Background: Endothelial dysfunction (ED) has been suggested to be associated with myxomatous mitral valve disease (MMVD) in dogs. Tetrahydrobiopterin (BH4) is an important cofactor for production of the endothelium-derived vasodilator nitric oxide (NO). Under conditions of oxidative stress, BH4 is oxidized to the biologically inactive form dihydrobiopterin (BH2). Thus, plasma concentrations of BH2 and BH4 may reflect ED and oxidative stress.

Objective: To determine plasma concentrations of BH2 and BH4 in dogs with different degrees of MMVD.

Animals: Eighty-four privately owned dogs grouped according to ACVIM guidelines (37 healthy control dogs including 13 Beagles and 24 Cavalier King Charles Spaniels [CKCSs], 33 CKCSs with MMVD of differing severity including 18 CKCSs [group B1] and 15 CKCSs [group B2], and 14 dogs of different breeds with clinical signs of congestive heart failure [CHF] because of MMVD [group C]).

Methods: Dogs underwent clinical examination including echocardiography. Plasma concentrations of BH2 and BH4 were measured using high-performance liquid chromatography with fluorescence detection.

Results: Higher plasma BH4 and BH2 concentrations were found with dogs in CHF compared with all other groups (control, B1 and B2; \( P \leq .001 \)). Females had higher concentrations of BH4 and BH4/BH2 (\( P \leq .0003 \)). BH4/BH2 was found to decrease with age (\( P < .0001 \)). Cardiovascular risk factors in humans such as passive smoking (\( P \leq .01 \)) and increased body weight (\( P \leq .009 \)) were associated with lower BH4 concentrations.

Conclusions and Clinical Importance: Age, sex, body weight, passive smoking, and cardiac status are associated with plasma biopterin concentration in dogs. Additional studies should clarify the clinical implications of the findings.

Key words: Endothelial dysfunction; Mitral regurgitation; Oxidative stress; Tetrahydrobiopterin.

Myxomatous mitral valve disease (MMVD) is the most common cardiovascular disease in dogs.\(^1\)\(^2\) The Cavalier King Charles Spaniel (CKCS) is a predisposed breed in which MMVD causes greater cardiac-specific morbidity and mortality compared with other breeds.\(^3\)\(^4\) MMVD is a slowly progressive disease, but duration of the preclinical period often is unpredictable.\(^5\) The uncertain pathogenesis of MMVD and medical treatment restricted to management of clinical signs\(^5\)\(^6\) warrant additional research to identify new diagnostic, therapeutic, and prognostic targets.

Impaired endothelial function and oxidative stress have long been associated with cardiovascular disease.

From the Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Frederiksberg, Denmark (Reimann, Mortensen, Lykkesfeldt, Falk, Olsen); the Department of Clinical Sciences, Faculty of Veterinary Medicine and Animal Science, Swedish University of Agricultural Sciences, Uppsala, Sweden (Haggstrom); and the Department of Cardiology, Odense University Hospital, Odense, Denmark (Moller).

This study was performed at the Departments of Veterinary Clinical and Animal Sciences and Veterinary Disease Biology, University of Copenhagen, Denmark, and Din Veterinær Animal Hospital, Helsingør, Denmark. Preliminary results were presented as an oral presentation at the 2013 European College of Veterinary Internal Medicine—Companion Animals (ECVIM-CA) Forum, Liverpool, 12–14th September 2013.

Corresponding author: L.H. Olsen, Prof., DVM, DVS, Department of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, 9 Ridebanevej, 1870 Frederiksberg C, Denmark; e-mail: lisbeth.hoier@sund.ku.dk.

Submitted December 19, 2013; Revised June 2, 2014; Accepted June 30, 2014.

Copyright © 2014 by the American College of Veterinary Internal Medicine.

DOI: 10.1111/jvim.12425

Abbreviations:

ACE-I angiotensin-converting enzyme inhibitor
ACVIM American College of Veterinary Internal Medicine
BCS body condition score
BH2 dihydrobiopterin
BH4 tetrahydrobiopterin
BW body weight
CBC complete blood count
CHF congestive heart failure
CKCS Cavalier King Charles Spaniel
CV coefficient of variation
DAP diastolic arterial pressure
DTE 1,4-Dithioerythritol
ED endothelial dysfunction
eNOS endothelial nitric oxide synthase
FMD flow-mediated vasodilatation
FS fractional shortening
IVSD\(_N\) interventricular septal thickness in diastole normalized for body weight
IVSS\(_N\) interventricular septal thickness in systole normalized for body weight
LA/Ao left atrial-to-aortic root ratio
LVI\(_D\)\(_D\)\(_N\) left ventricular end-diastolic diameter normalized for body weight
LVI\(_D\)\(_S\)\(_N\) left ventricular end-systolic diameter normalized for body weight
LV left ventricular
LVP\(_F\)\(_W\) left ventricular free wall thickness in diastole normalized for body weight
LVP\(_W\)\(_S\)\(_N\) left ventricular free wall thickness in systole normalized for body weight
MAP mean arterial pressure
MMVD myxomatous mitral valve disease
MR mitral regurgitation
NO nitric oxide
NS nonsignificant
SAP systolic arterial pressure
including heart failure and valvular disease in human patients. Endothelial dysfunction (ED) also has been associated with cardiovascular risk factors in humans including hypertension, hypercholesterolemia, aging, male sex, and cigarette smoking and has been suggested to have prognostic potential for human cardiovascular disease.

Veterinary studies have reported altered antioxidant status and biomarkers for oxidative stress in dogs with both spontaneous and experimentally induced cardiovascular disease including mitral valve disease (MMVD). Other studies of ED examining both circulating biomarkers and functional measurements suggest that ED may play a role in the pathogenesis of MMVD in dogs. Thus, oxidative stress and ED may serve as future targets with regard to improved diagnosis, treatment, or prognosis for dogs with MMVD.

Nitric oxide (NO) generated by the vascular endothelium is a key regulator of vascular homeostasis and normal endothelial function that inhibit disease processes such as inflammation, thrombosis, and oxidative stress. Tetrahydrobiopterin (BH4) is an important cofactor for endothelial NO synthase (eNOS) and thereby for production of NO. Under conditions of oxidative stress, BH4 in the vascular tissue is oxidized to dihydrobiopterin (BH2). BH2 lacks the ability to donate electrons to eNOS and may even decrease BH4 activity by competitive binding to eNOS. Insufficiency of BH4 causes eNOS to uncouple and produce superoxide rather than NO, a situation that further exacerbates oxidative stress and ED.

Thus, the aim of the present study was to determine plasma concentrations of BH2 and BH4 and the BH4/BH2 ratio in dogs with different degrees of MMVD and to investigate if concentrations correlated with disease severity and selected cardiovascular risk factors known to occur in humans.

**Materials and Methods**

We recruited healthy privately owned dogs ≥ 4 years with varying severity of MMVD. Written informed consent was obtained from all owners and the study was approved by the Danish Animal Experiments Inspectorate, license no. 2012-15-2934-00700. All dogs lived in private homes and were fed commercial diets, homemade diets, or a combination of these. Dogs receiving medical treatment apart from cardiac treatment and dogs with a history or signs of noncardiac organ-related disease were excluded. All dogs were subjected to the following protocol: interview with the owner, collection of blood samples, clinical examination, and echocardiography. Passive smoking was defined as dogs living in a home where the owner smoked indoors. Body condition score was graded 1–9. Left apical systolic murmur intensity was graded 1–6.

Blood pressure was measured using high-definition oscillometry equipment on the proximal part of the tail. Measurements were repeated 5 times as previously described. Thoracic radiographs (lateral and dorsoventral) were performed on dogs in congestive heart failure (CHF) (except in 4 dogs because of logistic reasons) to rule out concomitant pulmonary disease. All dogs were part of a previous study that evaluated variation in mitral regurgitation (MR).

**Blood Sampling**

Blood samples were drawn from the jugular vein using a vacutainer system connected to a 21-G butterfly catheter. Dogs were fasted 6–18 hours before blood sampling. Analysis included a complete blood count (CBC) and serum biochemistry profile. For biopterin analysis, blood was collected in K$_3$ EDTA tubes (4 mL) with 100 μL freshly made 1,4-Dithioerythritol (DTE) solution (40 mg DTE in 1,000 μL Milli-Q [18.2 MΩ] water) added to minimize oxidation during analysis. Samples were mixed gently before centrifugation (3,000 x g, 3 minutes, at 4°C) and plasma was frozen immediately at –80°C until analysis. Plasma BH4 and BH2 concentrations were measured by high-performance liquid chromatography with fluorescence detection employing iodine oxidation as described by Fukushima and Nixon.

After individual analysis, plasma from the 5 highest and 5 lowest BH2- and BH4-containing samples (regardless of group) was pooled and analyzed 5 times per day for 5 consecutive days to determine within-day and between-day variation. Within-day and between-day coefficients of variation of BH4 and BH2 measurements were below 7% (Table 1).

**Classification of Disease Severity**

Dogs were staged based on American College of Veterinary Internal Medicine (ACVIM) consensus statement classification guidelines: control group (healthy dogs with no auscultatory heart murmur including 13 Beagles and 24 CKCSs), group B1 (CKCSs with auscultatory heart murmur and LA/Ao ≤ 1.5), group B2 (CKCSs with auscultatory heart murmur and LA/Ao > 1.5), and group C (dogs in CHF including 9 CKCSs and 1 of each of the following breeds: Crossbreed, Springer Spaniel, Dachshund, Basset Hound, and Poodle).

**Table 1.** Within-day and between-day variation in plasma BH4, plasma BH2, and total biopterin results.

|           | BH4     | BH2     | Total Biopterin |
|-----------|---------|---------|-----------------|
| Within-day CV% |         |         |                 |
| High      | 2.62    | 3.18    | 1.49            |
| Low       | 6.11    | 1.77    | 4.32            |
| Between-day CV% |       |         |                 |
| High      | 1.92    | 2.94    | 1.08            |
| Low       | 6.38    | 4.87    | 3.75            |

High, pool of the 5 samples highest in BH2 and BH4, respectively; Low, pool of the 5 samples lowest in BH2 and BH4. BH4, tetrahydrobiopterin; BH2, dihydrobiopterin; CV, coefficient of variation.
Dachshund, Bull Terrier, Yorkshire Terrier). Dogs with CHF were defined as having clinical signs of CHF (eg, cough, dyspnea, nocturnal restlessness, exercise intolerance), echocardiographic changes compatible with CHF and response to diuretic treatment. Cardiac medication was given to all dogs in group C (Table 2).

**Statistical Analysis**

Data were analyzed using statistical software and for statistical analysis, a significance level of $P < 0.05$ was used.

For descriptive statistics, continuous variables were tested for Gaussian distribution within groups using a Shapiro-Wilk test. When significant associations were observed, a boxcox analysis was used to find the optimal transformation. BH4 results were transformed to BH4 $+$ $+$ $+$ and BH2 to BH2 $–$. Subsequently, the multiple linear regression models were reduced through backward selection based on $P$ values. For class variables that remained significant, differences between groups were investigated by performing posthoc testing using Tukey-Kramer adjustment when appropriate for multiple testing.

**Results**

Three dogs were excluded because of abnormalities in serum biochemistry ($n = 1$), heart disease other than MMVD diagnosed by echocardiography ($n = 1$), and medical treatment for CHF without echocardiographic signs compatible with CHF ($n = 1$). Twelve dogs (8 CKCs and 4 Beagles) received diet supplements including fish oil, glucosamine, algae, or some combination of these. The final study population consisted of 84 dogs allocated in ACVIM groups as follows: control group ($n = 37$), group B1 ($n = 18$), group B2

### Table 2. Canine characteristics and conventional echocardiographic variables.

| ACVIM Group | Control | B1 | B2 | C |
|-------------|---------|----|----|---|
| Total number| 37      | 18 | 15 | 14 |
| Cardiac treatment | 0 | 0 | 0 | 14* |
| Sex (female/male) | 21/16 | 13/5 | 8/7 | 2/12 |
| Age (years) | 5.9C (4.9–7.6) | 7.2C (6.3–8.5) | 8.1C (5.3–9.0) | 11.0Control,B1,B2 (9.8–13.0) |
| BW (kg) | 9.3 (8.5–12.3) | 9.1 (8.0–10.0) | 9.1 (8.0–10.4) | 11.5 (10.1–13.6) |
| BCS 3/4/5/6/7 | 0/7/19/11/0 | 0/3/7/6/1 | 0/3/6/4/2 | 1/1/3/4/3 |
| Cholesterol (mmol/L) | 5.8C (4.5–6.8) | 6.9 (5.4–8.2) | 6.3 (4.7–6.8) | 8.0Control (6.4–8.9) |
| Smoking (n/y) | 31/6 | 13/5 | 11/4 | 11/3 |
| SAP (mmHg) | 149.45 (140.5–162.5) | 154.5 (148.8–162.5) | 147.3 (137.5–154.9) | 150.5 (143.0–162.3) |
| DAP (mmHg) | 79.9 (70.5–87.3) | 77.0 (74.8–86.5) | 77.5 (75.3–81.6) | 84.8 (78.8–91.2) |
| MAP (mmHg) | 104.6 (99.5–111.0) | 106.5 (100.0–109.0) | 101.5 (98.4–106.6) | 108.0 (101.5–115.5) |
| Storage time (days) | 319.0 (275.0–333.0) | 326.5 (298.0–371.0) | 319.0 (297.5–337.0) | 309.0 (287.0–349.0) |
| MR (no/mi/mo or se) | 26/11/0 | 2/10/6 | 0/3/12 | 0/0/14 |
| LA/Ao | 1.4B2C (1.3–1.4) | 1.4B2C (1.4–1.5) | 1.7Control,B1,C (1.6–1.8) | 2.2Control,B1,B2 (2.0–2.4) |
| LVIDDN | 1.5B2C (1.4–1.6) | 1.6C (1.4–1.7) | 1.8Control,C (1.6–1.8) | 2.1Control,B1,B2 (1.9–2.2) |
| LVIDSN | 1.1 (1.0–1.1) | 1.0 (1.0–1.1) | 1.1 (1.0–1.2) | 1.2 (1.1–1.3) |
| FS (%) | 25.8B2C (22.5–32.0) | 27.8 (23.2–35.4) | 36.1Control (27.7–39.4) | 42.0Control (33.5–45.7) |
| LVPWDN | 0.5 (0.4–0.5) | 0.5 (0.4–0.5) | 0.5 (0.4–0.5) | 0.5 (0.4–0.5) |
| LVPWSN | 0.6 (0.5–0.6) | 0.6 (0.5–0.6) | 0.6 (0.6–0.7) | 0.6 (0.6–0.6) |
| IVSDN | 0.4 (0.4–0.5) | 0.4 (0.4–0.5) | 0.4 (0.4–0.5) | 0.4 (0.4–0.4) |
| IVSSN | 0.5 (0.4–0.6) | 0.5 (0.5–0.6) | 0.5 (0.4–0.6) | 0.6 (0.5–0.7) |

Within each row, superscripts $^{Control,B1,B2,C}$ represent the group from which there is statistically significant difference. Values reported are median and interquartiles. ACVIM, American College of Veterinary Internal Medicine; BW, body weight; BCS, body condition score; SAP, systolic arterial pressure; DAP, diastolic arterial pressure; MAP, mean arterial pressure; MR, mitral regurgitation using jet area method where no = no, mi = mild, mo = moderate, se = severe (no: <20%, mi: 20–50%, mo or se: >50%); 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; FS, fractional shortening; IVSDN, interventricular septal thickness in diastole normalized for BW; IVSSN, interventricular septal thickness in systole normalized for BW; LVPWDN, left ventricular free wall thickness in diastole normalized for BW; LVPWSN, left ventricular free wall thickness in systole normalized for BW.

$^{+}$Diuretics (Diu) ($n = 1$), Diu+Pimobendan (Pimo) ($n = 3$), Diu+Angiotensin converting enzyme inhibitor (ACE-i) ($n = 3$), Diu+ACE-i+Pimo ($n = 3$), Diu+ACE-i+Digoxin (Dig) ($n = 1$), Diu+ACE-i+Pimo+Dig ($n = 1$), Diu+ACE-i+Pimo+Dig+Hydralazine (Hyd)+Potassium gluconate supplementation ($n = 1$).
(n = 15), and group C (n = 14). Baseline characteristics and conventional echocardiographic results of the final study population are given in Table 2. Sex differed among groups (P = .01), but passive smoking did not (P = .7). No differences in baseline characteristics and conventional echocardiographic values were found between CKCSs and other dog breeds in group C.

Factors with influence on plasma concentrations of BH4, BH2, and BH4/BH2 are summarized in Table 3. Plasma concentrations of BH4 and BH2 were associated with MMVD severity (Figs 1, 2). Higher concentrations of BH4 and BH2 were found in ACVIM group C compared with the other groups. Additionally, both BH4 and BH2 correlated positively with LA/Ao ratio and degree of MR. Lower BH4 plasma concentrations were found in male dogs and in dogs from homes with indoor smoking. In the LA/Ao model, dogs with BCS 6 had lower plasma BH4 than dogs with BCS 5 (P = .04). In the ACVIM disease group model and the MR model, BCS was no longer significant in posthoc analysis after Tukey-Kramer adjustment. Finally, BH4 plasma concentrations decreased with increasing BW, but increased with increasing serum cholesterol concentration. BH2 was positively correlated with age (but only in the statistical models including LA/Ao or degree of MR as

Table 3. Overall P values of biopterin results in the multivariate statistical models including ACVIM disease group, LA/Ao, and MR jet area as disease severity variables in 84 dogs with or without MMVD.

| Explanatory Factors | BH4 \(0.5^C\) | BH2 \(^{-1}\) | BH4/BH2 Ratio |
|---------------------|---------------|---------------|---------------|
| Models with ACVIM group as disease variable | | | |
| Group | <.0001 | <.0001 | NS |
| Age (years) | NS | NS | <.0001 |
| Sex | .0001 | NS | <.0001 |
| Smoking | .01 | NS | NS |
| BW (kg) | .0004 | NS | NS |
| BCS | .03 | NS | NS |
| Cholesterol (mmol/L) | .005 | .04 | NS |
| SAP (mmHg) | NS | NS | |
| Storage time (days) | NS | NS | NS |
| Models with LA/Ao as disease variable | | | |
| LA/Ao | <.0001 | .005 | NS |
| Age (years) | NS | .005 | <.0001 |
| Sex | .0003 | NS | <.0001 |
| Smoking | .01 | NS | NS |
| BW (kg) | .001 | NS | NS |
| BCS | .03 | NS | NS |
| Cholesterol (mmol/L) | <.0001 | .007 | NS |
| SAP (mmHg) | NS | NS | NS |
| Storage time (days) | NS | NS | NS |
| Models with degree of MR as disease variable | | | |
| Degree of MR | <.0001 | .01 | NS |
| Age (years) | NS | .005 | <.0001 |
| Sex | .0003 | NS | <.0001 |
| Smoking | .01 | NS | NS |
| BW (kg) | .009 | NS | NS |
| BCS | .049 | NS | NS |
| Cholesterol (mmol/L) | .0005 | .03 | NS |
| SAP (mmHg) | NS | NS | NS |
| Storage time (days) | NS | NS | |

Final models with ACVIM groups as disease variable have an adjusted \(R^2\) of 0.57 (BH4), 0.42 (BH2), and 0.37 (BH4/BH2). Final models with LA/Ao as disease variable have an adjusted \(R^2\) of 0.48 (BH4), 0.38 (BH2), and 0.37 (BH4/BH2). Final models with MR as disease variable have an adjusted \(R^2\) of 0.50 (BH4), 0.37 (BH2), and 0.37 (BH4/BH2). ACVIM, American College of Veterinary Internal Medicine; BW, body weight; BCS, body condition score; SAP, systolic arterial pressure; LA/Ao, left atrial-to-aortic root ratio; MR, mitral regurgitation; NS, Nonsignificant.
disease variable) and serum cholesterol concentration. The BH4/BH2 ratio was not associated with disease severity, but the ratio decreased with increasing age and females had a higher BH4/BH2 ratio than males (Fig 3). No influence of SAP or sample storage time was found. Identical statistical ACVIM disease group models were performed with the control group divided into Beagles and CKCSs and the same explanatory variables reached significance.

Discussion

In the present study, we found significantly increased concentrations of BH4 and BH2 in dogs in CHF and a positive association with mitral valve disease severity, suggesting development of altered endothelial function and an association with oxidative stress as disease advances. Furthermore, several cardiovascular risk factors in humans including age, sex, smoking, BW, BCS, and serum cholesterol concentration influenced biopterin status in dogs.

The presence of increased plasma BH2 concentration in relation to CHF suggests that oxidative stress and ED are present in dogs in the late stages of MMVD. This finding agrees with findings of a study in humans in which a negative relationship between flow-mediated vasodilatation (FMD) and plasma BH2 concentration was found. Increased oxidative stress has been shown to be involved in the pathogenesis and progression of ED because eNOS-mediated NO production in the endothelium decreases as BH4 is oxidized to BH2 in exchange for increased superoxide production.

However, the observed increasing plasma concentrations of BH4 with increasing disease severity and in CHF dogs were unexpected. Although higher plasma BH4 concentrations were found in these dogs, the ability to produce NO may not be increased equally because BH2 has been shown to competitively bind to eNOS. BH4 concentrations most likely reflect the complex balance among compensatory de novo synthesis, oxidative loss, salvage pathways, and transport between extra- and intracellular compartments. Thus, a possible explanation for increased BH4 concentrations may be an adaptive increase in synthesis with disease progression perhaps in response to receptor downregulation, decreased receptor sensitivity, or decreased bioavailability. Another possibility is that increased plasma BH4 concentrations result from increased recycling from BH2 as disease develops. It could also be speculated that a leakage of BH4 from the endothelium occurs as disease progresses and that increased plasma BH4 concentrations thus reflect decreased intracellular concentrations of BH4. This hypothesis is supported by a study in humans that found decreased endothelial function in patients with high plasma BH4 concentrations and an inverse relationship between plasma and vascular BH4.

Increasing age was shown to be associated with increased BH2 and decreased BH4/BH2 ratio. This is an expected finding because both MMVD and ED in dogs are conditions that progress with age. Similarly, age and BH4/BH2 ratio have been suggested to be negatively associated in a study in humans. In older individuals, an age-associated accumulated oxidative damage may have influenced the results.

Females had significantly higher plasma BH4 concentrations and BH4/BH2 ratio than males, similar to findings in humans, suggesting that female sex has a protective effect on oxidative stress and ED. This finding supports other studies in dogs that have observed more severe MMVD in male dogs than in females of the same age and a study in humans that found decreased FMD in male patients.

In this study, BW was negatively associated with BH4 consistent with the risk profile in humans of obesity as a risk factor in cardiovascular disease. Accordingly, BCS, which better describes obesity or underweight body condition, also was associated with plasma BH4 concentration because 1 statistical model found significantly lower BH4 concentrations in dogs with BCS 6 compared with BCS 5. Dogs included in this study were estimated as having BCSs between 3 and 7 (with only 1 dog categorized as BCS 3), meaning that no severely obese or cachexic dogs were included.

Active smoking and passive smoking represent cardiovascular risk factors in humans and have been shown to be associated with plasma biopterin status and ED in human patients. We found that dogs from homes with indoor smoking had significantly lower plasma BH4 concentrations, suggesting that a higher degree of BH4 oxidation occurs with passive smoking exposure in dogs. The importance of passive smoking as a risk factor for disease progression in dogs is unknown. To the authors’ knowledge, circulating concentrations of BH4 have not been evaluated in passive smoking in humans.

Hypercholesterolemia represents another cardiovascular risk factor in humans, but there are discrepancies in research concerning the association with ED. In this study, serum cholesterol concentrations correlated positively with plasma BH4 and BH2 concentrations in dogs, but none of the dogs had severe

![Fig 3. Plot of plasma BH4/BH2 ratio as a function of age with fitted linear regression lines for males and females. ▲, males; ●, females. BH4/BH2 ratio was negatively associated with age. Males had significantly lower BH4/BH2 ratio.](image)
hypercholesterolemia. Furthermore, the lipoprotein composition in dogs differs from that of humans.\textsuperscript{49} In dogs, the high-density lipoproteins are predominant and the main carriers of cholesterol, whereas in humans, low-density lipoproteins constitute the major lipoprotein.\textsuperscript{50,51} This species difference may be of importance and it is uncertain whether or not hypercholesterolemia is a cardiovascular risk factor in dogs.

Limitations of the study include the use of privately owned dogs because potential selection bias cannot be avoided. Although the CKCSs were selected at random in a database, this database may not be representative of the CKCSs population because it is mainly based on dogs used for breeding. Different dietary regimens and dietary supplements may have influenced results because commercial dog foods are enriched with different amounts of antioxidants. Although all dogs had been fasted 6–18 hours before examination, the exact duration of fasting could have influenced results. Moreover, it cannot be ruled out that cardiac treatment in group C, which for a number of dogs included angiotensin-converting enzyme inhibitors, pi-mobendan, or both, could have influenced ED and oxidative stress and thereby BH2 and BH4 concentrations because these substances have been suggested to alter NO availability.\textsuperscript{52,53} However, for ethical reasons, we did not attempt to stop or alter treatment. Finally, minor deviations from reference in CBC and serum biochemistry results among dogs in group C may be of importance because plasma BH2 concentration has been shown to be increased and BH4/BH2 ratio decreased with increasing severity of renal failure in humans.\textsuperscript{54} Hence, we cannot discount the possibility that renal status might have influenced the results.

Conclusion

Myxomatous mitral valve disease severity and certain cardiovascular risk factors of humans including age, sex, BW, BCS, and passive smoking are associated with bioterin status in dogs, indicating the presence of ED and oxidative stress. Additional studies are warranted to investigate the clinical implications of these findings.

Footnotes

\textsuperscript{a} Vivid i echocardiograph; GE-medical, Milwaukee, WI
\textsuperscript{b} EchoPAC PC. Version 112; GE Vingmed Ultrasound AS, Horten, Norway
\textsuperscript{c} R studio, version 0.97.336, © 2009-2012 RStudio, Inc., Boston, MA

Acknowledgments

The authors thank Christina Tirsdal Kjemppf, Joan Elisabeth Frandsen, and Susanne Kronborg at the Department of Veterinary Disease Biology and Dennis Jensen at the Department of Veterinary Clinical and Animal Sciences, University of Copenhagen, Denmark, for excellent technical assistance. The study was supported financially by fellowship grants from the Lifepharm Centre for In Vivo Pharmacology to MJR and AM and grants from the Danish National Research Council (Project no. 271-08-0998) and Simon Fougher Hartmann’s family Foundation.

Conflict of Interest Declaration: The authors disclose no conflict of interest.

References

1. Detweiler DK, Patterson DF. The prevalence and types of cardiovascular disease in dogs. Ann N Y Acad Sci 1965;127:481–516.
2. Buchanan JW. Chronic valvular disease (endocardiosis) in dogs. Adv Vet Sci Comp Med 1977;21:75–106.
3. Egnevall A, Bonnett BN, Haggstrom J. Heart disease as a cause of death in insured Swedish dogs younger than 10 years of age. J Vet Intern Med 2006;20:894–903.
4. Haggstrom J, Hansson K, Kvart C, Swenson L. Chronic valvular disease in the Cavalier King Charles Spaniel in Sweden. Vet Rec 1992;131:549–553.
5. Atkins C, Bonagura J, Ettinger S, et al. Guidelines for the diagnosis and treatment of canine chronic valvular heart disease. J Vet Intern Med 2009;23:1142–1150.
6. Cunningham SM, Rush JE, Freeman LM. Systemic inflammation and endothelial dysfunction in dogs with congestive heart failure. J Vet Intern Med 2012;26:547–557.
7. Nakamura M, Yoshida H, Arakawa N, et al. Endothelium-dependent vasodilatation is not selectively impaired in patients with chronic heart failure secondary to valvular heart disease and congenital heart disease. Eur Heart J 1996;17:1875–1881.
8. Fischer D, Rossa S, Landmesser U, et al. Endothelial dysfunction in patients with chronic heart failure is independently associated with increased incidence of hospitalization, cardiac transplantation, or death. Eur Heart J 2005;26:65–69.
9. Sugamura K, Keaney Jr. Reactive oxygen species in cardiovascular disease. Free Radic Biol Med 2011;51:978–992.
10. Heitzer T, Schlinzig T, Krohn K, et al. Endothelial dysfunction, oxidative stress, and risk of cardiovascular events in patients with coronary artery disease. Circulation 2001;104:2673–2678.
11. Ruef J, Marz W, Winkelmann BR. Markers for endothelial dysfunction, but not markers for oxidative stress correlate with classical risk factors and the severity of coronary artery disease. (A subgroup analysis from the Ludwigshafen Risk and Cardiovascular Health Study). Scand Cardiovasc J 2006;40:274–279.
12. Celermajer DS, Sorensen KE, Bull C, et al. Endothelium-dependent dilation in the systemic arteries of asymtomatic subjects relates to coronary risk factors and their interaction. J Am Coll Cardiol 1994;24:1468–1474.
13. Egashira K, Inou T, Hirooka Y, et al. Impaired coronary blood flow response to acetylcholine in patients with coronary risk factors and proximal atherosclerotic lesions. J Clin Invest 1993;91:29–37.
14. Benjamin EJ, Larson MG, Keyes MJ, et al. Clinical correlates and heritability of flow-mediated dilation in the community: The Framingham Heart Study. Circulation 2004;109:613–619.
15. Inaba Y, Chen JA, Bergmann SR. Prediction of future cardiovascular outcomes by flow-mediated vasodilatation of brachial artery: A meta-analysis. Int J Cardiovasc Imaging 2010;26:631–640.
16. Prasad K, Gupta J, Kalra J, et al. Oxidative stress as a mechanism of cardiac failure in chronic volume overload in canine model. J Mol Cell Cardiol 1996;28:375–385.
17. Nishijima Y, Sridhar A, Bonilla I, et al. Tetrahydrobiop- 
terin depletion and NO2 uncoupling contribute to heart failure-
induced alterations in atrial electrophysiology. Cardiovasc Res 
2011;91:71–79.
18. Freeman LM, Rush JE, Milbury PE, Blumberg JB. Anti-
oxidant status and biomarkers of oxidative stress in dogs with 
congestive heart failure. J Vet Intern Med 2005;19:537–541.
19. Pedersen HD, Schutt T, Sondergaard R, et al. Decreased 
plasma concentration of nitric oxide metabolites in dogs with 
untreated mitral regurgitation. J Vet Intern Med 2003;17:178–184.
20. de Laforcade AM, Freeman LM, Rush JE. Serum nitrate 
and nitrite in dogs with spontaneous cardiac disease. J Vet Intern 
Med 2003;17:315–318.
21. Pedersen LG, Tarnow I, Olsen LH, et al. Body size, but 
neither age nor asymptomatic mitral regurgitation, influences 
plasma concentrations of dimethylarginines in dogs. Res Vet Sci 
2006;80:336–342.
22. Moesaegh SG, Falk T, Teerlink T, et al. Brain-natriuretic 
peptide and cyclic guanosine monophosphate as biomarkers of 
myxomatous mitral valve disease in dogs. Vet J 2011;189:349–352.
23. Jones ID, Fuentes VL, Boswood A, et al. Ultrasonographi-
ic measurement of flow-mediated vasodilation in dogs with 
chronic valvular disease. J Vet Cardiol 2012;14:203–210.
24. Moesaegh SG, Kloppergard C, Zois NE, et al. Flow-
mediated vasodilation measurements in Cavalier King Charles 
Spaniels with increasing severity of myxomatous mitral valve dis-
case. J Vet Intern Med 2012;26:61–68.
25. Schmidt T3, Alp NJ. Mechanisms for the role of tetrahy-
drobiopetin in endothelial function and vascular disease. Clin Sci (Lond) 2007;113:47–63.
26. Tayeb MA, Marlella MA. Macrophage oxidation of 
L-arginine to nitric oxide, nitrite, and nitrate. Tetrahydrobiopet-
rin is required as a cofactor. J Biol Chem 1989;264:19654–19658.
27. Mortensen A, Lykkesfeld J. Does vitamin C enhance 
nitric oxide bioavailability in a tetrahydrobipterin-dependent 
manner? In vitro, in vivo and clinical studies. Nitric Oxide 
2014;36:51–57.
28. Milsien S, Katusic Z. Oxidation of tetrahydrobiopetin by 
peroxynitrite: Implications for vascular endothelial function. Biochim Biophys Acta 1999;1461:681–684.
29. Vasquez-Vivar J, Martasek P, Whitsett J, et al. The ratio 
between tetrahydrobiopetin and oxidized tetrahydrobiopetin 
controls superoxide release from endothelial nitric oxide synthase: An EPR spin trapping study. Biochim Biophys Acta 
2002;162:732–739.
30. Landmesser U, Dikalov S, Price SR, et al. Oxidation of 
tetrahydrobiopetin leads to uncoupling of endothelial cell nitric 
oxide synthase in hypertension. J Clin Invest 2003;111:1201–1209.
31. Stroes E, Hijmering M, van Zandvoort M, et al. Origin of 
superoxide production by endothelial nitric oxide synthase. FEBS 
Lett 1998;438:161–164.
32. Ettinger SJ. The physical examination of the dog and cat. 
In: Ettinger SJ, Feldman EC, eds. Textbook of Veterinary Intern-
al Medicine: Diseases of the Dog and the Cat, 7th ed. St. Louis, 
MO: Saunders Elsevier; 2010:1–9.
33. Gompf RE. The clinical approach to heart disease: His-
tory and physical examination. In: Fox PR, ed. Canine and Feline Cardiology. New York, NY: Churchill Livingstone; 
1988:29–42.
34. Brown S, Atkins C, Bagley R, et al. Guidelines for the 
identification, evaluation, and management of systemic hyperten-
sion in dogs and cats. J Vet Intern Med 2007;21:542–558.
35. Reimann MJ, Moller JE, Hagstrom J, et al. R-R interval 
variations influence the degree of mitral regurgitation in dogs 
with myxomatous mitral valve disease. Vet J 2014;199:348–354.
36. Mortensen A, Lykkesfeldt J. Kinetics of acid-induced deg-
radation of tetra- and dihydrobiopetin in relation to their rele-
vance as biomarkers of endothelial function. Biomarkers 
2013;18:55–62.
37. Fukushima T, Nixon JC. Analysis of reduced forms of 
biopetin in biological tissues and fluids. Anal Biochem 
1980;102:176–188.
38. Thomas WP, Gaber CE, Jacobs GJ, et al. Recommendations 
for standards in transthoracic two-dimensional echocardio-
graphy in the dog and cat. Echocardiography Committee of the 
Speciality of Cardiology, American College of Veterinary Internal 
Medicine. J Vet Intern Med 1993;7:247–252.
39. Haggstrom J, Hansson K, Karlberg BE, et al. Plasma con-
centration of atrial natriuretic peptide in relation to severity of 
mitral regurgitation in Cavalier King Charles Spaniels. Am J 
Vet Res 1994;55:698–703.
40. Cornell CC, Kittleson MD, Della Torre P, et al. Allomet-
ric scaling of M-mode cardiac measurements in normal adult 
dogs. J Vet Intern Med 2004;18:311–321.
41. Takeda M, Yamashita T, Shinohara M, et al. Plasma tet-
rahydrobiopetin/dihydrobiopetin ratio: A possible marker of 
endothelial dysfunction. Circ J 2009;73:955–962.
42. Cai H, Harrison DG. Endothelial dysfunction in cardio-
vascular diseases: The role of oxidant stress. Circ Res 
2000;87:840–844.
43. Antoniades C, Shirodaria C, Crabtree M, et al. Altered 
plasma versus vascular biopers in humans atherosclerosis reveal 
relationships between endothelial nitric oxide synthase coupling, 
endothelial function, and inflammation. Circulation 
2007;116:2851–2859.
44. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxi-
dants, and the degenerative diseases of aging. Proc Natl Acad Sci 
U S A 1993;90:7915–7922.
45. Swenson L, Haggstrom J, Kvart C, Junega R. Relationship 
between parental cardiac status in Cavalier King Charles Spaniels 
and prevalence and severity of chronic valvular disease in off-
spring. J Am Vet Med Assoc 1996;208:2009–2012.
46. Olsen LH, Fredholm M, Pedersen HD. Epidemiology 
and inheritance of mitral valve prolapse in Dachshunds. J Vet Intern 
Med 1999;13:448–456.
47. Shinozaki K, Hirayama A, Nishio Y, et al. Coronary 
endothelial dysfunction in the insulin-resistant state is linked to 
abnormal pereidive metabolism and vascular oxidative stress. J 
Am Coll Cardiol 2001;38:1821–1828.
48. Celermajer DS, Adams MR, Clarkson P, et al. Passive 
smoking and impaired endothelium-dependent arterial dilatation 
in healthy young adults. N Engl J Med 1996;334:150–154.
49. Maldonado EN, Romero JR, Ochoa B, Avendano MI. Lipid 
and fatty acid composition of canine lipoproteins. Comp 
Biochem Physiol B Biochem Mol Biol 2001;128:719–729.
50. Lehmann R, Bhargava AS, Gunzel P. Serum lipoprotein 
pattern in rats, dogs and monkeys, including method comparison 
and influence of menstrual cycle in monkeys. Eur J Clin Chem 
Clin Biochem 1993;31:633–637.
51. Mahley RW, Weisgraber KH. Canine lipoproteins and 
atherosclerosis. I. Isolation and characterization of plasma lipo-
proteins from control dogs. Circ Res 1974;35:713–721.
52. Mombouli JV, Illiano S, Nagaoo T, et al. Potentiation of 
endothelium-dependent relaxations to bradykinin by angiosten-
in I converting enzyme inhibitors in canine coronary artery involves 
both endothelium-derived relaxing and hyperpolarizing factors. 
Circ Res 1992;71:137–144.
53. Iwasaki A, Matsumori A, Yamada T, et al. Pimobendan 
inhibits the production of proinflammatory cytokines and gene 
expression of inducible nitric oxide synthase in a murine model of 
viral myocarditis. J Am Coll Cardiol 1999;33:1400–1407.
54. Yokoyama K, Tajima M, Yoshida H, et al. Plasma pteri-
dine concentrations in patients with chronic renal failure. Neph-
rol Dial Transplant 2002;17:1012–1036.