Received: 16 March 2021 | Accepted: 15 September 2021

DOI: 10.1111/jvim.16277

STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access

ACVIM American College of Veterinary Internal Medicine

# Polymorphisms in the serotonin transporter gene and circulating concentrations of neurotransmitters in Cavalier King Charles Spaniels with myxomatous mitral valve disease

Maria J. Reimann[1] | Merete Fredholm[1] | Signe E. Cremer[2] | Liselotte B. Christiansen[1] | Kathryn M. Meurs[3] | Jacob E. Møller[4] | Jens Häggström[5] | Jens Lykkesfeldt[1] | Lisbeth H. Olsen[1]

[1]Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark

[2]Department of Veterinary Clinical Sciences, University of Copenhagen, Frederiksberg, Denmark

[3]Department of Clinical Sciences, North Carolina State University, Raleigh, North Carolina, USA

[4]Department of Cardiology, Copenhagen University Hospital Rigshospitalet, Copenhagen, Denmark

[5]Department of Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden

**Correspondence**
Maria J. Reimann, Department of Veterinary and Animal Sciences, University of Copenhagen, 9 Ridebanevej, 1870 Frederiksberg C, Denmark.
Email: mreimann@sund.ku.dk

**Funding information**
Det Frie Forskningsråd, Grant/Award Number: 7017-00131B

## Abstract

**Background:** The neurotransmitter serotonin (5-HT) affects valvular degeneration and dogs with myxomatous mitral valve disease (MMVD) exhibit alterations in 5-HT signaling. In Maltese dogs, 3 single nucleotide polymorphisms (SNPs) in the 5-HT transporter (SERT) gene are suggested to associate with MMVD.

**Hypothesis/Objectives:** Determine the association of SERT polymorphisms on MMVD severity and serum 5-HT concentration in Cavalier King Charles Spaniels (CKCS). Additionally, investigate the association between selected clinical and hematologic variables and serum 5-HT and assess the correlation between HPLC and ELISA measurements of serum 5-HT.

**Animals:** Seventy-one CKCS (42 females and 29 males; 7.8 [4.7;9.9] years (median [Q1;Q3])) in different MMVD stages.

**Methods:** This prospective study used TaqMan genotyping assays to assess SERT gene polymorphisms. Neurotransmitter concentrations were assessed by HPLC and ELISA.

**Results:** TaqMan analyses identified none of the selected SERT polymorphisms in any of the CKCS examined. Serum 5-HT was associated with platelet count ($P < .001$) but not MMVD severity, age or medical therapy and did not correlate with serum concentration of the 5-HT metabolite, 5-hydroxyindoleacetic acid. The ELISA serum 5-HT correlated with HPLC measurements ($\rho = .87$; $P < .0001$) but was lower (mean difference $= -22$ ng/mL; $P = .02$) independent of serum 5-HT concentration ($P = .2$).

**Abbreviations:** 5-HIIA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; ACVIM, American College of Veterinary Internal Medicine; BW, body weight; CHF, congestive heart failure; CI, confidence interval; CKCS, Cavalier King Charles Spaniel; CV, coefficient of variation; DBP, diastolic blood pressure; DOPAC, 3,4-dihydroxyphenylacetic acid; Emax, early E wave transmitral peak velocity; F, female; FS, fractional shortening; HPLC, high performance liquid chromatography; HVA, homovanillic acid; LA/Ao, left atrial to aortic root ratio; LVIDDN, left ventricular end diastolic diameter normalized for BW; LVIDSN, left ventricular end systolic diameter normalized for BW; M, male; MBP, mean blood pressure; MHGP, 3-methoxy-4-hydroxyphenylglycol; MMVD, myxomatous mitral valve disease; MR, mitral regurgitation; SBP, systolic blood pressure; SERT, serotonin reuptake transporter; SNP, single nucleotide polymorphism; TCP, thrombocytopenia.

**Conclusions and Clinical Importance:** Selected SERT SNPs associated with MMVD in Maltese dogs were not found in CKCS and only platelet count influenced serum 5-HT concentration. These SNPs are unlikely to be associated with MMVD pathophysiology or serum 5-HT concentration in CKCS. HPLC and ELISA serum 5-HT demonstrated good correlation but ELISA systematically underestimated 5-HT.

**KEYWORDS**
dogs, ELISA, genetic variation, heart disease, HPLC, serum serotonin

## 1 | INTRODUCTION

Myxomatous mitral valve disease (MMVD) is a common cardiovascular disorder in dogs, particularly among Cavalier King Charles Spaniels (CKCS).[1-4] Serotonin (5-hydroxytryptamine, 5-HT) is an important neurotransmitter for cardiac valvular development and function[5-7] and recent studies in dogs and humans have focused on the association between 5-HT and MMVD.[8] The protein 5-HT-reuptake transporter (SERT) controls uptake, storage and metabolism of circulating 5-HT[8] and downregulation of SERT has been linked to MMVD because a reduction of its expression, activity or both in platelets could increase free plasma 5-HT.[9] In dogs with MMVD, decreased SERT expression and decreased SERT protein levels have been reported,[10,11] and higher 5-HT concentrations in serum, platelet-rich plasma and platelets have been reported in dogs with MMVD compared to healthy controls and in CKCS compared to other breeds.[12-18] There is large individual variation in circulating 5-HT concentration in CKCS,[17] which could be because of differences in SERT genotype. In Maltese dogs, there are higher serum 5-HT concentrations compared to other breeds,[16] and furthermore, SERT gene single nucleotide polymorphisms (SNPs) are associated with MMVD.[19] Three SNPs were suggested to be damaging to SERT protein function and structure and thus, it is possible that certain SERT polymorphisms are associated with development of severe MMVD and higher circulating 5-HT concentration in dogs.

The roles of different neurohormonal systems are well-described in MMVD.[20] These systems are activated in response to disease development in order to modulate physiological responses and temporary hemodynamic support. Monoamine neurotransmitters and their metabolites can be classified into (a) the serotonergic system consisting of 5-HT and its major metabolite 5-hydroxyindoleacetic acid (5-HIAA), (b) the adrenergic system consisting of adrenalin, noradrenaline and its metabolite 3-methoxy-4-hydroxyphenylglycol (MHGP), and (c) the dopaminergic system consisting of dopamine and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA).[21] In humans, circulating concentration of 5-HIAA has been suggested as a marker for diagnosing and monitoring neuroendocrine 5-HT producing tumors,[22] but in dogs with MMVD, circulating 5-HIAA concentration has not been investigated previously. However, in CKCS with preclinical MMVD, urinary 5-HIAA concentration is not associated with MMVD severity or serum 5-HT concentration.[18] Higher circulating concentrations of other neurotransmitters including adrenaline and noradrenaline occur in dogs with congestive heart failure (CHF) because of MMVD.[23-25] However, concomitant investigation of the serotonergic, adrenergic, and dopaminergic systems has not been undertaken in dogs. The aim of the present study was to take such an approach in CKCS and to investigate if SERT polymorphisms previously identified in Maltese dogs are present and associated with serum 5-HT concentration, MMVD severity or both. In addition, it was investigated (a) if serum 5-HT concentrations measured by high performance lipid chromatography (HPLC) were associated with MMVD severity, selected clinical variables (age, sex, platelet count in EDTA-anticoagulated blood, and medical therapy) or correlated with serum 5-HIAA, (b) if serum 5-HT concentrations measured by HPLC and ELISA were correlated, and (c) if the concentration of additional selected serum neurotransmitters (5-HIAA, adrenaline, noradrenaline, MHGP, dopamine, DOPAC, and HVA) measured by HPLC, differed between MMVD stages.

## 2 | MATERIALS AND METHODS

### 2.1 | Dogs

The study was performed at the Department of Veterinary and Animal Sciences, University of Copenhagen from October 25, 2018 to May 23, 2019 and approved by the Danish Animal Experiments Inspectorate (license no. 2016-15-0201-01074). Client-owned CKCS with no MMVD or different stages of MMVD were prospectively enrolled in the study and included dogs examined for breeding approval, voluntary examinations and invited dogs. None of the dogs were related at the parental level. Written informed consent forms were signed by all owners. Dogs with cardiac disorders other than MMVD, unsuccessful blood collection as well as dogs with considerable deviations from reference intervals on complete blood count and a standard serum biochemistry profile were excluded.

All CKCS were examined by a standardized protocol including: interview with the owner, venous blood collection, physical examination with focus on the cardiovascular system, blood pressure measurement and echocardiography. Mitral regurgitation murmur intensity was graded 1-6/6.[26] High definition oscillometry equipment (Vet HDO monitor [Memodiagnostic], S + B medVET GmbH, Babenhausen, Germany) on the proximal part of the tail was used to determine blood pressure[27] and an average of 5 repetitive measurements was used as previously described.[28]

### 2.2 | Blood sampling

Blood was collected from the jugular vein in fasted dogs with a vacutainer system connected to a 21-G butterfly catheter into plain

tubes and tubes containing EDTA. Manual platelet count was performed after adding 20 μL of EDTA-stabilized whole blood to 380 μL of stromatolytic solution.[29] EDTA-stabilized whole blood was stored at −20°C until batched analysis of SERT genotype (TaqMan). Serum was prepared after 30 minutes of collection by centrifugation of blood from plain tubes at 3000*g* for 10 minutes at 4°C. Samples of serum and EDTA-stabilized whole blood were analyzed at the Veterinary Diagnostic Laboratory (University of Copenhagen) for a complete blood count and a standard serum biochemistry profile.

Several aliquots of serum were stored in cryotubes at −80°C until batched analysis of 5-HT concentration (ELISA and HPLC) and additional neurotransmitters. All samples were analyzed within 17 months after collection.

## 2.3 | TaqMan

DNA was isolated from 200 μL EDTA-anticoagulated blood with a DNA extraction kit (Epicenter Kit, Nordic Biolabs, Sweden) and kept at −20°C. Three selected SNPs in the SERT gene with possible functional impact and association with MMVD were: c.814insG (p.Lys272Arg), c.1193delT (p.Val397Gly), c.1324G>A (p.Gly442Ser; Table 1).[19] Single nucleotide polymorphism genotyping was performed by TaqMan assays established based on sequence information extracted from Ensembl, comprising 100 to 200 bp sequences on both sides of the polymorphisms. Primers and probes were designed for wild type and mutated sequences, respectively, by Thermo Fischer Scientific, Denmark using VIC for wild type alleles and FAM for variant alleles. TaqMan real-time PCR was carried out as follows on the Mx3005P platform (Agilent, Glostrup, Denmark): 1 μL of genomic DNA (20-25 ng/μL) was amplified in a total volume of 10 μL reaction mastermix containing 5.0 μL TaqMan Universal Mix, 0.25 μL TaqMan enzyme (Thermo Fischer Scientific, Denmark) and 3.75 μL $H_2O$. PCR conditions were: 1 cycle of 50°C for 2 minutes, 1 cycle of 95°C for 10 minutes, and 45 cycles of 92°C for 20 seconds followed by 60°C for 1 minute. Water was included as negative control and results were analyzed by MxPro qPCR Software (Agilent, Glostrup, Denmark).

## 2.4 | ELISA

Serum 5-HT concentration was measured with a 5-HT ELISA kit (RE59121, IBL International GMBH, Hamburg, Germany) that was previously validated in dogs.[12,14] All samples were analyzed in duplicate, and 3 interassay controls were included on each plate. Results were calculated as the mean value of the duplicate measurements. Samples with a coefficient of variation (CV) above 10% were reanalyzed.

## 2.5 | HPLC

Serum concentrations of 5-HT and the additional serum neurotransmitters (5-HIAA, adrenaline, noradrenaline, MHGP, dopamine, DOPAC, and HVA) were measured by HPLC using electrochemical detection. Briefly, 175 μL serum was acidified with 20 μL 1 N HCl and diluted with 530 μL 0.5 M ammonium acetate. The solution was put on a weak cation exchange solid phase extraction column (WCX-SPE part no. 186002503; Waters, Denmark) preconditioned with 1 mL methanol and 2 mL water. The column was washed with 1 mL 20 mM ammonium acetate and 1 mL of methanol and the analytes were eluted with 400 μL 85% acetonitrile containing 2% formic acid. The eluate was dried in a vacuum concentrator (Heto-Holten, Denmark) for 2 hours and resuspended in 35 μL analysis buffer, centrifuged (1 minute at 15 000*g*) and finally, 20 μL was injected onto the HPLC column. Separation and quantification was achieved essentially as described and validated previously.[21] All samples were run in duplicate.

## 2.6 | Echocardiography and electrocardiography

The echocardiographic examinations (VividE95 echocardiograph with a 5Sc transducer, GE Healthcare Denmark A/S) were performed by 2 experienced operators. Images were analyzed offline (EchoPAC PC. Version 202, GE Medical systems, Brøndby, Denmark) by 1 operator blinded to the identity of dogs and clinical characteristics. Transthoracic echocardiographic examination followed a standardized echocardiographic protocol modified from Reimann et al.[30] Echocardiographic measurements were assessed as the mean of at least 3 cardiac cycles. Mitral regurgitation (MR) severity was evaluated from left apical 4-chamber view by color

**TABLE 1** Probes used in TaqMan assays

| Genetic variation | Location | Dye | Sequence | Variant |
|---|---|---|---|---|
| SLC6A4_814wt | Insertion of G. Chromosome 9, bp: 44242179 | FAM | TGTTTTGACACCTTTCCAG | Wildtype |
| SLC6A4_814insG | | VIC | TGTTTTGACACCTcTTCCAG | Insertion |
| SLC6A4_1193delT | Deletion of T. Chromosome 9, bp: 44239810 | FAM | CATCTTTGGCCCCTCAG | Deletion |
| SLC6A4_1193wt | | VIC | ATCTTTGGCCaCCTCAG | Wildtype |
| SLC6A4_1324A[a] | G/A. Chromosome 9, bp: 44237789 | FAM | CCCTCCAAGCtTGCAAA | Substitution |
| SLC6A4_1324G[a] | | VIC | CCCTCCAAGCcTGCAAA | Wildtype |

[a]A typographical error in the reported amino acid alteration have been confirmed in a personal communication by Lee et al.[19] The reported amino acids in the c1324 location (Gly > Arg) should have been Gly > Ser.

**TABLE 2** Dog characteristics, echocardiographic variables, and circulating serotonin concentrations in 71 Cavalier King Charles Spaniels classified in stages of myxomatous mitral valve disease (MMVD) modified from the American College of Veterinary Internal Medicine guidelines

| | MMVD stage Am (n = 19 ) | MMVD stage B1 (n = 22) | MMVD stage B2 (n = 17) | MMVD stage C (n = 13) | Overall *P*-value |
|---|---|---|---|---|---|
| Sex (F/M, %) | 16/3 (84%/16%) | 11/11 (50%/50%) | 7/10 (41%/59%) | 8/5 (62%/38%) | .04 |
| Age (years) | 4.1 [1.7;4.7]$^{B1,B2,C}$ | 8.4 [5.1;9.8]$^{A}$ | 8 [7.3;10.6]$^{A}$ | 10.5 [9.6;10.8]$^{A}$ | <.001 |
| BW (kg) | 8.3 [7.6;9.3] | 9.2 [8.5;9.8] | 9.1 [8.2;10.9] | 9.4 [8.6;9.7] | .11 |
| SBP (mm Hg) | 132 [127;136] | 152 [132;166] | 151 [129;162]$^{16}$ | 134 [123;144]$^{12}$ | .04 |
| DBP (mm Hg) | 76 [70;82] | 84 [76;88]$^{C}$ | 80 [72;90]$^{16}$ | 71 [68;76]$^{B1,12}$ | .01 |
| MBP (mm Hg) | 94 [91;100]$^{B1}$ | 109 [95;115]$^{A}$ | 106 [96;115]$^{16}$ | 93 [88;103]$^{12}$ | .007 |
| MR severity (1/2/3) | 19/0/0 | 0/13/9 | 0/1/16 | 0/0/13 | Not tested |
| LA/Ao | 1.2 [1.2;1.3] | 1.3 [1.3;1.5] | 1.8 [1.7;1.9] | 2.4 [2.1;2.7] | Not tested |
| LVIDSN | 1 [0.9;1]$^{B1,B2,C}$ | 1.1 [1;1.1]$^{A}$ | 1.1 [1;1.2]$^{A}$ | 1.1 [1.1;1.3]$^{A}$ | .0003 |
| LVIDDN | 1.4 [1.3;1.5] | 1.6 [1.5;1.7] | 1.9 [1.8;2.1] | 2.2 [2.1;2.4] | Not tested |
| FS (%) | 28 [26;32]$^{B2,C}$ | 33 [28;36]$^{B2,C}$ | 38 [35;45]$^{A,B1}$ | 44 [41;46]$^{A,B1}$ | <.001 |
| Emax (m/s) | 0.7 [0.7;0.8]$^{B2,C,17}$ | 0.8 [0.7;0.9]$^{C,21}$ | 0.9 [0.9;1.1]$^{A,15}$ | 1.2 [1.1;1.4]$^{A,B1}$ | <.001 |
| PLT ($\times 10^6$ platelets/mL) | 152 [94;273] | 166 [141;328] | 99 [84;267] | 293 [272;377] | .13 |
| TCP (y/n, %) | 5/14 (26%/74%) | 5/17 (23%/77%) | 9/8 (53%/47%) | 2/11 (15%/85%) | .12 |
| Serum 5-HT, HPLC (ng/mL) | 386 [298;510] | 380 [302;536] | 350 [251;429] | 393 [365;463] | .75 |
| Serum 5-HT, ELISA (ng/mL) | 372 [297;507] | 383 [249;534] | 329 [249;383] | 344 [336;414] | .72 |
| Serum 5-HIAA (nM) | 0.79 [0.69;1] | 0.91 [0.75;1.21] | 0.89 [0.65;1.14] | 0.87 [0.62;1.1] | .80 |
| Serum adrenaline (nM) | 0.84 [0.63;1.3] | 0.65 [0.43;1.08] | 0.67 [0.32;1.13] | 0.74 [0.6;1.16] | .74 |
| Serum noradrenaline (nM) | 2.91 [2.27;3.69] | 3.01 [2.01;4.51] | 4.13 [2.29;4.61] | 3.95 [3.71;4.6] | .16 |
| Serum MHGP (nM) | 133 [111;159] | 123 [101;182] | 154 [107;189] | 136 [107;186] | .93 |
| Serum dopamine (nM) | 0.52 [0.19;1.27] | 0.75$^{20}$ [0.24;1.4] | 0.76 [0.44;1.31] | 0.61 [0.42;1.93] | .68 |
| Serum DOPAC (nM) | 4.12 [3.08;5.69] | 4.29 [3.00;5.63] | 3.23 [2.87;4.08] | 3.11 [2.55;4.19] | .21 |
| Serum HVA (nM) | 2.21 [1.81;2.43] | 2.04 [1.38;3.16] | 1.77 [1.33;2.34] | 2.05 [1.4;2.19] | .54 |

*Note*: Values reported are median and interquartiles. Within each row, superscripts $^{A,B1,B2,C}$ represent the group from which there is statistically significant difference assessed by Wilcoxon rank sum test with Bonferroni adjustment. Superscript numbers refer to reduced number of observations (serum dopamine was below detection limit in 2 dogs). Categorical variables were tested by Fisher's exact test. Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, serotonin; BW, body weight; DBP, diastolic blood pressure; DOPAC, 3,4-dihydroxyphenylacetic acid, Emax, early E wave transmitral peak velocity; F, female; FS, fractional shortening; HVA, homovanillic acid; LA/Ao, ratio of left atrium-to-aortic root; LVIDDN, left ventricular end diastolic diameter normalized for BW; LVIDSN, left ventricular end systolic diameter normalized for BW; M, male; MBP, mean blood pressure; MHGP, 3-methoxy-4-hydroxyphenylglycol; MR severity, mitral regurgitation severity assessed by jet area method where 1 = no or minimal MR (<20%), 2 = mild MR (20%-50%), 3 = moderate-severe MR (>50%); PLT, platelet count in EDTA-anticoagulated blood; SBP, systolic blood pressure; TCP, thrombocytopenia defined as dogs with platelet count <100 $\times$ $10^6$ platelets/mL in EDTA-anticoagulated blood.

Doppler based on the regurgitant jet area relative to left atrial (LA) area (gain set just below the color sparkling artifact in myocardium and the Nyquist limit of −0.56;−0.8 m/s).[31] Left atrial-to-aortic root ratio (LA/Ao) 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.[32,33] Furthermore, the end-systolic and diastolic diameters of the left ventricle were measured at chordae tendineae level (identified in a 2D image before placement of the M-mode cursor) in M-mode.[34] These variables were used to calculate fractional shortening and normalized to bodyweight.[35] Finally, the early E wave transmitral peak velocity was measured at the tip of the mitral valve leaflets from left apical 4-chamber view by pulsed-wave Doppler.[36]

Severity of MMVD was classified as MMVD stages modified from American College of Veterinary Internal Medicine consensus statement guidelines as follows: stage A modified (stage Am), CKCS with no auscultatory heart murmur and no or minimal MR (MR <20%); stage B1, CKCS with auscultatory heart murmur or MR ≥20% and echocardiographic evidence of MMVD but no cardiac enlargement; stage B2, CKCS with current or previous echocardiographic evidence of cardiac enlargement but no current or previous clinical signs of CHF; stage C, CKCS with CHF.[37,38] Thus, the MMVD stage Am included dogs with mild structural disorder of the heart (MR <20%) because such minimal MRs have been demonstrated not necessarily to have clinical or prognostic significance for MMVD in CKCS.[39] Cardiac enlargement was defined as LA/Ao ≥1.6 and left ventricular end-diastolic diameter normalized to bodyweight ≥1.7.[38] In addition to presence of MR, the diagnosis of MMVD was based on the presence of mitral valve thickening, prolapse or both. The diagnosis of CHF was based on a history of MMVD, previous or current clinical signs of CHF (eg, cough, dyspnea, tachypnea, nocturnal restlessness, and exercise intolerance), echocardiographic changes compatible with severe MMVD and response to diuretic treatment. Thoracic radiographs were not included in the examination.

**TABLE 3** Medical treatment for 71 included Cavalier King Charles Spaniels

| Stages | No. of dogs receiving medication | Medical treatment |
|---|---|---|
| MMVD stage Am (n = 19) | 0 dogs | |
| MMVD stage B1 (n = 22) | 5 dogs | Benazepril hydrochloride (n = 2)[a], cefadroxil monoydrate (n = 1), deslorelin (n = 1), levothyroxine sodium (n = 2), pregabalin (n = 1) |
| MMVD stage B2 (n = 17) | 6 dogs | Estriol (n = 1), furosemide (n = 2), pimobendan (n = 6), robenacoxib (n = 1) |
| MMVD stage C (n = 13) | 12 dogs | Benazepril hydrochloride (n = 3), clonazepam (n = 1), meloxicam (n = 1), oclacitinib (n = 1), furosemide (n = 8), pimobendan (n = 10), sildenafil (n = 1), spironolactone (n = 2) |

[a]In 1 of the dogs treatment ended 14 days before the examination.
Abbreviation: MMVD, myxomatous mitral valve disease.

## 2.7 | Statistical analysis

Data were analyzed by statistical software (R studio, version 1.1.383, 2009-2017 RStudio, Inc, Boston, Massachusetts) and a *P*-value <.05 was considered significant unless otherwise stated.

No previous data were available estimating the prevalence of the selected SERT polymorphisms in CKCS to perform sample size calculation. Instead, a sample size calculation was performed a priori by previously published data comparing serum 5-HT concentration in dogs with no MMVD (median, 657 ng/mL) and severe MMVD (median, 513 ng/mL).[14] These data indicated that a sample size of minimally 17 dogs per group was necessary to demonstrate a statistical significant difference in 5-HT between groups with an alpha of 0.05 and power of 0.80.

Descriptive data are presented as medians and interquartiles and overall group differences were investigated by a nonparametric Kruskal-Wallis test because many groups did not follow a normal distribution. Wilcoxon rank sum test with Bonferroni adjustment was used to assess differences among MMVD stages (except for LA/Ao and LVIDDN as these variables were used to allocate dogs into

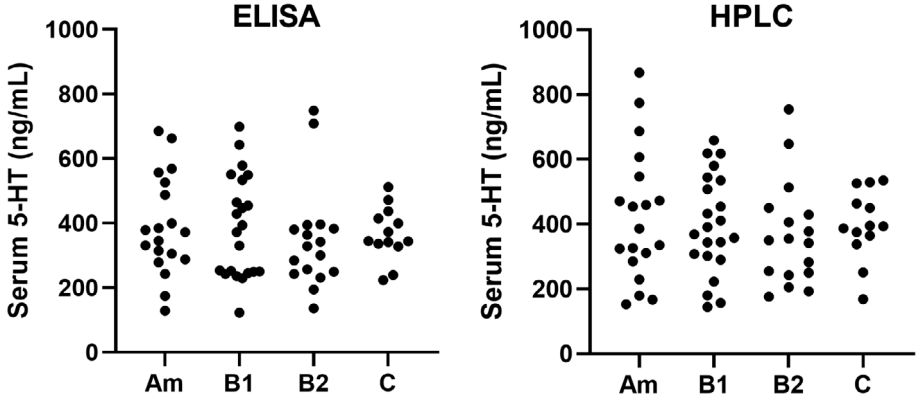


**FIGURE 1** Raw data plot showing serum serotonin (5-HT) concentration measured by ELISA (left) and HPLC (right) in 71 included Cavalier King Charles Spaniels allocated into stages of myxomatous mitral valve disease modified from American College of Internal Veterinary Medicine consensus guidelines

MMVD stages). Categorical data were compared between groups by Fisher's exact test (except for MR severity as this variable was used to allocate dogs into MMVD stages).

Univariable analyses were used to investigate associations between serum 5-HT concentration and dog characteristics/clinical variables (MMVD severity, age, sex, platelet count in EDTA-anticoagulated blood, and medical therapy [yes/no]).

Multivariable regression analyses were performed with ELISA and HPLC 5-HT concentration as response variables. Explanatory variables included the dog characteristics/clinical variables from univariable analyses. Covariance of the explanatory variables was assessed by visual inspection of scatter plots and calculation of the variance inflation factor. Disease severity variables included MMVD stage and LA/Ao, and because of high covariance, they were tested in separate multivariable models. Model residuals were tested for homogeneity of variation based on visual inspection, QQ plots, and the Shapiro-Wilk test. Backward stepwise elimination was based on *P*-values.

Correlation between serum 5-HT and serum 5-HIAA concentrations was evaluated by Pearson's correlation.

Intraassay and interassay CV% were calculated as (SD/mean) × 100 for ELISA 5-HT measurements.

Correlation between ELISA and HPLC serum 5-HT measurements were tested by Spearman's rank correlation because of nonnormal distribution and visualized by Bland-Altman plot. Because the differences between ELISA and HPLC measurements were normally distributed, a paired *t* test was used to determine if the 2 methods differed significantly. To determine if there was a concentration dependent difference, the correlation between the mean and difference of ELISA and HPLC measurements was tested by Spearman's rank correlation.

## 3 | RESULTS

A total of 80 CKCS were recruited, but 9 were excluded because serum biochemistry analysis indicated considerable systemic disease (n = 2) or lack of sufficient blood for analyses (n = 7). The final population of 71 CKCS were allocated to the following MMVD stages: stage Am (n = 19), stage B1 (n = 22), stage B2 (n = 17) and stage C (n = 13). Three CKCS (MMVD stage C: n = 1; MMVD stage B2: n = 2) treated with pimobendan were classified as having cardiac enlargement despite having LA/Ao < 1.6, LVIDDN <1.7 or both because it had been demonstrated at previous echocardiographic examinations before onset of cardiac therapy.[40]

A summary of descriptive data for the 4 MMVD stages is given in Table 2. No difference between stages in any of the included serum neurotransmitters was found (Figure S1). In Table 3, the medical treatment for included dogs is summarized.

All enrolled dogs were successfully genotyped for the c.814insG, c.1193delT, and 70 dogs (99%) for the c.1324G>A. All dogs were homozygous for the wildtype allele in all 3 SNPs. Thus, it was not possible to statistically test the association between genotype and MMVD stage or serum 5-HT concentration nor to include the interaction between genotype and MMVD stage in the statistical analysis. But it was concluded that MMVD stage and serum 5-HT concentration were not associated with genotype.

**TABLE 4** *P*-values from univariable analyses evaluating the effect of selected variables on serum serotonin (5-HT) measured with ELISA and HPLC, respectively, in 71 Cavalier King Charles Spaniels

| | Response variables | |
|---|---|---|
| | Serum 5-HT, HPLC | Serum 5-HT, ELISA |
| MMVD stage | 0.78 | 0.82 |
| LA/Ao | 0.52 | 0.8 |
| Age | 0.51 | 0.78 |
| Sex | 0.034 | 0.13 |
| PLT count | <0.001 | <0.001 |
| Medical therapy | 0.96 | 0.93 |

Abbreviations: MMVD, myxomatous mitral valve disease; PLT, platelet count in EDTA-anticoagulated blood.

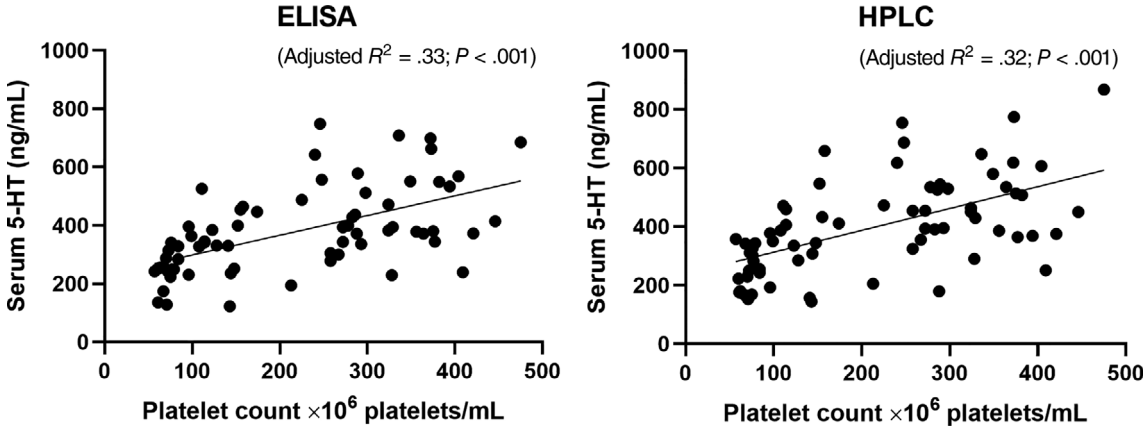


**FIGURE 2** Association between serum serotonin (5-HT) concentration measured by ELISA (left) and HPLC (right) and platelet count in EDTA-anticoagulated blood in 71 Cavalier King Charles Spaniels

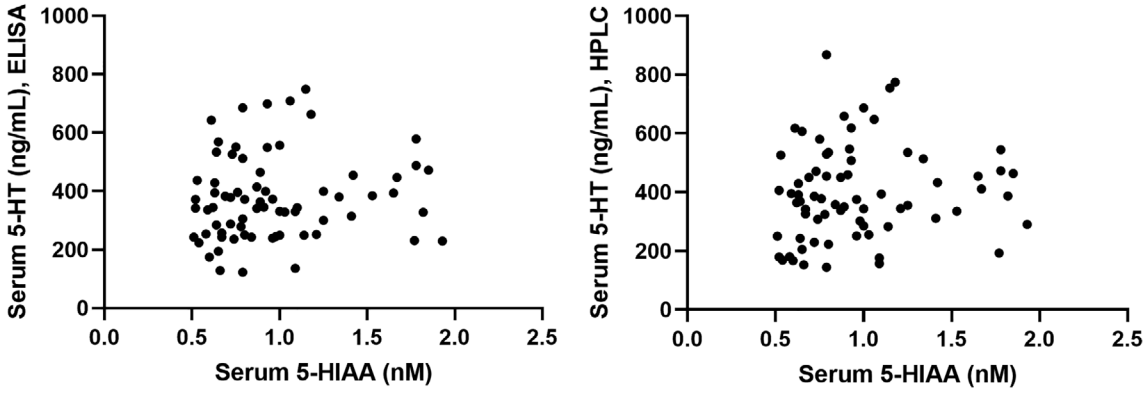


**FIGURE 3** Raw data plot of serum serotonin (5-HT) concentration measured by ELISA (left) and HPLC (right) and the metabolite 5-hydroxyindoleacetic acid (5-HIAA) in 71 Cavalier King Charles Spaniels

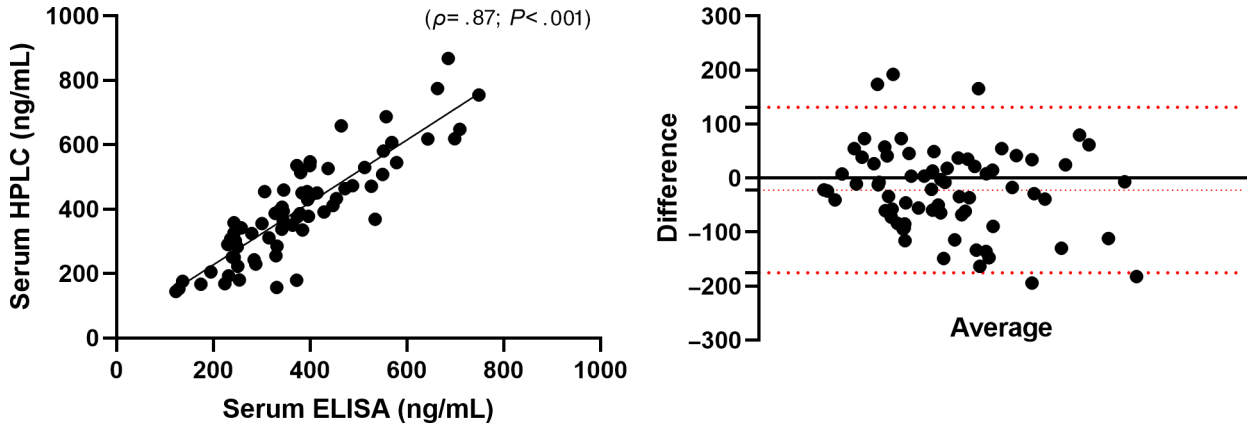


**FIGURE 4** Raw data plot (left) showing the correlation between serum serotonin concentration measured by ELISA and HPLC in 71 included Cavalier King Charles Spaniels. Bland-Altman plot (right) comparing HPLC and ELISA measurements. The outer dotted lines represent 95% limits of agreement. Upper limit of agreement: 131.35, confidence interval (CI) [99.55;163.15], lower limit of agreement: −175.36, CI [−207.16;−143.56]. The intermediate dotted line represents the mean of the deviation −22.00, CI [−40.52;−3.49]

In Figure 1, the serum 5-HT concentration is presented for the different MMVD stages. In univariable analyses, a positive association was found between platelet count and serum 5-HT measured by both HPLC and ELISA (Figure 2, Table 4). Females (399 ng/mL [326;471], median [quartile 1;quartile 3]) had higher serum 5-HT concentration compared to males (343 ng/mL [223;508]) but only when measured by HPLC. Multivariable regression analysis confirmed the effect of platelet count on serum 5-HT concentration. Disease severity (both MMVD stage and LA/Ao), age, sex, and medical therapy did not influence serum 5-HT concentration.

Neither serum 5-HT measured by HPLC nor ELISA correlated with serum 5-HIAA ($\rho = .11$; $P = .35$ and $\rho = .11$; $P = .36$, respectively; Figure 3).

The ELISA 5-HT concentration was reanalyzed in 12 dogs because of intraassay coefficients of variation (CV) >10% and results with the lowest CV were used in the data analysis. After reanalysis, intraassay CV from in-house validation was 4.1%. Interassay CV was 14.6%. Correlation analyses showed that serum 5-HT concentration measured by HPLC correlated with ELISA measurements ($\rho = .87$; $P < .001$) but ELISA measurements were lower (mean difference = −22.00 ng/mL; $P = .02$) indicating a constant systematic difference (Figure 4). The difference was independent of serum 5-HT concentration ($\rho = -.17$, $P = .2$).

## 4 | DISCUSSION

The 3 selected polymorphisms in the SERT gene that have been associated with MMVD in Maltese dogs were shown to be monomorphic in CKCS with MMVD. Serum 5-HT was positively associated only with platelet count and serum 5-HT concentration measured by HPLC and ELISA correlated well, independent of serum 5-HT concentration. Yet, ELISA values were slightly albeit significantly lower than HPLC results indicating a systematic underestimation. No difference in the selected serum neurotransmitters between the MMVD stages was detected.

The etiology and pathogenesis of MMVD in dogs have not been fully elucidated but recent research has focused on the possible influence of 5-HT. Different lines of evidence suggest an association

between 5-HT and MMVD as reviewed by Oyama et al.[8] Briefly, these include the ability of 5-HT to activate human and dog valve interstitial cells, increased transcription and expression of 5-HT pathway components in affected mitral valve tissue, increased 5-HT concentration (valvular and circulating) in dogs with MMVD, development of valve lesions secondary to either exogenous 5-HT, 5-HT-producing tumors or serotonergic drugs, and finally the ability of 5-HT receptor antagonists to block valvular interstitial cell activation and mitigate histological changes of MMVD in experimental models.[8]

Previous studies indicate higher platelet and circulating 5-HT concentration, with high individual variation, in certain breeds such as CKCS and Maltese dogs compared to other breeds[12-17] and high platelet and plasma 5-HT concentrations have been suggested to represent a familial trait among CKCS, which could result in the early onset of MMVD in the CKCS.[13] Circulating 5-HT concentration is controlled by SERT, and SERT downregulation has been associated with valvular disease. Development of valvular fibrosis was found in SERT knockout mice[41] and downregulation of SERT expression was shown in aortic and mitral valves of rats treated with 5-HT SC for 7 days.[42] It has been hypothesized that genetic variations of SERT can alter elimination of 5-HT and thereby affect susceptibility to valvular disease.[43]

Alterations in SERT expression and protein levels in dogs with MMVD have also been reported.[10,11] In Maltese dogs, 3 SNPs in the SERT gene have been associated with MMVD and predicted to be damaging to SERT protein function and structure.[19] Yet, these selected SNPs in the SERT gene were not present in any of the included CKCS in the current study and are therefore highly unlikely to be associated with MMVD pathophysiology or serum 5-HT concentration in this breed. This finding is supported by previous genomic research in the CKCS, which has not identified associations between the SERT gene and MMVD in CKCS.[44-47] Neither did a subsequent genome-wide association study in Maltese dogs.[48]

Previous studies have indicated higher serum 5-HT concentration in mild stages of MMVD in dogs compared to later stages, suggesting a role of 5-HT in the early development of disease.[14,17] Yet, in accordance with others,[18,49,50] the current study found large group variation in serum 5-HT and no association between circulating 5-HT and MMVD severity in CKCS was detected. The conflicting results might be a result of differences in disease classification or study population and a potential role of 5-HT in MMVD in dogs is not yet clear.

Approximately one-third of CKCS present with inherited macrothrombocytopenia that usually does not precipitate clinical signs of disease,[29,51,52] which is interesting as 5-HT is stored in platelets.[53] Indeed, platelet count was the only explanatory variable that affected serum 5-HT concentration in the current study, for which a weak to moderate positive association was found. Similar findings were reported in a previous study with CKCS, where CKCS with thrombocytopenia, defined as platelet count $<100 \times 10^6$ platelets/mL, had lower median serum 5-HT (482 ng/mL) compared to non-thrombocytopenic CKCS (731 ng/mL).[17] In contrast, 2 previous studies found no association between serum 5-HT concentration and platelet size and count.[12,14] The disagreement between these findings is uncertain but may be explained by different methods of quantifying platelets, variable presence of macroplatelets or different means of classifying dogs as having or not having thrombocytopenia. The clinical significance of lower serum 5-HT concentrations in CKCS with thrombocytopenia warrants further research.

Some previous studies have reported higher serum 5-HT concentrations in female dogs compared with males[14,15] or found a rather weak negative association with age.[12,17] In the present study, the same tendency was seen for female dogs. In univariable analysis, sex influenced serum 5-HT measured by HPLC, but this effect could not be confirmed in multivariable analysis. Thus, in accordance with others, the present study found no effect of sex[16,17] or age[14-16] on serum 5-HT concentration.

Median serum 5-HT concentration was considerably lower (approximately 50%) than concentrations reported in previous studies by the same ELISA assay.[12,14,16-18] In contrast, serum 5-HT concentrations are comparable to those recently reported by Höglund et al. in a study concerning interbreed 5-HT variation.[15] It was speculated that storage time or sample handling (samples were kept at −20°C and had been thawed and refrozen once) might explain this difference. However, it seems unlikely in the present study, since storage time of serum was relatively short (<17 months), samples were kept at −80°C and were not thawed and refrozen. Another study demonstrated that storage time did not affect serum 5-HT concentration in samples stored up to 2 years at −80°C.[14] Analytical error does not seem likely because both of the analytical methods (ELISA and HPLC) found similar serum 5-HT concentrations. Although ELISA results were lower than HPLC results, the difference was rather small (−22.00 ng/mL in average) and not nearly enough to reach similar ELISA serum 5-HT concentrations as described by others. Serum sample preparation involves clot formation leading to a variable degree of platelet aggregation and activation, which in turn may result in large variability in serum 5-HT measurements,[54] yet sample preparation protocol was strictly standardized and the process was not apparently different from sample preparation in previous research. The association between serum 5-HT and platelet count could also explain divergence from previously reported serum 5-HT concentrations, yet, the proportion of dogs with macrothrombocytopenia in the current study (30%) was not higher than the previous studies[12,14] or what has been reported as normal for the CKCS breed[29,51,52] and platelet count was similar to previous studies.[16-18] Hence, the reason for the lower serum 5-HT concentration in the current study remains unclear.

In humans, serum 5-HT and urinary 5-HIAA are used in the diagnosis and monitoring of 5-HT producing neuroendocrine tumors.[22] Serum 5-HIAA concentration may represent a future tool for diagnosing and monitoring in these patients and an association between serum 5-HT with serum 5-HIAA has been reported in humans.[55] Yet, a recent study in dogs with MMVD did not find an association with urinary 5-HIAA nor between urinary 5-HIAA and serum 5-HT,[18] which is in accordance with the current study.

Neurohormal activation involving various neurohormonal systems likely play an important role in MMVD.[20] Circulating concentrations of few of these neurotransmitters have been associated with MMVD

severity in dogs previously. Studies have reported that CHF because of MMVD in dogs is associated with higher circulating concentrations of noradrenaline and in some studies also adrenaline,[23-25] whereas this association has not been found in dogs with less severe MMVD.[56] In the current study, the serum concentration of none of the included monoamine neurotransmitters or their metabolites (5-HIAA, adrenaline, noradrenaline, MHGP, dopamine, DOPAC, and HVA) differed between MMVD stages including stage C. However, it is worth considering that most dogs in MMVD stage C in the present study presented with mild clinical signs and were treated with low doses of diuretics at enrolment. Therefore, results do not necessarily reflect dogs with more severe or uncontrolled CHF.

Several methods are available for measuring circulating 5-HT.[57] Serum 5-HT concentrations have been measured by ELISA in dogs[12-16] while HPLC is more widely used in human studies and to the authors' knowledge, HPLC has not been used previously to measure serum 5-HT in dogs.[57] The correlation between ELISA and HPLC measurements in the current study was good although ELISA results were lower than HPLC. However, the difference between the 2 methods was small and not concentration dependent and likely without clinical relevance. Therefore, ELISA measurement of serum 5-HT does seem an appropriate alternative if HPLC is not available.

The present study has limitations. Diets and supplements were not standardized and certain food items such as banana, chocolate, and nuts are rich in the 5-HT precursor, tryptophan, which may increase circulating 5-HT concentration.[58] Although these items are rarely included in dog diets and all dogs were food fasted 6 to 18 hours before blood sampling to minimize the effect of food intake, we cannot exclude variations in serum 5-HT caused by dietary intake.

Diverse medication profiles of the dogs is another limitation. Individual dogs respond differently to cardiac treatment and therefore, it was not considered ethically justifiable to standardize cardiac medications of dogs with MMVD. Few dogs were medicated for noncardiac conditions and were included in the study because such a study group represent a good reflection of the normal population of CKCS with MMVD. Yet, several drugs have been associated with valvular disease through 5-HT related pathways.[8,43] No dogs were treated with drugs with known serotonergic effect (yet 1 dog was treated with NSAID) and in regression analyses medical therapy was not found to influence serum 5-HT concentration, but an effect of medical treatment cannot be excluded.

Limitations related to group size apply. According to sample size calculations, a minimum of 17 dogs should have been included in each group. However, only 13 dogs in stage C were included which may have limited the power and increased the risk of type II errors when concluding that serum 5-HT did not differ between MMVD stages.

Dogs in the different MMVD stages were not matched with regards to age and sex. As mentioned, previous studies have reported diverging results regarding the influence of age and sex on serum 5-HT. In univariable analysis of the current study, a higher serum 5-HT measured with HPLC was found in females compared to males, but in multivariable analyses, no associations were found between serum 5-HT and age or sex, respectively, suggesting that age and sex differences among the study groups did not influence study results.

Finally, thoracic radiographs were not included as part of the criterion for diagnosing dogs as MMVD stage C which may potentially have caused a misclassification of some dogs.

## 5 | CONCLUSIONS

Previously identified SERT SNPs possibly associated with MMVD in Maltese dogs were not found in CKCS and are therefore unlikely to be associated with MMVD pathophysiology or serum 5-HT concentration in this breed. Serum 5-HT was associated with platelet count but not MMVD severity and did not correlate with serum 5-HIAA. The HPLC and ELISA serum 5-HT measurements indicated good correlation although ELISA slightly but systematically underestimated 5-HT concentrations compared to HPLC. None of the included serum neurotransmitters (5-HIAA, adrenaline, noradrenaline, MHGP, dopamine, DOPAC, and HVA) differed between MMVD stages.

### ACKNOWLEDGMENT

The study was supported financially by a research grant from the Independent Research Fund Denmark (Project no. 7017-00131B). Authors thank Marianne Kjestine Petersen, Tina Bahrt Neergaard Mahler, Charlotte Bjørner Larsen and Ricki Thanning at the Department of Veterinary and Animal Sciences, University of Copenhagen, Denmark for excellent technical assistance. Preliminary results were presented at the European College of Veterinary Internal Medicine—Companion Animals (ECVIM-CA) Congress 2020, 3-5th September 2020.

### CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

### OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

### INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approved by the Danish Animal Experiments Inspectorate (license no. 2016-15-0201-01074).

### HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

### ORCID

*Maria J. Reimann* https://orcid.org/0000-0002-7824-3323
*Jens Häggström* https://orcid.org/0000-0003-3402-023X

### REFERENCES

1. Egenvall A, Bonnett BN, Häggström J. Heart disease as a cause of death in insured Swedish dogs younger than 10 years of age. *J Vet Intern Med*. 2006;20(4):894-903.
2. Darke PG. Valvular incompetence in Cavalier King Charles Spaniels. *Vet Rec*. 1987;120(15):365-366.

3. Häggström J, Hansson K, Kvart C, Swenson L. Chronic valvular disease in the Cavalier King Charles Spaniel in Sweden. *Vet Rec*. 1992; 131(24):549-553.
4. Buchanan JW. Chronic valvular disease (endocardiosis) in dogs. *Adv Vet Sci Comp Med*. 1977;21:75-106.
5. Pavone LM, Norris RA. Distinct signaling pathways activated by "extracellular" and "intracellular" serotonin in heart valve development and disease. *Cell Biochem Biophys*. 2013;67(3):819-828.
6. Buskohl PR, Sun ML, Thompson RP, Butcher JT, Butcher JT. Serotonin potentiates transforming growth factor-beta3 induced biomechanical remodeling in avian embryonic atrioventricular valves. *PLoS One*. 2012;7(8):e42527.
7. Fligny C, Fromes Y, Bonnin P, et al. Maternal serotonin influences cardiac function in adult offspring. *FASEB J*. 2008;22(7):2340-2349.
8. Oyama MA, Elliott C, Loughran KA, et al. Comparative pathology of human and canine myxomatous mitral valve degeneration: 5HT and TGF-β mechanisms. *Cardiovasc Pathol*. 2020;46:107196.
9. Ayme-Dietrich E, Lawson R, Da-Silva S, Mazzucotelli JP, Monassier L. Serotonin contribution to cardiac valve degeneration: new insights for novel therapies? *Pharmacol Res*. 2019;140:33-42.
10. Disatian S, Orton EC. Autocrine serotonin and transforming growth factor beta 1 signaling mediates spontaneous myxomatous mitral valve disease. *J Heart Valve Dis*. 2009;18(1):44-51.
11. Scruggs SM, Disatian S, Orton EC. Serotonin transmembrane transporter is down-regulated in late-stage canine degenerative mitral valve disease. *J Vet Cardiol*. 2010;12(3):163-169.
12. Arndt JW, Reynolds CA, Singletary GE, Connolly JM, Levy RJ, Oyama MA. Serum serotonin concentrations in dogs with degenerative mitral valve disease. *J Vet Intern Med*. 2009;23(6):1208-1213.
13. Cremer SE, Singletary GE, Olsen LH, et al. Serotonin concentrations in platelets, plasma, mitral valve leaflet, and left ventricular myocardial tissue in dogs with myxomatous mitral valve disease. *J Vet Intern Med*. 2014;28(5):1534-1540.
14. Ljungvall I, Höglund K, Lilliehöök I, et al. Serum serotonin concentration is associated with severity of myxomatous mitral valve disease in dogs. *J Vet Intern Med*. 2013;27(5):1105-1112.
15. Höglund K, Häggström J, Hanås S, et al. Interbreed variation in serum serotonin (5-hydroxytryptamine) concentration in healthy dogs. *J Vet Cardiol*. 2018;20(4):244-253.
16. Roels E, Krafft E, Antoine N, et al. Evaluation of chemokines CXCL8 and CCL2, serotonin, and vascular endothelial growth factor serum concentrations in healthy dogs from seven breeds with variable predisposition for canine idiopathic pulmonary fibrosis. *Res Vet Sci*. 2015; 101:57-62.
17. Cremer SE, Kristensen AT, Reimann MJ, et al. Plasma and serum serotonin concentrations and surface-bound platelet serotonin expression in Cavalier King Charles Spaniels with myxomatous mitral valve disease. *Am J Vet Res*. 2015;76(6):520-531.
18. Christiansen LB, Cremer SE, Helander A, et al. Urine 5-hydroxyindoleacetic acid in Cavalier King Charles Spaniels with preclinical myxomatous mitral valve disease. *Vet J*. 2019;250:36-43.
19. Lee C-M, Han J-I, Kang M-H, Kim S-G, Park H-M. Polymorphism in the serotonin transporter protein gene in Maltese dogs with degenerative mitral valve disease. *J Vet Sci*. 2018;19(1):129-135.
20. Oyama MA. Neurohormonal activation in canine degenerative mitral valve disease: implications on pathophysiology and treatment. *J Small Anim Pract*. 2009;50(suppl 1):3-11.
21. Schou-Pedersen AMV, Hansen SN, Tveden-Nyborg P, Lykkesfeldt J. Simultaneous quantification of monoamine neurotransmitters and their biogenic metabolites intracellularly and extracellularly in primary neuronal cell cultures and in sub-regions of Guinea pig brain. *J Chromatogr B Anal Technol Biomed Life Sci*. 2016;1028:222-230.
22. Oberg K, Couvelard A, Delle Fave G, et al. ENETS consensus guidelines for the standards of care in neuroendocrine tumors: biochemical markers. *Neuroendocrinology*. 2017;105(3):201-211.
23. Marcondes Santos M, Strunz CMC, Larsson MHMA. Correlation between activation of the sympathetic nervous system estimated by plasma concentrations of norepinephrine and Doppler echocardiographic variables in dogs with acquired heart disease. *Am J Vet Res*. 2006;67(7):1163-1168.
24. Uechi M, Shimizu A, Mizuno M. Heart rate modulation by sympathetic nerves in dogs with heart failure. *J Vet Med Sci*. 2002;64(11): 1023-1029.
25. Ware WA, Lund DD, Subieta AR, Schmid PG. Sympathetic activation in dogs with congestive heart failure caused by chronic mitral valve disease and dilated cardiomyopathy. *J Am Vet Med Assoc*. 1990; 197(11):1475-1481.
26. Gompf RE. The clinical approach to heart disease: history and physical examination. In: Fox PR, ed. *Canine and Feline Cardiology*. New York: Churchill Livingstone; 1988:29-42.
27. Hanzlicek AS, Baumwart RD, Payton ME. Systolic arterial blood pressure estimated by mitral regurgitation velocity, high definition oscillometry, and Doppler ultrasonography in dogs with naturally occurring degenerative mitral valve disease. *J Vet Cardiol*. 2016;18(3): 226-233.
28. Acierno MJ, Brown S, Coleman AE, et al. ACVIM consensus statement: guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. *J Vet Intern Med*. 2018; 32(6):1803-1822.
29. Eksell P, Häggström J, Kvart C, Karlsson A. Thrombocytopenia in the Cavalier King Charles Spaniel. *J Small Anim Pract*. 1994;35(3): 153-155.
30. Reimann MJ, Moller JE, Häggström J, et al. R-R interval variations influence the degree of mitral regurgitation in dogs with myxomatous mitral valve disease. *Vet J*. 2014;199(3):348-354.
31. Pedersen HD, Häggström J, Falk T, et al. Auscultation in mild mitral regurgitation in dogs: observer variation, effects of physical maneuvers, and agreement with color Doppler echocardiography and phonocardiography. *J Vet Intern Med*. 1999;13(1):56-64.
32. Häggström J, Hansson K, Karlberg BE, Kvart C, Olsson K. Plasma concentration of atrial natriuretic peptide in relation to severity of mitral regurgitation in Cavalier King Charles Spaniels. *Am J Vet Res*. 1994; 55(5):698-703.
33. Hansson K, Häggström J, Kvart C, Lord P. Left atrial to aortic root indices using two-dimensional and M-mode echocardiography in Cavalier King Charles Spaniels with and without left atrial enlargement. *Vet Radiol Ultrasound*. 2002;43(6):568-575.
34. Lombard CW. Normal values of the canine M-mode echocardiogram. *Am J Vet Res*. 1984;45(10):2015-2018.
35. Cornell CC, Kittleson MD, Della Torre P, et al. Allometric scaling of M-mode cardiac measurements in normal adult dogs. *J Vet Intern Med*. 2004;18(3):311-321.
36. Larouche-Lebel É, Loughran KA, Oyama MA. Echocardiographic indices and severity of mitral regurgitation in dogs with preclinical degenerative mitral valve disease. *J Vet Intern Med*. 2019;33(2):489-498.
37. Keene BW, Atkins CE, Bonagura JD, et al. ACVIM consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs. *J Vet Intern Med*. 2019;33(3):1127-1140.
38. Boswood A, Häggström J, Gordon SG, et al. Effect of pimobendan in dogs with preclinical myxomatous mitral valve disease and cardiomegaly: the EPIC study—a randomized clinical trial. *J Vet Intern Med*. 2016;30(6):1765-1779.
39. Reimann MJ, Møller JE, Häggström J, et al. Mitral regurgitation severity and left ventricular systolic dimension predict survival in young Cavalier King Charles Spaniels. *J Vet Intern Med*. 2017;31(4):1008-1016.
40. Boswood A, Gordon SG, Häggström J, et al. Longitudinal analysis of quality of life, clinical, radiographic, echocardiographic, and laboratory variables in dogs with preclinical myxomatous mitral valve disease receiving pimobendan or placebo: the EPIC study. *J Vet Intern Med*. 2018;32(1):72-85.

41. Mekontso-Dessap A, Brouri F, Pascal O, et al. Deficiency of the 5-hydroxytryptamine transporter gene leads to cardiac fibrosis and valvulopathy in mice. *Circulation*. 2006;113(1):81-89.
42. Elangbam CS, Job LE, Zadrozny LM, et al. 5-Hydroxytryptamine (5HT)-induced valvulopathy: compositional valvular alterations are associated with 5HT2B receptor and 5HT transporter transcript changes in Sprague-Dawley rats. *Exp Toxicol Pathol*. 2008;60(4–5):253-262.
43. Fortier JH, Pizzarotti B, Shaw RE, Levy RJ, Ferrari G, Grau J. Drug-associated valvular heart diseases and serotonin-related pathways: a meta-analysis. *Heart*. 2019;105(15):1140-1148.
44. Madsen MB, Olsen LH, Häggström J, et al. Identification of 2 loci associated with development of myxomatous mitral valve disease in Cavalier King Charles Spaniels. *J Hered*. 2011;102(suppl 1):S62-S67.
45. French AT, Ogden R, Eland C, et al. Genome-wide analysis of mitral valve disease in Cavalier King Charles Spaniels. *Vet J*. 2012;193(1):283-286.
46. Meurs KM, Friedenberg SG, Williams B, et al. Evaluation of genes associated with human myxomatous mitral valve disease in dogs with familial myxomatous mitral valve degeneration. *Vet J*. 2018;232:16-19.
47. Bionda A, Cortellari M, Bagardi M, et al. A genomic study of myxomatous mitral valve disease in Cavalier King Charles Spaniels. *Animals*. 2020;10(10):1895.
48. Lee CM, Song DW, Ro WB, Kang MH, Park HM. Genome-wide association study of degenerative mitral valve disease in Maltese dogs. *J Vet Sci*. 2019;20(1):63-71.
49. Cremer SE, Moesgaard SG, Rasmussen CE, et al. Alpha-smooth muscle actin and serotonin receptors 2A and 2B in dogs with myxomatous mitral valve disease. *Res Vet Sci*. 2015;100:197-206.
50. Mangklabruks T, Surachetpong SD. Plasma and platelet serotonin concentrations in healthy dogs and dogs with myxomatous mitral valve disease. *J Vet Cardiol*. 2014;16(3):155-162.
51. Brown SJ, Simpson KW, Baker S, Spagnoletti MA, Elwood CM. Macrothrombocytosis in Cavalier King Charles Spaniels. *Vet Rec*. 1994;135(12):281-283.
52. Pedersen HD, Häggström J, Olsen LH, et al. Idiopathic asymptomatic thrombocytopenia in Cavalier King Charles Spaniels is an autosomal recessive trait. *J Vet Intern Med*. 2002;16(2):169-173.
53. Fidalgo S, Ivanov DK, Wood SH. Serotonin: from top to bottom. *Biogerontology*. 2013;14(1):21-45.
54. Lee GS, Simpson C, Sun BH, et al. Measurement of plasma, serum, and platelet serotonin in individuals with high bone mass and mutations in LRP5. *J Bone Miner Res*. 2014;29(4):976-981.
55. Lindström M, Tohmola N, Renkonen R, Hämäläinen E, Schalin-Jäntti C, Itkonen O. Comparison of serum serotonin and serum 5-HIAA LC-MS/MS assays in the diagnosis of serotonin producing neuroendocrine neoplasms: a pilot study. *Clin Chim Acta*. 2018;482:78-83.
56. Pedersen HD, Olsen LH, Mow T, Christensen NJ. Neuroendocrine changes in Dachshunds with mitral valve prolapse examined under different study conditions. *Res Vet Sci*. 1999;66(1):11-17.
57. Szeitz A, Bandiera SM. Analysis and measurement of serotonin. *Biomed Chromatogr*. 2018;32(1):e4135.
58. Chauveau J, Fert V, Morel AM, Delaage MA. Rapid and specific enzyme immunoassay of serotonin. *Clin Chem*. 1991;37(7):1178-1184.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Reimann MJ, Fredholm M, Cremer SE, et al. Polymorphisms in the serotonin transporter gene and circulating concentrations of neurotransmitters in Cavalier King Charles Spaniels with myxomatous mitral valve disease. *J Vet Intern Med*. 2021;35(6):2596-2606. doi: 10.1111/jvim.16277