Serum Concentrations of Leptin and Adiponectin in Dogs with Myxomatous Mitral Valve Disease

H.-S. Kim, J.-H. Kang, E.-B. Jeung, and M.-P. Yang

Background: The concentrations of circulating adipokines in dogs with myxomatous mitral valve disease (MMVD) have not been investigated in detail.

Objectives: To determine whether serum concentrations of adipokines differ between healthy dogs and dogs with MMVD and whether circulating concentrations depend on the severity of heart failure resulting from MMVD.

Animals: In the preliminary study, 30 healthy dogs and 17 client-owned dogs with MMVD, and in the subsequent study, 30 healthy dogs and 46 client-owned dogs with MMVD.

Methods: Prospective case-controlled observational study. In the preliminary study, serum concentrations of leptin, adiponectin, resistin, visfatin, interleukin (IL)-1β, IL-6, IL-10, IL-18, and tumor necrosis factor-α were measured. In the subsequent study, MMVD dogs were divided into three groups according to the International Small Animal Cardiac Health Council (ISACHC) classification, and serum concentrations of leptin and adiponectin were measured.

Results: In the preliminary study, serum leptin and adiponectin concentrations differed significantly between dogs with MMVD and healthy dogs. Serum leptin (P = .0013) concentrations were significantly higher in dogs with MMVD than in healthy dogs, whereas adiponectin (P = .0009) concentrations were significantly lower in dogs with MMVD. However, we observed no significant differences in the other variables. In the subsequent study, dogs classified as ISACHC class 3 had higher serum concentrations of leptin (P = .0022) than healthy dogs but ISACHC class 1 or 2 dogs did not. Serum adiponectin concentrations were significantly lower in ISACHC class 1 (P < .0001) dogs than in healthy dogs, whereas adiponectin concentrations in ISACHC class 3 dogs were significantly higher than in ISACHC class 1 dogs (P = .0081).

Conclusions and Clinical Importance: Circulating concentrations of leptin and adiponectin might be altered in dogs with MMVD.

Key words: Adipokine; Canine; Cardiology; Heart failure; Valvular disease.

Myxomatous mitral valve disease (MMVD) accounts for 75–80% of cardiac disease in small dog breeds.1 Progressive degeneration of the mitral valve can cause chronic volume overload with left atrial dilatation and left ventricular eccentric hypertrophy.1 Initially, myocardial remodeling resulting from adaptive changes can be beneficial, but eventually these compensatory mechanisms are not sufficient to overcome cardiovascular dysfunction, and congestive heart failure (HF) can develop.2 Although the development and progression of HF traditionally have been viewed as hemodynamic disorders caused by the associated structural changes, heart failure also may progress as a consequence of dysregulation of biologically active molecules.3–6

Adipokines, biologically active substances derived mainly from adipose tissue,7 play important roles in the pathophysiology of obesity and related conditions in humans.8 Several studies in humans and rodents showed that several adipokines affect cardiovascular functions, as well as many other physiological processes including regulation of energy metabolism, immune function, and inflammation.8 In veterinary medicine, the most well-characterized adipokines are leptin and adiponectin.7 Leptin modulates the immune system, exerts proinflammatory effects, and regulates energy homeostasis.8–10 In addition, results of recent experimental animal studies suggest that leptin regulates the baseline physiology of the heart, including myocyte contractility, hypertrophy, apoptosis, and metabolism, thereby contributing to the pathogenesis of HF and progressive left ventricular dysfunction.11–13 Recent studies in humans indicated that serum leptin concentrations in HF patients increase with the severity of HF and that high concentrations of leptin

Abbreviations:

| Abbreviation | Description               |
|--------------|---------------------------|
| BCS          | body condition score      |
| CHF          | congestive heart failure  |
| CI           | confidence interval       |
| HF           | heart failure             |
| IL           | interleukin               |
| ISACHC       | International Small Animal Cardiac Health Council |
| MMVD         | myxomatous mitral valve disease |
| Ob-R         | leptin receptor           |
| TNF-α        | tumor necrosis factor-α   |

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predict adverse clinical outcomes. In dogs, however, only serum leptin mRNA concentrations have been correlated with the severity of cardiac disease. Conversely, adiponectin is capable of slowing the progression of cardiovascular diseases such as cardiac hypertrophy, ischemic injury, and atherosclerosis in humans. High plasma adiponectin concentrations in healthy human beings are associated with low cardiovascular risk. In dogs, dilated cardiomyopathy is associated with higher adiponectin concentration compared to healthy and MMVD dogs. However, no information is available on the roles of leptin and adiponectin in dogs with cardiac disease. Hence, we sought to examine differences in serum concentrations of adipokines between healthy dogs and dogs with MMVD and subsequently to determine whether circulating concentrations of leptin and adiponectin varied with the severity of HF. In addition, we measured the mRNA levels of leptin and adiponectin receptors from canine myocardial tissues.

Materials and Methods

Case Selection

This case-controlled observational study consisted of 2 parts, a preliminary study and a subsequent study. Both studies were approved by the University Ethics Committee of Chungbuk National University, and informed consent was obtained from the dog owners. Figure 1 shows the flow diagram for enrollment of cases in the studies. Initially, dogs with any other cardiovascular disease and dogs that had ever been treated for MMVD were excluded from both studies.

In the preliminary study, 46 dogs with MMVD diagnosed between January 2012 and January 2013 were enrolled. Because we suspected that adipokines might be correlated with body fat mass in dogs, we selected 24 dogs with a body condition score (BCS) of 5 on a 9-point scale. Among these 24 dogs, 3 suffering from chronic kidney disease were excluded. At follow-up after enrollment, one dog was removed because of a diagnosis of hyperadrenocorticism. Three intact bitches also were excluded because the production of adipokines may be affected by the estrous cycle. Ultimately, 17 dogs with MMVD were included in the preliminary study.

Fig 1. Flow diagram of the enrollment of cases in the preliminary and subsequent studies. MMVD, myxomatous mitral valve disease; BCS, body condition score; CKD, chronic kidney disease; HAC, hyperadrenocorticism; AP, acute pancreatitis, ISACHC, International Small Animal Cardiac Health Council.
In the subsequent study, based on the results obtained from the preliminary study, more dogs with MMVD were recruited to evaluate leptin and adiponectin concentrations in relation to the severity of clinical signs. A total of 103 dogs diagnosed with MMVD between March 2013 and October 2014 were enrolled, of which 72 dogs with a BCS of 5/9 were selected. Of those, 8 suffering from chronic kidney disease were excluded. At follow-up after enrollment in this study, 10 dogs were excluded because of concurrent diseases including acute pancreatitis (n = 7) and hyperadrenocorticism (n = 3). Eight intact bitches also were excluded. Ultimately, 46 dogs with MMVD were included in the subsequent study. The preliminary and subsequent studies included the same 30 healthy dogs. Healthy dogs with a BCS of 5/9 were recruited as a control group from dogs presented for health examination at the same veterinary medical center. The dogs were considered to be healthy based on physical examination, indirect measurement of systolic blood pressure, fecal flotation results, heartworm antigen testing, CBC, serum biochemical analysis, urinalysis, adrenocorticotrophic hormone stimulation testing, thyroid function testing, electrocardiography, and diagnostic imaging including survey radiography and abdominal ultrasonography. After recruitment, these dogs were examined by echocardiography to confirm the absence of cardiac disease.

**Diagnosis of MMVD**

A diagnosis of MMVD was established based on echocardiographic evidence of color flow mitral regurgitation and valvular lesions of the mitral valve apparatus (mitral leaflet thickening and prolapse or both), as previously described. The following examinations were performed in all dogs diagnosed with MMVD: fecal flotation, heartworm antigen test, CBC, serum biochemical analysis, adrenocorticotrophic hormone stimulation testing, thyroid function testing, electrocardiography, and diagnostic imaging including radiography, ultrasonography, and echocardiography.

Thoracic radiographs identified cardiac and respiratory diseases. On thoracic radiographs, cardiac size was quantified using the vertebral heart scale method. An increase in cardiac silhouette size accompanied by enlargement of the left atrium and left ventricle led to suspicion of MMVD. Left-sided CHF was identified on thoracic radiographs. Congestive heart failure was evaluated by the following radiological changes: pulmonary venous hypervascular pattern with a hazy interstitial pattern, progressing to an alveolar pattern. A diagnosis of CHF was made if a dog had clinical signs of congestion (e.g., cough, dyspnea) and exercise intolerance. In addition, dogs had to show subjective signs of cardiomegaly including left atrial enlargement, as well as cardiogenic pulmonary edema, on thoracic radiographs.

Echocardiographic examinations included 2D, and color flow Doppler, were performed by a single experienced echocardiographer using an echocardiographic unit with 5S transducers. All examinations were performed in conscious unsedated dogs. If a dog had severe clinical signs of HF requiring hospitalization, echocardiographic examination was performed after treatment was carried out until respiratory distress resolved sufficiently to allow the dog to tolerate echocardiographic examination. Mitral valve structure and presence of mitral regurgitation were assessed from a right parasternal long-axis view and a left apical four-chamber view using color flow Doppler, respectively, as described elsewhere. The left atrial to aortic root (LA/Ao) ratio was measured in early ventricular diastole from the 2D right parasternal short-axis view. M-mode measurements of the left ventricle were performed using standard techniques on images obtained from the right parasternal short-axis view. M-mode values were used to derive end-diastolic left ventricular internal dimension (LVIDd), end-systolic left ventricular internal dimension (LVIDs), and fractional shortening (FS). Fractional shortening was calculated by subtracting the left ventricular systolic dimension from the diastolic dimension and dividing by the diastolic dimension. Expected normal dimensions were calculated as previously described: LVIDd (body weight^{0.294} × 1.53) and LVIDs (body weight^{0.151} × 0.95). Values for the percentage increases of end-diastolic left ventricular internal dimension (LVIDdmax) and end-systolic left ventricular internal dimension (LVIDsmin) were calculated as follows: observed dimension – expected normal dimension)/expected normal dimension × 100.

**Grouping**

In the preliminary study, to compare serum concentrations of adipokines with those of the healthy dogs, 17 dogs diagnosed with MMVD were divided into 2 groups: 8 dogs without HF and nine dogs with HF. In the subsequent study, to evaluate serum concentration of leptin and adiponectin according to clinical severity of HF, 46 dogs diagnosed with MMVD were classified into 3 groups according to the International Small Animal Cardiac Health Council (ISACHC) grading system.

**Analysis of Serum Adipokine Concentration**

All dogs were fasted ≥ 12 hours before blood collection. Blood was collected from the jugular or a peripheral vein into tubes without anticoagulant. Sera were separated from clotted whole blood by centrifugation at 1,200 × g for 10 minutes within 1 hour of blood collection. Samples were stored at –80°C before assays and then batch analyzed. In the 30 healthy dogs, serum adipokine concentrations were measured separately in the preliminary study and subsequent study.

The following adipokines were analyzed as described elsewhere: leptin, adiponectin, resistin, visfatin, interleukin (IL)-1ß, IL-6, IL-10, IL-18, and tumor necrosis factor (TNF)-α. Serum leptin concentrations were analyzed using a canine-specific ELISA kit; intra-assay variability was 4%, interassay variability was 6%, and assay sensitivity was 0.4 ng/mL. Serum adiponectin concentrations were analyzed using a canine-specific ELISA kit; intra- and interassay variabilities were <5 and <3%, respectively, and assay sensitivity was 0.03 μg/mL. Serum resistin concentrations were analyzed using a canine-specific ELISA kit (Canine Resistin ELISA kit); intra- and interassay variabilities were <5 and <7%, respectively. Serum visfatin concentrations were analyzed using a canine-specific ELISA kit (Canine Visfatin ELISA kit); the intra- and interassay variabilities were 3 and 9%, respectively, and the assay sensitivity was 0.2 ng/mL. Serum IL-1ß concentrations were analyzed using a canine-specific ELISA kit (Canine Cytokine/Chemokine MAGNETIC kit). Intra- and interassay variabilities of all these assays were <5 and <15%, respectively, and the assay sensitivities for IL-6, IL-10, IL-18, and TNF-α were 3.7, 8.5, 5.8, and 6.1 pg/mL, respectively. The assays were quantitated using a Luminex system.

**RT-PCR Assays**

When an owner consented to donate their dog after euthanasia, cardiac tissues obtained from the dog were used in this study.
Three dogs euthanized because of noncardiac disease were included. Cardiac tissue samples, including left ventricle (LV), left atrium (LA), interventricular septum (IVS), right ventricle (RV), and right atrium (RA), were collected. Total RNA was extracted using TriZol® reagent. Single-stranded cDNA was synthesized from 1 μg of total RNA by reverse transcription using random primers and the M-MLV RT kit. PCR amplification was used to simultaneously amplify cDNAs encoding the canine long-form leptin receptor (Ob-R), adiponectin receptor 1, adiponectin receptor 2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the internal control. Primers were designed based on published sequences in the National Center for Biotechnology Information GenBank database, and the primer sequences are listed in Table 1. PCR conditions consisted of an initial denaturation step at 95°C for 5 minutes, followed by 40 cycles at 95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, and a final extension step 72°C for 7 minutes. The amplified PCR products were analyzed by electrophoresis on a 1.5% agarose gel using ethidium bromide staining.

**Statistical Analyses**

All statistical analyses were carried out using a commercially available statistical program. The D’Agostino–Pearson omnibus test was performed to determine whether data were normally distributed. In the preliminary study, basic characteristics were compared between 2 groups (healthy and MMVD group) using Mann-Whitney U-tests and Fisher’s exact tests. Mann-Whitney U-tests were used to compare differences in serum adipokine concentrations between the healthy and MMVD groups in the preliminary study, and data are expressed as medians (interquartile ranges). P-values were calculated for 2-tailed tests, and 95% confidence intervals (CIs) were determined for differences between medians. In the subsequent study, basic characteristics were compared among 4 groups (1 healthy, and ISACHC 1, 2, and 3 groups) using Kruskal-Wallis tests and Fisher’s exact tests. On the basis of results from the preliminary study, serum leptin and adiponectin concentrations were compared between 2 groups (healthy and MMVD group) using Mann-Whitney U-tests. In addition, after MMVD dogs were divided into 3 groups according to ISACHC classification, Kruskal-Wallis tests were conducted to examine the differences of serum leptin and adiponectin concentrations among ISACHCs classes and healthy control groups. If a significant difference was detected, a pairwise comparison also was performed using the Mann-Whitney U-test with Bonferroni adjustment, for which a value of P < .0083 was considered significant, and the data were expressed as medians (interquartile ranges).

Simple linear regression analyses were used to evaluate associations among the adipokines (leptin or adiponectin) concentrations, age, sex, body weight, ISACHC classes, and echocardiographic variables (LA/AO ratio, FS, LVIDd, LVIDs). Natural logarithmic transformation was performed for dependent variables (leptin, adiponectin) to achieve normal distribution. Categorical data (sex, ISACHCs classes) were transformed to dummy variables. A value of P < .05 was considered statistically significant.

**Results**

**Serum Adipokine Concentrations in Healthy Dogs and Dogs with MMVD: Preliminary Study**

The median serum concentration of leptin (95% CI for difference between medians = 0.73–3.24; P = .0013) was significantly higher in dogs with MMVD (3.72 ng/mL [1.65–4.65]) than in healthy dogs (1.44 ng/mL [0.46–2.04]; Fig 2), whereas the median serum concentration of adiponectin (95% CI for difference between medians = −8.37 to −2.42; P = .0009) was significantly lower in dogs with MMVD (6.20 μg/mL [4.30–6.70]) than in healthy dogs (11.37 μg/mL [5.56–17.14]). The median visfatin concentration (95% CI for difference between medians = −0.06 to −0.00; P = .11) between healthy dogs (0.35 ng/mL [0.33–0.43]) and dogs with MMVD (0.34 ng/mL [0.32–0.35]) and resistin concentration (95% CI for difference between medians = −13.84 to 6.44; P = .49) between healthy dogs (29.07 ng/mL [17.60–41.04]) and dogs with MMVD (28.05 ng/mL [15.35–38.61]) did not differ.

Serum concentrations of IL-1β, IL-6, and IL-10 could not be quantitated in >60% of dogs, whereas IL-18 could be quantitated >80% of dogs (Table 2). Although internal controls and calibrations were valid, all dogs had TNF-α concentrations below the detection threshold. Therefore, IL-1β, IL-6, IL-10, and TNF-α were excluded from statistical analysis. The IL-18 concentrations did not differ significantly between healthy dogs and dogs with MMVD (P = .16).

**Serum Leptin and Adiponectin Concentrations According to the ISACHC Classification: Subsequent Study**

In the subsequent study, as expected, the median serum concentration of leptin (95% CI for difference between medians = 0.70–2.75; P = .0005) was significantly higher in dogs with MMVD (2.89 ng/mL [1.30–4.57]) than in healthy dogs (1.45 ng/mL [0.41–2.07]; Fig 3A and C), whereas the median serum concentration of adiponectin (95% CI for difference between medians = −8.37 to −2.42; P = .0009) was significantly lower in dogs with MMVD (6.20 μg/mL [4.30–6.70]) than in healthy dogs (11.37 μg/mL [5.56–17.14]).

**Table 1. Primers for leptin and adiponectin receptors.**

| Gene           | Accession Number | Product Size (bp) | Primers                                | Cycles |
|----------------|------------------|-------------------|----------------------------------------|--------|
| Leptin receptor | NM_001024634.1   | 175               | F – CACCCAGAATGATGCAAGGGTCT            | 40     |
|                |                  |                   | R – GCTCAAATGTGCTGCTGGTCTG             |        |
| ADIPOR1        | XM_843263.1      | 202               | F – GACAGAAGCAAGGAGTGGTTCC             | 40     |
|                |                  |                   | R – CTGAGAATCCAGCAGCAT                 |        |
| ADIPOR2        | XM_534929.2      | 192               | F – CCCCCTTCTTCTTAAATTTGG             | 40     |
|                |                  |                   | R – CTGATATGAGGGGTAGCA                 |        |

ADIPOR1, adiponectin receptor 1; ADIPOR 2, adiponectin receptor 2.
To further investigate whether serum concentrations of leptin and adiponectin differed according to the severity of HF, all dogs with MMVD were divided into 3 groups using the ISACHC classification (Fig 3B and D). Baseline characteristics and conventional echocardiographic results are given in Tables 3 and 4, respectively. We observed no significant differences in baseline characteristics such as sex, weight, and age. An overall significant difference ($P = .0040$) was found between the four groups (healthy, and ISACHC class 1, 2, and 3 groups) and leptin concentration. Serum leptin concentration was significantly higher in ISACHC class 3 (4.18 ng/mL [1.28–8.54]) than in healthy dogs (1.45 ng/mL [0.51–2.26]; 95% CI for difference between medians = 0.85–5.83; $P = .0022$). However, serum leptin concentrations in ISACHC class 3 (4.18 ng/mL [1.28–8.54]) did not significantly differ from those in ISACHC class 1 (3.19 ng/mL [1.74–4.88]; 95% CI for difference between medians = −1.36 to 5.53; $P = .53$) or class 2 (2.28 ng/mL [1.20–3.74]; 95% CI for difference between medians = −0.14 to 4.91; $P = .07$). An overall significant difference ($P = .0007$) was found between the 4 groups and adiponectin concentration. Serum adiponectin concentrations were significantly lower in ISACHC class 1 (3.01 μg/mL [0.88–5.04]; 95% CI for difference between medians = −13.18 to −4.32; $P < .0001$) than in healthy dogs (11.37 μg/mL [5.56–17.14]), whereas the concentration in class 3 (7.53 μg/mL [4.20–16.79]; 95% CI for difference between medians = 1.51–11.47; $P = .0081$) was significantly higher than that in ISACHC class 1 (3.01 μg/mL [0.88–5.04]). Serum adiponectin concentrations did not differ between ISACHC class 1 (3.01 μg/mL [0.88–5.04]) and class 2 (5.56 μg/mL [4.35–12.60]; 95% CI for difference between medians = 0.90–7.59; $P = .0181$) or between ISACHC class 2 (5.56 μg/mL [4.35–12.60]) and class 3 (7.53 μg/mL [4.20–16.79]; 95% CI for difference between medians = −2.19 to 4.43; $P = .54$).

Natural logarithm-transformed adiponectin concentrations increased as FS values increased ($R^2 = .134, B = .033, P = .013$), whereas they decreased with increasing LVIDs values ($R^2 = .170, B = −.058, P = .005$; Fig 4). Otherwise, natural logarithm-transformed leptin concentrations were not associated with any variables.

**Expression of Leptin and Adiponectin Receptors in Cardiac Tissues**

Reverse-transcriptase PCR confirmed mRNA expressions of leptin and adiponectin receptors in cardiac tissues. As shown in Figure 5, Ob-R (leptin receptor) and
adiponectin receptors 1 and 2 were expressed in cardiac tissue. Each cardiac region expressed mRNA of leptin and adiponectin receptors; we did not compare the mRNA levels among different regions.

**Discussion**

We found that circulating concentrations of serum leptin and adiponectin were altered in dogs with MMVD and that dogs in ISACHC class 3 (severe HF) had the highest concentrations of both leptin and adiponectin. In particular, significant associations between serum adiponectin concentrations and echocardiographic variables (LVIDs and FS) were shown. In addition, our confirmation of mRNA expression of leptin and adiponectin receptors in cardiac tissues raises the possibility that circulating leptin and adiponectin directly affect cardiomyocytes in dogs with MMVD.

### Table 2. Serum interleukin (IL) and tumor necrosis factor (TNF)-α concentrations in healthy dogs, dogs with MMVD without CHF, and dogs with MMVD and CHF in the preliminary study.

|                | Healthy Dogs (n = 30) | MMVD Dogs without CHF (n = 17) | MMVD Dogs without CHF (n = 8) | MMVD Dogs with CHF (n = 9) |
|----------------|----------------------|---------------------------------|-------------------------------|-----------------------------|
| IL-1β (pg/mL)  | 0 (0–0)              | 15.41 (0–28.19)                | 15.47 (3.01–26.82)            | 15.41 (0–54.66)             |
| [d/t]          | [3/30]               | [12/17]                         | [6/8]                         | [6/9]                       |
| IL-6 (pg/mL)   | 0 (0–0)              | 5.61 (2.16–9.36)               | 5.01 (1.081–8.48)            | 7.71 (2.81–15.40)           |
| [d/t]          | [3/30]               | [13/17]                         | [6/8]                         | [7/9]                       |
| IL-18 (pg/mL)  | 22.45 (0–26.00)      | 19.77 (14.33–22.55)            | 15.35 (11.88–22.03)          | 20.60 (17.55–23.15)         |
| [d/t]          | [23/30]              | [16/17]                         | [7/8]                         | [9/9]                       |
| IL-10 (pg/mL)  | 17.29 (0–36.30)      | 6.17 (7.40–14.65)              | 6.59 (1.51–8.22)             | 13.00 (7.40–29.60)          |
| [d/t]          | [17/30]              | [14/17]                         | [6/8]                         | [8/9]                       |
| TNF-α (pg/mL)  | 0 (0–0)              | 0 (0–0)                         | 0 (0–0)                       | 0 (0–0)                     |
| [d/t]          | [0/30]               | [0/17]                          | [0/8]                         | [0/9]                       |

Data are reported as medians (interquartile ranges). MMVD, myxomatous mitral valve disease; CHF, congestive heart failure; d/t, detected sample numbers/total sample numbers; Concentrations below assay detection of limit were set to zero.

![Fig 3](image-url)

**Fig 3.** Serum concentrations of leptin (A and B) and adiponectin (C and D) in healthy dogs (n = 30) and dogs with myxomatous mitral valve disease (MMVD) (n = 46) according to the International Small Animal Cardiac Council (ISACHC) classification in the subsequent study. Horizontal bars indicate medians and interquartile ranges. Outliers > 1.5 interquartiles from the medians are shown as squares. *P < .0083 (Mann–Whitney U-test (A and C) and Kruskal–Wallis test (B and D)).
Table 3. Characteristics in 30 healthy dogs, 17 dogs with myxomatous mitral valve disease (MMVD) in the preliminary study and 46 dogs with MMVD in the subsequent study.

|                  | Healthy Dogs (n = 30) | Dogs with MMVD in the Preliminary Study (n = 17) | Dogs with MMVD in the Subsequence Study (n = 46) |
|------------------|-----------------------|-----------------------------------------------|-----------------------------------------------|
| Age, years       | 11 (9–13)            | 11 (8–16)                                     | 11 (9–13)                                     |
|                  | (median; range)       | (median; range)                               | (median; range)                               |
| Sex (IF/NF/IM/NM)| 0/18/3/9             | 0/8/2/7                                       | 0/6/2/3                                      |
|                  | (median; range)       | (median; range)                               | (median; range)                               |
| Body weight, kg  | 4.6 (2.6–6.5)        | 4.4 (2.5–8.8)                                 | 4.4 (3.2–6.7)                                 |
|                  | (median; range)       | (median; range)                               | (median; range)                               |
| Breed            | Maltese 4            | 3                                             | 3                                             |
|                  | Miniature Schnauzer 7| 2                                             | 1                                             |
|                  | Mixed breed 7        | 3                                             | 1                                             |
|                  | Shih-Tzu 3           | 4                                             | 2                                             |
|                  | Others 9             | 4                                             | 2                                             |

Data are shown as medians (range).

IF, intact female; NF, neutered female; IM, intact male; NM, neutered male; BW, body weight; ISACHC, International Small Animal Cardiac Health Council; MMVD, myxomatous mitral valve disease.

Table 4. Echocardiographic data in healthy dogs and dogs with myxomatous mitral valve disease (MMVD).

|                  | Healthy Dogs (n = 30) | ISACHC 1 (n = 11) | ISACHC 2 (n = 20) | ISACHC 3 (n = 15) |
|------------------|-----------------------|-------------------|-------------------|-------------------|
| LA/AO            | 1.53 (1.37–1.64)      | 1.70 (1.45–2.00)  | 1.95 (1.68–2.66)  | 2.46 (2.05–3.10)  |
| LVIDd (mm)       | 23.45 (21.40–25.60)   | 25.47 (19.29–30.92) | 28.99 (19.39–32.22) | 30.36 (19.30–34.43) |
| LVIDs (mm)       | 14.13 (12.41–14.94)   | 11.48 (7.41–16.19) | 12.17 (9.05–15.49) | 12.18 (7.56–16.57) |
| iLVIDd (%)       | 8.64 (2.89–25.75)     | 24.67 (11.95–39.00) | 26.26 (17.43–37.72) | 30.19 (17.60–49.34) |
| iLVIDs (%)       | 9.13 (5.49–11.55)     | 18.04 (9.32–27.33) | 20.24 (8.43–29.94) | 25.27 (11.35–33.56) |
| FS (%)           | 41.04 (40.08–42.57)   | 47.25 (41.53–58.53) | 54.20 (51.45–59.60) | 54.55 (50.50–59.75) |
| HR (bpm)         | 127.5 (103.3–145.5)   | 140 (120–156)     | 160 (132–174)     | 170 (138–180.5)    |
| SAP (mmHg)       | 90 (75–115)           | 150 (142–180)     | 134 (121–157.5)   | 124 (99.5–165)     |

Values are reported as medians (interquartile ranges).

ISACHC, International Small Animal Cardiac Health Council; LA/AO, ratio of left atrium to aortic root; LVIDd, end-diastolic left ventricular internal dimension; LVIDs, end-systolic left ventricular internal dimension; iLVIDd, percentage increase in end-diastolic left ventricular internal dimension; iLVIDs, percentage increase in end-systolic left ventricular internal dimension; FS, fractional shortening; HR, heart rate; bpm, beat per minute; SAP, systolic arterial pressure.

Fig 4. Linear associations between natural logarithm-transformed adiponectin concentrations and (A) FS and (B) LVIDs. The dotted lines indicate 95% confidence intervals. FS, fractional shortening; LVIDs, end-systolic left ventricular internal dimension.
Our results suggest that alterations of circulating leptin and adiponectin concentrations might be related in the progression to CHF in MMVD dogs although a cause-and-effect relationship between the adipokines and the development of CHF in MMVD dogs cannot be inferred.

In humans, adipokines have been implicated in the development and progression of cardiovascular disease.14,16,28–32 Although the pathophysiology of HF typically follows similar courses in different animals, cardiac diseases that lead to HF differ among species. Therefore, a cause-and-effect relationship between adipokines and the development and progression of CHF caused by MMVD might be difficult to clearly establish. We found that serum leptin concentrations were upregulated in dogs with MMVD and that serum leptin concentration was highest in dogs with severe HF, indicating that leptin upregulation might be related to progression of HF in association with MMVD. These results are similar to those obtained in humans.31,32 Consistent with this finding, a recent study in dogs reported that the expression of circulating leptin mRNA varies with severity of cardiac disease.15 Circulating concentrations of leptin strongly correlate with body mass index and tissue adiposity in dogs.33 In our study, because the body condition score of the dogs in both the healthy and MMVD groups was matched (at a BCS of 5/9), it was unlikely that the adiposity of the dogs influenced serum leptin concentrations. On the other hand, because the primary function of circulating leptin is regulation of energy expenditure and energy storage according to metabolic needs, alterations of circulating leptin concentrations are likely to be related to metabolic status.34 In human medicine, patients with HF are characterized by metabolic abnormalities associated with progressive catabolic syndrome, and the neurohormonal activations resulting from HF are associated with an imbalance between anabolic and catabolic metabolism.35,36 Ultimately, the progressive catabolic syndrome in advanced HF leads to functional alteration of skeletal muscle, contributing to exercise intolerance in HF patients.37,38 Therefore, the catabolic condition arising from an increased serum leptin concentration might exacerbate the clinical signs of HF.

Indeed, a previous study reported that patients with HF and severe exercise intolerance had significantly higher serum concentrations of leptin than did patients with moderate exercise intolerance or healthy subjects.36 This observation in humans is consistent with our finding that serum leptin concentrations increased with the severity of CHF caused by MMVD.

On the other hand, several studies in humans identified correlations between increased circulating leptin concentrations and unfavorable outcomes of cardiac disorders.14 In the context of HF with neurohormonal activation, hyperleptinemia directly results in enhanced sympathetic activation in response to central stimulation of hypothalamic leptin receptors.39 Although this neurohormonal activation might initially serve to compensate for impaired cardiac function, increased norepinephrine concentrations in patients with chronic HF are associated with poor outcome and higher risk of mortality.40 Therefore, increased serum leptin concentration has been proposed as a risk factor for HF in humans.41,42 In MMVD dogs with CHF, it is possible that a high concentration of serum leptin also might predict a poor outcome, but further study is required to confirm the association between serum leptin concentration and clinical prognosis.

In our study, adiponectin concentrations were significantly lower in dogs with MMVD than in healthy dogs, but increased according to severity of cardiac clinical signs as determined by the ISACHC classification. Generally, in contrast to the effects of leptin, the best characterized effects of adiponectin in humans include enhancement of insulin sensitivity,43 anti-inflammatory properties,44 and inhibition of atherosclerosis.17 In particular, a stepwise increase of adiponectin with increasing HF severity has been described in humans,45,46 and recent studies also demonstrated an association between an increased serum adiponectin concentration and increased risk of mortality in HF patients.45 However, the mechanisms underlying the increase in serum adiponectin concentration in severe HF in humans remain undetermined. One possible mechanism is that the upregulation of circulating adiponectin concentrations in HF might be related to a counter-regulatory protective response, similar to the action of B-type natriuretic...
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peptide as a counter-regulatory hormone in HF pathophysiology. Adiponectin, as a cardioprotectant, regulates cardiac injury by modulating anti-inflammatory and prosurvival reaction and inhibiting cardiac remodeling, thereby protecting against the progression of myocardial injury. Therefore, our findings suggest that upregulation of adiponectin concentrations in dogs with severe MMVD might be a counter-regulatory protective response. However, because in our study, the serum adiponectin concentrations of asymptomatic dogs in ISACHC class 1 were lower than those of healthy dogs or dogs in ISACHC class 3, it is also possible that patients with low adiponectin concentrations might have a high cardiovascular risk. A recent study showed that there was no significant difference in adiponectin concentrations of asymptomatic dogs in ISACHC class 1, which was lower than those of healthy dogs or dogs in ISACHC class 3. This discrepancy might be due to differences in the population of enrolled dogs, such as age and neutering status. Therefore, further studies will be necessary to clarify the relationship between the change of adiponectin concentrations and MMVD severity.

To the best of our knowledge, no information currently is available regarding the presence of leptin and adiponectin receptors in cardiac tissue of dogs. We demonstrated the expression of leptin and adiponectin receptor mRNA in canine cardiac tissue, suggesting that serum leptin and adiponectin molecules act directly on canine cardiomyocytes. The effects of circulating leptin are mediated by its leptin receptor, which generally are referred to as Ob-R. In the mouse, this receptor is expressed abundantly in many different cells, including cardiomyocytes and intact myocardium. Previous studies reported that leptin produces marked hypertrophy in cultured neonatal rat ventricular myocytes, and hyperplastic hypertrophy was manifested as increased cell size, increased protein synthesis, and upregulation of a number of genetic hypertrophic markers. Direct action of leptin on cardiomyocytes included a decrease in cardiomyocyte apoptosis, activation of fatty acid oxidation, increased fatty acid uptake, and increased protein synthesis. In dogs, leptin concentrations of leptin in patients with HF might exert a direct effect on cardiomyocytes, and such an activity might contribute to the progression of HF. Therefore, the presence of leptin receptor in cardiomyocytes of dogs indicated that circulating leptin might also act as a prohypertrophic factor on the heart in dogs. Further characterization of leptin receptor (Ob-R) isoforms indicated that cardiac tissue expresses several isoforms of Ob-R including Ob-R, Ob-Rb, and Ob-Re. Murine cardiac tissues exhibit differences in the cardiac regional distribution of Ob-R isoforms, but no information about leptin receptor isoforms in dogs is currently available. Additional studies regarding leptin receptor expression and postreceptor leptin signaling will be necessary to determine the direct effects of leptin on the cardiac tissues of dogs. Two specific adiponectin receptors have been cloned: adiponectin receptor 1 is primarily expressed in skeletal muscle, whereas adiponectin receptor 2 is highly expressed in the liver. In humans, the cardioprotective effect of adiponectin is mediated by its ability to prevent (through adiponectin receptors 1 and 2) activation of the AMPK and ERK 1/2-NF-κB signal transduction pathways. Our study also identified the expression of mRNAs encoding both adiponectin receptors in cardiac tissues of dogs. However, quantitative analyses of protein and mRNA expression will be necessary to identify the missing link in the causal relationship between activation of receptors by the 2 adipokines and the pathogenesis of MMVD in dogs.

Visfatin, also known as pre-B-cell colony-enhancing factor, exerts beneficial effects in the context of metabolic disorders. However, growing clinical evidence indicates that visfatin is a biomarker or even a predictor of inflammation and endothelial injury in several metabolic diseases. Several approaches have determined that visfatin exerts direct deleterious effects on the cardiovascular system, including cell proliferation, leukocyte adhesion, inflammation, and vascular inflammation and remodeling. In our study, serum visfatin concentrations did not differ in MMVD dogs as compared to healthy dogs. Because few data are available regarding visfatin in dogs, additional studies are required to fully elucidate the role of visfatin in dogs with CHF and MMVD. Resistin concentrations did not differ between dogs with MMVD and healthy dogs. Resistin received its name based on the original observation that it induced insulin resistance in murine adipocytes. In mice, resistin is expressed mainly by adipocytes, whereas in humans, it appears to be produced primarily by vascular myocytes and monocytes/macrophages. Resistin concentration is positively correlated with indicators of inflammation and endothelial activation such as leukocyte counts, and high-sensitivity C-reactive protein and endothelin-1 concentrations in blood. This observation suggests that resistin is a strong risk factor for cardiovascular diseases, including vascular disease, diabetes, and atherosclerosis. Resistin also is associated with markers of inflammation, including TNF-α and IL-6, which have in turn been shown to predict HF incidence in atherosclerosis. However, the pathogenesis of MMVD is associated with deposition and overproduction of extracellular matrix, rather than inflammation associated with vascular or ischemic injury. Because inflammatory cells are absent on histological evaluation of diseased valves and myocardium, the degenerative changes that cause mitral regurgitation are considered to be noninfectious and noninflammatory, suggesting that serum resistin does not exert direct effects on the severity of MMVD in dogs. Our study population was too small to assess the impact of this adipokine. Consequently, further study is required to confirm the role of resistin in cardiac disease in dogs.

In our study, concentrations of IL-1β, IL-6, IL-10, and TNF-α could not be quantitated in most dogs. Although the IL-18 concentration could be measured in most dogs, it did not differ significantly between healthy
dogs and dogs with MMVD. However, increased myocardial cytokine expression and increased concentrations of circulating cytokines are characteristic of chronic HF in humans implying that overexpression of proinflammatory cytokines contributes to the progression of HF. These discrepant findings might be due to differences in the etiology of HF among species. Most studies in humans and mice have focused on HF secondary to coronary artery disease or dilated cardiomyopathy related to inflammation of the myocardium or vasculature whereas MMVD, the most common chronic cardiac disease in small dog breeds, generally is not caused by infiltration of the myocardium by inflammatory cells. Nevertheless, we are unable to explain why we observed no significant increases in inflammatory cytokines such IL-1β, IL-6, IL-18, and TNF-α in dogs with clinically severe MMVD. A recent study reported that monocyte chemoattractant protein-1 is involved in the pathogenesis of CHF caused by MMVD in dogs, whereas downregulation of IL-2, IL-7, and IL-8 is evident in Cavalier King Charles Spaniels with increasing severity of MMVD. Future studies using larger cohorts of MMVD dogs with refractory or chronic CHF will improve our understanding of the role of alteration of circulating cytokines in the progression of MMVD.

Our study had several limitations. One limitation was the small number of dogs with MMVD, which constrains the reliability of the negative findings. Another limitation is the possibility that ISACHC groups do not perfectly reflect the severity of MMVD, because the ISACHC grading system is a classification system for assessing the severity of HF but not that of MMVD specifically. To address this issue, a more accurate method for classifying the severity of MMVD as mitral regurgitation scores could have been applied. Future studies using regression models are necessary to elucidate the associations between the leptin and adiponectin concentrations and the severity of MMVD. Finally, our findings address only a small part of the complex pathophysiology of CHF in dogs with MMVD. Confirmation of these results will require additional studies using larger cohorts of dogs.

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Footnotes

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