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Original article

Iron status in dogs with myxomatous mitral valve disease

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Abstract

In humans, iron deficiency represents a relevant occurrence in heart failure (HF), with or without anaemia, and is associated with the worst outcome. Moreover, chronic kidney disease (CKD) is a well-known comorbidity of HF and is strongly associated with the risk of developing anaemia. The most common cause of HF in dogs is myxomatous mitral valve disease (MMVD). To the best of our knowledge, no studies have examined the iron status in dogs with HF, with and without CKD. The aim of this retrospective study was to evaluate the iron status in dogs affected by MMVD and how strong is the relation with HF.

The retrospective study included 54 dogs with complete case records, echocardiography and laboratory analyses. Iron status was evaluated by measuring serum iron concentration (SIC), unsaturated iron binding capacity (UIBC), total iron binding capacity (TIBC), and percentage of saturation (%SAT).

The prevalence of dogs showing low serum iron concentration (SIC) was 18% in the whole population, 33% in symptomatic patients, 100% in dogs with acute decompensated HF. No significant differences in SIC, UIBC, TIBC and %SAT median values were found among dogs classified in different ACVIM (American College of Veterinary Internal Medicine) classes, between symptomatic and non-symptomatic patients, and among IRIS (International Renal Interest Society) classes. Azotemic and non-azotemic patients presented a significant difference in SIC mean values ($p=0.02$). Generalised linear model (GLM) revealed that dogs with low SIC were at higher risk of being included in a higher ACVIM class (OR=6.383, p-value=0.014).

Log-rank analysis showed shorter survival in dogs with low SIC ($p=0.020$), multivariate Cox analysis revealed that only HF symptoms can affect survival.

Key words: iron, dog, myxomatous mitral valve disease, cardiorenal syndrome, cardiovascular-renal disorder

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Introduction

In human medicine, recent studies have highlighted the role of iron deficiency (ID) in heart failure (HF) patients. Dysregulation of iron metabolism was generally considered relevant only in association with anaemia, which have a prevalence of approximately 50% (Okonko et al. 2011, Arora et al. 2014). Nevertheless, recent studies have found that the prevalence of serum iron deficiency (SID) in HF can be higher than the prevalence of anaemia and varies from 37% to 61% (Wong et al. 2016). In these patients, ID can also be present in the absence of anaemia and is associated with the worst symptoms (Klip et al. 2013, Rangel et al. 2014, Fitzsimmons et al. 2015). ID represents an increased risk of mortality and morbidity itself (Klip et al. 2013, Rangel et al. 2014, Fitzsimmons et al. 2015). Moreover, ID is responsible for exercise intolerance, reduction in quality of life, and can promote cardiac remodelling, as shown in murine animal models (Nayto et al. 2009, Wong et al. 2016). Myocites and cardiomyocites are in fact cells with elevated energy demand, for which the maintenance of a correct iron metabolism is fundamental. Alterations in this metabolism can promote impairment in oxidative and cellular energy metabolism (Arora et al. 2014).

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

ID can be defined as absolute or functional and both can be found in patients affected by heart diseases (Jankowska et al. 2013).

In fact, impaired absorption from the gastrointestinal (GI) tract, chronic blood loss and decrease of dietary intake can mediate absolute ID, but since HF is a chronic condition that promotes inflammatory status of the organism, functional ID due to cytokines and hepcidin overexpression can be present (Wong et al. 2016).

To asset iron status, in human medicine, ferritin can be used. Indeed, ferritin has an important role in the inflammatory cycle and represents both a well-known marker of iron stores as well as an important biomarker of inflammation, that could be present in chronic diseases (Bohn 2015). It has been reported that ferritin levels can be high in the presence of several pathologies such as acute respiratory distress syndrome, coronary artery disease, hypertension and myocardial infarction (Kell et al. 2014).

Moreover, immunoassays for human serum ferritin cannot be used in other species, so individual assays still must be developed and calibrated for each species. (Andrews 2010)

Chronic kidney disease (CKD) is a well-known comorbidity of HF and the presence of both conditions is known as Cardiorenal Syndrome, or Cardiovascular-renal disorder (Pouchelon et al. 2015). Anaemia can be associated with both HF and CKD in human patients, which is commonly referred to as Cardiorenal-anaemia syndrome (Martinelli et al. 2016).

The most common acquired heart disease affecting dogs and leading to HF is Myxomatous Mitral Valve Disease (MMVD), also known as endocardiosis, that can reach a prevalence close to 100% in elderly small breed dogs (Borgarelli et al. 2012).

To the best of our knowledge, no studies have examined iron status in dogs affected by MMVD, with or without CKD. Therefore, the aims of this study were to determine the iron status in dogs affected by MMVD, to analyse whether any differences in serum iron values were present between the American College of Veterinary Internal Medicine (ACVIM) classes, between symptomatic and non-symptomatic patients for HF and with respect to selected echocardiographic parameters. Moreover, we investigated the possible existence of any difference in SIC among patients presenting MMVD and azotemia as a model of cardiovascular renal disorder.

A final aim was to evaluate the presence of an association between SID and patient's survival time.

Materials and Methods

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

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

A full echocardiographic examination was performed using standardized thoracic imaging planes according to the recommendations of the American Society of Echocardiography (Thomas et al. 1993, Bonagura et al. 2000). From 2D view we obtained: Aortic root (Ao) and Left atrial (LA) dimension (right

parasternal short-axis view). Mitral valve inflow (E peak velocity, E/A ratio) and peak velocity of mitral and tricuspid regurgitations (MR and TR) were obtained through colour and spectral Doppler. We then calculated: left atrial-to-aortic root ratio (LA/Ao), End systolic volume index (ESVI), End diastolic volume index (EDVI), according to the Teichholz formula normalized to body surface area (BSA).

The MMVD patients were categorized based on the ACVIM classification (Atkins et al. 2009). As recommended by the ACVIM guidelines, stage B included dogs affected by MMVD without signs of CHF (namely, in stage B1, dogs without evidence of cardiac enlargement; in B2, dogs with signs of cardiac remodelling as LA and/or LV enlargement); stage C dogs with clinical signs of CHF due to MMVD, and stage D subjects presenting both clinical signs of CHF and refractoriness to standard medical treatments. (Atkins et al. 2009, Boswood et al. 2016).

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

The International Renal Interest Society (IRIS) guidelines were used to classify patients affected by CKD, and dogs were considered azotemic if their serum creatinine (sCr) values were ≥ 1.4 mg/dl. According to IRIS, patients were classified as IRIS 2 (sCr between 1.4 and 2 mg/dl), IRIS 3 (sCr between 2.1 and 5.0 mg/dl) and IRIS 4 (sCr >5.0 mg/dl). Sub staging was performed based on BP and the presence of proteinuria. Proteinuria was investigated trough urine creatinine to protein ratio (UPC) and considered present when UPC ≥ 0.5 (IRIS 2015).

Blood samples were collected from the brachiocephalic vein during routine clinical evaluation. For CBC, EDTA was used as anticoagulant, whereas for biochemical evaluation, the blood was placed in tubes without anticoagulant and centrifuged at 3250 x g for 5 minutes. The CBC was performed using an automated laser haematology analyser (Sysmex XT-2000iV; Sysmex corporation; Japan). For every CBC, red blood cells (RBC), haematocrit (Ht), haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin concentration (MCHC), red distribution width (RDW) and platelet count were considered. Simultaneous evaluation of the blood smear was performed and the degree of anisocytosis and poikilocytosis was reported, together with a description of the main erythrocytes' alterations. Anaemia was defined as a haematocrit value ≤ 37% (Tvedten et al. 2010).

Routine biochemistry was carried out with an automated chemistry analyser (Cobas Mira; Roche diagnostics, Switzerland) with reagents provided by Hagen diagnostic system (Italy). The serum samples were kept frozen (-20° C) until the iron analyses were performed.

As described by Zaldivar-Lopez et al (2014), iron status was evaluated by measuring SIC (normal value 90 - 200 mcg/dL) and total iron-binding capacity (TIBC, normal value 270-496 μg/dL); the TIBC was calculated by adding the unsaturated iron binding capacity (UIBC) to the SI. Percentage transferrin saturation (%SAT, normal value > 23%) was obtained using the formula %SAT = SI/TIBC x 100 (Andrews et al. 2010).

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

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

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

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

Survival was calculated as days between diagnosis and death (all causes of mortality) or between diagnosis and last contact with the owners (control visit or phone call). Subjects lost to follow-up were included in the survival analysis up until the last time at which they were known to be alive and then were thereafter censored in the analysis. The Kaplan-Meier method was used to estimate the survival function and plot time to event curves in the different groups; the equality of

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

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

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

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

survival distributions was tested using the Log-Rank method, and then univariate and multivariate Cox proportional hazard analysis were used to evaluate the influence of different variables on survival. The variables considered were: presence/absence of clinical signs of CHF (dichotomous variable), age, sex, body weight, ACVIM class, RBC, Hb, Ht, RDW, SIC, low SIC (dichotomous variable), sCr, IRIS class, ESVI, EDVI, MR peak velocity, TR peak velocity, presence/absence of pulmonary hypertension (dichotomous variable), normalized left ventricular end diastolic and sistolic diameter calculated according to Cornell's method of allometric scaling, La/Ao, E/A, peak velocity of E wave.

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

Results

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

The mean age of the dogs was 11 years (±3.2 SD), and median body weight was 11 kg (IQR 6 – 22). Most of the patients included were intact males (n=23, 43%), followed by neutered females (n=17, 31%), neutered males (n=8, 15%) and intact females (n=6, 11%). The most represented breeds were mongrels (n=24, 44%), Dachshund (n=4, 7%) and Cavalier King Charles (n=3, 6%). Breeds with less than three dogs were grouped and listed as "others" (n=21, 39%). Twenty-two dogs were classified as ACVIM B1 (41%), 14 as ACVIM B2 (26%), 15 as ACVIM C (28%) and 3 as ACVIM D (5%). Sixtyseven % (n=36) of the dogs were asymptomatic for CHF (ACVIM classes B1 and B2), and 33% were symptomatic (ACVIM classes C and D) (n=18). Most of the dogs were classified as IRIS class 1 (80%, 44/54), 7 as class 2 (14%) and only 3 as IRIS class 3 (6%).

No dogs were classified as IRIS class 4. Non-azotemic dogs represented the 80% of the population (n=44), whereas 20% presented azotemia (n=10). The mean values of SIC, TIBC, UIBC and %SAT for different ACVIM classes and in symptomatic or non-symptomatic patients for CHF are reported in Table 1. The mean values of SIC, TIBC, UIBC and %SAT for different IRIS classes are reported in Table 2. The mean values of SIC, TIBC, UIBC and %SAT for

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

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

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

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

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

azotemic and non-azotemic dogs are reported in Table 3.

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

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

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

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

Only 1 patient (2%) presented low SIC and concurrent anaemia. The prevalence of anaemia in the whole MMVD population was 6% (3/54). Analysis of the blood smear revealed no substantial alterations in dimension or morphology of the erythrocytes, except microcytosis in 2/10 (20%) dogs with low SIC. The only patient presenting a mild degree of poikilocytosis with presence of codocytes and schistocytes was the dog with low SIC and concurrent anaemia. MCV was below the reference interval in 1/54 dogs (2%), with concurrent microcytosis highlighted at the blood smear analysis. MCHC was in the reference range for all the dogs included. RDW was above the reference interval for 3/54 dogs (5%), one of them presenting low SIC and concurrent anaemia.

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

Statistical analysis by GLM revealed that dogs with SID were at higher risk of being included in a higher ACVIM class when compared with dogs with normal levels of iron (OR=6.383, p-value=0.014). The data were adjusted for age, sex and body size (Table 4). In contrast, SID (adjusted for age, sex and size) was neither associated with an increase in IRIS classes nor considered a risk factor for azotemia.

Mean values for different echocardiographic parameters of the dogs included in the study are presented in Table 5. ANOVA revealed that statistical significant differences were present between different ACVIM

Table 5. Mean (± SD) values in selected echocardiographic parameters for different ACVIM classes

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

* One-Way ANOVA, Brown and Forsythe Test

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

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

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

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

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

Statistical analysis by GLMs revealed that dogs with SID were at higher risk of have certain echocardiographic parameters altered, including a higher La/Ao ratio (OR=1.50, p=0.018) and TR peak velocity (OR=1.88, p=0.023), and a lower MR peak velocity (OR=0.49, p=0.002).

Log-rank analysis showed shorter survival in MMVD dogs with SID (p-value=0.020). The median survival times (MST) were 201 days for dogs presenting low SIC and 296 days for dogs with normal SIC. Univariate Cox analysis showed statistical significant results in determining survival for some of the considered variables. Hazard ratios are reported in Table 6. Multivariate Cox analysis revealed that only age and the presence of CHF symptoms could affect the dogs' survival (Table 7).

Discussion

In our population, dogs diagnosed with MMVD and presenting low SIC had a prevalence of 18%.

The prevalence of SID among dogs presented with HF due to MMVD (ACVIM C and D) was 33%. The 100% of the dogs admitted with acute decompensated CHF presented SID.

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

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

Old, male, small size breed dogs with low SIC are at higher risk of being included in a higher ACVIM class.

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

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

In veterinary medicine, anorexia, like it is seen at late stage of HF, is a quite concerning topic for owners and a study from Mallery et al. (1999) reported that is one of the most common reasons for owners to choose euthanasia during late-stage HF (Mallery et al. 1999). Anorexia leading to low SIC is not something common in veterinary medicine. Moreover, dogs are normally fed balanced diets, which help to prevent any kind of dietary insufficiency. Dogs' diets are usually rich in meat products, making dietary haeme, an important iron source released from dietary myoglobin and haemoglobin, more bioavailable. (Harvey 2008)

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

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

On the other hand, anorexia in symptomatic CHF, in conjunction with GI oedema and the inflammatory status that is related to the presence of CHF could offer a possible explanation for SID in dogs symptomatic for HF, where is more prevalent.

It is known that HF is a chronic condition that promotes inflammatory status of the organism, and this can alter iron metabolism inducing functional ID, due to cytokines and hepcidin overexpression. TIBC and %SAT values were used to this purpose, and the results seem to suggest that, in our dogs, ID is more frequently absolute (primary) than functional (secondary to inflammatory conditions). In fact, in real iron deficiency, TIBC is usually normal or slightly over the reference interval, and %SAT is lower, in functional iron deficiency both TIBC and %SAT are lower than the reference intervals (Harvey 2008, Andrews 2010).

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

Although a statistically significant difference was found in the median iron values between azotemic and non-azotemic patients, it is important to highlight that the 80% of dogs that presented SID were classified as IRIS class 1; SID does not appear to be an exclusive finding of renal complication of cardiovascular disease (cardiorenal syndrome), and we should consider it as possible comorbidity in HF alone (Rangel et al. 2014, Fitzsimmons et al. 2015).

Regarding survival, MST, calculated trough Kaplan-Meier, were significantly different in dogs with or without SID. Univariate Cox regression revealed the impact of changes in echocardiographic variables related to severity of MMVD in shortening survival, in particular increase in EDVI, La/Ao ratio, ejection fraction and E wave peak velocity and presence of pulmonary hypertension. Moreover, increase in age and in sCr were associated with shorter survival. Finally, presence of clinical symptoms of CHF decrease survival times. SIC was not associated with shorter survival, but RDW was, suggesting that alteration in RBC dimensions, that can be mediated by low SIC, and consequent reduced oxygen

transport can alter energetic metabolism and heart work and shorten patients' survival. Still, in a multivariate Cox analysis, only increase in age and the presence of clinical symptoms are related to shorter survival. It is important to remind that survival results should be interpreted with caution due to the small number of dogs included in the study and the small percentage of dogs that experienced death in our sample.

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

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

There are several limitations in our study. We are aware that the measurement of serum iron represents a small fraction of the total body iron and therefore we decided to also measure transferrin (TIBC) and percentage of saturation. Indeed, the main limitation can be found in the impossibility, at the moment, to measure ferritin and hepcidin, which are fundamental in the diagnostic algorithm and can be useful markers to characterize SID. Ferritin in particular is considered one of the diagnostic cornerstones of all the study about iron deficiency in cardiovascular diseases in human medicine. Moreover, limitations have to be searched in the retrospective nature of the study. Finally, the limited sample numbers, in particular for the most severe ACVIM class (D), make it harder to draw conclusions for this class of patients. Strict inclusion criteria were applied to improve reliability, that decreased sample size. Small sample size and underrepresentation of ACVIM class C and D could have biased some of the results.

In conclusion, SID is a relatively frequent condition associated to MMVD in dogs; it is present in almost 20% of patients affected by MMVD, but the percentage raise to the 33% in symptomatic patients and to 100% in patients presenting acute decompensated CHF.

SID represents a risk factor for dogs affected by MMVD: low SIC is associated with a 6.3-times higher risk of being included in a higher ACVIM class. Moreover, dogs presenting low SIC have a 1.5 times higher risk of presenting an increase in La/Ao ratio.

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

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

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