# ABSTRACT

## *New insights concerning histopathologic morphometry and transcriptomics to evaluate myocardial dysfunction in dogs with chronic heart failure*

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Degenerative mitral valve disease (DMVD) is the leading cause of congestive heart failure in small breed dogs. Patent ductus of arteriosus (PDA) and pulmonary stenosis (PS) are also the most common canine congenital cardiovascular abnormalities in most epidemiological studies, eventually resulting in heart failure. In chronic heart diseases, the accurate evaluation of myocardial function is important, since it could be a guideline when planning medical treatment and formulating prognosis. In DMVD, however, the clinical assessment of myocardial function is challenging due to the fact that mitral insufficiency induces hemodynamic circumstance of high-volume and low-pressure, misguiding echocardiographic measurements. The myocardial dysfunction basically accrues from histopathologic

degeneration, so that the myocardial decompensation results in clinical symptoms. However, there is little knowledge on how the myocardial degeneration is microscopically progressed in DMVD. In order to better understand pathophysiologic progression of heart diseases, clinically numerous ancillary tests have been investigated in terms of diagnostic and prognostic ability in dogs and cats with heart diseases. Furthermore, some of those cardiac biomarkers have been being practically applied in small animal clinics. However, currently available cardiac biomarkers still have many drawbacks, such as being influenced by concurrent systemic diseases, and their unreliable biostability. In this study, histopathologic and genetic approaches are introduced in an attempt to develop clinically reliable and sensitive cardiac biomarkers in chronic heart diseases.

In DMVD (n=117), histopathologic alterations were assessed in left atrium (LA) and left ventricle (LV) (fatty replacement, myocardial vacuolization, infiltrated immune cells, increased perinuclear space, enlarged nuclei, hypertrophy, interstitial fibrosis), as well as in lung (alveolar septal thickening, heart failure cells and hyperplasia of type II pneumocytes), by using either semi-quantitative scoring or computer-based digitizing system. The quantified pathologic findings were analyzed in view of their relationship with various clinical indices. There was no significant difference among all groups of severity (International Small Animal Cardiac Health Council, ISACHC), where pathologically severe progression was found, except for the heart failure cells that significantly increased only in class III ($P<0.0001$). The LA had more highly degenerated myocardial lesions than the LV on the findings such as myocardial fatty replacement, immune cell infiltration, and interstitial fibrosis ($P<0.0001$). Meanwhile, cardiomyocytes in the LV were more hypertrophied than

those in the LA ($P$<0.0001). Multivariate regression analysis revealed that left ventricular internal dimension in diastole was well associated with myocardial changes in the LA: fatty replacement ($P$=0.033, $R^2$=0.584) and myocardial vacuolization ($P$=0.003, $R^2$=0.588). Interstitial fibrosis was negatively related to ejection fraction in both the LA ($P$=0.012, $R^2$=0.231, $\beta$ =-0.251) and the LV ($P$=0.036, $R^2$=0.205, $\beta$ =-0.207). Thus, myocardial and pulmonary pathologic degenerations are evident even in the early stages of DMVD, but there were discrepancies on the pathologic findings between the LA and the LV. In addition, although the conventional clinical indices were partly related to the histopathologic outcomes, it seems that the traditional echocardiographic parameters are still limited to soundly reflect pathologic degeneration, resulting in myocardial dysfunction.

Sarcoplasmic reticulum $Ca^{2+}$-ATPase and its regulatory proteins are major determinants of myocardial contraction and relaxation. The lowered density of the sarcoplasmic reticulum $Ca^{2+}$-ATPase has been well identified as a main cause of myocardial dysfunction in cardiac diseases. The genes linked to sarcoplasmic reticulum calcium uptake were reported not only being expressed in peripheral blood but serving as potential cardiac biomarkers in dogs with chronic mitral regurgitation, such as sarcoplasmic reticulum $Ca^{2+}$ adenosine triphosphatase isoform 2α (*SERCA2α*), phospholamban (*PLN*), and HS-1 associated protein X-1 (*HAX-1*). The aim of this study is to determine whether the target genes expressed in the blood will be translatable to the myocardial setting as cardiac biomarkers. The mRNA expression levels of the target genes (*SERCA2α*, *PLN*, *HAX-1*) from biopsied LV and peripheral blood mononuclear cells (PBMC) in 129 dogs undergoing mitral valve repair surgery were estimated with quantitative real-time PCR by using comparative Ct method with

GAPDH. The gene expression levels in the LV and PBMC were compared and their clinical relationships were evaluated. The diagnostic power of the gene expressions in PBMC was analyzed by comparing to those in 33 normal dogs. The mRNA expression levels of the target genes in both the LV and PBMC were progressively and significantly reduced in line with the severity of DMVD (ISACHC system, $P<0.01$). Particularly, clinical indices to suggest hemodynamic change such as left ventricular internal systolic (LVIDs) and diastolic dimensions (LVIDd) have a negative effect on the expression level of *SERCA2α* and *PLN* in the LV (*SERCA2α* with LVIDs, $P=0.0251$, adjusted $R^2=0.524$; PLN with LVIDs $P=0.012$, adjusted $R^2=0.530$ and with LVIDd, $P=0.037$, adjusted $R^2=0.573$). Additionally, the target genes expressed in the LV and PBMC were highly correlated each other ($P< 0.0001$; *SERCA2α*, $r=0.7425$, $R^2=0.5513$; *PLN*, $r=0.7720$, $R^2=0.5959$; *HAX-1*, $r=0.6598$, $R^2=0.4353$), although the LV and PBMC showed different expression levels in a pair-wise comparison ($P< 0.0001$). When it comes to diagnostic values, *SERCA2α* and *PLN* expressed in the PBMC could clearly discriminate all ISACHC groups from the control (n=33; $P< 0.0001$). Moreover, all target genes in the PBMC showed high area under the curve (AUC) values in receiver-operating characteristic analysis ($P<0.0001$; *SERCA2α*, AUC=0.9212; *PLN*, AUC=0.8936; *HAX-1*, AUC=0.8797). Therefore, the target genes expressed in the peripheral blood may be translatable to the myocardial setting as cardiac biomarkers.

Although evaluating the cardiac specific genes, expressed in PBMC may be able to predict myocardial distress in DMVD, the previous simple cross-sectional diagnostic study is insufficient to identify that hemodynamic overload is a primary determinant which leads to the altered expression level of the target genes, such as

*SERCA2α*, *PLN*, and *HAX-1*. This is because the population consisted of DMVD has various confounding factors that can influence on hemodynamics such as occult comorbidities, age-dependent senescence, and medical treatment provided to individuals. To expand and clarify the previous work, the candidate genes (*SERCA2α* and *PLN*) were investigated in younger population with patent ductus of arteriosus (PDA; n=8) and with pulmonic stenosis (PS; n=5) and compared with healthy laboratory beagles (n=24). None of patients have been treated previously with cardiovascular medication and had concurrent other diseases. The expression levels of *SERCA2α* and *PLN* in PBMC were longitudinally and pair-wisely evaluated before and one week after surgical intervention. In parallel, NT-proBNP was also measured as a reference cardiac biomarker. Both transcriptomic cardiac biomarkers significantly decreased in PDA ($P$<0.001; fold change: 0.50±0.17 for *SERCA2α* and 0.41±0.21 for *PLN*) as well as in PS ($P$<0.001; fold change: 0.47±0.20 for *SERCA2α* and 0.35±0.10 for *PLN*). In addition, their expression levels were significantly recovered in one week after surgical correction in both PDA ($P$<0.01; fold change: 0.74±0.22 for *SERCA2α* and 0.68±0.24 for *PLN*) and PS ($P$<0.01; fold change: 0.74±0.18 for *SERCA2α* and 0.65±0.20 for *PLN*). Meanwhile, plasma levels of NT-proBNP measured at before and after surgery in PDA (before surgery, 761.0±579.2 pmol/L; one week after surgery, 679.9±437.2 pmol/L) were not different from control group (521.5±203.1 pmol/L). On the contrary, NT-proBNP level in PS was significantly higher than the control ($P$<0.05). However, there were no significant difference between the plasma concentration of NT-proBNP measured at before and one week after pulmonary valvectomy (before surgery, 867.2±560.2 pmol/L; one week after surgery, 863.4±523.1 pmol/L). Additionally, both *SERCA2α* and *PLN* were strongly correlated to fractional shortening in PDA ($P$<0.001; *SERCA2α*,

$R^2$=0.6058; *PLN*, $R^2$=0.5477) and peak pressure gradient in pulmonary artery in PS (*P*<0.01; *SERCA2α*, $R^2$=0.6798; *PLN*, $R^2$=0.6565). Moreover, AUC values of *SERCA2α* (*P*<0.01; 0.95±0.04 in PDA; 0.95±0.04 in PS) and *PLN* (*P*<0.01; 1.00±0.0 in PDA; 0.98±0.03 in PS) in receiver-operating characteristic analysis were remarkably higher than those of NT-proBNP in both PDA and PS.

In conclusion,

1. It is suggested that severe pathologic changes are probably progressed from very early stage of heart failure with significant pathologic disparity between the LA and the LV. Conventional diagnostic imaging techniques might be limited to properly predict the progression of the myocardial and pulmonary degeneration in DMVD.

2. Transcriptomic alterations of the calcium uptake related genes (*SERCA2α*, *PLN*, *HAX-1*) expressed in PBMC may be reliable cardiac biomarkers that reflect myocardial dysfunction resulted from the disturbance of intra-myocardial calcium handling.

3. *SERCA2α* and *PLN* expressed in PBMC may be able to sensitively manifest myocardial distressful environment, primarily determined by hemodynamic change. High diagnostic power of *SERCA2α* and *PLN* was successfully reproduced even in the young animals with sub-clinical stage of congenital cardiac diseases.