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Veterinary Medicine and Multi-Omics Research for Future Nutrition Targets: Metabolomics and Transcriptomics of the Common Degenerative Mitral Valve Disease in Dogs

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Abstract

Canine degenerative mitral valve disease (DMVD) is the most common form of heart disease in dogs. The objective of this study was to identify cellular and metabolic pathways that play a role in DMVD by performing metabolomics and transcriptomics analyses on serum and tissue (mitral valve and left ventricle) samples previously collected from dogs with DMVD or healthy hearts. Gas or liquid chromatography followed by mass spectrophotometry were used to identify metabolites in serum. Transcriptomics analysis of tissue samples was completed using RNA-seq, and selected targets were confirmed by RT-qPCR. Random Forest analysis was used to classify the metabolites that best predicted the presence of DMVD. Results identified 41 known and 13 unknown serum metabolites that were significantly different between healthy and DMVD dogs, representing alterations in fat and glucose energy metabolism, oxidative stress, and other pathways. The three metabolites with the greatest single effect in the Random Forest analysis were γ-glutamylmethionine, oxidized glutathione, and asymmetric dimethylarginine. Transcriptomics analysis identified 812 differentially expressed transcripts in left ventricle samples and 263 in mitral valve samples, representing changes in energy metabolism, antioxidant function, nitric oxide signaling, and extracellular matrix homeostasis pathways. Many of the identified alterations may benefit from nutritional or medical management. Our study provides evidence of the growing importance of integrative approaches in multi-omics research in veterinary and nutritional sciences.

Introduction

Degenerative mitral valve disease (DMVD) affects approximately 9% of all dogs, increasing with age such that the overall cumulative incidence is greater than 40% (Atkins et al., 2009; Buchanan, 1999; Olsen et al., 2010; Rush and Cunningham, 2014). The histological changes associated with DMVD include extracellular matrix (ECM) changes, such as proteoglycan deposition and disorganization and disruption of collagen filaments (Olsen et al., 2010; Orton et al., 2012; Oyama and Levy, 2010). Although the echocardiographic, pathological, and histological changes have been well documented, the molecular changes contributing to DMVD remain unclear. Heredity appears to play a major role in risk for development of the disease and specific molecular and metabolic changes may alter the onset or progression of disease in predisposed dogs, including changes in serotonin, transforming growth factor-beta (TGF-β), nitric oxide (NO) signaling pathways, and collagen disorders (Olsen et al., 2010; Oyama and Levy, 2010; Sisson et al., 1999).

The ECM is a dynamic network that is constantly being remodeled to achieve balance between synthesis and degradation of matrix components. Matrix metalloproteinases (MMPs) are the driving force for ECM degradation, while tissue inhibitors of metalloproteinases (TIMPs) balance the degradation (Li et al., 2000). Aberrant expressions of MMPs and TIMPs genes have been implicated in maladaptive ECM remodeling in a variety of cardiovascular diseases, including DMVD (Li et al., 2000). Both serotonin and TGF-β signaling have been implicated in regulating ECM turnover in canine

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DMVD, potentially via upregulation of downstream effector genes, including MMPs and TIMPs (Orton et al., 2012; Oyama and Chittur, 2006). Experimental evidence also suggested an association between NO activities and DMVD pathogenesis (Moesgaard et al., 2007a; 2007b; Oyama and Chittur, 2006; Siney and Lewis, 1993).

A hallmark of transition from fetal to adult cardiac metabolism is the switch in energy substrate preference from carbohydrates to fatty acids (Rajabi et al., 2007). This transition is accompanied by changes in expression patterns of genes and proteins (Dirkx et al., 2013; Rajabi et al., 2007). One of the prominent features of the biomechanically stressed heart is the return to the fetal gene program and the pattern of fetal metabolism, in which carbohydrates once again become the predominant energy substrate (Rajabi et al., 2007; Stanley et al., 2005). This adaptive mechanism is thought to help maintain heart function under stress.

Genomics, transcriptomics, proteomics, and metabolomics are the major omics platforms in today's biomedical research. Multi-omics integrative analysis has become increasingly common in studying nutrition and functional food components (Kato et al., 2011), or in dissecting regulatory networks for complex disease trait (Civelek and Lusis, 2014). Past studies have demonstrated that the multi-omics study at the systems level can provide more powerful and valuable insights into the biological mechanism than a single platform analysis. The current study applied advanced metabolomics and transcriptomics methods to characterize molecular and metabolic pathway derangements that might play a role in the pathogenesis and progression of DMVD. The objective was to identify pathways that might ultimately be modified via nutritional or pharmaceutical options to prevent, reverse or manage DMVD.

Materials and Methods

Canine tissue and serum samples

The study protocol was reviewed and approved by Nestlé Purina's Institutional Animal Use and Care Committee and complied with Nestlé Purina's animal welfare guidelines. Serum and tissue samples were from dogs categorized as having either a healthy heart or heart affected by DMVD based on echocardiography performed or evaluated by a board-certified veterinary cardiologist, pathological examination of the heart, or both. For the metabolomics experiments, serum from 18 dogs with DMVD and 11 age- and gender-matched healthy control dogs had been previously collected and was stored at −80°C until analysis. Tissues for the transcriptomics study were from different dogs humanely euthanized for reasons unrelated to this study. Mitral valve (MV) samples were obtained from three control dogs with no evidence of heart disease and three dogs with DMVD. Tissue samples from the free wall of left ventricles (LV) were collected from four control dogs and two dogs with DMVD. All tissue samples had been collected within 30 min of euthanasia, frozen immediately, and stored at −80°C until use.

Metabolomics experiment

Serum samples were processed by a commercial laboratory (Metabolon, Inc., Durham, NC) using a standard extraction protocol (Evans et al., 2009). The extracts were then split into equal parts for analysis using gas or liquid chromatography, followed by mass spectrophotometry. Metabolites were identified by matching the ions' chromatographic retention index and mass spectral fragmentation signatures with reference library entries. For ions that were not covered by the standards, additional library entries were added based on their unique ion signatures.

Total RNA extraction and RNA-seq

Total RNA was extracted, and quality control and quantitation were performed using standard methods. Total RNA was converted into a library of template cDNA molecules suitable for high throughput DNA sequencing. Library preparation and subsequent deep sequencing were conducted at the Tufts University Genomics Core Facility. An average of 25 million 50-base single-end reads was obtained per sample.

Quantitative PCR validation assay

In order to confirm the results from high throughput sequencing, a selected group of 15 target genes plus housekeeping gene, TATA box binding protein (TBP), were further evaluated using a two-step RT-qPCR procedure performed on the LV samples. The targets were selected based on their biological relevance to the disease. For example, ECM turnover, fatty acid metabolism, nitric oxide signaling, and TGF-β signaling are known to be involved in heart remodeling in humans and dogs. All targets were standard from Taqman® Gene expression Assay (Applied Biosystems/Life Technologies, Grand Island, NY) except for HOPX. The sequences for forward primer, reverse primer, and probe for HOPX were (from 5′ to 3′): TCAGCATAGTGCTATGTGTTTCATTGT, CAGCTTCCTATAGTTATTTCTTTCCTAGTTATATAC ATT, and TAGGTGTCCTGCTATTTAAC, respectively. The assay was performed as previously described (Middleton et al., 2013). Reverse transcriptase reactions were performed in duplicate for each sample and quantitative PCR reactions were performed in triplicate. All target values were normalized against the TBP.

Metabolomics data analysis

Data analyses consisted of two parts, significance tests and classification analyses. Two sample t-tests were used to test the null hypothesis that the means of two groups were equal. The Welch's correction was applied to allow for unequal variances between the groups. A p value ≤ 0.05 was considered statistically significant. Random Forest analysis, which is a supervised machine learning technique based on decision trees, was used to classify the metabolites and to predict the presence or absence of DMVD (Breiman et al., 2001). Decision trees were first built using a subset of samples with class information and then used to predict a different subset of samples. The predicted classes were compared to the true class information to estimate unbiased prediction accuracy. The metabolites were ordered by the strength of their influence on the accuracy of prediction for separation between the control and DMVD groups.

RNA-seq data analysis

The canine gene annotation file, genome index file, and the whole genome sequence file were downloaded from

Illunina's iGenome FTP site (http://support.illumina.com/sequencing/sequencing_software/igenome.html). The canine genome sequence was the CanFam3.1 assembly produced in September 2011.

The quality of sequencing reads was initially assessed using standard software (Fast QC, Babraham Bioinformatics, Cambridge, UK). Illumina adaptor sequences were trimmed and data analysis followed the workflow described by Trapnell et al., using Bowtie and TopHat to align sequencing reads and TopHat to identify transcript splice sites (Trapnell et al., 2012). Differentially expressed transcripts (DETs) between control and DMVD groups were identified using Cufflinks, a standard statistical analysis tools for RNA-seq (Trapnell et al., 2012), with the selection criteria of p value <0.01 and magnitude of fold changes ≥2. Canine Ensembl gene IDs for unannotated DETs were used to search Ensembl database (http://useast.ensembl.org) for human and rodent orthologs (Kinsella et al., 2011). Corresponding human or rodent orthologous gene names were used for unannotated canine counterparts.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis

Gene ontology annotates genes into biological or molecular terms in a hierarchically structured way, whereas KEGG assigns genes to functional pathways. Annotation maps for GO (Carlson, GO.db, version 2.10.1) and KEGG (Carlson, KEGG.db, version 2.10.1) were obtained and analyses of over-represented GO and KEGG categories on the gene expression data were performed using the GOseq software package (Young et al., 2010). In RNA-seq experiments, long or highly expressed transcripts have greater likelihoods to be detected as DETs than their shorter or less expressed counterparts. GOseq takes this bias into consideration when performing systems biology analyses.

RT-qPCR data analysis

Student's *t*-tests were performed to test the null hypothesis that the means between control and diseased groups were not different. *P* values and fold changes were calculated using a standard statistical software package R (R Core Team, 2013).

Results

Metabolomics analysis

Metabolomics analysis of serum identified 41 known metabolites (Table 1) representing numerous metabolic pathways and 13 unknown metabolites that were significantly ($p < 0.05$) different between the healthy and DMVD dogs. Glucose was significantly lower ($p = 0.018$) and lactate higher in dogs with DMVD compared to controls. Succinyl- ($p = 0.029$), and hexanoyl-carnitine ($p = 0.033$), which facilitate entry of activated acyl-CoA into the mitochondria, were higher in dogs with DMVD, while deoxycarnitine ($p = 0.049$) was significantly lower in the dogs with DMVD compared to healthy controls. Concentrations of three other acyl carnitines, lauroyl-, palmitoyl-, and oleoyl-carnitine, also were lower in dogs with DMVD, but these did not reach statistical significance ($p > 0.05$). Markers of oxidative status were significantly different in serum from dogs with DMVD: oxidized glutathione (GSSG) was significantly higher ($p < 0.001$) and asymmetric dimethylarginine (ADMA), an inhibitor of nitric oxide synthase (NOS), was significantly lower ($p = 0.039$) in dogs with DMVD compared to healthy controls.

Analysis of serum metabolites using Random Forest decision trees generated a model that indicated good separation between the samples from healthy dogs and those with DMVD, with an ''out of bag'' error rate of 20.69% (Table 2). The Random Forest analysis identified 30 metabolites that contributed significantly to the model: the three metabolites with the greatest single effect on accuracy of separation were γ-glutamylmethionine, GSSG, and dimethylarginine (Fig. 1). Two of the serum metabolites, trans-4-hydroxyproline and 3-hydroxy-ethylpropionate, were important in the Random Forest separation model despite the means not being significantly different between DMVD and control samples: dogs with DMVD had a 1.5-fold higher concentration of 3-hydroxy-ethylpropionate ($p = 0.060$) and a 2.-fold lower concentration of trans-4-hydroxyproline ($p = 0.065$) compared to healthy controls.

Transcriptomics analysis

Validation using RT-qPCR confirmed 13 of 15 (87%) selected genes were differentially expressed ($p < 0.05$) in the LV samples of dogs with DMVD compared to those of controls (Table 3), including a 3-fold higher expression level of natriuretic peptide B in the LV of dogs with DMVD. The remaining two transcripts, TGF-ß3 and fatty acid desaturase-1, were numerically higher but this was not statistically significant ($p = 0.09$ and $p = 0.12$, respectively).

Using predetermined selection criterion based upon both magnitude of expression changes (≥2-fold) and statistical significance ($p < 0.01$), 812 LV and 263 MV DETs were identified by global transcriptomics analysis (Supplementary Tables S1 and S2; supplementary material is available online at www.liebertpub.com/omi). 114 transcripts were found to be differentially expressed in both tissues (Supplementary Table 3). The sequences have been deposited to the NCBI's Gene Expression Omnibus public repository under the accession number GSE64544. Significantly over-represented GO terms found in both LV and MV included response to chemical stimulus (GO:0042221), response to lipid (GO:0033993), and response to stress (GO:0006950), among others (Table 4). Over-represented KEGG pathways (FDR <0.05) common to both LV and MV include several signaling pathways (Table 5). Many of the DETs grouped into energy metabolism, antioxidant function, NO signaling, and ECM homeostasis pathways (Table 6).

Energy metabolism and antioxidant status

Numerous changes in gene expression related to energy metabolism were observed in dogs with DMVD (Table 6). Changes were noted in cell-surface fatty acid transporters: expression of fatty acid binding protein 4 was lower, while fatty acid transporter protein 6 was significantly higher in the MV of dogs with DMVD. Expression of acyl CoA synthase long chain family member 1, the enzyme for activating long chain fatty acids (LCFAs) to their CoA ester in the cell, was significantly lower in both LV and MV of dogs with DMVD. The gene for phytanoyl-CoA hydroxylase, important for utilization of branched-chain fatty acids as an alternative cellular energy source, was significantly lower in the MVs of dogs with DMVD. Additionally, acyl-CoA thioesterase 6, an

Table 1. Heat Map of Differentially Expressed Identifiable Serum Metabolites in Dogs with Degenerative Mitral Valve Disease (DMVD) and Healthy Controls

*RanF**	*Biochemical name*	*FC*	*Pathway*	*Sub pathway*
Y	Methionine	0.68‡	Amino acid	Cysteine, methionine, S-adenosylmethionine, taurine metabolism
	Glutamate	1.29§	Amino acid	Glutamate metabolism
Y	Glutathione, oxidized	2.32‡	Amino acid	Glutathione metabolism
Y	Threonine	0.71‡	Amino acid	Glycine, serine and threonine metabolism
	Beta-hydroxypyruvate	0.81§	Amino acid	Glycine, serine and threonine metabolism
	Sarcosine (N-Methylglycine)	0.73§	Amino acid	Glycine, serine and threonine metabolism
	Serine	0.84§	Amino acid	Glycine, serine and threonine metabolism
Y	Mannosyltryptophan	1.32‡	Amino acid	Tryptophan metabolism
Y	Dimethylarginine (asymmetric and symmetric dimethylarginine)	0.85§	Amino acid	Urea cycle; arginine and proline metabolism
	Valine	0.82§	Amino acid	Valine, leucine and isoleucine metabolism
	Beta-hydroxyisovalerate	1.18§	Amino acid	Valine, leucine and isoleucine metabolism
Y	N-acetylneuraminate	1.88‡	Carbohydrate	Aminosugar metabolism
	Erythronate‖	1.25§	Carbohydrate	Aminosugar metabolism
	Glucose	0.91§	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
	Lactate	1.32§	Carbohydrate	Glycolysis, gluconeogenesis, pyruvate metabolism
	Cis-aconitate	1.30§	Energy	Krebs cycle
	Malate	1.30§	Energy	Krebs cycle
Y	Succinylcarnitine	1.50§	Energy	Krebs cycle
	Hexanoylcarnitine	1.70§	Lipid	Carnitine metabolism
Y	Deoxycarnitine	0.85§	Lipid	Carnitine metabolism
Y	12-Hydroxyeicosatetraenoic acid	1.19§	Lipid	Eicosanoid
	Methyl palmitate	1.49§	Lipid	Fatty acid, branched
Y	2-Hydroxyoctanoate	0.61‡	Lipid	Fatty acid, monohydroxy
Y	Glycerophosphorylcholine	1.64‡	Lipid	Glycerolipid metabolism
	Pentadecanoate (15:0)	1.36§	Lipid	Long chain fatty acid
Y	Margarate (17:0)	1.57§	Lipid	Long chain fatty acid
Y	1-Palmitoleoylglycerophosphocholine‖	0.49‡	Lipid	Lysolipid
Y	1-Linoleoylglycerophosphocholine	0.53‡	Lipid	Lysolipid
	1-Oleoylglycerophosphocholine	0.62‡	Lipid	Lysolipid
	1-Docosapentaenoylglycerophosphocholine‖	0.45‡	Lipid	Lysolipid
	2-Linoleoylglycerophosphocholine‖	0.48§	Lipid	Lysolipid
Y	2-Oleoylglycerophosphocholine‖	0.42§	Lipid	Lysolipid
Y	1-Eicosadienoylglycerophosphocholine‖	0.60§	Lipid	Lysolipid
Y	1-Arachidonoylglycerophosphocholine‖	0.55§	Lipid	Lysolipid
	1-Stearoylglycerophosphoinositol	1.57§	Lipid	Lysolipid
Y	N6-Carbamoylthreonyladenosine	1.25§	Nucleotide	Purine metabolism, guanine containing
Y	Cytidine	1.39§	Nucleotide	Pyrimidine metabolism, cytidine containing
Y	Gamma-glutamylmethionine	0.57‡	Peptide	gamma-glutamyl
	Pantothenate	1.49§	Vitamin	Pantothenate and CoA metabolism
Y	4-Hydroxymandelate	0.61‡	Xenobiotic	Benzoate metabolism
Y	N-Glycolylneuraminate	2.51‡	Xenobiotic	Food/plant component

*RanF: Y = Random Forest Analysis identified this as important for separating samples between dogs with DMVD and healthy controls

†Fold Change in concentration of metabolites in serum samples from dogs with DMVD and healthy controls. Red and green indicate a significantly upregulated and downregulated metabolite, respectively. A number greater than 1 reflects a higher concentration in dogs with DMVD; less than 1 reflects a lower concentration in dogs with DMVD.

‡,§Statistical significance where ‡ = $p < 0.01$ and § = $p < 0.05$. ‖Indicates that compounds have been identified based on mass, retention time, and fragmentation pattern rather than based on a standard.

auxiliary enzyme for alpha-oxidation in peroxisomes (Hunt et al., 2012; Westin et al., 2007), was significantly lower (3.3-fold) in the MVs of dogs with DMVD.

Expression of 3-oxoacid CoA transferase 1, which converts acetoacetate to acetoacetyl-CoA ester before entering the Kreb's cycle, was significantly lower in the MV of dogs with DMVD. Expression of cellular glucose transporters (GLUT) also were altered in DMVD. Compared to controls, GLUT3 expression was significantly higher in MV and LV and GLUT6 expression was significantly higher in MV.

Antioxidant enzymes also were altered in DMVD (Table 6). The expression of GSTP1, a π class isoform of glutathione S-transferase expressed in heart and brain, and sirtuin-5, which desuccinylates and activates superoxide dismutase, were both significantly lower in the MV of dogs with DMVD. Expression of the constitutive enzyme, endothelial nitric

Table 2. Random Forest Accuracy of Decision Tree Prediction Model to Separate Dogs into Diseased or Healthy Group Based on Serum Metabolites

		Predicted group		
Random Forest		DMVD	Healthy	Class error*
Actual group	DMVD	14	4	0.22
	Healthy	2	9	0.18

DMVD, Degenerative mitral valve disease
*The overall out of box (OOB) error rate was 20.69%.

oxide synthase (eNOS or NOS3), which synthesizes nitric oxide from L-arginine, was significantly higher in dogs with DMVD. However, expression of the inducible NOS enzyme, NOS2, was significantly lower in the LV of these dogs.

Enzymes involved in ECM homeostasis and muscle function

Numerous expression changes in the a disintegrin and metalloprotease with thrombospondin repeats (ADAMTS) family of ECM metalloproteinases, as well as MMPs, were observed in tissues of dogs with DMVD. ADAMTS1, ADAMTS4, ADAMTS9, and ADAMTS28 were significantly higher in LV or MV, while ADAMTS7 was significantly lower in the LV from dogs with DMVD compared to healthy controls (Table 6). The expression of MMP8, MMP9, and the inhibitor, TIMP1, were significantly higher, while MMP11 and MMP15 were significantly lower in the LV of dogs with DMVD. Also changed in the LV tissue of dogs with DMVD was expression of muscle myosin heavy chain (MYH), with significantly lower expression of MYH1, MYH4, MYH7B, MYH8, and MHY13, but significantly higher expression of the myosin light chain (MYL)1. Additionally, MYH8, MYL2, and MYL3 all showed significantly decreased expression in the MV of dogs with DMVD compared to that of healthy controls.

Discussion

This study identified numerous metabolomics and transcriptomics changes that reflect altered energy metabolism, oxidative status, inflammatory mediators, and changes in ECM metabolism in dogs with DMVD.

Consistent with previous research on dogs with heart failure (Qanud et al., 2008), both lipid and glucose metabolism were altered in dogs with DMVD in the current study. Gene expression changes suggested that LCFA β-oxidation, branched-chain fatty acid α-oxidation, and ketolysis were compromised, while glucose uptake and glycolysis increased. The normal heart meets its high energy demand mostly by LCFA β-oxidation, which requires the LCFA to be actively transported into the cytoplasm and mitochondria.

The current study showed that the expression of several genes involved in transport of LCFA to the cytoplasm, including fatty acid translocase, plasma membrane fatty acid binding protein, and fatty acid transporter proteins- 1 and -6, were altered in cardiac tissue from dogs with DMVD. In addition, the expression of acyl CoA synthase long chain family member 1, the enzyme responsible for activating LCFA to its CoA ester in the cell, was decreased in DMVD, and the serum concentrations of three long chain acyl carnitines were numerically, although not significantly, lower in dogs with DMVD. Branched-chain fatty acids, such as phytanic acid, can provide an alternative source of energy. Activation of α-oxidation of phytanic acid requires phytanoyl-CoA hydroxylase, which was decreased in dogs with DMVD. Ketones can be a major energy source for many tissues, including the heart, when glucose sources are limited. Expression of 3-oxoacid CoA transferase 1, the rate-limiting enzyme in ketolysis was decreased in the MV from dogs with DMVD.

In contrast, expression of genes involved in glucose uptake and anaerobic glycolysis were increased. Increased expression of GLUT3 in both MV and LV, and of GLUT6 in MV, was observed. GLUT3 has higher affinity for glucose and greater glucose transport capacity compared to other isomers of the GLUT gene family. Consistent with this, the serum concentration of glucose was lower and that of lactate higher in dogs with DMVD compared to controls. These data support the fetal gene program hypothesis where the stressed or diseased heart switches to anaerobic metabolism, decreasing fatty acid oxidation while increasing glycolysis (Dirkx et al., 2013; Rajabi et al., 2007; Stanley et al., 2005). Results from the current study indicate disturbances in energy metabolism.

Expression of eNOS was increased and ADMA, an inhibitor of NOS, was decreased in dogs with DMVD. Nitric oxide functions in vasodilation and vasoprotection. Under physiological conditions, it is produced in small amounts by the constitutive enzyme, eNOS. Released NO activates guanylate cyclase to generate cGMP, which mediates cGMP-dependent cellular signaling. Increased NO release and NOS activities were reported in mild porcine DMVD (Moesgaard et al., 2007b) and increased NOS expression was previously observed in canine DMVD (Oyama and Chittur, 2006). In addition, elevated serum NO concentrations have been found in dogs with DMVD (de Laforcade et al., 2003). The observations of significantly elevated expression of eNOS in both the LV and MV of dogs with DMVD in the current study, combined with the decreased serum level of the inhibitor ADMA, provide additional evidence for the involvement of NO signaling in DMVD in dogs.

Increased NO production is usually accompanied by increased reactive oxygen species (Pfeilschifter et al., 2003), consistent with our observations of increased GSSG. Studies have shown that when NO concentrations reach medium to high concentration, an alternative redox-sensitive signaling pathway is activated (Grisham et al., 1999; Pfeilschifter et al., 2003). Nitric oxide reacts with oxygen molecules or superoxide to generate a very potent nitrosating agent, N_2O_3. Many transcription factors and expression of genes such as MMPs and TIMPs, are regulated via nitrosation, nitration, and oxidation by this indirect NO signaling (Grisham et al., 1999; Gu et al., 2002; Pfeilschifter et al., 2001; 2003). The expressions of metallothionein-1 and metallothionein-2, both of which were implicated as targets of redox-based NO signaling, also were significantly increased in the LV and MV of dogs with DMVD (Pearce et al., 2000). Hence, we hypothesize that ECM homeostasis is, at least in part, regulated by an indirect redox-based NO signaling pathway in canine DMVD.

Inducible NOS2 expression was significantly lower in the myocardium of dogs with DMVD. This change, which was confirmed by RT-qPCR, is opposite to that reported in human heart failure, where NOS2 is increased (Speranza et al., 2012; 2013). NOS2 appears to be upregulated

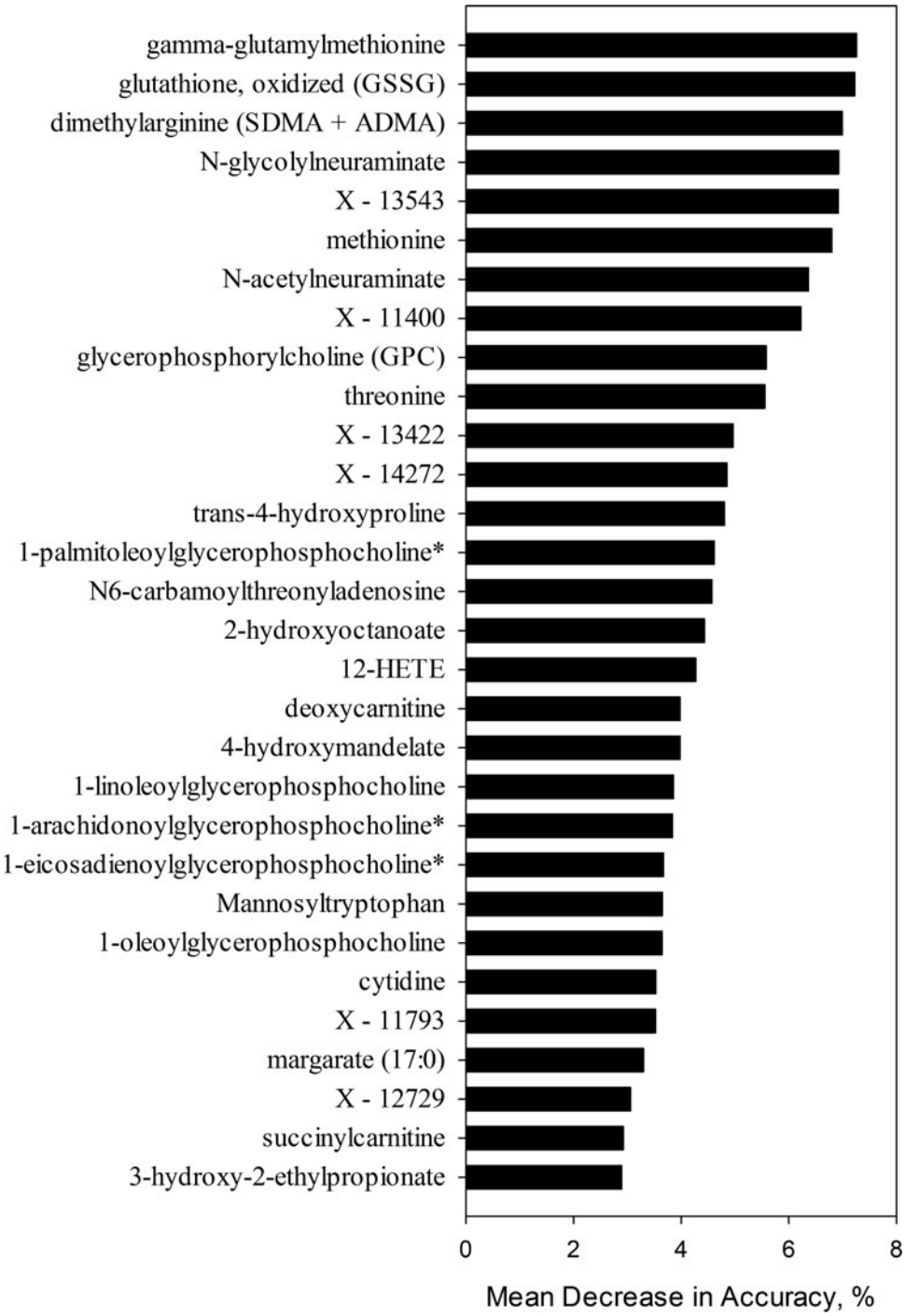

FIG. 1. Random Forest ranking of serum metabolites from dogs with degenerative mitral valve disease or healthy hearts. All metabolites shown, including both known and unidentified metabolites, contribute significantly to the model differentiating between groups. Metabolites are listed in decreasing order of importance for prediction of segregation of the groups as indicated by a larger mean decrease in accuracy. Elimination of individual metabolites from the model reduces the accuracy of prediction by the amount shown. * indicates that compounds have been identified based on mass, retention time, and fragmentations pattern rather than based on a standard.

by reactive oxygen species (Speranza et al., 2013; Zhen et al., 2008) and downregulated by increased NO concentration (Yoshioka et al., 2010). In the current study, it is possible that increases in NO concentrations produced by eNOS suppressed NOS2. However, more research is needed in order to understand the role of NOS2 in canine DMVD.

The KEGG pathway analysis revealed enrichment of several signaling pathways responsible for generating innate and adaptive immune defense responses and driving pro-

Table 3. Heat Map Display of 15 Target Genes of Left Ventricle Tissues in RT-qPCR Validation Assay

Gene symbol	*Ensembl ID*	P *value*	*Fold change*	*Description*
ACSL1	ENSCAFG00000007662	0.0015	−2.17	acyl-CoA synthetase long-chain family member 1
COL14A1	ENSCAFG00000000934	0.0150	−1.94	collagen, type XIV, alpha 1
FADS1	ENSCAFG00000025083	0.1224	−2.11	fatty acid desaturase 1
HOPX	ENSCAFG00000031330	0.0000	3.15	HOP homeobox
LIPE	ENSCAFG00000004813	0.0133	−1.79	lipase, hormone-sensitive
MLYCD	ENSCAFG00000019971	0.0042	−1.75	malonyl-CoA decarboxylase
MMP15	ENSCAFG00000008544	0.0007	−1.73	matrix metallopeptidase 15
MMP8	ENSCAFG00000023335	0.0000	155.13	matrix metallopeptidase 8
MMP9	ENSCAFG00000009905	0.0041	256.22	matrix metalloproteinase-9 precursor
NOS2	ENSCAFG00000018642	0.0061	−11	nitric Oxide Synthase, inducible
NOS3	ENSCAFG00000004687	0.0000	6.4	nitric Oxide Synthase, endothelial
NPPA	ENSCAFG00000016539	0.0538	6.89	natriuretic peptides A Atrial natriuretic factor
PAI-1	ENSCAFG00000013909	0.0000	43.16	serpin peptidase inhibitor, clade E
TGFB3	ENSCAFG00000017101	0.0910	−1.35	transforming growth factor, beta 3
TIMP1	ENSCAFG00000015155	0.0000	51.77	metalloproteinase inhibitor 1 precursor

Red, green, and gray colors indicate a significant increase, decrease and nonsignificance in gene expression, respectively. A positive number reflects increased expression in dogs with DMVD; a negative number reflects decreased expression in dogs with DMVD.

inflammatory cytokine production. Upregulation of several pro-inflammatory cytokines and their receptors were observed in this study. Although the results of at least one prior study suggested that DMVD was unlikely to be caused by inflammation (Orton et al., 2012), other studies (Speranza et al., 2013) and our data demonstrate increased expression of the innate/adaptive inflammatory defense system. The KEGG pathways, such as ECM-receptor interaction, cell adhesion molecules, and focal adhesion stress the important interplays between cell adhesion molecules, ECM components, and the cytoskeleton.

In both humans and dogs, the dynamic homeostasis in the ECM network is important for maintaining normal valvular structure and function (Aupperle and Disatian, 2012). Collagen and proteoglycan are two major constituents of the ECM proteins, accounting for 90% of the dry weight in human valves (Aupperle and Disatian, 2012). The Gly-Pro-Hyp repeat is the most common collagen triplet and the Hyp residue plays a critical role in maintaining the stability of collagen triple helix (Ramshaw et al., 1998; Shoulders and Raines, 2009).

In the current study, decreased Hyp was observed in the serum of dogs with DMVD, which may reflect either decreased turnover and greater ECM stability or, more likely, decreased fibrillar collagen synthesis and compromised ECM stability. Pathological changes associated with DMVD include an accumulation of proteoglycan consistent with a relative decrease in fibrillar collagen in the ECM (Orton et al., 2012). Elevated serum sialic acids have been associated with increased cardiovascular mortality and heart failure (Crook et al., 1997). The sialic acid linkage patterns were shown to be different in mitral valves of normal pigs and pigs affected by endocardiosis (Amoresano et al., 2000). Our observation of increased sialic acid metabolites, N-acetylneuraminate and N-glycolylneuraminate, suggests that this also may contribute to the changes in ECM structure and function in dogs with DMVD.

Matrix metalloproteinases are the driving force for ECM degradation, whereas TIMPs are their endogenous inhibitors (Li et al., 2000). Aberrant expressions of MMPs and TIMPs genes have been implicated in maladaptive ECM remodeling that contributes to a variety of cardiovascular diseases in humans (Li et al., 2000). In both humans and

Table 4. Over-represented Gene Ontology (GO) Common in Both Left Ventricle and Mitral Valve from Dogs with Degenerative Mitral Valve Disease

GO ID	*GO term**	*Gene ontology*
GO:0002376	Immune system process	BP
GO:0006950	Response to stress	BP
GO:0009611	Response to wounding	BP
GO:0032501	Multicellular organismal process	BP
GO:0033993	Response to lipid	BP
GO:0042221	Response to chemical stimulus	BP
GO:0044707	Single-multicellular organism process	BP
GO:0050896	Response to stimulus	BP
GO:0051239	Regulation of multicellular organismal process	BP
GO:0065008	Regulation of biological quality	BP
GO:0044421	Extracellular region part	CC
GO:0005576	Extracellular region	CC

BP, biological process; CC, cellular component; *All terms have a false discovery rate <1.00E-07.

Table 5. Over-represented Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathways Common in Both Left Ventricle and Mitral Valves from Dogs with Degenerative Mitral Valve Disease

KEGG ID	*KEGG pathway**
4610	Complement and coagulation cascades
4380	Osteoclast differentiation
4060	Cytokine-cytokine receptor interaction
4621	NOD-like receptor signaling pathway
4620	Toll-like receptor signaling pathway
4512	ECM-receptor interaction
4514	Cell adhesion molecules
4640	Hematopoietic cell lineage
4510	Focal adhesion

*All pathways have a false discovery rate <0.05

Table 6. Heat Map of Differentially Expressed Transcripts from RNA-seq Study on Left Ventricle and Mitral Valve Tissues from Dogs with Degenerative Mitral Valve Disease (DMVD) and Control Dogs with Healthy Hearts

Symbol	Functional role	Mitral valve Fold change from control	Left ventricle Fold change from control	Description
GLUT3	EM	7.49	16.51	Solute carrier family 2, facilitated glucose transporter member 3
GLUT6	EM	11.7	NS	Solute carrier family 2, facilitated glucose transporter member 6
ACOT6	EM	−3.33	NS	Acyl-CoA thioesterase 6
ACSL1	EM	−2.57	−2.98	Acyl-CoA synthetase long-chain family member 1
FABP4	EM	−2.91	NS	Homolog to human fatty acid binding protein 4, adipocyte
FATP6	EM	4.01	NS	Solute carrier family 27 (fatty acid transporter), member 6
PHYH	EM	−3.01	NS	Phytanoyl-CoA hydroxylase-like
OXCT1	EM	−2.5	NS	3-oxoacid CoA transferase 1
SIR5	OS	−2.31	NS	NAD-dependent protein deacylase sirtuin-5, mitochondrial
GSTP1	OS	−2.56	NS	Glutathione S-transferase pi 1
MT1	OS/NO	3.87	24.1	Metallothionein-1
MT2	OS/NO	7.24	134	Metallothionein-2
NOS2	NO	NS	−21.84	Nitric oxide synthase, inducible
NOS3	NO	4.62	5.72	Nitric oxide synthase, endothelial
MMP8	EC	NS	162	Matrix metallopeptidase 8 (neutrophil collagenase)
MMP9	EC	NS	256	Matrix metalloproteinase-9
MMP11	EC	NS	−6.2	Matrix metallopeptidase 11 (stromelysin 3)
MMP15	EC	NS	−3.4	Matrix metallopeptidase 15 (membrane-inserted)
TIMP1	EC	NS	47.7	Tissue inhibitors of metalloproteinases -1
ADAMTS1	EC	NS	4.23	A disintegrin and metallopeptidase with thrombospondin repeats, 1
ADAMTS4	EC	7.45	13.4	A disintegrin and metallopeptidase with thrombospondin repeats, 4
ADAMTS7	EC	NS	−4.12	A disintegrin and metallopeptidase with thrombospondin repeats, 7
ADAMTS9	EC	NS	13.8	A disintegrin and metallopeptidase with thrombospondin repeats, 9
ADAM28	EC	3.15	NS	ADAM metallopeptidase domain 28
MYH1	SP	NS	−4.13	Myosin-1
MYH4	SP	NS	−48.34	Myosin-4
MYH7B	SP	NS	−3.13	Myosin, heavy chain 7B, cardiac muscle, beta
MYH8	SP	−5.08	−2.43	Myosin-8
MYH13	SP	NS	−60.39	Myosin, heavy chain 13, skeletal muscle
MYL1	SP	NS	11.51	Myosin, light chain 1, alkali; skeletal, fast
MYL2	SP	−122.02	NS	Myosin light chain 2, regulatory, cardiac, slow
MYL3	SP	−36.45	NS	Myosin, light chain 3, alkali; ventricular, skeletal, slow

EC, energy metabolism; EM, extracellular matrix; NO, nitric oxide signaling; NS, nonsignificance; OS, oxidative stress; SP, sarcomeric proteins.

Red, green, and gray colors indicate a significant increase, decrease, and nonsignificance in gene expression, respectively. A positive number reflects increased expression in dogs with DMVD; a negative number reflects decreased expression in dogs with DMVD.

All are significant ($p < 0.01$) unless otherwise indicated.

dogs, the expression patterns of MMPs and TIMPs in both normal and degenerated mitral valves have been examined (Aupperle and Disatian, 2012; Aupperle et al., 2009; Oyama and Chittur, 2006). One study showed no expression changes in MMPs from the mitral valves of DMVD (Oyama and Chittur, 2006), consistent with results from the current study. Another canine study showed no change in MMP1, MMP2, or MMP9, but did show an increase in MMP3 in valves from dogs with DMVD (Obayashi et al., 2011).

To our knowledge, the current study was the first to evaluate the gene expression changes of MMPs and TIMPs in LV tissues of dogs with DMVD. Our results show greater than 100 fold increases in expression in MMP8 and MMP9, while the expressions of MMP11 and MMP15 decreased in the LV of dogs

with DMVD. TIMP1 expression also was increased up to 50-fold. In addition, expression of other matrix metallopeptidases including metallopeptidases with ADAM and ADAMTS domains were significantly altered. Such changes may contribute to LV dilation and remodeling, which has been documented to occur in late-stage DMVD in both humans and dogs (Carabello, 2008; Hezzell et al., 2012; Van De Heyning et al., 2013).

While this study provides interesting new data regarding DMVD in dogs, there are some important limitations to this study. First, it was not possible to match the breeds involved in the study, resulting in an over-representation of Cavalier King Charles Spaniels in the DMVD group (data not shown). As this breed is predisposed to DMVD, the imbalance may have introduced bias to the data. A second limitation to this study was the small number of dogs from which cardiac tissues were collected (four control dogs and two dogs with DMVD). These samples were obtained from dogs euthanized for health or other reasons unrelated to the current study, which limited the number of samples available. Future studies with larger sample sizes are needed. Third, mRNA expression levels do not always reflect protein expressions. However, previous studies have shown that the protein concentrations correlated with the abundances of their corresponding mRNA levels in both prokaryotic and eukaryotic cells, with a correlation coefficient of $\sim$63% (Vogel and Marcotte, 2012). Higher correlations have also been reported (de Sousa Abreu et al., 2009; Maier et al., 2009). The transcriptomics data is useful in providing potential candidates for the next-step proteomics study. Finally, this was an observational study so medical or nutritional management factors were not controlled or evaluated. Nonetheless, results from our multi-omics integrative analysis are consistent with previous findings and provide new insights on DMVD in dogs. This study highlights the emerging importance of integrative research in veterinary medicine and animal nutrition science.

Conclusions

This study documented altered energy metabolism in cardiac tissue from dogs with DMVD; specifically, evidence of compromised LCFA β-oxidation, branched chain fatty acid α-oxidation, and ketolysis, as well as increased glucose uptake and glycolysis was observed. These data suggest that the fetal gene program hypothesis, wherein the stressed heart switches to anaerobic metabolism, decreasing fatty acid oxidation, and increasing glycolysis may apply also to dogs. Other changes included an increase in markers of oxidative stress, altered nitric oxide metabolism, and changes in the extracellular matrix and sarcomeric proteins. Many of the observed disturbances may benefit from nutritional or medical management, but additional research is needed to more fully understand these alterations and their possible treatment.

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Abbreviations Used

ADAMTS = A disintegrin and metalloprotease with thrombospondin repeats
ADMA = Asymmetric dimethylarginine
DET = Differentially expressed transcript
DMVD = Degenerative mitral valve disease
ECM = Extracellular matrix
eNOS = Endothelial nitric oxide synthase
GLUT = Glucose transporters
GO = Gene ontology
GSSG = Oxidized glutathione
KEGG = Kyoto Encyclopedia of Genes and Genomes
LCFA = Long chain fatty acid
LV = Left ventricle
MV = Mitral valve
MMP = Matrix metalloproteinases