex differences in UAldo:C in a single-breed population, 3) confirm the effect of Chihuahua breed on UAldo:C, comparing this parameter between Chihuahuas and a healthy multiple-breed population.

Materials & Methods

Animals and study timeline

The present study was part of the research project "Identification of breed-specific reference values for echocardiographic parameters and neurohormonal biomarkers in Chihuahua breed", approved by the Animal Welfare Organisation of the Università degli Studi di Milano (OPBA_88_2021).

The study was conducted with informed consent of the owners. Subjects enrolled were recruited among private owned dogs referred to the Veterinary Teaching Hospital – Università degli Studi di Milano (January-December 2021) for cardiological screening.

Inclusion and exclusion criteria

Chihuahuas enrolled in the present study had to be healthy and older than 12 months. Female dogs had to be neutered or in anoestrus (i.e, at least 3 months after the last oestrus and before the subsequent proestrus), in order to exclude any possible influence of high progesterone/oestrogens states on NT-proBNP and UAldo:C. Subjects with any known disease, any clinical signs or subjected to any pharmacological treatment at the time of examinations were excluded.

Each dog underwent the following procedures in this order: collection of medical history (including diet information), indirect blood pressure measurement, complete physical examination, echocardiography, blood sampling (for complete blood count, biochemistry profile and plasma NT-proBNP measurement) and collection of a free-catch urine sample (for urinalysis and urinary aldosterone determination). A dog was considered healthy if the aforementioned procedures did not reveal any significant alteration.

Urinary aldosterone-to-creatinine ratio of healthy Chihuahuas was compared with that of a healthy population derived from an authors' previous study[390], in order to estimate the impact of Chihuahua breed on this parameter. For this purpose, the comparison was performed between intact males only. The healthy group of the previous study was also deprived of all Chihuahuas and Cavalier King Charles Spaniels. This choice was based on previous results[390]. For convenience, the new group was named healthy intact males multiple-breed (HIM-M) group. Inclusion and exclusion criteria of HIM-M subjects are reported in the related study[390].

Systemic arterial pressure and echocardiography

Indirect systemic arterial pressure measurements were carried out with a veterinary high-definition oscillatory device (VET-HDO ®- MONITOR, S + B medVET GmbH, Babenhausen, Germany), following published guidelines[346].

Echocardiography was performed on conscious dogs by two experienced echocardiographers (AG, CL) using an ultrasonographic unit (Esaote MyLabOmega) equipped with two different multifrequency phased array probes (1-9 and 2-5 Mhz respectively) and following published standards[347].

Sample collection, storage and analysis

Blood was drawn by peripheral venepuncture at least 6 hours after meal and collected into EDTA and serum gel tubes. After centrifugation (3750 rpm x 5 minutes), serum was used for biochemical analysis (urea, creatinine, glucose, total protein, albumin, alanine aminotransferase, alkaline phosphatase). Blood collected into EDTA tubes was used for complete blood count and then immediately centrifuged (3750 rpm x 5

minutes). Plasma samples were stored at -80°C and subsequently sent at IDEXX laboratory (Korwestheim, Germany) for plasma NT-proBNP measurement by a second-generation ELISA (Cardiopet® proBNP test, IDEXX Laboratories, Westbrook, ME).

Urine samples were collected by spontaneous micturition and standard urinalysis was performed (i.e., dipstick chemistry test and refractometer). Samples were then immediately centrifuged at 1250 rpm for 5 minutes and supernatant was obtained. Urinary protein and urinary creatinine were determined by a standard colorimetric assay assessment. Residual supernatant was stored at -80°C until urinary aldosterone analysis.

Urinary aldosterone concentrations were determined by a commercially available species-independent ELISA kit (Enzo Life Sciences Aldosterone ELISA kit, Enzo Life Sciences Inc., Farmingdale, NY, USA), as previously described[390].

Statistical analysis

Statistical analysis was performed with a commercially available statistical software (IBM SPSS® Statistics 28). Normality was tested using the Kolmogorov-Smirnov test. Normally distributed variables were reported as mean ± standard deviation and non-normally distributed variables were presented as median and interquartile range. Parametric data were compared by unpaired t-test and non-parametric data were compared by unpaired Mann-Whitney test. A P value <0.05 was considered statistically significant.

Reference intervals (expressed as 2.5th [lower limit] and 97.5th [upper limit] fractiles) and 90% confidence intervals (CI) of each limit were calculated with an open-source application (Reference Value Advisor 2.1)[429]. In accordance with published guidelines[430], the RI obtained with the robust method (on raw or Box-Cox transformed data) was the first choice; if the robust method did not produce a valid RI, the one calculated with the non-parametric method was reported. Potentials outliers were identified by the same software via the combination of visual inspection, Tukey test and Dixon-Reed test. However, in accordance with the American Society for Veterinary

Clinical Pathology guidelines[430], as all dogs included were selected randomly from a well-defined population and their health was confidently established, no outliers were removed from the analysis.

Values of NT-proBNP below the limit of detection (250 pmol/L) were reported as half of the lower limit for the statistical analysis.

Results

Animals

The study population consisted of 43 healthy Chihuahuas of which 26 were intact females, 14 were intact males and 3 were neutered males. The median age was 2.32 (1.19-4.11) years, and the median BW was 2.71 (2.39-3.10) kg. Thirty-eight dogs were fed a normal-sodium commercial diet (0.34% [OASY One Animal Protein Adult/Small Mini Salmon; OASY, Republic of San Marino], 0.4% sodium [Royal Canin Mini Adult; Royal Canin, Aimargues, France] and 0.2% sodium [Monge All Breeds Adult Rabbit with Rice and Potatoes/Monge Grain Free - Duck with Potatoes - Mini Adult; Monge & C S.p.a, Monasterolo di Savigliano, Cuneo, Italy]); 5 dogs were fed an unspecified commercial diet. Body condition score was 3/9 in 1 dog, 4/9 in 18 dogs, 5/9 in 18 dogs, 6/9 in 4 dogs and 7/9 in 2 dogs. Mean systolic arterial pressure was 137.72 ± 16.76 mmHg. Echocardiography, complete blood count, biochemistry profile and urinalysis were unremarkable in all dogs.

Body weight was significantly higher in males (3.13 ± 0.75 kg) compared to females (2.62 [2.35-2.84] kg; P=0.020). No significant differences in age and systolic arterial pressure were found between males and females.

The group of intact male Chihuahuas had a mean age of 3.51 ± 2.13 years and a mean BW of 2.97 ± 0.44 kg.

The HIM-M group consisted of 10 intact male dogs. Mean age and BW were 4.80 ± 2.35 years and 29.3 ± 16.22 kg respectively. The following breeds were included: 2 American Staffordshire terriers, 1 Toy Poodle, 1 Standard Poodle, 1 Great Dane, 1 German Shepherd, 1 Bichon Frisè, 1 Rhodesian Ridgeback, 1 Springer Spaniel and 1

mixed-breed. Body Weight was significantly higher in HIM-M group compared to intact male Chihuahuas (P=0.001). Age was not significantly different between the two groups (P=0.173).

Laboratory parameters

Results of plasma NT-proBNP and urinary aldosterone concentrations are reported in Table 8. Eleven dogs (25.6%) had a value of NT-proBNP below the limit of detection (<250 pmol/L). One NT-proBNP (2248 pmol/L) and two UAldo:C (13.93 µg/g and 23.80 µg/g respectively) samples were detected as outliers. There were no significant differences in any reported parameters between females and males.

Table 8. Laboratory parameters

	All	Females	Males	P
NT-proBNP (pmol/L)	347 (125-515)	347 (255-547)	347 ± 229	0.408
UAldo(pg/dl)	4139 (2810-7194)	4938 (2926-7405)	3242 (2672-7146)	0.449
UAldo:C (µg/g)	2.59 (1.57-4.61)	3.08 (1.92-5.60)	1.86 (1.51-4.32)	0.124

NT-proBNP N-terminal pro-B-type natriuretic peptide, UAldo urinary aldosterone, UAldo:C urinary aldosterone-to-creatinine ratio.
Normally distributed variables are presented as mean ± standard deviation. Non-normally distributed variables are presented as median and interquartile range.
The Males group includes both intact and neutered males.

Reference intervals for NT-proBNP and UAldo:C in healthy Chihuahuas are reported in Table 9.

Table 9. Reference intervals for NT-proBNP and UAldo:C in healthy Chihuahuas.

	Median	Min-Max	Lower RI	90% CI	Upper RI	90% CI
NT-proBNP (pmol/L)	347	125-2248	125	125-125	2121.4	941.6-2248
UAldo:C (µg/g)	2.54	0.57-23.80	0.6	0.5-0.9	16.8	10.9-27.4

NT-proBNP N-terminal pro-B-type natriuretic peptide, UAldo:C urinary aldosterone-to-creatinine ratio, RI reference interval, CI confidence interval.
Median and reference interval for NT-proBNP was calculated via the non-parametric method.
Median and reference interval for UAldo:C was calculated via the robust method on Box-Cox transformed data.

Urinary aldosterone-to-creatinine ratio was significantly higher in intact male Chihuahuas (1.81 [1.51-3.15] µg/g) compared to HIM-M group (0.68 [0.54-1.62] µg/g; P=0.019).

Discussion

N-terminal pro-B-type natriuretic peptide has been reported to have a significant breed-to-breed variability, and breed-specific reference ranges have been recommended[12-14]. To the author's knowledge, a specific reference interval for NT-proBNP in Chihuahuas had not yet been established[12,214]. In the present study, Chihuahuas showed a median value of NT-proBNP equal to 347 pmol/L, with a calculated 95% RI of 125-2121.4 pmol/L. The median value was in line or lower than those reported in other small size breed dogs[12,214]. The calculated RI was wide, but it was not an unexpected result. Rather, it likely reflects the high biological variability of NT-proBNP that has been previously documented in dogs, in the form of both intra-individual and within-group variation[12,13,211,213,214]. Wide RIs were already found in healthy Labrador Retrievers (275-2100 pmol/L)[13] and in a healthy population of different small size breeds dogs (157-2842 pmol/L), where inter-individual coefficient of variation (CV) ranged from 62% in CKCSs to 100% in Miniature Poodles[214]. In the present study, the inter-individual CV was 87%, confirming a relevant inter-individual variability of NT-proBNP even within the same breed.

The interpretive criteria indicated by the manufacturer propose a cut-off of 900 pmol/L as a screening tool in dogs with suspected heart diseases (presence of heart murmur or at-risk breed)[219]. Ninety-one percent (39/43) of Chihuahuas enrolled in this study had an NT-proBNP < 900 pmol/L; the remaining 4 dogs showed a value of 907, 961, 982 and 2248 pmol/L, respectively. Based on these results, the generic cut-off of 900 pmol/L appear to be suitable for this breed, although a comparison with Chihuahuas with heart diseases would be necessary to establish an accurate breed-specific cut-off. Exceeding values could occur also in healthy Chihuahuas, likely because of the normal biological variation of NT-proBNP. This characteristic is more problematic in patients with heart diseases, because it can make difficult to interpret disease progression and response to therapy in face of changes in NT-proBNP[211,212]. Thus, individual serial monitoring should be performed in dogs with heart diseases, and previous studies

suggested that a change of approximately 50-70% (depending on ACVIM stage) is required to detect a disease progression[211,212].

There were no significant differences in NT-proBNP between females and males. This result is in contrast with previous reports in dogs, which found significantly higher NT-proBNP in females compared to males[201,214], and with reports in humans, where women showed significantly higher NPs concentrations than men[215-218]. The mechanism behind the influence of sex on NPs is not completely clear, but it is supposed to be related to a combined effect of different sexual hormones (e.g., oestrogens, progesterone and androgens)[218,419,421,422,424,425]. The reason of a different result between the present study and the previous ones[201,214] might be related to a smaller sample size and/or more homogeneous population (i.e., single breed and restricted ranges of age and BW); even different levels of sexual hormones could have played a role, since they were not measured in any of these studies and all intact females in the present one were in anoestrus (i.e., low progesterone and oestrogens state). Measuring NT-proBNP along with sexual hormones in different sexes/neuter status and in different phase of reproductive cycle will help elucidate the relationship between NPs and gender.

The assessment of RAAS activity through the measurements of its components could be of great aid in the monitoring and therapeutic management of dogs with heart diseases. Aldosterone represents the last effector of the neurohormonal cascade and has acquired a relevant role in the pathophysiology of congestive heart failure, becoming a therapeutic target in dogs with symptomatic MMVD[6,7,63]. Urinary aldosterone-to-creatinine ratio derived from spot urine samples has been proven to correlate well with 24h urinary aldosterone excretion[124]; thus, it can be a valid indicator of the overall RAAS activity. Several studies have investigated this parameter in healthy dogs, finding values <1.0 µg/g; however, they were carried out on research dogs (hound-type dogs or beagles) and in a controlled experimental setting[97]. A recent study has reported a median value of 0.64 (0.54-0.83) µg/g and a RI of 0.23 (90% CI 0.17-0.32)-1.82 (90% CI 1.27-2.5) µg/g, derived from 31 healthy client-owned dogs with a median BW of 22.2 kg (17 spayed females and 14 neutered males; breed not specified)[99]. The

median value and the breed-specific RI found in the present study were much higher and wider, respectively. Two main differences may have contributed to this discrepancy: sex and breed.

All female dogs included in the study of Ames et al (2022)[99] were spayed, while those enrolled in the present one were all intact. An authors' previous study has found significantly higher UAldo:C in intact females compared to males and spayed females[390]. Recently, Adin et al have found significantly higher angiotensin-converting-enzyme activity in intact females (not in oestrus, not pregnant and not lactating) Dobermann Pinscher compared to spayed female and intact male of the same breed; even angiotensin-II, angiotensin-I and renin activity were higher, but not significantly[431]. In the present study, UAldo:C was higher in intact females compared to males, although the difference between groups was not statistically significant. In the authors' previous study, phase of reproductive cycle was unknown[390], while in the present one all female dogs were in anoestrus. Sex differences in RAAS activity are primarily attributed to the effect of female sexual hormones[361-363,366-369,390,401,412,431,432]. It can be presumed that high progesterone/oestrogens states (e.g., dioestrus, oestrus) enhance the discrepancy in RAAS activity between sexes/neuter status, while this difference is blunted in case of low concentrations of these hormones (e.g., anoestrus). However, although oestradiol and progesterone concentrations in intact females-anoestrus and spayed females seem to overlap[433], the production of ovarian hormones is completely absent in spayed females and males. In contrast, ovaries are not quiescent during anoestrus and hormones fluctuations could occur[434]; if they are sufficient to cause an increase in RAAS activity compared to spayed females or males remains to determine. The study of Adin et al. (2023) seems to support this hypothesis[431]. The present results appear to follow the same direction, although the lack of statistical significance and other factors (i.e., absence of spayed females, low number of neutered males and lack of sexual hormones measurements) does not permit to draw conclusions. Moreover, although the effect of sex is mainly related to female sexual hormones[361-366-369,390,401,412,431,432], testosterone has been reported to increase renin levels

and angiotensin converting enzyme activity in rats[432]. Further investigations in the field of sex-RAAS relationship in dogs are warranted.

Breed was the other main difference between the two RIs. In the study of Ames et al. (2022), breed of healthy dogs was not specified, but they were likely of medium/large size considering the reported BW[99]. In the authors' previous study, Chihuahuas and Cavalier King Charles Spaniels showed significantly higher UAldo:C than other breeds [390]. The impact of Chihuahua breed on UAldo:C appear to be confirmed by the present study, which showed significantly higher UAldo:C in healthy intact male Chihuahuas compared to healthy intact males of other breeds. The reason of these elevated UAldo:C values in Chihuahuas remains to determine. Polymorphisms of genes encoding for RAAS components could be a possible explanation[355,413,435]. However, other countless and unexplored breed-specific physiological factors could have played a role, considering the relevant between-breed genetic variation reported in canine species[436]. For examples, breed differences in heart rate, systemic blood pressure and catecholamine concentrations have been reported in dogs[12,437-439].

Overall, these sex and breed differences may have led to both higher absolute values and higher inter-individual variability of UAldo:C in the present study compared to that of Ames et al (2022)[99]. In support of this hypothesis, even median Ualdo:C of intact males of other breeds (HIM-M group; 0.68 µg/g) was much closer to the median values of Ames et al (0.64 µg/g) than those of the present study. Also other factors such as circadian aldosterone variations[428], dietary sodium intake[84], electrolytes and blood pressure fluctuations, hydration status and changes in sympathetic activity may contribute to both within and between studies variability[3,99]. Additional comparisons are difficult to make also because, in contrast to NT-proBNP, UAldo:C does not have a standardized assay, lack of interpretative criteria and its use in patients with heart diseases is supported by much less studies. All these factors limit the diagnostic power of UAldo:C as a point-of-care biomarker, as well as the utility of a reference interval in a clinical setting. As previously suggested, an individual longitudinal monitoring should be preferred at the current state of knowledge[99,390].

The present study has several limitations. First of all, the low number of subjects may have limited the statistical power of the study; in particular, the ideal number of subjects for the calculation of a RI is ≥ 120 in veterinary medicine[430]. Secondly, because of the biological variability of NT-proBNP and UAldo:C[211-213,428], multiple measurements (i.e, daily and weekly) and a more standardized urine and blood sampling time would have improved the accuracy of the results. Thirdly, serum electrolytes were not assessed and diet information (i.e., sodium intake) were missing for 5 Chihuahuas and for the HIM-M group; both factors represent a potential source of RAAS activity variability[3,84]. Lastly, absence of spayed females, low number of neutered males and lack of sexual hormones measurements limit the considerations on the relationship between sex and both NT-proBNP and UAldo:C, and further investigations in this field are encouraged.

In conclusion, the present study assessed Chihuahua-specific preliminary RIs for NT-proBNP and UAldo:C, which represent different aspects of the same neurohormonal system. Median values, reference interval and inter-individual CV of NT-proBNP are in line with those previously reported in other small-size breed dogs[12,214]. The proposed non-breed specific cut-off of 900 pmol/L[219] seems to be valid for most healthy Chihuahuas, although markedly higher values in normal subjects might occur even in this breed. In contrast to previous studies[201,214], no sex differences in NT-proBNP were detected. The results of the present study appear to confirm a relevant impact of Chihuahua breed on UAldo:C. At the current state of literature, the use of a RI for UAldo:C seems to be worthless in a clinical setting, and an individual longitudinal approach should be pursued.

STUDY 4: Impact of haemoconcentration on survival in dogs hospitalized for acute left-sided congestive heart failure – a retrospective study

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Phd student activity: design of the study, acquisition of clinical data, performing statistical analysis and drafting the manuscript.

Abstract

Introduction Worsening renal function and electrolytes disorders are the major complications of diuretic therapy in patients hospitalized for acute heart failure. Recent human literature highlighted the importance of adequate decongestion, even in the face of WRF, and the negative prognostic value of electrolytes abnormalities. The aim of this study was to retrospectively evaluate the effect of selected clinical and laboratory variables upon admission and discharge on survival in dogs hospitalized for left-sided CHF.

Methods This was a retrospective study. Selected clinical and laboratory parameters upon admission had to be available for all dogs included. For dogs with subsequent laboratory analysis, only the last one performed before discharge was considered. Haemoconcentration (HC) was defined as a concomitant increase in microhematocrit (mHtc) and total protein (TP) above the admission values; WRF was defined as creatinine increase ≥ 0.3 mg/dL between admission and discharge. All-cause and cardiac mortality were considered. Relationship between variables and survival was assessed by Cox proportional hazard regression.

Results Fifty-one dogs were included. Discharge laboratory parameters were available in 37 of 51 dogs. On admission, creatinine was predictive of all-cause mortality (HR 1.91; P=0.037) and number of previous CHF episodes (n°-CHF) was predictive of cardiac mortality (HR 2.137; P=0.041). At discharge, admission creatinine (HR 2.233; P=0.040) and loop diuretic dose administered in the first 24h (HR 1.083; P=0.005) were associated with an increased risk of all-cause mortality, while n°-CHF (HR 2.601; P=0.043) and loop diuretic dose administered in first 24h (HR 1.091; P=0.004) were predictive of cardiac mortality. Haemoconcentration was associated with a lower risk of all-cause and cardiac mortality (HR 0.270 [P=0.003] and HR 0.336 [P=0.028]). Hypochloraemia was the most common electrolyte disorder both on admission and discharge, but none of the electrolytes were associated with survival.

Conclusions Number of previous CHF episodes was associated with an increased risk of cardiac mortality. Loop diuretic dose administered in first 24h was predictive of all-cause and cardiac mortality. Presence of HC at discharge was associated with lower all-cause and cardiac mortality, supporting the importance of adequate decongestion.

Introduction

Diuretics represent the cornerstone of in-hospital acute HF therapy, allowing to rapidly relieve fluid congestion and dyspnoea[7,10]. However, these drugs have potentially harmful effects, especially in the setting of an aggressive diuresis, such as excessive volume depletion, impairment of renal perfusion, electrolyte disorders and increase in RAAS activity[127,254,255,328,440-445]. Several laboratory parameters allow to monitor the impact of diuretics on the cardiovascular-renal system, but their prognostic value is still not completely clear, especially in veterinary medicine.

Worsening renal function, usually defined as an increase in serum creatinine ≥ 0.3 mg/dL, is one of the major complications reported during in-hospital diuretic treatment [17,298-300,310]. Historically, WRF has been associated with adverse outcomes in people hospitalized for acute HF[299,303-307]. Therefore, the occurrence of WRF commonly

affects therapeutic decisions and limits the aggressive use of diuretics, with the risk of residual congestion at discharge[16]. However, several human studies have recently questioned the risks of aggressive diuresis and the prognostic significance of WRF during hospitalization, highlighting instead the predominant role of fluid overload on survival. Indeed, WRF showed to have a prognostic value only in the presence of congestion; WRF alone did not impact on survival or did not offset the benefits of haemoconcentration, used as marker of decongestion[17-19,263,310-314,322]. Moreover, obtaining an adequate decongestion resulted to be more prognostically important than improving cardiac indexes, and congestion appeared to be one of the primary causes of WRF, thus revising the negative role of volume depletion on renal function[262,263,310,312,319,446,447]. Lastly, WRF was not associated with tubular injury in people with acute HF[320]. These observations have raised the possibility that in-hospital aggressive diuresis, even in the face of WRF, can affect post-discharge survival positively and that haemoconcentration can be a therapeutic target[17,19,310,312,320]. In a recent study, the occurrence of WRF at first outpatient re-evaluation did not impact on survival in dogs hospitalized for left-sided CHF; however, effect of haemoconcentration in this species has not yet been investigated[300].

The maintenance of an electrolytic balance could be another important goal during hospitalization. Considering the action of diuretics on Na+, K+ and Cl- transporters, electrolyte disorders represent a common side effect of heart failure therapy[86,301,328,329,442,448]. Electrolyte abnormalities can impact on heart and kidney functions through different mechanisms, such as RAAS dysregulation and pro-arrhythmogenic effects[328]. Hyponatremia, dyskalemia and hypochloraemia have been identified as risk factors for an adverse outcome in people with acute HF[20,21,328,329-332]. In contrast, controversial results have been reported in dogs hospitalized for CHF. In one study, hyponatremia has been associated with worse outcome at discharge[333], while another one has found a positive correlation between in-hospital development of hypokalaemia and survival at discharge[301]. In a more recent study, none of electrolytes

(sodium, potassium, chloride) assessed upon admission had an impact on long-term survival[334].

A better understanding of the prognostic meaning of these parameters in dogs would help improve the therapeutic strategy in CHF patients. The aim of this study was to retrospectively evaluate the impact of selected clinical and laboratory parameters on long-term mortality in dogs hospitalized for acute CHF and subjected to diuretic treatment.

Materials & Methods

Case selection & Data collection

This was a retrospective longitudinal study. Database of the emergency and critical care (ECC) unit of the Ospedale Veterinario San Francesco (Milan, Italy) was searched to identify dogs hospitalized for acute left-sided CHF between January 2016 and December 2019. Diagnosis of acute left-sided CHF was confirmed by the presence of tachypnoea (≥36 breaths/min at rest) and/or dyspnoea (not explained by other diseases according to clinical judgment of ECC staff clinicians) and compatible medical imaging. Presence of pulmonary oedema on admission was confirmed by thorax radiography or point-of-care lung ultrasound depending on patient clinical stability[449]. Cardiac disease diagnosis was confirmed by complete echocardiography, performed following published standards[347]. If patient was clinically unstable, a preliminary evaluation on admission was performed by point-of-care cardiac ultrasound[450] and a complete echocardiography was carried out only after patient stabilization. Both dogs with first acute CHF episode and dogs with a history of previous acute CHF episodes were included. All dogs were required to have the following blood parameters available on admission: creatinine (Cr), sodium (Na), potassium (K), chloride (Cl), microhematocrit (mHtc) and plasma total protein (TP). For dogs with subsequent in-hospital laboratory evaluations, only the last one performed before discharge or death/euthanasia was retained for data analysis. For dogs that were discharged, the time

of the last analysis was upon the discretion of intensive care clinicians (e.g., clinical status, results of previous measurements). Exclusion criteria were clinically relevant systemic diseases (eg, neoplastic, metabolic, endocrine or any systemic disease potentially affecting animal's lifespan) at time of admission and administration of oral/parenteral corticosteroids, non-steroidal anti-inflammatory drugs and thiazide diuretics at the time of admission or during hospitalization. Data collected included signalment (sex, breed, age, body weight), cardiac disease diagnosis, number of previous episodes of acute CHF, results of laboratory analysis, length of in-hospital stay and outcome. Detailed information regarding -in hospital therapy were registered: total loop diuretic dose (furosemide and/or torsemide) received during first 24 hours, administration of ACE-I and pimobendan (either IV or PO). For purpose of analysis, torsemide administration was accounted for by multiplying torsemide dosage as suggested by the most recent pharmacokinetics and pharmacodynamics study in dogs[451]: by 10 for doses up to 0.2 mg/kg/day, by 16.67 for doses equal to 0.3 mg/kg/day and by 20 for doses equal to or above 0.4 mg/kg/day. Follow-up survival information were obtained from medical records as well as from owners by phone or email. Cardiac death was defined as death or euthanasia secondary to CHF or sudden death at home without other evident causes. Non-cardiac death was defined as death or euthanasia secondary to non-cardiac diseases or death of unknown cause (i.e., reason of death was not recorded in the medical records and owner did not respond to phone call/email).

Laboratory analysis

Blood samples were obtained by venepuncture. Creatinine and electrolytes were assessed by venous blood gas analysis (VBGA). For VBGA, blood was directly collected into heparinized syringe and analysed by an auto-analyzer (Stat Profile Prime Plus® VET, Nova Biomedical). Residual heparinized blood was transferred into capillary tubes and centrifuged at 1250 rpm x 5 minutes; after mHtc (42-54%) reading, plasma was used to determine TP (5.9-7.8 g/dL) by refractometer. Reference intervals were respectively 0.7-1.8 mg/dL for Cr, 142-149 mmol/L for Na, 3.65-4.70 mmol/L for K and 112.2-118.1 mmol/L for Cl. Corrected Cl was calculated as previously

described[452]. Admission variables were named with the "ad-" prefix (e.g., ad-Na), while discharge variables with the "ds" prefix (e.g., ds-Na). Worsening renal function was defined as a creatinine increase ≥0.3 mg/dL between the admission and the last creatinine evaluation before discharge[16]. Haemoconcentration at discharge was defined as a concomitant increase in mHtc and TP above the admission values[453].

Statistical analysis

Statistical analysis was performed with commercially available statistical software (IBM SPSS® Statistics 28). All dogs included in the study had the selected admission variables available, and they formed the admission group. Among dogs included in the admission group, those with both selected admission and discharge variables available formed the discharge group. All analyses were performed separately on the admission group (only admission data were considered) and the discharge group (both admission and discharge data were considered). Within the discharge group, descriptive statistics was repeated separately for its own admission and discharge variables. In addition, dogs included in the discharge group were then divided based on the presence or absence of HC at discharge, and descriptive statistics was repeated also for these two subgroups.

Distribution of variables was tested for normality using the Kolmogorov-Smirnov test. Normally distributed variables were presented as mean ± SD and compared by unpaired or paired t-test as appropriate; non-normally distributed variables were presented as median and IQR and compared by unpaired Mann-Whitney test or paired-sample Wilcoxon signed-rank test as appropriate. To compare a normally distributed variable with a non-normally distributed one, unpaired Mann-Whitney or paired-sample Wilcoxon signed-rank test was used as appropriate. Categorical variables were expressed as numbers and percentages and were compared as follows: by Pearson's chi-squared test (unpaired variables when frequency of all cells were >5%), Fisher's exact test (unpaired variables when frequency of at least one cell was ≤5%) or McNemar's test (paired variables). For purpose of statistical analysis, number of acute

CHF episodes was divided in two categories: 0=first acute CHF episode, 1=second, third, etc acute CHF episodes. The variable was named n°-CHF.

Survival time was calculated from the admission to the date of death or end of follow-up period (November 2020). The study endpoint was long-term all-cause mortality. A secondary endpoint was long-term cardiac mortality. Survival analysis performed on the admission group evaluated the effect of selected variables on survival upon admission; survival analysis performed on the discharge group evaluated the effect of selected variables on survival at discharge. The effect of selected variables on survival was analysed using multivariable backward stepwise Cox proportional hazards regression. Proportional hazards assumption was tested including time-dependent covariates in the Cox model. For each covariate, the related time-dependent covariate was generated by the product of time and selected covariate. Each covariate and the related time-dependent covariate were then included in the Cox model using a hierarchical regression strategy (i.e., model 1 composed by original covariates, followed by a model 2 composed by time-dependent covariates). If no significant ($P>0.05$) improvement of fit from model 1 to model 2 was seen (i.e., all time-dependent covariates are not significant), the proportional hazard assumption was considered satisfied. Collinearity was evaluated using variance inflation factor, and values <5 were considered acceptable. Dogs that were alive at the end of the study period were censored. In addition, dogs dying from non-cardiac causes were censored as well in the cardiac-mortality analysis. Hazard ratios (HR) >1 was indicative of increased risk of outcome, while HR <1 was indicative of decreased risk of outcome. Variables included in the survival analysis were selected based on their likely clinical relevance and evidence from current literature in patients with acute HF.

A P value <0.05 was considered significant.

Results

Review of medical records between January 2016 and December 2019 identified 108 dogs hospitalized for acute left-sided CHF. Fifty-seven dogs were excluded because of the aforementioned inclusion and exclusion criteria. Thus, 51 dogs were included in the study.

Admission group

All 51 dogs formed the admission group. Demographic, hospitalization and laboratory data are reported in Table 10. The admission group was composed by the following breed: mixed-breed (n=12; 23.5%), Cavalier King Charles Spaniel (n=10; 19.6%), Dachshunds (n=4; 7.8%), Chihuahua (n=4; 7.8%), Pinscher (n=4; 7.8%), Beagle (n=3; 5.9%), Toy Poodle (n=3; 5.9%); Shih-tzu (n=2; 3.9%), Yorkshire Terrier (n=2; 3.9%), Pekingese (n=2; 3.9%), Maltese (n=2; 3.9%) and Bolognese, Volpino, Boston Terrier (n=1; 2% each). Underlying heart disease was myxomatous mitral valve disease for most dogs (n=50; 98.04%); one dog had a diagnosis of dilated cardiomyopathy (n=1; 1.96%). Twenty-five dogs (49%) experienced the first acute CHF episode; current decompensation was the second acute CHF episode for 23 dogs (45.1%) and the third one for 3 dogs (5.9%).

There were no significant differences between ad-Cl and ad-Cl_cor (P=0.345). Prevalence of hyperchloremia (P=0.219) and hypochloraemia (P=0.552) did not significantly change using ad_Cl or ad-Cl_cor. The most common electrolyte abnormality upon admission was hypochloraemia (45.1%). See Table 1 for more details on electrolytes disorders.

Admission creatinine was above the upper reference limit in 4 of 51 dogs (7.84%).

Table 10. Demographic, hospitalization and laboratory data of the admission group

	Dogs n=51	
	Variable	**Value**
Demographic data	Age (years)	11 (10-13)
	Body weight (kg)	7.2 (4.3-10)
	Sex (F/M)	21 (41.2%) / 30 (58.8%)
Laboratory data (admission)	ad-mHtc (%)	49.22 ± 8.38
	ad-TP (g/dl)	7 ± 0.85
	ad-Cr (mg/dl)	0.9 (0.7-1.30)
	ad-Na (mmol/L)	146.52 ± 3.24
	ad-K (mmol/L)	4.04 ± 0.50
	ad-Cl (mmol/L)	113.14 ± 3.93
	ad-Cl_cor (mmol/L)	112.38 ± 4.11
	ad-Hyper/Hyponatremia	11 (21.6%) / 6 (11.8 %)
	ad-Hyper/Hypokalaemia	4 (7.8%) / 13 (25.5%)
	ad-Hyper/Hypochloraemia	8 (15.7%) / 23 (45.1%)
	ad-Hyper/Hypochloraemia_cor	4 (7.8%) / 26 (51%)
Hospitalization data	First acute CHF (YES/NO)	25 (49%) / 26 (51%)
	Hours	26 (20-42)
	Loop diuretic dose 24h (mg/kg)	12 (10-15)
	ACE-I (YES/NO)	32 (62.7%) / 19 (37.3%)
	Pimobendan (YES/NO)	47 (92.2%) / 4 (7.8%)

F, females; M, males; CHF, congestive heart failure; mHtc, micro haematocrit; TP, total protein; Cr, creatinine; Na, sodium; K, potassium; Cl, chloride; Cl_cor, corrected chloride; Loop diuretic dose 24h, estimated dose of furosemide during first 24 hours of hospitalization; ACE-I, angiotensin converting enzyme inhibitor. Normally distributed variables are reported as mean ± standard deviation; non-normally distributed variables are reported as median (interquartile ranges). Categorical variables are expressed as number and percentages.

Discharge group

Discharge laboratory parameters were available in 37 of 51 dogs, and they formed the discharge group. Demographic, hospitalization and laboratory data are reported in Table 11. The discharge group was composed by the following breed: mixed-breed (n=9; 24.3%), Cavalier King Charles Spaniel (n=5; 13.5%), Dachshunds (n=4; 10.8%), Chihuahua (n=4; 10.8%), Beagle (n=3; 8.1%), Toy Poodle (n=2; 5.4%); Shih-tzu (n=2; 5.4%), Yorkshire Terrier (n=2; 5.4%) and Pekingese, Maltese, Bolognese, Pinscher, Volpino, Boston Terrier (n=1; 2.7% each). Underlying heart diseases included myxomatous mitral valve disease (n=36; 97.3%) and dilated cardiomyopathy (n=1; 2.7%). Twenty dogs (54.1%) experienced the first acute CHF episode; current decompensation was the second acute CHF episode for 15 dogs (40.5%) and the third one for 2 dogs (5.4%).

There were no significant differences between ad-Cl and ad-Cl_cor (P=0.537), as well as between ds-Cl and ds-Cl_cor (P=0.409). Prevalence of hyperchloremia and hypochloraemia did not significantly change using Cl or Cl_cor both on admission (P=0.174 and P=0.815 respectively) and discharge (P=1.0 and P=0.782 respectively).
The most common electrolyte abnormality was hypochloraemia, both on admission (43.2% Cl) and at discharge (78.4%). Prevalence of hypokalaemia (P=0.049), hypochloraemia (P=<0.001) and hypochloraemia_corrected (P=0.007) was significantly higher at discharge compared to admission. Creatinine and TP were significantly higher at discharged compared to admission (both with P<0.001), while Na (P=0.018), K (P=0.018), Cl (P<0.001) and Cl_cor (P<0.001) were significantly lower.
Prevalence of creatinine above the upper reference limit was significantly different (P=0.004) between admission (n=2 dogs; 5.41%) and discharge (n=11 dogs; 29.73%).

Table 11. Demographic, hospitalization and laboratory data of the discharge group

	Dogs n=37	
	Variable	**Value**
Demographic data	Age (years)	11 (9-13)
	Body weight (kg)	7.2 (4.55-11.1)
	Sex (F/M)	16 (43.2%) / 21 (56.8%)
Laboratory data (admission)	ad-mHtc (%)	50.08 ± 8.81
	ad-TP (g/dl)	7.11 ± 0.83
	ad-Cr (mg/dl)	0.9 (0.7-1.25)
	ad -Na (mmol/L)	146.30 ± 3.02
	ad -K (mmol/L)	3.99 ± 0.41
	ad -Cl (mmol/L)	113.58 ± 4.10
	ad -Cl_cor (mmol/L)	112.98 ± 4.11
	ad-Hyper/Hyponatremia	6 (16.2%) / 4 (10.8%)
	ad-Hyper/Hypokalaemia	2 (5.4%) / 10 (27%)
	ad-Hyper/Hypochloraemia	7 (18.9%) / 16 (43.2%)
	ad-Hyper/Hypochloraemia_cor	3 (8.1%) / 17 (45.9%)
Laboratory data (discharge)	ds-mHtc (%)	50.7 ± 7.34
	ds-TP (g/dl)	8 (6.40-7.95)
	ds-Cr (mg/dl)	1.4 (1-2.05)
	ds-Na (mmol/L)	144.15 ± 5.56
	ds-K (mmol/L)	3.62 (3.5-4.13)
	ds-Cl (mmol/L)	108.27 ± 5.99
	ds-Cl-cor (mmol/L)	109.29 ± 4.49
	ds-Hyper/Hyponatremia	6 (16.2%) / 11 (29.7%)
	ds-Hyper/Hypokalaemia	2 (5.4%) / 19 (51.4%)
	ds-Hyper/Hypochloraemia	3 (8.1%) / 29 (78.4%)
	ds-Hyper/Hypochloraemia_cor	2 (5.4%) / 28 (75.7%)

	HC (YES/NO)	20 (54.1%) / 17 (45.9%)
	WRF (YES/NO)	25 (67.6%) / 12 (32.4%)
Hospitalization data	First acute CHF (YES/NO)	20 (54.1%) / 17 (45.9%)
	Hours	27 (22.5-44)
	Loop diuretic dose 24h (mg/kg)	13 (10.5-16)
	ACE-I (YES/NO)	24 (64.9%) / 13 (35.1 %)
	Pimobendan (YES/NO)	35 (94.6 %) / 2 (5.4%)

F, females; M, males; CHF, congestive heart failure; mHtc, micro haematocrit; TP, total protein; Cr, creatinine; Na, sodium; K, potassium; Cl, chloride; Cl_cor, corrected chloride; HC, haemoconcentration (increase of mHtc and TP above admission values); WRF, worsening renal function (increase of Cr ≥0.3 mg/dl compared to admission); Loop diuretic dose 24h, estimated dose of furosemide during first 24 hours of hospitalization; ACE-I, angiotensin converting enzyme inhibitor. Normally distributed variables are reported as mean ± standard deviation; non-normally distributed variables are reported as median (interquartile ranges). Categorical variables are expressed as number and percentages.

Haemoconcentration vs non-haemoconcentration

Variables comparison between dogs with and without HC are summarized in Table 12. Dogs without HC had significantly higher ad-mHtc (P<0.001), ad-TP (P=0.002) and ds-mHtc (P=0.045) compared to dogs with HC. No other variables were significantly different between the two groups.

In dogs with HC, mHtc (P<0.001), TP (P<0.001) and creatinine (P=0.003) were significantly higher at discharge compared to admission, while Na (P=0.037), Cl (P=0.002), and Cl_cor (P=0.022) were significantly lower at discharge compared to admission. Prevalence of hyponatremia was significantly higher at discharge compared to admission (P=0.031).

In dogs without HC, mHtc (P=0.061) and TP (P=0.490) were not significantly different between admission and discharge. Creatinine was significantly higher at discharge compared to admission (P=0.001). Chloride and Cl_cor were significantly lower at discharge compared to admission (P<0.001 and P=0.001 respectively). Prevalence of hypochloraemia and hypochloraemia_cor were significantly higher at discharge compared to admission (P=0.008 and P=0.016 respectively).

Prevalence of creatinine above the upper reference limit was not significantly different between HC and non-HC dogs, both on admission (P=1.0) and at discharge (P=0.719).

Table 12. Demographic, laboratory and hospitalization data in haemoconcentration vs no-haemoconcentration

	No haemoconcentration n=17	Haemoconcentration n=20	P value
Demographic data			
Age (years)	10.88 ± 2.28	10.50 (10-12.75)	0.729
Body weight (kg)	7.3 (4.1-9.85)	9.31 ± 5.92	0.662
Sex (F/M)	10 (58.8%) / 7 (41.2%)	6 (30%) / 14 (70%)	0.078
Laboratory data (admission)			
ad-mHtc (%)	56.18 ± 8.16	44.90 ± 5.44	<0.001*
ad-TP (g/dl)	7.53 ± 0.72	6.76 ± 0.77	0.002*
ad-Cr (mg/dl)	0.8 (0.7-1.2)	0.9 (0.8-1.28)	0.232
ad-Na (mmol/L)	146.20 ± 3.27	146.37 ± 2.87	0.867
ad-K (mmol/L)	3.40 ± 0.41	3.98 ± 0.43	0.913
ad-Cl (mmol/L)	114.67 ± 3.81	112.65 ± 4.21	0.137
ad-Cl_cor (mmol/L)	114.17 ± 4.40	111.98 ± 3.65	0.107
ad-Hyper/Hyponatremia	2 (11.8%) / 3 (17.6%)	4 (20%) / 1 (5%)	0.667/0.315
ad-Hyper/Hypokalaemia	1 (5.9%) / 4 (23.5%)	1 (5%) / 6 (30%)	1.0/0.725
ad-Hyper/Hypochloraemia	4 (23.5%) / 6 (35.3%)	3 (15%) / 10 (50%)	0.680/0.368
ad-Hyper/Hypochloraemia_cor	3 (17.6%) / 7 (41.2%)	0 / 10 (50%)	0.088/0.591
Laboratory data (discharge)			
ds-mHtc (%)	52.76 ± 7.26	47 (44-53.50)	0.045*
ds-TP (g/dl)	8 (7.4-8.5)	7.79 ± 0.62	0.598
ds-Cr (mg/dl)	1.74 ± 0.93	1.53 ± 0.68	0.443
ds-Na (mmol/L)	144.44 ± 5.79	143.9 ± 5.49	0.775
ds-K (mmol/L)	3.6 (3.5-4.19)	3.80 ± 0.41	1.0
ds-Cl (mmol/L)	108.45 ± 5.19	108.12 ± 6.73	0.871
ds-Cl_cor (mmol/L)	109.24 ± 2.74	109.33 ± 5.65	0.949
ds-Hyper/Hyponatremia	2 (11.8%) / 4 (23.5%)	4 (20%) / 7 (35%)	0.667/0.447
ds-Hyper/Hypokalaemia	1 (5.9%) / 9 (47.4%)	1 (5%) / 10 (50%)	1.0/0.858
ds-Hyper/Hypochloraemia	1 (5.9%) / 14 (82.4%)	2 (10%) / 15 (75%)	1.0/0.701
ds-Hyper/Hypochloraemia_cor	0 / 14 (82.4%)	2 (10%) / 14 (70%)	0.489/0.462
WRF (YES/NO)	12 (70.6%) / 5 (29.4%)	13 (65%) / 7 (35%)	0.717
Hospitalization data			
First acute CHF (YES/NO)	11 (64.7%) / 6 (35.3%)	9 (45%) / 11 (55%)	0.231
Hours	25 (22.50-50.50)	32.15 ± 13.02	0.775
Loop diuretic dose 24h (mg/kg)	13 (11-15.50)	13 (10-16.75)	0.916
ACE-I (YES/NO)	10 (58.8%) / 7 (41.2%)	14 (70%) / 6 (30%)	0.478
Pimobendan (YES/NO)	16 (94.1%) / 1 (5.9%)	19 (95%) / 1 (5%)	1.0

ad, admission; ds, discharge; females; M, males; CHF, congestive heart failure; mHtc, micro haematocrit; TP, total protein; Cr, creatinine; Na, sodium; K, potassium; Cl, chloride; Cl_cor, corrected chloride; HC, haemoconcentration (increase of mHtc and TP above admission values); WRF, worsening renal function (increase of Cr ≥0.3 mg/dl compared to admission); Loop diuretic dose 24h, estimated dose of furosemide during first 24 hours of hospitalization; ACE-I, angiotensin converting enzyme inhibitor. Normally distributed variables are reported as mean ± standard deviation; non-normally distributed variables are reported as median (interquartile range). Categorical variables are expressed as numbers and percentages. * P value <0.05.

Survival analysis

Admission group

Median survival time of dogs included in the admission group was 271 (17-510) days. At the end of follow-up period, 43 dogs were dead (84.31%) and 8 dogs were still alive (15.69%). Thirty-one of 43 (72.1%) dogs died of cardiac causes, while 12 dogs (27.91%) died of non-cardiac causes. Six out of 43 dogs died during hospitalization because of cardiac causes. Cox regression final step for all-cause and cardiac mortality is reported in Table 13 and Table 14 respectively. The only variable found to be predictive of all-cause mortality on admission was creatinine. The only variable found to be predictive of cardiac mortality on admission was n°-CHF. Cox regression produced the same results using Cl_cor instead of Cl.

Table 13. Cox proportional hazards regression final step for the effect of admission variables on long-term all-cause mortality

	Dogs n=51		
Variable	**Hazard Ratio**	**95% CI**	**P value**
n°-CHF	1.767	0.943-3.313	0.076
ad-Cr	1.910	1.040-3.509	0.037*

CI, confidence interval; n°-CHF: number of previous heart failure episodes (first=0, second or more=1), ad, admission; Cr, creatinine admission. Variables included in the model: age, n°-CHF, ad-mHtc, ad-TP, ad-Cr, ad-Na, ad-K, ad-Cl.

Table 14. Cox proportional hazards regression final step for the effect of admission variables on long-term cardiac mortality

	Dogs n=51		
Variable	**Hazard Ratio**	**95% CI**	**P value**
n°-CHF	2.137	1.031-4.433	0.041*

CI, confidence interval; n°-CHF: number of previous heart failure episodes (first=0, second or more=1). Variables included in the model: age, n°-CHF, ad-mHtc, ad-TP, ad-Cr, ad-Na, ad-K, ad-Cl. *P value <0.05.

Discharge group

Among admission laboratory parameters (variables subjected to change during hospitalization), only ad-Cr resulted significantly associated with survival; thus, it was kept in the Cox regression model of the discharge group. Median survival time of dogs included in the discharge group was 362 (82-509) days. At the end of follow-up period, 32 dogs were dead (86.49%) and 5 dogs were still alive (13.51%). Twenty-three

(71.88%) of 32 dogs died of cardiac causes, while 9 dogs (28.13%) died of non-cardiac causes. Three out of 32 dogs died during hospitalization because of cardiac causes. Cox regression final step for all-cause and cardiac mortality is reported in Tables 15 and 16 respectively. Presence of haemoconcentration at discharge was associated with a significantly lower long-term all-cause and cardiac mortality (Figure 9). Median survival time was 233 (12-460.50) days in non-HC dogs and 454 (117-574.25) days in HC dogs.

Admission creatinine and loop diuretic dose 24h were significantly associated with an increased risk of long-term all-cause mortality, while n°-CHF and loop diuretic dose 24h were significantly associated with an increased risk of long-term cardiac mortality. Cox regression produced the same results using Cl_cor instead of Cl.

Table 15. Cox proportional hazards regression final step for the effect of discharge variables on long-term all-cause mortality

	Dogs n=37		
Variable	**Hazard Ratio**	**95% CI**	**P value**
n°-CHF	2.022	0.927-4.409	0.076
Loop diuretic dose 24h	1.083	1.024-1.144	0.005*
ad-Cr	2.233	1.039-4.797	0.040*
HC	0.270	0.115-0.634	0.003*

CI, confidence interval; n°-CHF: number of previous heart failure episodes (first=0, second or more=1); Loop diuretic dose 24h, estimated dose of furosemide during first 24 hours of hospitalization; ds, discharge; Cr, creatinine; HC, haemoconcentration. Variables included in the model: age, n°-CHF, Loop diuretic dose 24h, ds-Na, ds-K, ds-Cl, WRF (YES/NO), HC (YES/NO). *P value <0.05.

Table 16. Cox proportional hazards regression final step for the effect of discharge variables on long-term cardiac mortality

	Dogs n=37		
Variable	**Hazard Ratio**	**95% CI**	**P value**
n°-CHF	2.601	1.033-6.550	0.043*
Loop diuretic dose 24h	1.091	1.028-1.158	0.004*
HC	0.336	0.128-0.887	0.028*

CI, confidence interval; n°-CHF: number of previous heart failure episodes (first=0, second or more=1); Loop diuretic dose 24h, estimated dose of furosemide during first 24 hours of hospitalization; HC, haemoconcentration. Variables included in the model: age, n°-CHF, Loop diuretic dose 24h, ds-Na, ds-K, ds-Cl, WRF (YES/NO), HC (YES/NO). *P value <0.05.

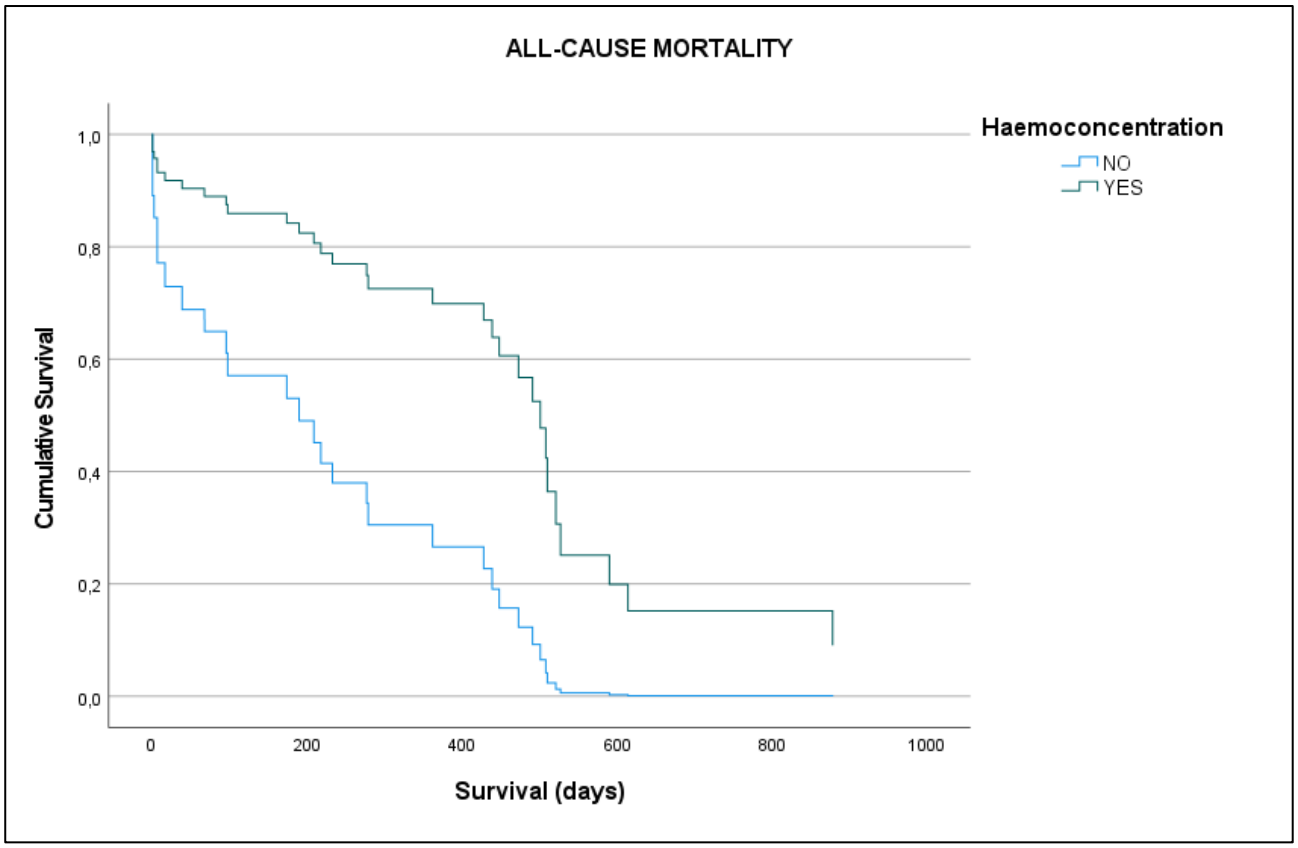

Figure 9. Multivariable survival curves according to haemoconcentration status considering all-cause mortality. Variables included in the model: age, n°-CHF, Loop diuretic dose 24h, ad-Cr, ds-Na, ds-K, ds-Cl, WRF (YES/NO), HC (YES/NO).

Discussion

In the present study, haemoconcentration at discharge was associated with a significantly lower risk of all-cause and cardiac mortality. This result is in line with recent human literature [17,86,310-312,453,454]. Although HC is not a perfect measure of decongestion and changes in absolute blood volume[16,454], several human studies have showed the association between increase in haemoconcentration parameters (e.g., haematocrit, total protein, albumin, haemoglobin) and decrease in markers of congestion (e.g., brain natriuretic peptide, N-terminal pro-B type natriuretic peptide, body weight, net fluid output, right atrial pressure, pulmonary capillary wedge pressure) [17,310,312,453,455]. Haemoconcentration can be an easily assessable indicator of

reduced congestion in patients with acute HF. As consequence, the positive effect of HC on survival might reflect the beneficial effects of achieving an adequate decongestion[310]. Volume overload, rather than low cardiac output, is the primary cause of HF hospitalizations in people, with dyspnoea, rales and peripheral oedema as the most common symptoms/clinical signs[456]. Moreover, the presence of residual congestion at discharge has been associated with worse outcome and higher incidence of re-hospitalizations[18,310,318,319,456-458]. Congestion is now known to have a predominant role in the progression of HF syndrome. Indeed, this hemodynamic abnormality is the primary driving force of several organs damage. Congestion is responsible of increased myocardial stretch and cardiomyocytes apoptosis, thus resulting in cardiac remodelling[456,459,460]. Repeated and sustained elevations in cardiac filling pressure and pulmonary capillary pressure lead to devastating lung injuries, which turn into impaired gas diffusion and pulmonary hypertension[2,243-245]. Elevated central venous pressure, rather than reduced cardiac output, is now known to be the primary determinant of deteriorating renal function in patients with HF, a condition named as congestive nephropathy[2,264]. Lastly, congestion is also the main responsible of liver and gastrointestinal dysfunctions[4,5,273,274,283]. These multi-organ derangements feed off each other, thus establishing a vicious circle and further promoting neurohormonal dysregulation, inflammation and oxidative stress[2,4,220-222]. It is important to highlight that only HC at discharge positively affected survival in people, while early HC during hospitalization did not[17,313]. This result is likely related to the fact that HC occurs when "fluid has been removed from the intravascular space faster than it could be replaced by extravascular fluid"[313]; as consequence, HC alone just reflects changes in intravascular volume and not the total body volume status[313]. In case of severe extravascular volume overload, intravascular volume overload can rapidly reoccur if fluid removal is not maintained to a sufficient degree[313]. However, the presence of HC (i.e., intravascular volume improvement) at discharge (i.e., presumable restoration of extravascular volume overload) can reflect an optimal overall volume status[313]. Dogs which responded to treatment with HC could be patients with a lower disease severity.

However, other results of this study appear not to support this hypothesis. Firstly, there were no significant difference in most of admission variables between dogs with and without HC. In particular, proportion of dogs at their first acute CHF episode was not significantly different between the two groups, as well as the prevalence of hypochloraemia, which has been suggested to be associated with MMVD severity[121]. The only different admission variables were mHtc and TP, which were significantly lower in HC dogs; this result is in line with previous studies in people[17,310,311] and could have paradoxically predicted a worse outcome for HC dogs. In a recent human study, plasma volume status (calculated as 100-hematocrit/haemoglobin) was used as marker of congestion on admission, and it was associated with increased risk of in-hospital mortality[461]. Moreover, 24h loop diuretic dose was not significantly different between HC and non-HC dogs, as well as the proportion of dogs receiving ACE-I and pimobendan during hospitalization.

Loop diuretic dose administered in first 24 hours was associated with an increased risk of all-cause and cardiac mortality. In a recent study, total in-hospital parental furosemide dosage was not associated with survival in dogs hospitalized for acute left-sided CHF[300]. In contrast, another previous study found a positive association between survival and furosemide dose≥6.7 mg/kg in dogs with advanced HF, but only 33% of dogs required hospitalization and diuretic dose was divided in two categories based on the median value registered before death/end of the study[462]; thus, a comparison might not be appropriate. On the other hand, previous studies in people have reported similar findings to ours, accounting for the maximal in-hospital daily dose or total in-hospital diuretic dose. In these studies, the well-known side effects of diuretics were suggested as possible determinants of the negative association with survival, but it was also stated that a direct cause-effect relationship cannot be fully established[463,464]. Indeed, the need of higher diuretic doses, especially in the first 24 hours, might also be a marker of a "sicker" patient (e.g., more advanced underlying cardiac disease, more severe pulmonary oedema). Even a lower diuretic response may trigger the increase of diuretic doses; this parameter has been associated with worse outcome and more advanced

cardiac disease in people with acute HF[16,321,466,467]. However, measures of diuretic response were not different between chronic stage C and stage D dogs with MMVD[121]. Moreover, a recent study in diuretic-naïve dogs hospitalized for the first acute CHF episode suggested that diuretic responsiveness could also be subjected to an individual variability, regardless of the disease severity and previous diuretic therapy[334,468]. The negative effect of high diuretic doses on survival might appear in contrast with the benefit of haemoconcentration. In two studies in people, patients with HC showed lower mortality and received significantly higher diuretic doses compared to those without HC; this result suggested that the benefits of high doses might overwhelm their side effects[310,313]. However, HC and diuretic doses may not be necessarily correlated. A given dose of diuretic might not produce the same amount of volume depletion in two different patients. In support of this hypothesis, a previous study reported significantly lower IV diuretic doses in people that achieved haemoconcentration compared to those that did not[311]. In the present study, there were no significant difference in 24h loop diuretic dose between dogs with and without HC, although the total dose administered during the hospitalization period was not collected and possible discrepancies could have been missed. The Diuretic Optimization Strategies (DOSE) trial investigated the effect on survival of a high-dose vs low-dose diuretic strategy in people with acute HF, but findings with respect to post-discharge outcome were "neutral"[314]. However, the subsequent exploratory analysis revealed interesting results[19]. Accounting for the total amount of loop diuretic revealed a beneficial effect of high-dose strategy on survival, but adjusting for multiple congestion markers removed this benefit. These finding suggest that positive effect of high doses are mediated by achieving adequate decongestion. Overall impact of high diuretic doses likely derives from a balance between potential harms and benefits[19]. If the goal of decongestion is achieved, the adverse effects are overwhelmed (e.g., diuretic-related decrease in renal function might be counterbalanced by relief of congestive nephropathy); if volume overload persists, the negative effects likely take over (e.g., extreme renin-angiotensin-aldosterone system stimulation). The concomitant presence

of 24h diuretic dose and HC as significant opposite predictors of outcome might reflect this precarious equilibrium. Thus, high or low diuretic doses is probably not the right question. Indeed, current human HF guidelines state that "the aim of diuretic therapy is to achieve and maintain euvolemia with the lowest diuretic dose"[10]. The "right" dose for a single patient likely depends on countless individual factors (e.g., degree of congestion/hypotension, neurohormonal activity, diuretic responsiveness, renal function, type of cardiac disease)[469]. For this reason, diuretic (± RAAS blockers) strategies titrated on the single patient and guided by multiple parameters need to be developed. In this context, haemoconcentration at discharge might be a driving parameter in people[299,453] and, maybe, in dogs, but the association with other markers is recommended. Among all, assessment of diuretic response has gained a lot of interest and is acquiring a central role in human acute HF therapy[16]. Combination of haemoconcentration and diuretic response improved the risk prediction for early re-hospitalization in people[470], highlighting the importance of a multimodal approach. Oyama MA et al (2023) recently published an exhaustive perspective on diuretic responsiveness and its assessment in veterinary medicine[272], laying the foundations for future investigations in this field.

Creatinine showed a significantly increase from admission to discharge in the entire discharge group and both in HC and non-HC dogs. Worsening renal function occurred in 67.6% of discharge dogs, which is a higher percentage compared to a previous study that used the same definition[300]. This difference could be related to the use of higher median loop diuretic dose in the present study (13 [10.5-16] mg/kg in 24 hours vs 7.7 [4.4-12.7] mg/kg during entire hospitalization period)[300], although Giorgi ME et al. (2022) accounted only parenteral route while some of our patients could have received OS administration even in the first 24 hours because of rapid stabilization after first IV doses. Outpatient re-evaluation was not an objective of the present study; thus, percentage of transient vs true WRF is not known. However, Giorgi ME et al. well documented that WRF developed during acute CHF hospitalization seems to be transient in most dogs[300]. Both creatinine at discharge and prevalence of WRF were not

significantly different between HC and non-HC dogs. This result is in contrast with previous studies in people, where WRF was significantly more common in HC patients[17,310,312]. However, WRF is not used as a marker of decongestion, and it can occur in both patients with and without congestion[18,312]. Moreover, elevated central venous pressure is a primary determinant of decreased renal function in patients with HF[2,264]. Thus, prevalence of WRF between the two groups could be balanced by a combination of greater volume depletion/relief of congestive nephropathy in HC dogs and less volume depletion/persistent congestion in non-HC dogs. Higher creatinine on admission was associated with worse outcome in the present study, in contrast to two previous studies in dogs which did not find any effect on survival[279,300]. In people hospitalized for acute HF, baseline renal insufficiency (estimated glomerular filtration rate <60 ml/min), but not WRF, was associated with increased risk of death and re-hospitalization[263]. Two other studies found a similar negative predictive value for creatinine >120 μmol/L (corresponding to 1.36 mg/dL) and >1.5 mg/dL on admission respectively[307,471]. Another study showed that all-cause mortality significantly increased with second and third tertile of cystatin C measured on admission[472]. These results can be interpreted from both a pure renal and cardio-renal perspective. Creatinine concentration is the driving parameter of renal disease staging and, used as a continuous variable, higher creatinine was associated with increased risk of death in dogs with different stages of chronic kidney disease[473]. Thus, higher baseline creatinine might reflect a worse intrinsic renal function regardless of cardiac disease severity, and play an additive negative prognostic role as an independent comorbidity[263,307]. On the other hand, higher creatinine on admission might reflect a more severe hemodynamic derangement (i.e., more severe congestion/hypoperfusion) and, maybe, a more severe cardiac disease and/or decompensation episode[263,307]. Even a combination of both conditions could occur. However, in the present study, it was not possible to categorize patients based on the presence or absence of increased creatinine because only 4 of 51 dogs had admission creatinine above the upper reference limit. For this reason, attributing different renal function to patients with different but within range creatinine

levels might not be appropriate. Further investigations with a creatinine-based stratification of patients are necessary.

Hypochloraemia was present in 23 of 51 dogs (45.1%) and was the most common electrolyte abnormality upon admission. Even considering dogs at their first acute CHF episode and dogs that experienced a reoccurrence separately, hypochloraemia persisted as the most common electrolyte disorder (32% and 57.7% respectively). This result is in line with two previous studies[301,334]. Roche-Catholy M et al investigated electrolyte disorders in dogs at their first acute CHF episode on admission and found hypochloraemia in 20% of dogs[334]. Gouta CM et al found hypochloraemia in 34.1% of dogs hospitalized for acute CHF (first or reoccurrence)[301]. Percentage differences between studies might be related to the administration of diuretic therapy before hospitalization (and its dose) and to the severity of the underlying cardiac disease, since both factors can contribute to hypochloraemia[121,329,334,474]. Indeed, the greater discrepancy occurs between dogs with reoccurrence of acute CHF in the present study and dogs included in the study of Roche-Catholy M et al (all were at their first acute CHF episode)[334]. In the discharge group, all electrolytes were significantly lower at discharge compared to admission. Moreover, prevalence of hypochloraemia, hypokalaemia and hyponatraemia approximately doubled at discharge compared to admission, although only hypokalaemia and hypochloraemia were statistically significant. These results are in a line with a previous study, and they are likely due to the intensive diuretic administration during hospitalization, as well as to the acute HF itself (e.g., extreme RAAS, vasopressin and sympathetic nervous system activity)[328,474]. Both on admission and at discharge, none of the electrolytes were associated with survival in the Cox regression. Two previous studies found the same result for admission electrolytes[301,334]. In contrast, another study found a significant lower admission sodium in dogs that did not survive to discharge; however, study design was largely different because patients were divided in survivors and non-survivors to discharge, and no follow-up was recorded[333]. Similarly, Gouta CM et al found that hypokalaemia during hospitalization was associated with improved outcome, but again

survival was considered until discharge[301]. In people with acute HF, hyponatremia, dyskalemia and hypochloraemia are primary electrolytes abnormalities on admission and at discharge and they are associated with worse short-term and long-term outcome[20,21,328-332,474-480]. Hyponatremia may reflect higher RAAS activity and a more advanced HF[328,481]. Potassium abnormalities determine pro-arrhythmogenic effects[328,481]. Hypochloraemia has gained a lot of interest in recent years and has been found to be responsible of derangements of fluid homeostasis, neurohormonal activity and diuretic responsiveness, thus contributing to the progression of HF[272,473]. However, as previously explained by Roche-Catholy M et al[334], human studies are characterized by a large number of patients which are categorized in classes of different electrolyte concentrations (e.g., hypo/hyper/normal, quartiles). This approach could be more performing in detecting differences in survival, but sample size of the present study was too small to apply this method. Overall, current literature seems to indicate that electrolytes disorders have no prognostic role on long-term outcome in dogs with acute CHF, but we believe that large scale investigations which permit a categorization based on electrolytes levels are necessary to confirm or deny this result.

Prior acute CHF hospitalization was associated with an increased risk of long-term cardiac mortality in the present study. It can be an intuitive but interesting result to discuss. This result is different but complementary to that reported in a previous study, which showed that dogs hospitalized at the time of diagnosis of advanced heart failure had shorter survival time compared to dogs managed as outpatients[462]. History of prior HF hospitalization has been associated with worse outcome in several studies in people with both chronic and acute HF[482-486]. However, reasons behind this association remain subject of debate even in human literature. A first hypothesis can be related to the fact that each acute HF episode represents a devastating hemodynamic and neurohormonal disorder in which multiple organs are subjected to severe distress. Thus, each event can potentially lead to multi-organ injury and dysfunction[5,221]. Intuitively, the more these acute episodes reoccur, the more organ damage can worse and become permanent, contributing to the progression of the HF syndrome. Even the potential side effects of

intensive doses of different drugs (e.g., diuretics, inotropes) might play a role in this association[254,482,487]. A second hypothesis is that acute HF reoccurrence is simply a marker of "sicker" patients with a more advanced HF syndrome (e.g., more severe cardiac disease, less diuretic responsiveness and presence of comorbidities such as pulmonary hypertension, systemic hypertension, decreased renal/liver/gastrointestinal function)[485,488]. A recent study in people sustained this hypothesis, since the association between prior HF hospitalization and mortality was lost after adjustment for patient baseline and in-hospital clinical characteristics (e.g., baseline medications, dyspnoea severity on admission, chronic lung disease)[488]. However, the two hypotheses could be related and coexist. For example, a patient may be "sicker" and have worse clinical characteristics also because of the deleterious effects derived from previous hospitalizations. We cannot exclude that non-biological factors might have played a confounding effect on the association between prior acute CHF hospitalization and cardiac mortality in the present study, such as early discharge because of financial limitations, increased propension for euthanasia after acute HF reoccurrence or poor adherence to prescribed therapy which can favour re-hospitalization and death[462,485,488]. Therefore, this result needs to be confirmed in prospective studies.

The present study has several limitations. First of all, the sample size of the present study was small, and all dogs included came from the same veterinary center. Secondly, due to the retrospective nature of the study and the consequent lack of several information, causality between the achievement of HC and improved survival cannot be proved. Thirdly, HC was defined with a method similar to those used in previous human studies[313,453] and using parameters that are routinely assessed in the emergency and intensive care unit. However, HC definition is not standardized[453] and the one used in the present study may not be stringent enough (e.g., adding haemoglobin and albumin could give different results) and be subjected to inter/intra-observer variability because of mHtc and TP manual reading; moreover, association with other markers of decongestion (e.g., natriuretic peptides, net fluid output) is encouraged. Fourthly, cardiac medications administered before and after hospitalization, as well as some

medications administered during hospitalization (e.g., spironolactone, dobutamine, vasodilators, antiarrhythmics, electrolytes supplements), were not recorded, and they potentially affected the results. Fifthly, cardiac medications during hospitalization, as well as timing of clinicopathologic assessments, were not standardized and they were upon the discretion of clinicians. Sixthly, route of administration of diuretics affects their bioavailability but it was not recorded; all dogs were subjected to IV diuretic administration on admission (boluses and/or CRI), but it is possible that some dogs which achieved a rapid stabilization switched to IM, SC or OS administration within the first 24 hours. Moreover, torsemide conversion might be inaccurate, especially for IV/IM administration. These factors might be responsible of inaccurate estimation of furosemide dose. Seventhly, the total in-hospital diuretic dose was not registered, and it could have shown a different association with survival, as well as it could have differed between HC and non-HC dogs. Lastly, thorax radiography is considered the gold standard for the diagnosis of acute left-sided CHF, but it was not performed in all cases. Because of these important limitations, our findings have no clinical applicability at the current state of knowledge. They should be considered hypothesis-generating and serve to stimulate further large-scale prospective multicenter studies in this field.

In conclusion, haemoconcentration was associated with lower long-term all-cause and cardiac mortality, supporting the predominant role of congestion in HF pathophysiology and the importance of adequate decongestion at discharge. Further investigations are necessary to replicate and validate this result and understand its potential clinical implications. Loop diuretic dose administered in first 24 hours was associated with worse outcome. Higher diuretic doses might reflect patients with more advanced HF or less diuretic responsiveness, although their side-effects could play a role in this negative association. Dogs with prior acute CHF hospitalization showed an increased risk of cardiac mortality. This association could be related to the potential deleterious effects of each acute decompensation, to the fact that patients with acute CHF reoccurrence are “sicker”, and to a combination of both. Lastly, the present study

confirmed hypochloraemia as the most common electrolyte abnormality in dogs with acute CHF, both on admission and at discharge. However, further investigations with stratification of a large cohort of patients in different electrolytes classes are necessary to re-evaluate the prognostic significance of electrolytes disorders in dogs with HF.

GENERAL CONCLUSIONS

We believe that the present project contributed to better understand the diagnostic value of different markers of the HF syndrome in dogs, as well as to improve their interpretation.

The first study revealed that UAldo:C was not significantly different among dogs with different stages of MMVD. However, this parameter was affected by several individual factors, such as gender, breed, age and therapy, making difficult its interpretation.

In particular, intact females showed the highest values of UAldo:C in study 1, and the second study further investigated the potential mechanisms behind this result. Indeed, a relationship between aldosterone and progesterone concentrations was found in canine species, as was already reported in humans and rats. In contrast, NT-proBNP was not affected by progesterone and did not vary between anoestrus and dioestrus; there were no previous reports in dogs about the assessment of this peptide in different phases of oestrus cycle and its potential relationship with progesterone.

Because breed-specific reference ranges for NT-proBNP have been encouraged by previous literature, the third study assessed a preliminary breed-specific reference interval for NT-proBNP in healthy Chihuahua, a breed whose most common causes of death are heart diseases and low respiratory tract disorders. The results revealed that the current proposed canine generic cut-off of NT-proBNP seems to be suitable for this breed. We did not find sex differences in this peptide, in contrast to what previously reported. In the same study, we confirmed the strong impact of Chihuahua breed on UAldo:C, which was suggested in study 1, as well as a high individual variability of this parameter. At the current state of knowledge, a reference interval for UAldo:C seems to be worthless in the clinical practice, and an individual longitudinal approach (i.e., progressive monitoring) should be preferred.

In the fourth study, we investigated another aspect of the HF syndrome, that is the multi-organ damage and, in particular, the dilemma of WRF in acute HF. The study showed that haemoconcentration, a sign of decongestion, was associated with improved survival, while WRF did not have an impact on outcome. This result is in

line with recent human literature. Despite the numerous limitations of a retrospective study, this finding can stimulate further prospective investigations in this field, in order to understand the potential clinical applicability.

The authors hopes that the present project may provide novel insights for future research in the field of HF syndrome.

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