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## Accepted Manuscript

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## Original Article

### Urine 5-hydroxyindoleacetic acid in Cavalier King Charles spaniels with preclinical myxomatous mitral valve disease

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## Highlights

- Urine 5-hydroxyindoleacetic acid (5-HIAA) concentrations predict progression of cardiac disease in some human conditions
- Urine 5-HIAA was measured in cavalier King Charles spaniels (CKCS) with preclinical myxomatous mitral valve disease (MMVD)
- Urine 5-HIAA concentrations were higher in clinic-collected samples than in morning, home-collected samples
- Female CKCS showed higher urine 5-HIAA concentrations than male dogs
- Urine 5-HIAA concentrations were not associated with disease severity in CKCS with preclinical MMVD

## Abstract

Higher concentrations of circulating serotonin have been reported in Cavalier King Charles spaniels (CKCS) compared to other dog breeds. The CKCS is also a breed highly predisposed to myxomatous mitral valve disease (MMVD). The aim of this study was to determine urine concentrations of 5-hydroxyindoleacetic acid (5-HIAA), the major metabolite and excretion product of serotonin, in a population of CKCS with preclinical MMVD, and to evaluate whether urine 5-HIAA concentrations were associated with MMVD severity, dog characteristics, setting for urine sampling, platelet count, and serotonin concentration in serum and platelet-poor plasma (PPP). The study population consisted of 40 privately-owned CKCS (23 females; 17 males) with and without preclinical MMVD as follows: American College of Veterinary Internal Medicine (ACVIM) group A ( $n=11$ ), ACVIM group B1 ( $n=21$ ) and ACVIM group B2 ( $n=8$ ).

Urine 5-HIAA concentrations were not significantly associated with preclinical MMVD disease, platelet count or circulating concentrations of serotonin (in serum and PPP;  $P>0.05$ ). Females had higher 5-HIAA concentrations than males in morning urine collected at home (females, 3.1 [2.9-3.7]  $\mu\text{mol}/\text{mmol}$  creatinine [median and quartiles]; males, 1.7 [1.2-2.2]  $\mu\text{mol}/\text{mmol}$  creatinine;  $P=0.0002$ ) and urine collected at the clinic (females, 3.5 [3.1-3.9]  $\mu\text{mol}/\text{mmol}$  creatinine; males, 1.6 [1.3-2.1]  $\mu\text{mol}/\text{mmol}$  creatinine;  $P<0.0001$ ). Five-HIAA concentrations in urine collected at home and at the clinic were significantly associated ( $P=0.0004$ ;  $r=0.73$ ), and higher concentrations were found in urine collected at the clinic ( $P=0.013$ ). Urine 5-HIAA concentration was influenced by sex and setting of urine sampling. Urine 5-

HIAA concentration was not associated with MMVD severity or circulating concentrations of serotonin in CKCS with preclinical disease.

*Keywords:* 5-HIAA; Biomarker; Canine; Mitral valve disease; Serotonin metabolite

ACCEPTED MANUSCRIPT

## Introduction

Myxomatous mitral valve disease (MMVD) is the most common heart disease in dogs (Egenvall et al., 2006) and an important cause of congestive heart failure. Cavalier King Charles spaniels (CKCS) are highly predisposed to develop MMVD (Darke, 1987; Häggström et al., 1992). Non-inflammatory, degenerative changes with proliferation of the extracellular matrix of the mitral valve apparatus are hallmarks of canine MMVD, and may lead to mitral valve prolapse (MVP) and mitral regurgitation (MR; Whitney, 1967; Buchanan, 1977; Fox, 2012). Structurally similar heart valve changes have been demonstrated following repeated serotonin injections in rats (Gustafsson et al., 2005) and oral serotonin in rabbits (Lancellotti et al., 2015). In human patients diagnosed with serotonin-producing neuroendocrine tumours of the gastrointestinal tract, and consequently showing high circulating serotonin concentrations, the tricuspid valve can be affected in a similar way (Pellikka et al., 1993).

A number of veterinary studies have investigated the role of serotonin in the development of MMVD in dogs as reviewed elsewhere (Oyama and Levy, 2010; Orton et al., 2012). Given the breed predisposition of MMVD in CKCS, a noteworthy finding is that CKCS have higher concentrations of circulating serotonin (i.e. in serum and platelet rich plasma) compared to many other breeds (Arndt et al., 2009; Ljungvall et al. 2013; Cremer et al., 2014; Höglund et al., 2018). While a number of studies have investigated possible associations with the development of MMVD, the exact role of increased circulating serotonin pools in the development of valvular disease is not yet clear. Higher concentrations of serotonin have been reported in dogs with mild pre-clinical MMVD compared to dogs with late-stage MMVD, suggesting a

potential causative role of high serotonin concentrations in the early development of canine MMVD (Ljungvall et al., 2013; Cremer et al., 2015a). Nevertheless, other studies reported no difference in circulating serotonin concentrations between dogs with mild and severe MMVD (Mangklabruks and Surachetpong, 2014; Cremer et al., 2015b). These conflicting results may be partly due to differences in methods of serotonin analysis, MMVD classification, and to breed, sex and age compositions of dog groups studied.

High biological variation in circulating serotonin concentrations challenges the diagnosis of neuroendocrine tumors in human patients (Feldman and O'Dorisio, 1986). To overcome this, urine concentration of the major metabolite of serotonin, 5-hydroxyindoleacetic acid (5-HIAA), is routinely used instead (Oberg et al., 2017). Notably, urine 5-HIAA concentration can predict progression of heart disease in human patients with neuroendocrine tumors (Møller et al., 2003; Dobson et al., 2014). Similar baseline concentrations of urine 5-HIAA (5–40  $\mu\text{mol/L}$ ) have been reported in humans and dogs (Some and Helander, 2002) but the applicability of urine 5-HIAA analysis in dogs with MMVD is unknown.

The aim of this study was to determine urine 5-HIAA concentrations in a population of CKCS with and without preclinical MMVD, and to evaluate if urine 5-HIAA concentrations were associated with severity of MMVD, dog characteristics, setting of urine sampling, platelet count and concentrations of serotonin in serum and platelet poor plasma (PPP).

## Material and methods

### *Dogs*

The study was performed at the Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark, after approval by the Danish Animal Experiments Inspectorate (Permission 2011/561-71; 7 October 2011). The study population consisted of privately owned pedigreed CKCS. Dogs >4 years with and without audible heart murmurs due to varying severity of preclinical MMVD were prospectively recruited by invitation. Written informed owner consent was obtained. Exclusion criteria were presence of heart disease(s) other than MMVD, presence of congestive heart failure, systemic medical treatment including heart medication, inability to obtain voided urine, and clinical signs or abnormalities of laboratory variables indicating systemic disease. The dogs were not subjected to food or water restrictions, except that they were fasted for 6 h to 18 h prior to the clinic visit. Before the scheduled clinical visit, urine collection equipment was sent to the dog owners. This included detailed instructions on how to collect urine from the first morning voiding at home and storage of samples protected from light and at low temperature, preferably in the refrigerator or on ice. On the same day, a second voided urine sample was collected at the clinic before the beginning of the clinical examination, which included blood sampling, cardiac auscultation, echocardiography and electrocardiography (ECG). Left apical systolic murmurs were graded from 1-6 (Gompf, 1988) by a single experienced observer (LHO). A voided urine sample was collected after the clinical examination if the sampling before the clinical examination was unsuccessful. The time intervals between urine sampling at home and sampling of urine and blood at the clinic were recorded.

*Echocardiographic examination*

Standardized transthoracic echocardiographic views (Thomas et al., 1993) were recorded from the right parasternal and left apical windows by a single trained examiner (LHO) using a Vivid E9 echocardiographic system with a 5Sc transducer (GE Healthcare). A standardized echocardiographic protocol using 2-D, M-mode, colour Doppler and spectral Doppler was followed as previously described (Reimann et al., 2014). The same examiner (LHO) performed the off-line analysis using EchoPac software (EchoPAC PC. Version 112, GE Healthcare), masked to the identity of the dog. MR severity grading was performed using colour Doppler with the gain set just below the colour sparkling artefact in air and a standardized Nyquist limit of 0.62 m/s from the left parasternal apical four-chamber view; this was determined based on the regurgitant jet area relative to left atrial (LA) area (Pedersen et al., 1999a; Reimann et al., 2014). Briefly, an area of MR <20% was regarded as no or mild MR; an area of 20-50% was classified as moderate MR; and an area of >50% was classified as severe MR. From the right parasternal long axis four-chamber view, the severity of MVP and mitral valve leaflet thickness were assessed (Olsen et al., 1999; Pedersen et al., 1999b; Birkegård et al., 2016). The ratio between LA diameter to aortic (Ao) diameter was measured in the right parasternal short axis view at the level of the aortic root at the first frame after closure of the aortic valve (Hägström et al., 1994; Hansson et al., 2002). By using 2-D guided M-mode at the right parasternal short axis view, the internal diameter of the left ventricle in diastole (LVIDD) was measured at chordae tendineae level identified in a 2D image prior to placement of the M-mode cursor and normalized (LVIDD<sub>N</sub>) to bodyweight, as previously described (Cornell et al., 2004).

CKCS were classified into clinical disease severity groups according to the American College of Veterinary Internal Medicine (ACVIM) guidelines (Atkins et al., 2009; Boswood et al., 2016). In brief, CKCS with no auscultatory murmur and/or normal echocardiogram ( $MR < 20\%$ ) were classified as ACVIM group A, while CKCS with a characteristic left apical systolic mitral regurgitation murmur and/or a  $MR \geq 20\%$  were classified as ACVIM group B1 if no remodelling of the heart was evident, or ACVIM group B2 if remodelling of LA ( $LA/Ao \geq 1.6$ ) and left ventricle ( $LVIDD_N \geq 1.7$ ) were detected (Boswood et al., 2016).

#### *Urine analyses*

At the clinic, voided urine samples were protected from light and kept at  $4\text{ }^\circ\text{C}$  until centrifuged at  $3,000\text{ g}$  for  $10\text{ min}$  at  $4\text{ }^\circ\text{C}$ . The supernatants were aliquoted to polypropylene tubes and stored at  $-80\text{ }^\circ\text{C}$  until batch analysis. The concentration of urine 5-HIAA was determined using high-performance liquid chromatography with electrochemical detection, as previously described (Helander et al., 1991). An intra-assay coefficient of variation of  $2.5\%$  was calculated after analyzing three urine samples in five replicates. All urine 5-HIAA concentrations were corrected for variations in urine concentration using the urine creatinine concentration measured by the Jaffe method (Bonsnes, 1945).

#### *Blood analyses*

A venous blood sample was drawn from the jugular vein using a  $21\text{ G}$  butterfly catheter connected to a vacutainer system into one plain tube, one  $K_2\text{EDTA}$  tube, and one tube containing  $3.2\%$  citrate. Within  $30\text{ min}$  (citrate stabilized blood) and after  $30\text{ min}$  (the plain tube), blood samples were centrifuged at  $3,000\text{ g}$  for  $10$

min at 4 °C. Supernatants, consisting of PPP and serum respectively, were aliquoted into polypropylene tubes and stored at -80 °C for later batch analyses. Samples of serum and EDTA-stabilized whole blood were delivered to the Veterinary Diagnostic Laboratory (University of Copenhagen) for a complete blood count and a standard serum biochemistry profile. A manual platelet count was performed after adding 20 µL of EDTA-stabilized blood to 380 µL of stromatolytic solution, as previously described (Eksell et al., 1994). All ELISA analyses were done in accordance with the manufacturer's instructions by one skilled technician using a Labtech LT-4000 microplate reader (Labtech International). All samples were performed in duplicate and a new standard curve was constructed for each assay. A commercial human ELISA kit (RE59121, IBL International) was used for quantitative measurements of serotonin in serum and in PPP, as previously described (Ljungvall et al., 2013; Cremer et al., 2015a). The assay was validated for use of canine PPP with regards to intra-assay and inter-assay coefficients of variation (CV) and estimated limit of detection (LD). For the PPP, samples used for analysis of analytical precision (median: 87.7 ng/mL; range: 61.6-107.4 ng/mL), the serotonin ELISA kit had an intra-assay CV of 13.0% and an inter-assay CV of 17.6%. For the serum samples, the assay was previously validated (Ljungvall et al., 2013) and samples used for analytical precision (median: 452.8 ng/mL; range: 342.9-687.5 ng/mL) performed in the present study with an intra-assay CV of 4.2% and an inter-assay CV of 6.1%. The LD was estimated at 17.8 ng/mL. Because of the lower concentration of PPP samples, the CVs were higher than serum, and all CVs were acceptable (<20%) for a manual assay<sup>1</sup>.

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<sup>1</sup> See: European Medicines Agency, 2011. Guideline on bioanalytical method validation. [http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2011/08/WC500109686.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf) (Accessed 9 May 2019).

### *Statistics*

Statistical analyses were conducted using statistical software (SAS version 9.4) with the significance level set at  $P < 0.05$ . Group data were tested for normal distribution using Shapiro-Wilks test and were reported as median and interquartile range (IQR) since all data were not normally distributed. Differences between the ACVIM groups (A, B1, B2) in age, bodyweight (BW), platelet count, LA/Ao, LVIDD<sub>N</sub>, urine 5-HIAA concentrations and serotonin concentrations in serum or PPP were investigated by one-way ANOVA followed by post hoc *t*-test if the overall *P*-value demonstrated a significant difference between groups. Differences between groups of categorical variables (sex, severity of MR, MVP and leaflet thickness) were tested using Fisher's exact test. Concentrations of 5-HIAA in urine collected at home and at the clinic were compared using paired Wilcoxon-signed rank test. Univariate regression analyses were used to evaluate the associations between urine 5-HIAA (home and clinic, respectively) and serotonin concentrations in PPP and serum. In addition, univariate regression analyses were used to individually evaluate associations between urine 5-HIAA concentrations (response variable) and dog characteristics (age, sex, BW), platelet count and disease variables (ACVIM group, LA/Ao, LVIDD<sub>N</sub> and severity of MR, MVP and mitral valve leaflet thickness). Thereafter, a backward stepwise multivariate regression analysis was performed including the explanatory variables with  $P < 0.20$  in the univariate analyses. Model residuals were tested for homogeneity of variation based on visual inspection of residual plots. Logarithmic transformation was used when appropriate.

## Results

### *Dogs*

Sixty-two CKCS were eligible for the study, but 22 were excluded due to lack of sufficient urine for analyses ( $n=18$ ), or use of cardiac medication ( $n=4$ ). The remaining 40 dogs were allocated to the following groups: ACVIM group A ( $n=11$ ), ACVIM group B1 ( $n=21$ ) and ACVIM group B2 ( $n=8$ ). A summary of descriptive statistics in the three groups, conventional echocardiographic variables, platelet count, circulating concentrations of serotonin and urine 5-HIAA concentrations, given as the ratio to creatinine to correct for differences in urine dilution, are shown in Table 1. Nine of the 40 dogs were neutered (7 females; 2 males). In 19 dogs, urine was collected both at home in the morning and later the same day at the clinic. The time interval between urine sampling at home and at the clinic was 4.9 h (3.5-5.9 h), and between urine and blood sampling at the clinic was 0.6 h (0.4-1.1 h). In three dogs, urine was collected after the clinical examination (0.8-0.9 h after blood sampling) because the attempt before the examination was unsuccessful. Serum and PPP samples for serotonin analysis were missing for one dog.

### *Regression analyses*

The univariate regression analyses did not reveal any significant associations between urine 5-HIAA concentrations collected at home and any of the MMVD disease variables (ACVIM group,  $P=0.31$ , Fig. 1; MR,  $P=0.38$ ; LA/Ao,  $P=0.61$ ; LVIDDN,  $P=0.75$ ; MVP,  $P=0.94$ ; mitral valve leaflet thickness,  $P=0.44$ ), age ( $P=0.33$ ) or platelet count ( $P=0.17$ ). Similarly, 5-HIAA concentrations measured in urine collected at the clinic were not significantly associated with MMVD disease severity (ACVIM group,  $P=0.16$ , Fig. 1; MR,  $P=0.56$ ; LA/Ao,  $P=0.16$ ), LVIDDN

( $P=0.14$ ), MVP ( $P=0.31$ ) and mitral valve leaflet thickness ( $P=0.15$ ), age ( $P=0.71$ ) or platelet count ( $P=0.98$ ). Female CKCS had higher concentrations of urine 5-HIAA than male CKCS at home (female: 3.1 [2.9-3.7]  $\mu\text{mol}/\text{mmol}$ ; male: 1.7 [1.2-2.2]  $\mu\text{mol}/\text{mmol}$ ;  $P=0.0002$ ) and at the clinic (female: 3.5 [3.1-3.9]  $\mu\text{mol}/\text{mmol}$ ; male: 1.6 [1.3-2.1]  $\mu\text{mol}/\text{mmol}$ ;  $P<0.0001$ ; Fig. 2). Urine concentration of 5-HIAA decreased with increasing BW ( $P=0.024$ ,  $r=0.46$  [home] and  $P=0.019$ ,  $r=0.40$  [clinic]). However, the association did not remain significant in the multivariate analysis when sex was considered. In the multivariate analyses, only sex had a significant effect on urine 5-HIAA concentrations ( $P<0.05$ ). Concentrations of 5-HIAA in urine samples collected at home and at the clinic were significantly associated ( $P=0.0004$ ,  $r=0.73$ ). However, higher concentrations were found in urine samples collected at the clinic ( $P=0.013$ ; Fig. 3). A subanalysis showed that the effect of sampling setting (home vs. clinic) on urine 5-HIAA concentration was significant in female dogs ( $P=0.0020$ ), but not in male dogs ( $P=0.65$ ).

Serum and PPP serotonin concentrations were also weakly associated ( $P=0.039$ ;  $r=0.33$ ; Fig. 4). However, urine 5-HIAA was not associated with serum or PPP serotonin concentration. There were no differences in serum and PPP serotonin concentrations between the three ACVIM groups ( $P>0.05$ ; Fig. 5).

## Discussion

This study is, to our knowledge, the first to evaluate the concentration of 5-HIAA in urine from dogs diagnosed with MMVD. The study, which included CKCS with preclinical MMVD, revealed no associations between urine 5-HIAA concentrations and MMVD disease severity. The excretion of 5-HIAA in urine was

higher in female CKCS than male CKCS, and was dependent on the situation (home vs. clinic) of urine collection.

It has been suggested that increased serotonin concentration are associated with the development of MMVD in dogs (Ljungvall et al., 2013). Although the classification schemes used in previous studies differ slightly from ours, it seems, based on the presence of clinical signs and echocardiographic variables, that the reported differences between disease groups are only found when comparing dogs with MMVD (corresponding to ACVIM group C and group D according to the ACVIM classification scheme; Atkins et al., 2009) with groups of at-risk and preclinical diseased dogs (ACVIM group A and B; Ljungvall et al., 2013; Cremer et al., 2014; Cremer et al., 2015a). The present study, which only included dogs in stages A, B1 and B2, did not demonstrate any differences in serotonin concentrations in serum or PPP and 5-HIAA concentrations in urine between the three groups. Considering that only eight dogs had signs of remodelling of the heart (B2) and no dogs with congestive heart failure were included, our results for serum and PPP serotonin concentrations are in agreement with previous reports of dogs with similar baseline characteristics and in similar stages of MMVD (Arndt et al., 2009; Ljungvall et al., 2013; Cremer et al., 2014; Cremer et al., 2015a; Cremer et al., 2015b).

In people suspect for increased circulating serotonin, the rationale for using 24 h urine 5-HIAA measurements as a diagnostic tool is to overcome the large intra-individual variations in circulating serotonin concentrations when only one blood sample is obtained (Feldman and O'Dorisio, 1986; Oberg et al., 2017). In the present study, 5-HIAA concentrations in spot urine samples did not provide additional

information compared to circulating serotonin concentrations regarding the possibility that serotonin is involved in the early development of MMVD in dogs. Whether collection of 24 h urine over spot samples may increase the sensitivity for urine 5-HIAA in dogs with MMVD, even when the latter are corrected for variations in urine dilution by using the ratio to creatinine, remains to be elucidated.

Human studies have shown that urine 5-HIAA concentration is superior to circulating serotonin concentrations in detecting neuroendocrine tumours located in the midgut, which are known to have a very high serotonin production (Feldman and O'Dorisio, 1986; Kema et al., 1994; Niederle et al., 2016). On the contrary, in tumours of the foregut, which produce low amounts of serotonin, 5-HIAA was not superior to circulating serotonin in detecting disease (Meijer et al., 2000). In dogs with MMVD, circulating serotonin concentrations are generally lower than those of human patients with serotonin producing tumours (Robiolio et al., 1995; Cremer et al., 2015a) and this may limit the use of urine 5-HIAA in dogs with MMVD. A recent study reported promising results regarding the use of plasma 5-HIAA rather than 24 h urine 5-HIAA and circulating serotonin in human patients (Carling et al., 2002). However, plasma 5-HIAA concentrations have not been implemented in human medicine, due to the lack of larger clinical studies (Oberge et al., 2017) and the technical demands of the assay (Carling et al., 2002). In dogs where 24 h urine collection is not possible, plasma 5-HIAA could be investigated to potentially increase the sensitivity of early MMVD identification.

Our finding of serotonin concentrations in serum that were on average 33 times higher than the plasma concentration corresponds with previous results (Cremer

et al., 2015a). It is generally accepted that the vast majority of circulating serotonin is actively taken up and stored in dense granules of platelets (Born and Gillson, 1959). This was also confirmed in dogs, where the serotonin concentration was 350-fold higher in platelet-rich plasma compared to PPP (Cremer et al., 2014). Due to the absence of anticoagulants in serum tubes, serum serotonin concentrations appear to mirror the total pool of serotonin released from dense granules on platelet activation and free circulating serotonin. The lower serotonin plasma concentration reflects solely the free unbound circulating serotonin. Preanalytical factors causing platelets to be partly activated may however increase in vitro plasma concentrations of serotonin (Brand and Anderson, 2011).

Urine 5-HIAA concentrations were not associated with circulating serum or PPP serotonin concentrations in blood samples collected at the clinic. We cannot provide an exact reason for this, but there are potential factors that might influence the results; including comparably large daily variations in circulating serotonin concentrations, as well as in individual variations in urine 5-HIAA excretion, as shown in human patients (Zuetenhorst et al., 2004; Oberg et al., 2017).

Platelets are not the only mechanism for clearing serotonin from the circulation in the organism. Experimentally, serotonin was rapidly cleared by the lungs and the liver following IV infusion of serotonin in dogs (Thomas and Vane, 1967). Whether serotonin cleared by the lungs and liver is subsequently taken up by platelets is not known, nor how serotonin metabolites from intracellular serotonin enter the urine. Radioactive labelled serotonin in platelets was shown to accumulate in tissues including gut, thyroid gland and adrenal glands in rat experiments (Osimo and

Wyllie, 1983). This indicates that there may be a delay in time from serotonin circulation in blood until excretion of 5-HIAA in the urine, and this may interfere with correlations between circulating serotonin and excretion of the metabolite. Although this topic remains speculative we gather that several biological processes might contribute to the absence of a correlation between circulating serotonin and 5-HIAA in urine in our study.

Interestingly, higher concentrations of 5-HIAA were found in urine samples collected at the clinic compared to samples collected earlier the same day at home environment. This finding raises the question if physical activity, stress and/or anxiety in conjunction with transport to the clinic may account for increasing concentrations of circulating serotonin followed by an increase in the urine excretion of 5-HIAA. Differences in other circulating plasma markers (i.e. nitrite, von Willebrand factor and L-arginine) and in urine catecholamine concentrations have previously been described in healthy dogs between samples obtained consecutively in the home environment and in a clinical setting (Moesgaard et al., 2007; Höglund et al., 2012).

As previously described, human guidelines for diagnosing neuroendocrine tumours recommend use of 24 h urine collection for to 5-HIAA analysis (Oberg et al., 2017), but this is rarely, if ever, possible for privately owned dogs in the home environment. Use of urine collection cages or hospitalization with catheterization of dogs would be required for collection of 24 h urine with the risk of stress biasing such results. A strong correlation was found in human patients between the 5-HIAA concentration in 24 h urine voids and spot samples obtained in the morning minimally 8 h after the last voiding of urine (Gedde-Dahl et al., 2013). The latter approach

resembles home collection used in our present study; however, the dog owners were not instructed to keep the dogs indoor for 8 h during the night. Urine sampled overnight may be relevant for future studies evaluating urine 5-HIAA concentrations in dogs with MMVD. Although the biomarker was not more sensitive than circulating serotonin in differentiating between early stages of MMVD in CKCS in the present study, a major advantage of a biomarker in urine is the possibility for serial collections in the home environment.

Lastly, in our study, urine 5-HIAA concentrations were higher in female CKCS than male CKCS. Higher serum serotonin concentrations in female CKCS than male CKCS have also been reported in other studies (Arndt et al., 2009; Ljungvall et al., 2013; Höglund et al., 2018). Urine 5-HIAA concentrations have been regarded in behavioural studies as an indicator of stress and anxiety in dogs without evidence of heart disease. Similarly, urine 5-HIAA concentrations were higher in female dogs compared to male dogs (Part et al., 2014). Other studies have reported similar serotonin concentrations in male and female dogs with MMVD (Cremer et al., 2015a) or higher serotonin concentrations in platelets from male dogs (Mangklabruks and Surachetpong, 2014). In the present study, some of the dogs were neutered or spayed, but the number in each group was too low to include in the analyses. To the best of our knowledge, no previous studies have reported whether neutering influences circulating serotonin and urine 5-HIAA concentrations in dogs; this requires further investigation.

The present study has some limitations. One is that the dogs were only restricted in their food intake 6-18 h prior to urine and blood sampling at the clinic.

Banana, kiwi fruit and nuts are rich in tryptophan, the precursor of serotonin. In human studies, these food items should be avoided for 3 days prior to 24 h urine collection (Oberg et al., 2017), as this could otherwise cause diet-induced increases in serotonin and 5-HIAA concentrations. Although those particular tryptophan rich foodstuffs are rarely included in canine diets, there was a lack of standardization of the diet prior to blood and urine sampling, which could have affected the concentrations of the biomarkers in our study. Methods have been described to analyse tryptophan content in diets (Yust et al., 2004). In future canine studies, the impact of dietary tryptophan on circulating serotonin concentrations and urine concentrations of the metabolite 5-HIAA, respectively, should be investigated.

Blood samples were collected only at one time point and only in the clinical setting, whereas urine was collected both at home in the morning and later the same day at the clinic. Additionally, the time between urine and blood sampling in the clinic was not standardized.

Colour Doppler regurgitant area was included as one of the indices of disease severity. This method is influenced by haemodynamic and technical factors including Doppler gain settings (Lancellotti et al., 2013). In order to limit the influence of this issue, Doppler gain settings were standardized in the present study and we used additional indices of disease severity, including ACVIM group.

Limitations related to group size apply. The numbers of dogs in each group were small and the risk of type II error cannot be excluded when concluding that there were no significant differences between disease groups in circulating serotonin and

urine 5-HIAA concentrations. The relatively high variability in urine and blood serotonin markers between dogs, even within the same breed, is worth considering in future studies.

Finally, disease groups were not matched according to age. The CKCS in ACVIM group B2 were older than in groups A and B1, probably because development of MMVD is related to age in dogs (Buchanan, 1977; Häggström et al., 1992; Fox, 2012). However, no associations were found between age and urine 5-HIAA concentrations in the study population, suggesting that age differences among the ACVIM groups did not influence our study conclusions.

## **Conclusions**

Urine 5-HIAA concentration was not associated with preclinical MMVD disease severity in CKCS. Higher 5-HIAA concentrations were found in urine collected at the clinic compared to at home, but the concentrations were significantly associated. Circulating levels of serotonin were not associated with urine 5-HIAA. Further studies evaluating urine 5-HIAA in dogs with MMVD are relevant, but the differences observed between male and female dogs, and between sampling settings must be considered.

## **Conflict of interest**

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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**Table 1.**

Dog characteristics, standard echocardiographic variables and serotonin markers in blood and urine from 40 Cavalier King Charles spaniels <sup>a</sup>.

	ACVIM Group A (n=11)	ACVIM Group B1 (n=21)	ACVIM Group B2 (n=8)	P
Sex (n; male/female)	4/7	11/10	2/6	NS
Age (years)	6.1 (4.4-7.4)	7.7 (6.8-8.7)	9.8 (9.0-11.1) <sup>c, d</sup>	0.0026
Bodyweight (kg)	8.8 (7.8-10.6)	8.9 (7.9-10.4)	8.5 (7.0-10.6)	NS
LA/Ao	1.2 (1.1-1.3)	1.3 (1.2-1.4)	1.7 (1.6-2.0) <sup>c, d</sup>	<0.0001
LVIDD <sub>N</sub> <sup>b</sup>	1.5 (1.4-1.6)	1.6 (1.4-1.6)	1.9 (1.7-2.2) <sup>c, d</sup>	<0.0001
MR severity	11/0/0	0/13/8	0/0/8	<0.0001
No or mild/ moderate/severe				
MVP severity	0/6/5/0	1/3/9/8	0/0/3/5	0.011
0/1/2/3				
Mitral valve leaflet thickness	0/7/4/0	0/7/12/2	0/0/7/1	0.041
No or mild/ moderate/severe				
Serotonin concentration Serum, ng/mL	782.8 (583.8- 1016.1)	688.1 (513.3- 981.8) n=20	694.1 (631.8- 745.1)	NS
Serotonin concentration PPP, ng/mL	42.9 (10.0-121.8)	27.8 (15.3-47.1) n=20	60.0 (14.6- 131.9)	NS
5-HIAA, home, µmol/mmol creatinine	2.6 (1.9-4.3) n=8	1.7 (1.3-3.0) n=11	2.9 (2.4-3.1) n=5	NS
5-HIAA, clinic, µmol/mmol creatinine	3.1 (2.2-3.7) n=9	2.1(1.5-3.5) n=18	3.1 (2.8-3.7)	NS
Platelet count ×10 <sup>6</sup> platelets/mL	302 (173-379) n=9	94 (61-255)	278 (112-337)	NS

ACVIM, American College of Veterinary Internal Medicine; LA/Ao, Ratio of left atrium to aortic root; LVIDN, Left ventricular internal diameter normalized for bodyweight; MR, Mitral regurgitation; MVP, Mitral valve prolapse; PPP, Platelet poor plasma; 5-HIAA, 5-hydroxyindoleacetic acid; NS, Not significant,

<sup>a</sup> Continuous variables are expressed as median (interquartile range).

<sup>b</sup> Logarithmic transformation to obtain variance homogeneity in the statistical model.

Within each row, italic numbers state reduced *n* in case of missing values and

superscripts denote significant difference between groups:

<sup>c</sup> Statistically significant difference with group A ( $P<0.05$ )

<sup>d</sup> Statistically significant difference with group B1 ( $P<0.05$ )

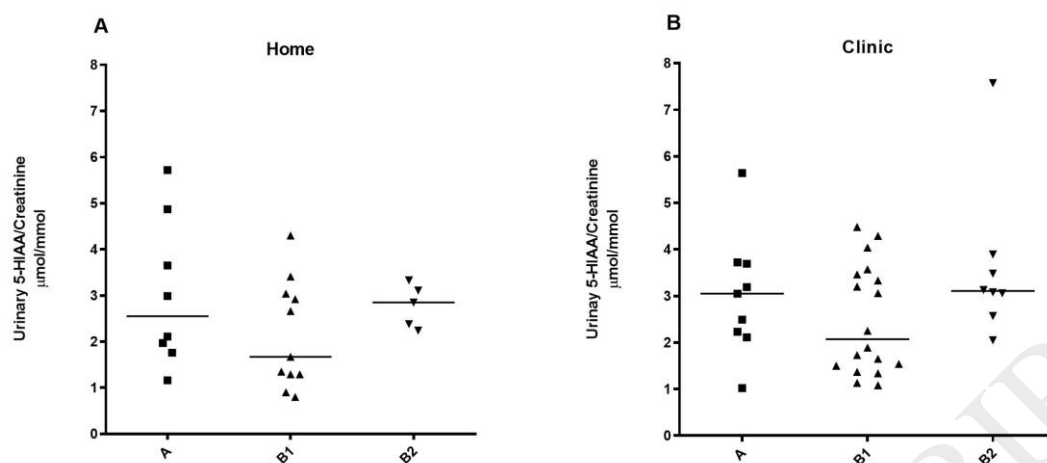


Fig. 1. Scatterplots of 5-hydroxyindoleacetic acid (5-HIAA) concentrations in (a) morning urine collected at home ( $n=24$ ) and (b) collected at the clinic ( $n=35$ ) on the same day from Cavalier King Charles spaniels with myxomatous mitral valve disease (MMVD) stage A (A), MMVD stage B1 (B1) and MMVD stage B2 (B2), according to ACVIM guidelines (Atkins et al., 2009). The horizontal bars indicate the median concentration. The 5-HIAA concentration is expressed as the ratio to urine creatinine, to compensate for variations in urine dilution.

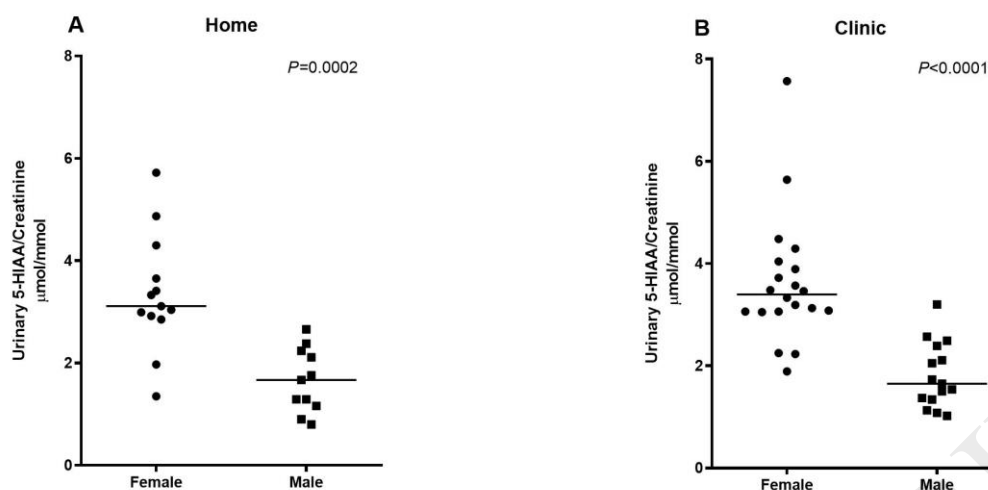


Fig. 2. Scatterplots showing higher 5-hydroxyindoleacetic acid (5-HIAA) concentrations in female Cavalier King Charles spaniels (CKCS) compared to male CKCS in samples collected (a) at home ( $n=24$ ) and (b) later the same day at the clinic ( $n=35$ ). The 5-HIAA concentration is expressed as the ratio to urine creatinine, to compensate for variations in urine dilution. Logarithmic transformation of 5-HIAA concentrations were used to obtain variance homogeneity in the statistical model. Horizontal lines indicate median concentrations.

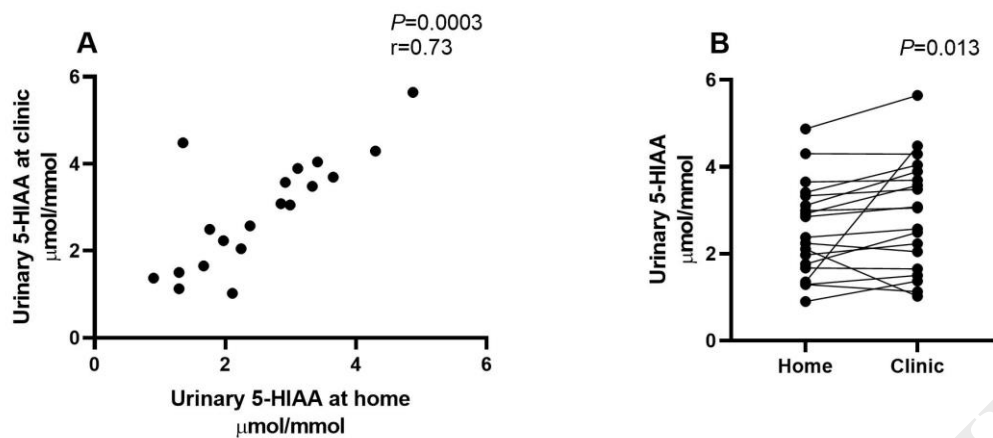


Fig. 3. Plots showing (a) significant association between 5-hydroxyindoleacetic acid (5-HIAA) in morning urine samples collected from 19 Cavalier King Charles spaniels at home and later the same day at the clinic, and (b) significantly higher concentrations of 5-HIAA in urine samples collected at the clinic compared to samples collected at home.

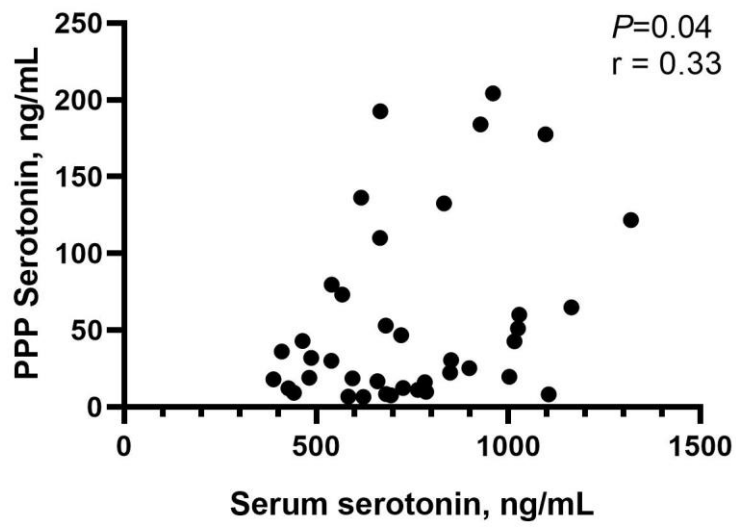


Fig. 4. Plot showing the association between serum and platelet poor plasma (PPP) serotonin concentrations in 39 Cavalier King Charles spaniels.

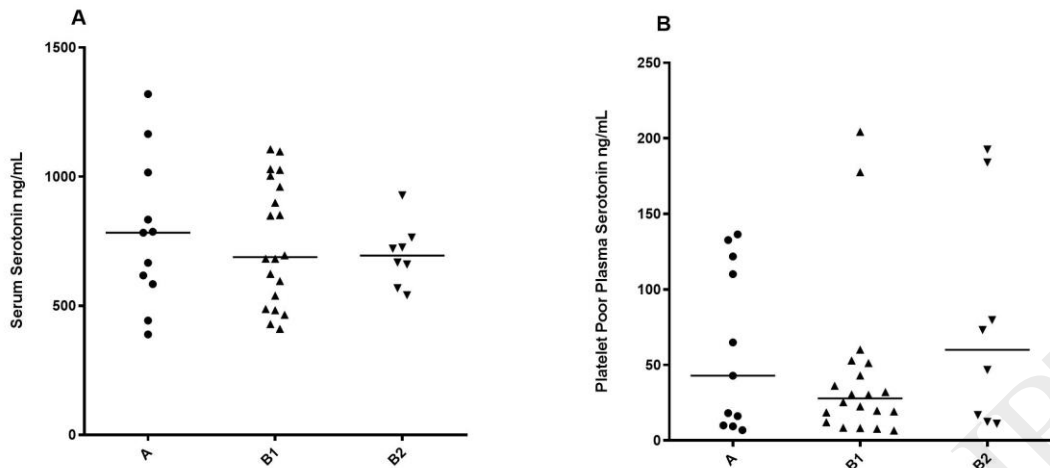


Fig 5. Scatterplots of (a) serum serotonin and (b) platelet poor plasma (PPP) concentrations in 39 Cavalier King Charles spaniels with myxomatous mitral valve disease (MMVD) stage A (A), MMVD stage B1 (B1) and MMVD stage B2 (B2) according to American College of Veterinary Internal Medicine (ACVIM) guidelines (Atkins et al., 2009). The horizontal bars indicate the median concentration.