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# The effect of myxomatous mitral valve disease severity on packed cell volume in dogs

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**Objectives:** The aim of this study was to examine whether associations between disease severity and packed cell volume exist in dogs with myxomatous mitral valve disease.

**Materials and Methods:** Data were selected from 289 dogs that had been examined at a research clinic (2004–2017) on multiple occasions (n=1465). American College of Veterinary Internal Medicine stage and echocardiographic measurements were entered in separate multivariable linear mixed effects models with packed cell volume as the dependent variable. Age, breed, sex, weight and blood urea nitrogen concentrations were additionally tested in these analyses to control for patient characteristics.

**Results:** Packed cell volume (% whole blood) in stages B1 and B2 (B1: 42.62 ± 0.27, P=0.001; B2: 41.77 ± 0.42, P < 0.001) was lower than stage A (44.57 ± 0.53). In stage C, packed cell volume was greater than both preclinical stages (C: 43.84 ± 0.46). When the administration of loop diuretics was included in statistical models, packed cell volume was inversely related to normalised left ventricular internal diameters (β: −2.37; 95% confidence intervals: −3.49, −1.25; P < 0.001).

**Clinical Significance:** Dogs with myxomatous mitral valve disease may develop reductions in packed cell volume as their disease progresses. Although this finding was statistically significant at a population level, it should be noted that the differences described are relatively small. This, along with other causes of variation in packed cell volume, means that changes would be challenging to appreciate within individual patients. Plasma volume depletion following diuretic administration may explain why findings differed in stage C.

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## INTRODUCTION

In human patients anaemia is a common comorbidity of chronic heart failure (ChHF). The World Health Organisation (WHO) defines anaemia as being present when haemoglobin (HgB) concentrations fall below established values (HgB < 13 g/dL in men, < 12 g/dL in women) and estimate global prevalence as 24.8% (WHO 2005; WHO 2011). In ChHF the prevalence is notably higher, ranging from 30% in stable patients, to 50% in patients hospitalised for management of their disease (Anand & Gupta 2018). When presented in comparison to disease severity, the prevalence of anaemia has been shown to increase alongside New York Heart Association (NYHA) functional heart failure scores, with the highest frequencies reported in patients with class III or IV symptoms (Coats 2004). The presence of anaemia in ChHF is considered a negative prognostic indicator as it has been associated with reduced survival, increased risk of hospitalisation and reduced quality of life. This has led researchers to question whether anaemia itself contributes to the syndrome of

 

heart failure, increasing the risk of experiencing an adverse outcome (da Tang & Katz 2008, Groenveld *et al*. 2008). To examine the causal nature of this relationship, a series of clinical trials have evaluated the effect of medical intervention with erythropoietin stimulating agents. The largest study in this series, a phase III randomised controlled trial, found that treatment with Darbepoetin Alpha did not affect patient outcome (Swedberg *et al*. 2013), suggesting that ChHF associated anaemia does not directly increase the risk of mortality. Instead, its role as a prognostic marker may better relate to its development with increasing disease severity.

There are several proposed mechanisms for the pathogenesis of heart failure associated anaemia. It is predominantly thought to reflect reduced erythropoiesis, either due to decreased production of endogenous erythropoietin (EPO), or a blunted response to EPO by bone marrow progenitor cells (Anand 2008). As pro-inflammatory cytokines are elevated in individuals with ChHF, it is hypothesised that chronic inflammation is the primary cause of these changes (Deswal *et al*. 2001). In the context of cardiovascular disease, it is worth noting that the dilutional effects of increased plasma volume can lead to patients meeting the diagnostic criteria for anaemia whilst having normal erythrocyte numbers (Abramov *et al*. 2008).

To date, there is a deficit of similar research into the presence and role of anaemia in dogs with cardiovascular disease, despite evidence that inflammation is implicated in the syndrome of canine heart failure (Reimann *et al*. 2016, Domanjko Petrič *et al*. 2018). Anaemia, which has traditionally been defined in veterinary medicine as a low packed cell volume (PCV), has been associated with prognosis in untreated dogs with functional heart failure secondary to myxomatous mitral valve disease (MMVD); the most common cause of cardiac disease in adult patients (Yu & Huang 2016, Keene *et al*. 2019). In this study the prevalence of anaemia was positively associated with NYHA functional scores, which were used to describe the severity of clinical signs of congestive heart failure (CHF). It is not clear whether changes in PCV also occur before the onset of CHF as when disease severity is assessed using the objective guidelines produced by the ACVIM, associations between MMVD and haematological markers are less apparent. In a single study, haematocrit concentrations were compared at different stages of disease and no association with severity was observed (Boswood & Murphy 2006). In addition, analyses of paired data have failed to demonstrate a difference in haematocrit or PCV associated with the development of CHF (Boswood & Murphy 2006, Boswood *et al*. 2018). Given the disparity between these findings and the breadth of evidence in human patients, we sought to establish whether an association between PCV and disease severity exists in dogs affected by MMVD using a large cohort of patients. The primary aim of this study was to examine, at a population level, whether and to what extent PCV and ACVIM stage are related.

## METHODS

### Study design and patient population

The study was a retrospective analysis of prospectively collected data. Clinical data were selected from a population of dogs that had been examined on more than one occasion at a research clinic held at two primary-care practices in *London* (*Beaumont Sainsbury Animal Hospital, Camden. Blue Cross Animal Hospital, Victoria*) between the years 2004 and 2017. The collection and storage of patient information was performed with owner consent and the study protocol received approval from the *Royal Veterinary College's* Ethics and Welfare Committee (URN: 2017 U155).

To be included in the study, dogs were required to have received an echocardiographic diagnosis of MMVD by a board-certified cardiologist (A.B.) and have a measurement of PCV available from the same examination. MMVD was diagnosed when there was evidence of mitral regurgitation on colour Doppler and degenerative lesions on the valve or its associated apparatus. Dogs with cardiac co-morbidities other than atrioventricular valve degeneration were excluded from the study population. Non-cardiac comorbidities that developed over the course of a dog's life were not considered cause for exclusion so that MMVD progression could be studied in a natural setting. Dogs underwent repeated examinations approximately every 6 months and at each visit a jugular blood sample was taken and placed in ethylenediaminetetraacetic acid (EDTA) anti-coagulant. PCV (% whole blood) and total solids (TS, g/L) were measured by postgraduate students or nurses associated with the research clinic from a non-heparinised microhaematocrit tube that had undergone high speed centrifugation (relative centrifugal field: 13,000 g) for 5 minutes (Hawksley Microhaematocrit Reader, Hawksley, Lancing, UK. Hawksley Dual Speed Vetspin, Hawksley, Lancing, UK). Blood urea nitrogen (BUN, mmol/L) concentration was measured from manual test strips (DiaSys Blood Urea Test Strips, DiaSys Diagnostic Systems GmBH, Holzheim, Germany). Standard right parasternal echocardiographic views were obtained, from which the left atrial to aortic root ratio (LA:Ao) and the normalised left ventricular internal diameter at end diastole (LVIDDN) were calculated (Cornell *et al*. 2004). Heart disease was staged according to ACVIM guidelines; however, the cut-off for LA:Ao was lowered to account for a systematic difference associated with the measurement technique used (Fig S1) (Keene *et al*. 2019). Unlike the current guidelines in which left atrial and aortic dimensions are measured in the first frame after aortic ejection (Hansson *et al*. 2002), in this study, LA:Ao had historically been measured at the onset of the P wave (Moonarmart *et al*. 2010). Thus, preclinical dogs were classified as being in stage B2 if they had an LA:Ao ≥1.50 and LVIDDN ≥1.70.

A population of normal, unaffected patients seen at the same research clinic were included in the study and classified as ACVIM stage A if they did not have an audible murmur when auscultated by a veterinary cardiologist (A.B.). Additional inclusion criteria for this control group were a minimum age of 7 years at the first visit and a maximum weight of 20 kg to match the affected cohort with a reasonable degree of similarity. In Cavalier King Charles Spaniels (CKCS), the minimum age of entry was lowered to 5 years to reflect the early age of onset of MMVD experienced by this breed (Serfass *et al*. 2006). Control dogs underwent the same data collection procedures as dogs with MMVD but did not receive an echocardiographic examination.

### Statistical analyses

All analyses were performed using commercially available software or freeware (R 4.0.2, R Foundation for Statistical Computing, Vienna, Austria. The R packages "lmer4" v1.1–23 (Bates *et al.* 2020), "emmeans" v1.5.0 (Lenth *et al.* 2020), "ggplot2" v3.3.2 (Wickham *et al.* 2020), "cowplot" v1.1.0 (Wilke 2020) and "ggpubr" v0.4.0 (Kassambara 2020). SPSS version 26.0 for Macintosh, released 2019, SPSS Inc. San Diego, USA). Descriptive statistics for categorical variables are reported as a frequency and proportion (%) and continuous variables are reported as the median value with upper and lower quartiles. The normality of continuous data was assessed by visually inspecting histograms and the findings were used to guide selection of appropriate methods. Continuous variables were screened for collinearity using correlation coefficients and variance inflation factors (VIF). Collinearity was considered if Spearman's rho exceeded 0.7 or VIF exceeded 5 for a given combination of variables.

The association between PCV and disease stage was assessed using data that had been collected over a number of visits. To account for repeated measures from the same individuals, a linear mixed effects model was used with patient case number included as a random effect. Additionally, a compound symmetry covariance structure was selected based on the Akaike information criterion (AIC) to model autocorrelation within individual patients. In this multivariable model ACVIM stage was entered as an independent variable alongside the demographic covariates age, breed (CKCS/ non-CKCS), sex and weight to control for any potential confounding. As weight was non-linearly associated with PCV, it was transformed into quartiles prior to inclusion in the multivariable model. BUN was additionally included as a possible confounder as previous studies have described associations between renal dysfunction, cardiac disease and anaemia (Pouchelon *et al.* 2015, Martinelli *et al.* 2016). Selection methods were used to identify covariates for adjustment as, for the larger pool of variables being tested, there was insufficient information available to specify a priori which ones would be required to adequately control confounding (Greenland & Pearce 2015). Linear mixed effects models were fitted to complete data and manual backwards stepwise elimination was conducted based on F tests of fixed effects and the stability of coefficients for the variable of interest, ACVIM stage. Stopping criteria were a significance level of 5% ($P < 0.05$) and/ or a change in estimate of greater than or equal to 20% (Dohoo *et al.* 2009). Two-way interaction terms were tested for each combination of variables that remained in the main effects model and retained if significant. Using these final models, estimated marginal means were calculated for categorical variables with continuous variables held constant at their mean value. Estimated marginal means were compared using Fisher's Least Significant Difference (LSD) test.

To explore the association with disease stage further, a second analysis was conducted using echocardiographic measurements of left atrial and ventricular size as an alternative method of assessing disease severity. LA:Ao and LVIDDN were entered as independent variables alongside the same demographic variables as the previous model: age, breed (CKCS/ non-CKCS), sex, weight and BUN concentration. Data from visits in stage A were not used in this analysis as echocardiography had not been performed in dogs without a heart murmur. Unlike the ACVIM stage analysis, where concerns about collinearity prohibited inclusion of cardiac medications in models, assessing echocardiographic dimensions allowed us to account for any changes in PCV associated with therapy. For each visit, treatment status with furosemide, pimobendan, angiotensin converting inhibitors (ACEi) and spironolactone was included as a separate variable for each drug. As before, backwards stepwise elimination was used to select a final multivariable model and two-way interaction terms were tested for significant variables. Following this analysis, the final main effects model was reproduced with furosemide dose in mg/kg per 24 hours in place of furosemide as a binary variable. The daily dosage was quantile transformed and entered as a categorical variable, so that PCV could be compared with individuals that were not currently receiving medication ("Not Receiving"). Dogs where dosage information was not available were encoded as "Missing" for the purpose of this analysis. Interaction terms were not reproduced so that the results of this analysis could be more easily interpreted. As a final method of exploring changes in PCV and their relation to other components of blood, the ACVIM and echocardiographic models were redeveloped with TS as the dependant variable. The assumption of linearity was met for all continuous factors, so weight was included as a continuous variable.

For both multivariable models, results are reported as the coefficient (β) and 95% confidence intervals. For continuous variables, this represents the average change in PCV associated with a one unit increase in a variable, according to its scale of measurement. For categorical variables, this represents the average difference in PCV when compared to a reference category. The following levels of categorical variables were chosen as reference groups to provide a suitable point of comparison: ACVIM, stage A; breed, non-CKCS; cardiac medications, not currently receiving; sex, male entire; weight, less than or equal to 7.4 kg. Estimated marginal means are reported as the mean predicted value of PCV ± its standard error.

## RESULTS

Data from 289 dogs were available for analysis, providing results from a total of 1465 visits. Amongst these data, 12.7% (n=186) of visits were from dogs in stage A, 64.4% (n=944) in stage B1, 10.0% (n=147) in stage B2 and 10.3% (n=151) in stage C. A small number of dogs (2.5%, n=37) had not been staged at the time of examination and had insufficient data available to complete this retrospectively. Cavalier King Charles Spaniels formed the majority breed having been assessed on 43.1% (n=631) of visits. The split across sexes was near even; 56.2% visits were from male dogs (n=824). Additional descriptive statistics are summarised in Table 1. Data had been recorded from a client owned population and some dogs had comorbidities at the time of examination (Table S1a). Degenerative joint disease was the most commonly recorded complaint (21.7%, n=318) and other chronic pro-inflammatory conditions included allergic skin disease (4.1%, n=60), chronic gastrointestinal disease (5.7%, n=84), dental disease (3.1%, n=45), chronic kidney disease (0.5%, n=7)

**Table 1. A summary of the clinicopathologic characteristics of the study population grouped according to ACVIM disease stage**

| | | Stage A (n=186) | | Stage B1 (n=944) | | Stage B2 (n=147) | | Stage C (n=151) | |
|---|---|---|---|---|---|---|---|---|---|
| **Factor** | | | **MD** | | **MD** | | **MD** | | **MD** |
| Age | | 10.00 (8.23, 11.78) | 1.6% (3) | 9.99 (8.04, 12.00) | 0.2% (2) | 10.21 (8.33, 11.93) | 0.7% (1) | 11.65 (9.61, 13.51) | |
| BCS | Underweight | 5.9% (11) | 2.7% (5) | 9.9% (93) | 0.3% (3) | 14.3% (21) | 0.7% (1) | 35.8% (54) | 0.7% (1) |
| | Ideal | 63.4% (118) | | 66.2% (625) | | 63.9% (94) | | 52.3% (79) | |
| | Overweight | 28.0% (52) | | 23.6% (223) | | 21.1% (31) | | 11.3% (17) | |
| Breed | CKCS | 22.6% (42) | | 46.9% (443) | | 48.3% (71) | | 45.7% (69) | |
| BUN | | 6.00 (4.00, 8.00) | 8.1% (15) | 7.00 (4.00, 8.00) | 8.1% (76) | 6.00 (6.00, 8.00) | 10.9% (16) | 10.00 (7.00, 14.00) | 10.6% (16) |
| LA:Ao | | - | | 1.20 (1.11, 1.31) | 0.1% (1) | 1.63 (1.55, 1.73) | | 1.79 (1.58, 2.03) | 2.0% (3) |
| LVIDDN | | - | | 1.63 (1.46, 1.82) | 0.1% (1) | 2.07 (1.90, 2.28) | | 2.16 (1.95, 2.39) | 6.6% (10) |
| Medications | ACEi | 0.5% (1) | | 9.4% (89) | | 18.4% (27) | | 57.0% (86) | |
| | Furosemide | 1.1% (2) | | 6.9% (65) | | 15.6% (23) | | 85.4% (129) | |
| | Pimobendan | 0.5% (1) | | 9.9% (93) | | 28.6% (42) | | 72.8% (110) | |
| | Spironolactone | - | | 0.4% (4) | | 1.4% (2) | | 21.9% (33) | |
| PCV | | 46.00 (43.00, 50.00) | | 43.00 (39.00, 47.00) | | 41.00 (38.00, 45.00) | | 42.00 (38.00, 46.00) | |
| Sex | ME | 10.2% (19) | | 17.2% (162) | 0.2% (2) | 19.0% (28) | | 21.9% (33) | |
| | MN | 37.1% (69) | | 40.3% (380) | | 38.8% (57) | | 34.4% (52) | |
| | FE | 5.4% (10) | | 3.5% (33) | | 0.7% (1) | | 7.3% (11) | |
| | FN | 47.3% (88) | | 38.9% (367) | | 41.5% (61) | | 36.4% (55) | |
| TS | | 70.00 (64.00, 74.00) | 1.1% (2) | 70.00 (64.00, 74.00) | | 68.00 (64.00, 72.00) | | 70.00 (64.00, 74.00) | 0.7% (1) |
| Weight | | 8.40 (6.30, 12.13) | 3.8% (7) | 9.90 (7.79, 12.76) | | 9.90 (7.65, 12.33) | | 8.90 (6.20, 11.55) | 2.6% (4) |

Categorical variables are reported as the proportion (frequency). Continuous variables are reported as the median (lower quartile, upper quartile). N stands for the number of visits from which data were available. MD is the missing data for each variable across the stages of disease. Age is reported in years. ACEi stands for angiotensin converting enzyme inhibitor. Body condition score was assessed using the American Animal Hospital Association's five-point scale and grouped as follows: 0.5 to 2 are classed as underweight, 2.5 to 3.5 are classed as ideal and 4 to 5 are classed as overweight. Blood urea nitrogen (BUN) is reported in mmol/L. LA:Ao stands for the left atrial to aortic root ratio. LVIDDN is the left ventricular internal diameter in diastole (cm), normalised to bodyweight (kg) using the following equation: $LVIDD/(BW^{0.294})$. Packed cell volume (PCV) is expressed as a percentage of whole blood. Total solids (TS) are reported in g/L. Weight is reported in kg

and neoplasia (2.1%, n=31). Thirty-nine visits (2.7%) came from dogs with a medically controlled endocrine disorder. Amongst data from the study population, the presence of anaemia was noted at only 5.5% visits (n=80, PCV < 35%). Reported comorbidities at these visits are recorded in Table S1b.

When exploring differences in PCV associated with ACVIM stage, the following variables were retained in a multivariable model: ACVIM stage, age, breed (CKCS/ non-CKCS) and BUN concentration; indicating that ACVIM stage and PCV were related when controlling for patient characteristics (Table 2). A difference in PCV could be noted as early as stage B1 (Fig 1), where values were lower than in stage A patients (estimated marginal mean PCV in stage A: 44.57 ±0.53; B1: 42.62 ±0.27). In this study, PCV appeared to decrease with increasing severity of preclinical disease, producing a significant difference when stages B1 and B2 were compared (estimated marginal mean PCV stage B2: 41.77 ±0.42, P=0.034). This inverse relationship with ACVIM stage did not continue through to stage C. PCV in stage C was significantly greater than either preclinical stage, lying closer to that of the unaffected dogs (estimated marginal mean PCV stage C: 43.84 ±0.46). As well as ACVIM stage, several demographic factors also remained in the model. When grouped as CKCS or non CKCS, PCV was significantly lower in CKCS. The average value of PCV in this group was 6.68 units (%) lower when measured as a percentage of whole blood (95% CIs: −7.69 to −5.68, P < 0.001). Patient age and blood urea concentration were found to interact, indicating that the effect of either variable on PCV was influenced by the other. Graphical representations have been produced to illustrate this interaction (Fig S2a,b), showing that the association between BUN concentration and PCV became more strongly negative with increasing patient age. Similarly, the relationship between age and PCV was more strongly negative at higher concentrations of BUN.

In a second multivariable analysis, examining echocardiographic dimensions and cardiac medications in lieu of ACVIM stage, the following variables remained significant: LVIDDN, furosemide treatment status, age, breed, and BUN concentration (Table 3). The patient factors retained through backwards stepwise elimination were the same as in the ACVIM model. As well as agreement in results, this provides similar adjustment to interpret results from this alternative method of assessing disease severity. In this analysis, the association between LVIDDN and PCV was negative, indicating that PCV tended to be lower when left ventricular size increased (change in PCV per unit increase in LVIDDN: −2.37 ±0.57, 95% CIs: −3.49 to −1.25, P < 0.001). This relationship was observed when furosemide administration had been controlled for. The effect of furosemide on PCV interacted with BUN concentrations (Fig S2c) so, to examine the effect of diuresis in isolation, estimated values of PCV were calculated across the range of possible BUN concentrations. Above 4 mmol/L BUN, PCV was consistently greater in dogs receiving treatment with a loop diuretic (Table S2). When examining the effect of BUN on PCV, a negative association with PCV was only observed in patients that were not receiving treatment with a loop diuretic (Change in PCV per unit increase in BUN, no treatment: −0.16 ±0.04, 95% CIs: −0.24 to −0.09, P < 0.001; treatment: 0.05 ±0.06, 95% CIs: −0.07 to 0.17, P=0.438).

**Table 2. The results of a multivariable linear mixed effects model for the association between ACVIM stage and PCV**

| Factor | β (±SE) | 95% CI Lower bound | 95% CI Upper bound | P |
|---|---|---|---|---|
| Intercept | 51.70±1.15 | 49.44 | 53.95 | <0.001 |
| ACVIM (A) | - | - | - | - |
| ACVIM (B1) | −1.95±0.53 | −2.99 | −0.90 | <0.001 |
| ACVIM (B2) | −2.77±0.65 | −4.04 | −1.50 | <0.001 |
| ACVIM (C) | −0.73±0.69 | −2.09 | 0.62 | 0.288 |
| Age | −0.30±0.10 | −0.50 | −0.11 | 0.002 |
| Breed (CKCS) | −6.68±0.51 | −7.69 | −5.68 | <0.001 |
| Breed (non CKCS) | - | - | - | - |
| BUN | 0.10±0.11 | −0.13 | 0.32 | 0.389 |
| Age * BUN | −0.02±0.009 | −0.04 | −0.0004 | 0.045 |

ACVIM American College of Veterinary Internal Medicine, BUN Blood urea nitrogen, CI Confidence interval, CKCS Cavalier King Charles Spaniel, SE Standard error
Results are reported as the coefficient (β)±standard error and 95% confidence intervals. For continuous variables, this represents the average change in PCV associated with a one unit increase in a variable, according to its scale of measurement. For categorical variables, this represents the average difference in PCV when compared to a reference category. A hyphen (–) indicates that this level of a categorical variable was used as the reference group. Age was measured in years. BUN was measured in mmol/L, PCV was measured as a percentage of whole blood. An asterisk (*) is used to indicate a two-way interaction term

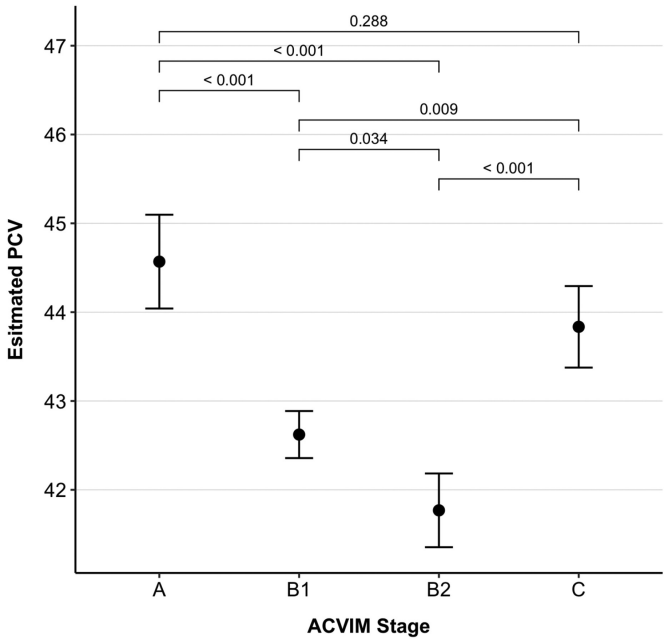


**FIG 1. Estimated values of PCV at different ACVIM stages of disease, adjusted for patient age, breed and BUN concentrations. Estimated marginal means for PCV were calculated using a multivariable linear mixed effects model where the other factors in the model were held at a constant. For continuous variables, this was their mean (age=10.24, BUN=7.68) and for categorical variables, this was the reference group (breed=non-Cavalier King Charles Spaniel). Dots are used to indicate the estimated mean value and error bars are used to indicate the standard error of the mean. Estimated marginal means were compared using Fisher's Least Significant Difference (LSD) test and P values are displayed above lines indicating the groups compared. PCV was measured as a percentage of whole blood. ACVIM American College of Veterinary Internal Medicine, BUN Blood urea nitrogen, PCV Packed cell volume**

This second model was reproduced to contain more specific information on furosemide dosage. In the study population, the median dosage of furosemide was 2.69 mg/kg/24 hours (1.84, 4.36). Amongst

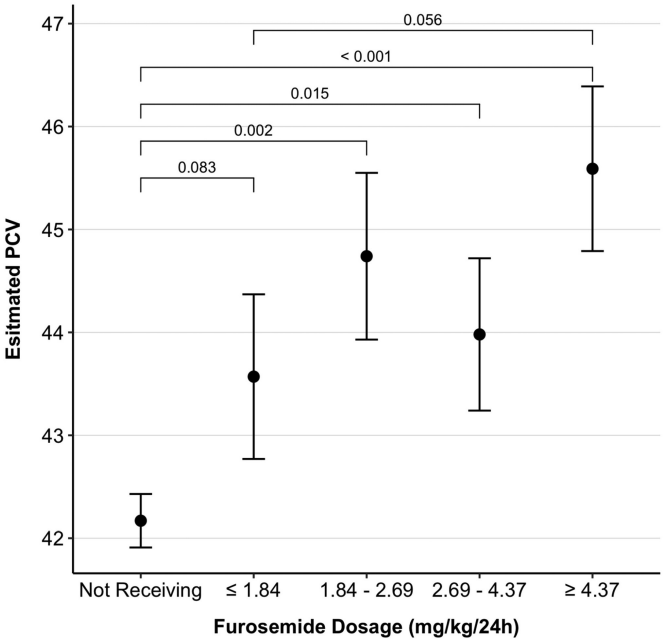


**FIG 2. Estimated values of PCV at different furosemide doses adjusted for LVIDDN, patient age, breed and BUN concentrations. Estimated marginal means for PCV were calculated using a multivariable linear mixed effects model where the other factors in the model were held at a constant. For continuous variables, this was their mean (age=10.26, BUN=7.78, LVIDDN=1.76) and for categorical variables, this was the reference group (breed=non-Cavalier King Charles Spaniel). Dots are used to indicate the estimated mean value and error bars are used to indicate the standard error of the mean. Estimated marginal means were compared using Fisher's Least Significant Difference (LSD) test and P values are displayed above lines indicating the groups compared. P values for dosage comparisons not shown above were as follows: ≤1.84 to 1.84–2.69, P=0.271; ≤1.84 to 2.69–4.37, P=0.681; 1.84–2.69 to 2.69–4.37, P=0.459; 1.84–2.69 to ≥4.37, P=0.419; 2.69–4.37 to ≥4.37, P=0.097. PCV was measured as a percentage of whole blood. BUN Blood urea nitrogen, PCV Packed cell volume**

the dogs receiving furosemide (n=185), dosage information was missing for 22.2% patients (n=41). Dogs receiving furosemide at dosages larger than 1.84 mg/kg/24 h had significantly greater values of PCV than dogs that were not receiving medication. As can be observed in Fig 2, dogs on higher total daily doses of furosemide tended to have greater values of PCV than dogs on lower total daily doses.

Finally, when TS was analysed as the dependant variable, associations in the model containing echocardiographic measurements were similar to those previously described for PCV (Table S3). Again, there was an inverse relationship between echocardiographic dimensions and TS, and values of TS were greater in patients receiving furosemide. When ACVIM stage was used as the measure of disease severity, no significant association was observed (Table S4).

## DISCUSSION

This study identified an inverse relationship between PCV and the severity of MMVD. Furthermore, by analysing several methods of assessing disease severity, this study identified factors that have the potential to confound results in the context of canine cardiovascular disease. When MMVD was assessed using

**Table 3. The results of a multivariable linear mixed effects model for the association between echocardiographic measurements and PCV**

| Factor | β (±SE) | 95% CI Lower bound | 95% CI Upper bound | P |
|---|---|---|---|---|
| Intercept | 54.74 ±1.10 | 52.58 | 56.89 | <0.001 |
| Age | −0.39 ±0.07 | −0.54 | −0.25 | <0.001 |
| Breed (CKCS) | −6.26 ±0.54 | −7.33 | −5.20 | <0.001 |
| Breed (non CKCS) | - | - | - | - |
| BUN | −0.16 ±0.04 | −0.24 | 0.09 | <0.001 |
| Furosemide (no) | - | - | - | - |
| Furosemide (yes) | 0.24 ±0.81 | −1.36 | 1.84 | 0.765 |
| LVIDDN | −2.37 ±0.57 | −3.49 | −1.25 | <0.001 |
| BUN * Furosemide | 0.21 ±0.07 | 0.07 | 0.35 | 0.003 |

BUN Blood urea nitrogen, CI Confidence interval, CKCS Cavalier King Charles Spaniel, LVIDDN Left ventricular internal diastolic diameter normalised to bodyweight (kg), SE Standard error

Results are reported as the coefficient (β) ±standard error and 95% confidence intervals. For continuous variables, this represents the average change in PCV associated with a one unit increase in a variable, according to its scale of measurement. For categorical variables, this represents the average difference in PCV when compared to a reference category. A hyphen (–) indicates that this level of a categorical variable was used as the reference group. Age was measured in years. BUN was measured in mmol/L. LVIDDN was measured in $cm/kg^{0.294}$, PCV was measured as a percentage of whole blood. An asterisk (*) is used to indicate a two-way interaction term

the ACVIM scheme, there was a tendency for PCV to decrease across stages, however, the presence of stage C disease modified the direction of this relationship. The results of a separate model containing LVIDDN and furosemide suggest that this may be due to diuresis, as dogs receiving furosemide had higher values of PCV. When the effect of medication was accounted for, PCV was negatively associated with LVIDDN suggesting that PCV decreases as MMVD advances. This study was conducted using data from a large number of examinations which improved our ability to detect these small differences associated with MMVD severity and other variables. When examining individual patients, it would be challenging to differentiate these changes from other sources of variation.

As measurements of PCV are relative to other components of whole blood, a simple explanation for these findings is a process of haemodilution. PCV may appear low if erythrocytes are present at a reduced concentration in the sample used for analysis (Hauptman *et al.* 2000, Hansen & DeFrancesco 2002). Using labelled blood products, haemodilution has been observed in human patients with ChHF and is attributed to compensatory increases in plasma volume (Abramov *et al.* 2008, Adlbrecht *et al.* 2008). Due to the invasive nature of such testing the prevalence of this "pseudoanaemia" is not well investigated in cardiovascular disease, although one human study found that it accounted for 46% of cases with low HgB concentrations (Androne *et al.* 2003). Differences in the pathophysiology of disease mean that this figure is not easily translated to dogs with MMVD. Previous studies in dogs have indirectly assessed haemodilution by measuring components of the neuroendocrine pathways involved in intravascular volume homeostasis. N-terminal pro-B-type natriuretic peptide (NT-proBNP); an established biomarker of left ventricular volume overload, is increased in preclinical MMVD, indicating that fluid retention may begin to occur in preclinical disease, as was suggested by our results in stage B dogs (Chetboul *et al.* 2009, Tarnow *et al.* 2009, Wolf *et al.* 2013, Fox *et al.* 2015). In contrast to human research our study found that PCV was greater in dogs diagnosed with heart failure. These conflicting findings likely reflect fundamental differences in the definition of heart failure which, in dogs, is considered when cardiogenic pulmonary oedema is present. Canine patients in CHF therefore require chronic diuresis to pharmaceutically manage their disease. Loop diuretics have been shown to contract intravascular volume in patients with CHF (Davidov *et al.* 1967, Feigenbaum *et al.* 2000, James *et al.* 2005) and this effect, by mitigating haemodilution, may explain why PCV was greater in ACVIM stage C. In a sub-analysis that included the effect of furosemide dosage, there was some suggestion of a dose–response relationship with PCV. In this study population the dogs receiving the highest daily doses of furosemide tended to have greater values of PCV than dogs on the lowest doses, though this was not statistically significant (P=0.056). It is possible that the ability to detect this difference was limited by a lack of statistical power. Finally, when analysing TS as the dependant variable, similar associations between LVIDDN, furosemide administration and TS were observed. Haemodilution would not be expected to affect PCV in isolation, so finding these results for another component of blood provides further support for our interpretation. Unlike PCV, a relationship with ACVIM stage was not appreciated for TS. Although there is some overlap in the causal factors involved in the pathogenesis of anaemia and hypoproteinaemia in heart failure (Arques & Ambrosi 2011), a parallel response from the two haematological variables would only be expected to occur if they were affected by all of the same factors at the same points throughout the course of disease progression.

When the effect of treatment was controlled for, PCV was inversely related to an echocardiographic marker of disease severity, LVIDDN. These findings highlight several points for further research. Firstly, it is possible that other mechanisms contributed to the decline in PCV observed in this study. Evidence of inflammation is appreciable in dogs with more advanced MMVD and may have impacted the response to EPO (Zois *et al.* 2012, Reimann *et al.* 2016). Support for this hypothesis comes from the observation that anaemia is a comorbidity of other chronic inflammatory diseases where haemodilution is not a component of the disease pathophysiology (Miller *et al.* 2009). As there are few viable ways of measuring absolute red blood cell counts, alternative methods could evaluate iron deficiency, which develops alongside anaemia secondary to inflammation (Okonko *et al.* 2011). On its own, iron deficiency is prevalent in ChHF and is associated with poorer outcomes, even in cases where HgB concentrations fall within normal limits (Klip *et al.* 2013, Anand & Gupta 2018). A second point is whether haemodilution itself may be of prognostic value. One study in dogs with MMVD found that low PCV was associated with a shorter survival time (Yu & Huang 2016) and furthermore, the percentage change in plasma volume following decongestion is a risk factor for adverse outcomes (Van Der Meer *et al.* 2013, Duarte *et al.* 2015, Hudson *et al.* 2016, Kobayashi *et al.* 2019). This can be estimated using an algorithm derived from values of haematocrit and haemoglobin (Duarte *et al.* 2015). As diuresis appeared to induce a

change in PCV it is possible that a similar metric could facilitate the management and prognostication of CHF.

It is important to understand that our results were derived from analyses conducted at a population level across a large number of visits. For the most part, values of PCV fell within conventional canine reference intervals (Table 1). Although our results describe relatively small changes in PCV, their utility relates to the fact that they to allow us to explore the phenotype of MMVD and compare findings to studies of human patients, potentially highlighting areas for further research. Given the magnitude of differences we noted, it may be challenging to observe similar results in individual patients. Certainly, studies examining paired data from dogs with MMVD that went on to develop CHF suggest that this is the case (Doxey & Boswood 2004, Boswood *et al*. 2018). It is well recognised that there are many other causes of variation in an individual's PCV which limits the application of these results in a clinical setting. In the present study, patient age, breed and BUN concentration were examples of such factors. Finding an inverse association between age and PCV was not unexpected, as it has been previously hypothesised that aging is accompanied by a decline in EPO production and a greater propensity for inflammation (Lawrence *et al*. 2013, Radakovich *et al*. 2017). For breed, specific differences in neurohormonal markers of volume retention have been observed in previous research and compensatory pathways appear to be activated to a greater degree in CKCS (Pedersen *et al*. 1995, Hezzell *et al*. 2012). An alternative explanation could be that changes reflect different haematological phenotypes in the two groups of dogs. CKCS have been shown to have significantly lower red blood cell counts, haematocrit and HgB when compared to other breeds (Lawrence *et al*. 2013). As the number of studies describing these effects increases, future research evaluating haematological variables could consider standardising measurements to account for breed differences. An important concept in human medicine is the interplay between cardiac disease and renal dysfunction, in the form of cardiorenal syndrome (CRS). There is a growing awareness that cardiac disease, renal disease and anaemia share common pathophysiological links and that the three conditions may exacerbate one another, leading to clinical deterioration (Silverberg *et al*. 2003). Our study found that BUN concentration was inversely related to PCV, though it did not interact with markers of MMVD severity in either model. In dogs receiving furosemide, this relationship was no longer significant but trended positive and, in descriptive data from the study population, dogs in stage C tended to have higher values of BUN. We suggest that the dogs on diuretics may have developed changes in renal function secondary to the action of these drugs on the kidney and the corresponding physiological response to that effect.

## Limitations

Retrospectively selecting patients for this study meant that the administration of cardiac medications was not standardised. The research clinic that provided data for this study captures observational data from dogs under the care of other veterinary practitioners. Thus, some dogs received cardiac medications at points that do not match the ACVIM guidelines. As previously outlined in this discussion, including dogs undergoing treatment with loop diuretics may be responsible for some of this study's findings. As patients were not sampled in experimental conditions, it is not possible to conclude that the findings were a direct consequence of treatment, although this hypothesis is supported by prior research, as well as the possible dose–response relationship we observed in our data. In addition to loop diuretics, some patients were receiving treatment with ACEis. As angiotensin II is a potent stimulant of renal EPO production, suppressing angiotensin converting enzyme (ACE) may contribute to the development of anaemia (Mrug *et al*. 1997, Häggström *et al*. 2013, Kim *et al*. 2014). Treatment with ACEis did not remain in multivariable models, so this study was unable to determine if ACEis negatively affected erythrocyte production. If research is conducted into the associations between cardiac disease and anaemia, the potential for drug effects should be considered and warrants investigation.

Retrospective selection also introduced some discrepancies in the way that MMVD was staged. Even though data had been captured according to a standardised protocol, the method of obtaining echocardiographic dimensions varied from those recommended in the most recent ACVIM consensus statement (Keene *et al*. 2019). Stage B2 was therefore classified using a different threshold for LA:Ao to account for systematic differences associated with this measurement technique. Given the reason for this adjustment, the methods we used would still identify preclinical cardiomegaly, providing a suitable group for this analysis. When the relationship between PCV and disease severity was further explored using continuous measures of cardiac size, similar associations were observed. A difference in the threshold used to stage preclinical disease is therefore unlikely to result in erroneous conclusions being drawn. As outlined in our methods, patients were classed as being in stage A using auscultation, as the type of dogs typically affected by MMVD are unlikely to have cardiac disease in the absence of a murmur. This method of assessment is still recommended by current guidelines (Keene *et al*. 2019). Despite this, the absence of cardiac disease was not echocardiographically confirmed and it is possible that a minority of individuals in the control population may have had subclinical disease.

Being a retrospective analysis, we were also limited in the data available from other factors that may account for additional variation in PCV. Pulmonary hypertension, which has been linked to haematological parameters in humans (Krasuski *et al*. 2011, Rhodes *et al*. 2011), could not be included in the present analysis as it had been infrequently assessed (< 50% visits). In addition, there was insufficient data to identify cases where the cause of pulmonary hypertension was MMVD. This information would be necessary to inform analytical methods as if pulmonary hypertension was secondary to MMVD, it would be considered an intermediate variable in the association between disease severity and PCV (Corraini *et al*. 2017). Information on comorbidities was only available if they were known to be present at the time of examination and recorded in the clinic's note system. It should be noted that hydration status was not assessed as part of standard data capture. Per

protocol, all dogs attending the clinic were systemically well at the time of examination though, as would be expected in an aging population, some patients were affected by chronic pro-inflammatory conditions or endocrine disorders. Whilst this increases the generalisability and external validity of our results, the presence of comorbidities may have introduced noise into the data, weakening the strength of the associations detected.

A final limitation was the method by which anaemia was assessed. PCV provides an approximation of absolute red blood cell numbers and it can be influenced by changes in the composition of plasma (Stockham & Scott 2008). It is suggested that this study was partially confounded by these changes. As fluid retention is anticipated in left sided heart disease, the findings were still considered meaningful; however, it should be acknowledged that plasma volume was not directly assessed. As previously discussed, at a patient level, similar factors can impact the interpretation of isolated measurements of PCV. Individual variation in the components of whole blood may be brought about by normal physiological mechanisms or as a consequence of concurrent comorbidities. In addition, artefactual changes in PCV are common and may occur secondary to inconsistencies in sample handling and processing (Breheny *et al*. 2017). Whilst there was a general tendency for values of PCV decrease with increasing disease severity, the distributions of results were broad and overlapped with one another, the relevance of which would be difficult to interpret in practice.

In conclusion, this study found evidence of an inverse relationship between disease severity and PCV in dogs with MMVD. By relating PCV to ACVIM stage, we were able to appreciate that PCV was greater in stage C, which suggests that the administration of potent diuretics alters this relationship by reducing haemodilution. When this diuretic effect was controlled for, PCV and left ventricular size were negatively correlated. It is therefore possible that mechanisms other than changes to plasma volume are responsible for some of our findings. This hypothesis would benefit from further research. All findings reported in this study are the result of analyses comparing PCV across a large number of examinations and we do not recommend that these results are applied to individual patients.

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## Conflict of interest

J. Wilshaw BVetMed MRCVS is currently undergoing a PhD studentship that is sponsored by Boehringer Ingelheim Animal Health GmbH.

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N. Lotter RVN has no conflicts of interest to declare.

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A. Boswood MA VetMB DVC DipECVIM-CA (Cardiology) MRCVS holds a consultancy with Boehringer Ingelheim Animal Health GmbH. He has received research funding from Boehringer Ingelheim, CEVA Animal Health and Zoetis.

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## Supporting Information

The following supporting information is available for this article:

**Appendix S1:** Supporting information