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Gene-expression profiling of endomyocardial biopsies from dogs with dilated cardiomyopathy phenotype☆

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Abstract *Introduction:* The employment of advanced molecular biology technologies has expanded the diagnostic investigation of cardiomyopathies in dogs; these technologies have predominantly been performed on postmortem samples, although the recent use of endomyocardial biopsy in living dogs has enabled a better premortem diagnostic approach to study the myocardial injury.
Animals, materials, and methods: Endomyocardial biopsies were collected in nine dogs with a dilated cardiomyopathy phenotype (DCM-p) and congestive heart failure and submitted to histologic examination, next-generation sequencing (NGS), and polymerase chain reaction analysis. Data from three healthy dogs (Fastq files) were retrieved from a previously approved study and used as a control group for ribonucleic acid sequencing.
Results: Histologic examination revealed endocardial fibrosis in 6 of 9 dogs, whereas lymphocytic interstitial infiltrates were detected in two of nine dogs, and lymphoplasmacytic and macrophage infiltrates were detected in one of nine dogs. On polymerase chain reaction analysis, two dogs tested positive for canine parvovirus two and one dog for canine distemper virus. Gene-expression pathways involved in cellular energy metabolism (especially carbohydrates-insulin) and cardiac structural proteins were different in all DCM-p dogs compared to those in the control group. When dogs with lymphocytic interstitial infiltrates were compared to those in the control group, NGS analysis revealed the predominant role of genes related to inflammation and pathogen infection.
Conclusions: Next-generation sequencing technology performed on *in vivo* endomyocardial biopsies has identified different molecular and genetic factors that could play a role in the development and/or progression of DCM-p in dogs.

Abbreviations

DCM	dilated cardiomyopathy
DCM-p	dilated cardiomyopathy-phenotype
EMB	endomyocardial biopsy
GO	gene ontology
NGS	next-generation sequencing
PCR	polymerase chain reaction
RNA-seq	ribonucleic acid sequencing

Introduction

Dilated cardiomyopathy (DCM) is a myocardial disorder characterized by left ventricular or biventricular dilation in association with impaired systolic function. The development of DCM is complex, with both genetic and non-genetic factors at play. Breed predispositions for DCM have long been reported, and several genetic mutations have been identified, though the role these mutations play in producing DCM is not completely understood [1—5]. Nongenetic forms of DCM can be associated with myocarditis, endocrinopathies, autoimmune diseases, exposure to drugs and toxins, or nutritional deficiencies [6—8].

In human medicine, several studies have highlighted the presence of an important inflammatory response associated or not associated with pathogenic agents responsible for myocardial injury. The development of inflammatory cardiomyopathy, defined as myocarditis in association with cardiac dysfunction and ventricular remodeling, can progressively culminate in DCM and end-stage heart failure [9—11].

In recent years, the application of advanced molecular biology technologies has improved the diagnostic investigation of cardiomyopathies in dogs; these technologies have predominantly been performed on postmortem samples, although the recent use of endomyocardial biopsy (EMB) *in vivo* has enabled a better diagnostic approach to study the myocardial injury (e.g. myocarditis) and for exploring the presence of myocardial pathogens [12,13].

In this context, next-generation sequencing (NGS), has significantly improved the knowledge on the etiopathogenesis of DCM. Friedenberg et al. [14] used a ribonucleic acid sequencing (RNA-seq) approach on postmortem cardiac tissue to identify differentially expressed genes and pathways in canine DCM. An expression of genes related to cardiac structural proteins and downregulation of

Table 1 Clinical, histological, and PCR findings of DCM-p dogs enrolled.

Sample ID	Age (month)	Weight (kg)	Breed	Sex	ECG Holter	Histology	PCR
S_002	48	21	Border Collie	M	PVC	Lymphocytic infiltrates, endomyocardial fibrosis cardiomyocyte hypertrophy	–
S_003	6	32	Mixed breed	M	VT	Interstitial and endomyocardial fibrosis cardiomyocyte hypertrophy	CPV 2
S_005	60	27.3	Mixed breed	M	VT	Lymphoplasmacytic-macrophage infiltrates, cardiomyocytes necrosis	CPV 2
S_007	56	33.5	Weimaraner	M	VT	Cardiomyocyte hypertrophy	–
S_008	6	19.8	Vizsla	M	FAT, VT	Lymphocytic infiltrates, interstitial and endomyocardial fibrosis	CDV
S_010	93	44	Corso	F	FAT, VT	Endomyocardial fibrosis	–
S_011	9	10	Mixed breed	M	VT	Interstitial fibrosis, cardiomyocyte hypertrophy	–
S_012	36	22.6	Mixed breed	F	FAT, VT	Endomyocardial fibrosis cardiomyocyte hypertrophy and necrosis	–
S_013	60	16.5	Whippet	F	PVC	Endomyocardial fibrosis cardiomyocyte hypertrophy	–

Abbreviations: CDV: canine distemper virus; CPV2: canine parvovirus 2; DCM-p: dilated cardiomyopathy phenotype; ECG: electrocardiography; F: female; FAT: focal atrial tachycardia; M: male; PCR polymerase chain reaction; PVC: premature ventricular contractions; SVT: supraventricular tachycardia; VT: ventricular tachycardia.

genes involved in cellular energy metabolism, particularly of carbohydrates and fats has been reported in canine DCM [14].

In the present study, the authors hypothesized that the use of NGS performed on EMB samples may be useful to (1) explore the gene expression pathways in dogs with DCM phenotype (DCM-p) and (2) compare the different gene-expression pathways involved in dogs presenting with DCM-p related and not related to inflammatory cardiomyopathy.

Animals, materials, and methods

The study protocol was approved by the Ethical Animal Care and Use Committee of the University of Naples Federico II (Protocol No. 67990/2015). Informed consent was obtained from all owners. Nine dogs diagnosed with a DCM-p were enrolled in the study. According to ethical principles to create a control group, data from healthy control dogs ($n = 3$) were retrieved from a previous approved study performed on heart tissue (NCBI BioProject PRJNA78827).

All animals received a complete clinical examination, thoracic radiograph, and hematobiochemical profile and were fed with home-made diet formulated by a veterinary nutritionist. The diagnosis of DCM-p was based on reported echocardiographic data[i] showing shortening fraction $<20\%$, left ventricular ejection fraction obtained with modified Simpson's method of discs in right parasternal four-chamber long-axis view $<40\%$, and left ventricular end systolic volume index and left ventricular end diastolic volume index obtained with modified Simpson's method of discs in right parasternal four-chamber long-axis view, respectively, >55 mL/m^2 and >95 mL/m^2 [15–17]. All the affected dogs had an arrhythmia identified on both 12-lead surface electrocardiography and Holter monitoring (Table 1); all dogs had previous severe signs of congestive heart failure. Based on clinical signs, radiographs, and echocardiography, all the dogs were classified as stage C according to Wess [15]. An appropriate therapy with furosemide, ACE-inhibitor, pimobendan, spironolactone, and antiarrhythmic medications was administered to each dog as clinically indicated.

Endomyocardial biopsy collection and polymerase chain reaction assay

Endomyocardial biopsies were collected as previously described [13]. Briefly, at least four biopsies were collected from the right ventricular apex and/or from the interventricular septum from each dog. Immediately after collection, the biopsies were submitted for histological examination (10% neutral buffered formalin) and collected in ribonuclease-free tubes with RNA later solution; then the biopsies were stored at -80 °C until NGS and qualitative polymerase chain reaction (PCR) analysis were

[i] GE Logic-9 Expert Machine, GE medical system, Phoenix, AZ, USA.

performed. Using specific primers, the PCR analysis was conducted for selected cardiotropic pathogens [13] (Supplementary Table A).

Histologic examination of endomyocardial biopsies samples

Formalin-fixed-paraffin-embedded EMB samples were processed by routine methods and sectioned at a thickness of 5 μm. Twenty-four serial sections were prepared for each EMB sample. Twelve samples were evaluated histologically, for a morphological evaluation, and 12 were used for immunohistochemical analysis. Sections were numbered sequentially, and the 12 sections for histologic examination were mounted on glass slides and stained with hematoxylin and eosin stain (slides 1, 6, 11, 16, 21, and 24) or with Masson trichrome stain (slides 2, 7, 12, 17, 22, and 23) to detect collagen deposition. Each EMB sample was assessed by a single pathologist for evidence of cardiomyocyte hypertrophy, sarcoplasm vacuolization, interstitial or replacement fibrosis, lymphocytic infiltration, and necrosis.

Total ribonucleic acid sequencing libraries preparation and sequencing

Endomyocardial biopsies were processed to perform total RNA-seq. Total RNA was extracted from a single biopsy using Norgen's RNA/Deoxyribonucleic Acid (DNA) Purification kit,[j] following the manufacturer's instructions. Indexed libraries were prepared from 7 ng of purified RNA with SMARTer Stranded Total RNA-seq kit v2-pico Input Mammalian[k] according to the manufacturer's instructions. The samples were sequenced using an Illumina NextSeq500 System[l] in a 2 × 75 paired-end read format at a final loading concentration of 1.7 pmol.

Ribonucleic acid sequencing data analysis

Fastq files from three control samples were downloaded from the NCBI BioProject PRJNA78827—a dataset of RNA-seq experiments from different tissue types, generated to create a comprehensive catalog of transcripts for the dog genome project. Only raw sequence files from cardiac tissue, with accession SRX111067, were used in the analysis.

All Fastq files were quality checked using FastQC software [18], then adapter sequences were removed and low-quality reads were filtered out using Trimmomatic software, version 0.39 [19] with the following parameters: LEADING:3, TRAILING:3, SLIDINGWINDOW:4:15 MINLEN:20. The high-quality reads were mapped to the dog reference genome (RefSeq accession: GCF_000002285.5) with STAR software version 2.7.4a [20] using default parameters. The number of reads mapping to each transcript was computed with featureCounts v2.0.0 [21]. The counts were then imported in R (version 3.6.3), and differential gene expression analysis was performed comparing sequenced samples from canine EMBs and control samples from the downloaded project—R package DESeq2 v1.26.0 [22]. Differential expression was reported as |a fold change of| $\geq$1.5 along with associated adjusted P values (false discovery rate $\leq$0.05), computed according to Benjamini—Hochberg [23,24]. Gene Ontology (GO) analysis of differentially expressed genes was performed with ShinyGO [25], and only functions and pathways showing a P-value cutoff false discovery rate $\leq$0.01 were considered.

Results

The characteristics of the study population are reported in Table 1. None of the animals enrolled had any clinical history of previous infectious diseases and their hematobiochemical profile were in the normal range (data not shown).

In total, 13 EMBs were obtained from nine dogs, with duplicate samples obtained from dogs _002, _007, _008, and _010. None of the dogs experienced complications related to the EMB procedure. Myocardial fibrosis was identified in six of the nine dogs. Endocardial fibrosis (Fig. 1A) was described in six of the nine dogs, in two of which (2/6) it was associated with interstitial fibrosis. Three dogs with fibrosis also showed the presence of inflammatory infiltrates that were confirmed by immunohistochemistry (data not shown): sample_005 showed lymphoplasmacytic and macrophage infiltrates (Fig. 1B), whereas samples_002 and _008 showed lymphocytic infiltrate (Fig. 1C). In six of the nine dogs, signs of hypertrophy of cardiomyocytes were detected; cardiomyocytes necrosis was present only in two of the nine samples (Table 1).

Furthermore, in three of twelve samples (_003, _005, _008) nucleic material of associated pathogens was detected via PCR analysis. Specifically, nucleic acids from canine parvovirus 2 was detected in two EMB samples (_003, _005), whereas

[j] RNA/DNA Purification kit, Norgen Biotek Corp., Thorold, Ontario, Canada.
[k] SMARTer Stranded Total RNA-seq kit v2-pico Input Mammalian, Takara Bio Inc., Kusatsu, Shiga, Japan.
[l] Illumina NextSeq500 System, Illumina Inc., San Diego, California, USA.

sample_008 was positive for canine distemper virus (Table 1).

The sequencing of RNA-seq libraries from EMB samples produced a total of 590,362,452 reads with an average of 45,412,496.31 reads per sample (range: 31,427,174—104,856,322 reads).

Bioinformatics analysis

The three control samples consisted of 61,373,812 reads in total, with an average of 20,457,937.33 reads per sample (range: 20,429,448—20,484,644 reads). The quality control and the alignment steps results are summarized in Supplementary Table B.

The expression profiles of all EMB samples were compared with those of control samples to detect expression changes in the two tested groups, identifying 2,422 differentially expressed transcripts, as shown in the volcano plot in Figure 2A.

The GO analysis showed the presence of several cellular pathways, as summarized in Figure 3 and Table 2.

For the 2,422 total differentially expressed genes, statistically significant correlations with distinct pathways were detected in heart disorders, as well as in tissue and cellular metabolism (Supplementary Table C). In Table 3, a selection of differentially expressed genes from the analysis of all EMBs samples versus controls is presented.

Further analysis was performed on the three cases that showed lymphocytic interstitial infiltrates with signs of inflammation compared to controls (Supplementary Table D). The analysis revealed 6,474 differentially expressed genes (3,718 up-regulated and 2,756 down-regulated) summarized in the volcano plot in Figure 2B.

The GO analysis revealed distinct pathways involved in cardiac activity and pathogen interaction, as well as in structural cell organization and metabolism (Table 4 and Fig. 4A). Concerning heart related diseases, 21 differentially expressed genes were linked to "viral myocarditis" and 82 differentially expressed genes to "diabetic cardiomyopathy" (false discovery rate = 0.006 and 1.62E−06 respectively).

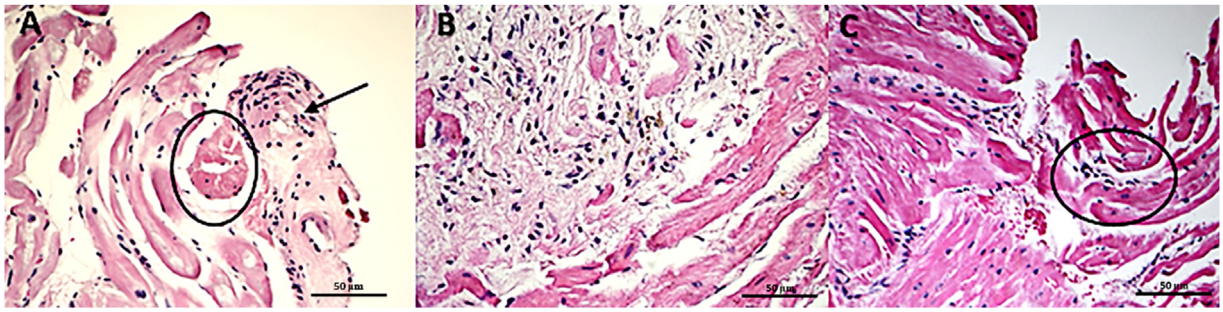

Figure 1 Histological sections stained with hematoxylin and eosin from three dogs with a dilated cardiomyopathy phenotype. A: Endomyocardial tissue from case_008 characterized by focal and moderate endocardial fibrosis associated with leukocyte infiltrate (arrow). Necrosis of some myocardial cells contiguous to the endocardium (oval). 20×. B: Portion of histological section from case_002 characterized by large area of replacement fibrosis associated with florid mixed leukocyte infiltration. 40×. C: Myocardial tissue from case_005 characterized by focal, mild, interstitial leukocyte infiltrate (oval). 20×.

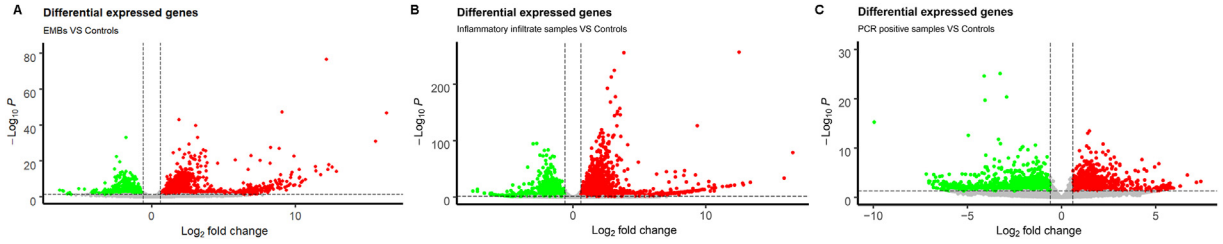

Figure 2 Volcano plot representing differentially expressed genes resulting from the comparison between (A) all endomyocardial biopsy samples, (B) the three samples with inflammatory infiltrates, and (C) three endomyocardial biopsies samples positive for polymerase chain reaction test versus control group. Up-regulated genes with a fold change of ≥1.5 are shown in red and down-regulated genes with fold change of ≤−1.5, in green. Genes associated with not significant expression values, according to adjusted P values (false discovery rate) threshold of 0.05, are reported in gray. On the x-axis and y-axis are reported Log2 fold change values and -Log10 P-values, respectively. EMBs: endomyocardial biopsies; PCR: polymerase chain reaction.

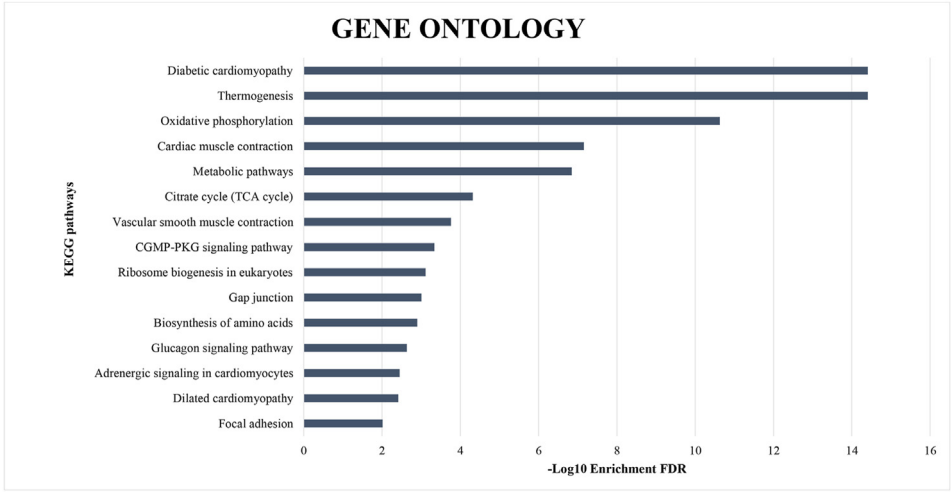

Figure 3 Significant Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway detected with ShinyGO online software, resulted from the comparison between all endomyocardial biopsy samples versus control group. On x-axis are reported KEGG pathways observed, on y-axis, the values of enrichment, expressed as −log10 of enriched false discovery rate (FDR).

Table 2 Gene Ontology results with the detected Kyoto Encyclopaedia of Genes and Genomes pathways from the analysis of 2,422 differentially expressed genes resulted from the comparison between all endomyocardial biopsies and control samples.

Pathway	N genes	Enrichment FDR	Fold enrichment
Diabetic cardiomyopathy	60	3.93E−15	3.294747716
Thermogenesis	64	3.93E−15	3.14969593
Oxidative phosphorylation	40	2.36E−11	3.477789256
Cardiac muscle contraction	27	7.03E−08	3.521261622
Metabolic pathways	190	1.42E−07	1.494977282
Citrate cycle (TCA cycle)	12	4.82E−05	4.637052342
Vascular smooth muscle contraction	28	0.000173995	2.394543422
CGMP-PKG signaling pathway	31	0.000457995	2.170700677
Ribosome biogenesis in eukaryotes	18	0.000771906	2.761773821
Gap junction	20	0.00098957	2.544723846
Biosynthesis of amino acids	18	0.001256747	2.645079153
Glucagon signaling pathway	21	0.002334941	2.330858757
Adrenergic signaling in cardiomyocytes	27	0.003550108	2.026625394
Dilated cardiomyopathy	20	0.0038386	2.293047861
Focal adhesion	32	0.009694615	1.76649613

Abbreviations: CGMP-PKG: cyclic guanosine monophosphate-dependent protein kinase G; FDR: false discovery rate; TCA: tricarboxylic acid.

In addition, the gene expression from the three samples positive for PCR was compared with controls, to evaluate transcriptomic responses to infections (Supplementary Table E). The differential expression analysis revealed 3,413 genes (1,782 and 1,631 up-regulated and down-regulated, respectively) plotted in Figure 2C.

On these genes, GO analysis was also performed showing significantly enriched functional pathways such as heart activity and metabolic processes, such as cardiac muscle and vascular smooth muscle contraction, oxidative phosphorylation, mitogen-activated protein kinase signaling pathway, and extracellular matrix receptor. All GO results are summarized in Table 5 and plotted in Figure 4B. Finally, in supplementary figure I, diabetic (A) and dilated (B) cardiomyopathy pathways are described as Kyoto Encyclopaedia of Genes and Genomes graph.[m]

[m] Kyoto Encyclopaedia of Genes and Genomes (KEGG), www.genome.gp/kegg/ Copyright 1995−2024 Kanehisa Laboratories Japan.

Table 3 Selected differentially expressed genes resulted from the analysis of all EMB samples vs controls.

Gene ID	FDR	Fold-change	Pathway
MYH7	5.69E−06	2.31	Dilated cardiomyopathy
TNNT2	1.71E−08	3.58	Dilated cardiomyopathy
TTN	6.59E−30	5.99	Dilated cardiomyopathy
PLN	1.65E−11	−3.52	Dilated cardiomyopathy
ACTC1	3.73E−07	−3.24	Dilated cardiomyopathy
ACSL1	0.030743204	−1.97	Metabolic
ATP1A3	0.00076937	−3.17	Cardiac muscle contraction
PIK3R1	0.014006	2.29	Dilated cardiomyopathy
CACNA2D3	0.029529842	16.41	Dilated cardiomyopathy
CACNA1S	0.006671291	13.03	Dilated cardiomyopathy
ATP2A2	1.83E-06	−2.81	Dilated cardiomyopathy, diabetic cardiomyopathy, CGMP-PKG signaling

False discovery rate (FDR) < 0.05, Fold-change value of 2,422 differentially expressed genes.
ACTC1: Actin Alpha Cardiac Muscle 1; ACSL1: Acyl-CoA Synthetase Long Chain Family Member 1 gene; ATP1A3: ATPase Na+/K+ Transporting Subunit Alpha 3 gene; ATP2A2: ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 2; CACNA1S: calcium voltage-gated channel subunit Alpha1 S; CACNA2D3: calcium voltage-gated channel auxiliary subunit Alpha2delta 3; CGMP-PKG: cyclic guanosine monophosphate-dependent protein kinase G; EMB: endomyocardial biopsy; MYH7: Myosin Heavy Chain 7; PIK3R1:Phosphoinositide-3-Kinase Regulatory Subunit 1 gene; PLN: phospholamban; TNNT2: Troponin T2, Cardiac Type; TTN: Titin.

Table 4 Gene Ontology differentially expressed table with the detected Kyoto Encyclopaedia of Genes and Genomes pathways from the analysis of 6,474 differentially expressed genes resulted from the comparison between the three cases with inflammatory infiltrates and control samples.

Pathway	N Genes	Enrichment FDR	Fold enrichment
Calcium signaling pathway	75	0.009536827	1.348349858
Cell adhesion molecules	46	0.009017418	1.479165373
Adherens junction	28	0.008431352	1.677946491
B cell receptor signaling pathway	27	0.008431352	1.695068394
Bacterial invasion of epithelial cells	31	0.008431352	1.634799295
Viral myocarditis	21	0.006087306	1.887689802
Leukocyte transendothelial migration	41	0.004790761	1.574383912
Tight junction	57	0.004490295	1.473490826
ECM-receptor interaction	34	0.003978895	1.680942824
Regulation of actin cytoskeleton	73	0.002929047	1.42933985
Oxidative phosphorylation	48	0.002204977	1.582063834
PI3K-Akt signaling pathway	112	0.002063393	1.342357192
Cardiac muscle contraction	35	0.002063393	1.730382318
Platelet activation	47	0.000995809	1.645066376
Adrenergic signaling in cardiomyocytes	56	0.000752444	1.593445588
CGMP-PKG signaling pathway	60	0.000533773	1.592681712
Gap junction	39	0.000160894	1.881112485
Focal adhesion	81	2.37E−06	1.695068394
Diabetic cardiomyopathy	82	1.62E−06	1.70696361
Metabolic pathways	436	5.74E−09	1.300489879

Abbreviations: CGMP-PKG: cyclic guanosine monophosphate-dependent protein kinase; ECM: extracellular matrix; FDR: false discovery rate; PI3K-Akt: phosphatidylinositol 3-kinase and protein kinase B.

Discussion

To the best of the authors' knowledge, this is the first study using an RNA-seq analysis on heart tissues obtained from live dogs with DCM-p. Post-mortem RNA analysis is quite challenging because of the instability and rapid denaturation of the RNA molecules. Thus, for this study, a deep characterization of myocardial abnormalities was achieved using the NGS technology, which allowed for the transcriptomic landscape of dogs with DCM-p to be assayed. Based on our study design, it appears difficult to discriminate the presence of genetic forms of DCM from non-genetic forms of

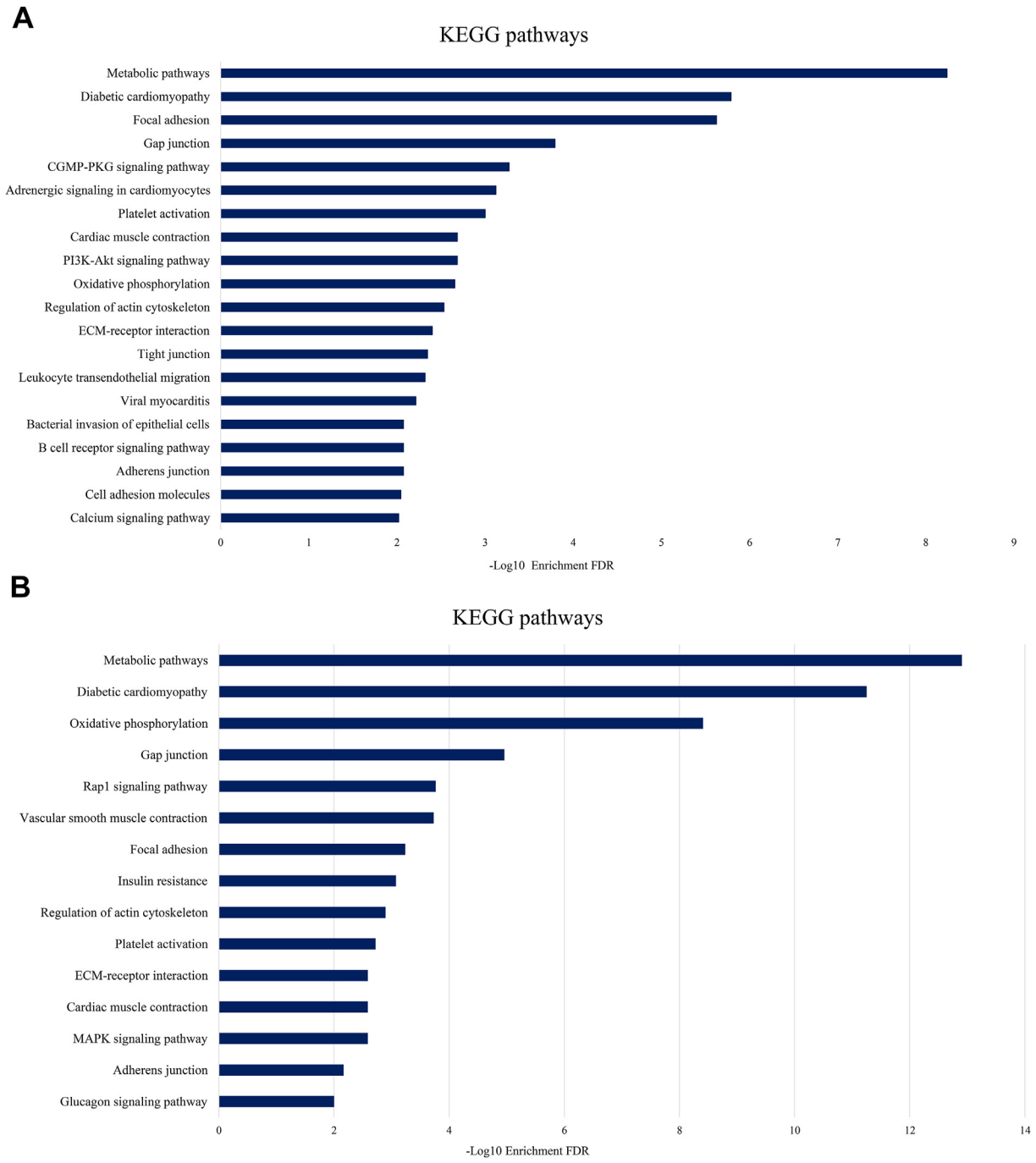

Figure 4 Kyoto Encyclopaedia of Genes and Genomes pathway (KEGG) detected with ShinyGO online software that resulted from the analysis of gene expression between (A) three samples with inflammatory infiltrates and (B) the three samples positive for polymerase chain reaction versus control group. On y-axis are reported KEGG pathways observed; on x-axis, the values of enrichment, expressed as −log10 of enriched false discovery rate (FDR).

DCM in the enrolled dogs. In dogs with genetically mediated DCM, several genes, and their mutations, have been studied in pure-breed dogs. These genes have been mainly related to the synthesis of proteins involved in the pathologic process, such as dystrophin [26], pyruvate dehydrogenase kinase isozyme 4, and striatin [27,28]. Our study did not show any correlation with these genes, probably due to a different experimental design: DNA vs RNA analysis and/or different breeds enrolled.

Instead, using the RNA-seq analysis, our results are similar to those reported by Friedenberg et al. [14]. In eight dogs with DCM-p with heart failure, showing the involvement of 17 genes encoding for structural and functional cardiac proteins and a

Table 5 Gene Ontology results. Significant pathways were reported with the false discovery rate value (≤0.01) and the number of differentially expressed genes involved in three samples positive for polymerase chain reaction and controls.

Pathway	N genes	Enrichment FDR	Fold enrichment
Metabolic pathways	288	1.24E−13	1.570853946
Diabetic cardiomyopathy	66	5.59E−12	2.576751648
Oxidative phosphorylation	44	3.90E−09	2.719904517
Gap junction	29	1.10E−05	2.62341123
Rap1 signaling pathway	50	0.0001713	1.854480353
Vascular smooth muscle contraction	35	0.000185164	2.110790645
Focal adhesion	46	0.000578182	1.805420026
Insulin resistance	29	0.000840296	2.08854098
Regulation of actin cytoskeleton	47	0.001275521	1.725952011
Platelet activation	30	0.001897501	1.969359666
MAPK signaling pathway	58	0.002589596	1.575968651
Cardiac muscle contraction	23	0.002589596	2.132652405
ECM-receptor interaction	23	0.002589596	2.132652405
Adherens junction	19	0.006811489	2.135462224
Glucagon signaling pathway	24	0.009941646	1.893937381

Abbreviations: FDR: false discovery rate; ECM: extracellular matrix; MAPK: mitogen-activated protein kinase; Rap1: Ras-related protein 1.

severe dysregulation of cellular energy metabolism; other genes, in common with our study, are myosin heavy chain 7, troponin T2 cardiac type, titin, phospholamban, and actin alpha cardiac muscle 1 [29—32]. It is interesting to note that these last genes were also included in the 14 canine candidates selected by Wiersma et al. [29] and were related to sarcomeric protein synthesis involved in DCM [28]. However, it is difficult to define whether the dysregulation of these genes represent primary or secondary factors in the pathogenesis of DCM.

Of all the genes observed, some were related to ion trafficking, whereas most of them were involved in the energetic metabolism such as Acyl-CoA Synthetase Long Chain Family Member 1 gene, ATPase Na+/K+ Transporting Subunit Alpha 3, and Phosphoinositide-3-Kinase Regulatory Subunit 1. The Acyl-CoA Synthetase Long Chain Family Member 1 gene is a key player in promoting the first step in the intracellular metabolism of fatty acids and consequent energetic balance [33]; as a member of the P-type cation transport ATPases family, the ATPase Na+/K+ Transporting Subunit Alpha 3 gene is an integral membrane protein that maintains the correct electrochemical gradients of ions across the plasma membranes. It also promotes the uptake of glucose into cells playing a fundamental role for cardiac contractility. Dysfunctions associated with dysregulation or mutations of this gene are also implicated in diabetic cardiomyopathy [34]. The Phosphoinositide-3-Kinase Regulatory Subunit 1 is involved in insulin signaling and the production of phosphatidylinositol 3,4,5-trisphosphate in glucose metabolism; its dysfunctions are associated with marked insulin resistance. According to Friendeberg et al. [14], genes related to the Phosphoinositide-3-Kinase family and ATP synthase complex play a key role in energy balance in cardiomyocytes, and dysregulation of these genes is strongly related to the pathogenesis of DCM in dogs. Similarly, other human studies have highlighted how energetic metabolism plays an important role in the development of DCM [5,35—37]; in our study, the involvement of energetic metabolism is supported by GO analysis, which clearly indicates a diabetic cardiomyopathy pathway.

The diabetic cardiomyopathy is associated with an increase in circulating fatty acids, hyperglycemia, and insulin resistance that promote the generation of reactive oxygen species, mitochondrial dysfunction, and impaired calcium metabolism. Together, these processes can trigger cardiac fibrosis, hypertrophy, and cardiomyocyte death, with consequent contractile dysfunction and endothelial cell damage. Among these factors, insulin resistance seems to play a key role in the development of heart muscle dysfunction. Recently, it has been reported that insulin resistance is also present in people with cardiomyopathy without diabetes, which suggests a potential bidirectional link between cardiomyopathy and insulin resistance [38].

In dogs, significant changes in the availability and production of energy in the myocardium are

noted in the later stages of DCM and other myocardial diseases [39]. Although the contractile capacity of the myocardium is dependent on efficient cellular energy metabolism, it cannot disregard proper transmembrane ion flow. Changes in mRNA expression levels of genes related to ion channels, such as calcium voltage-gated channel auxiliary subunit alpha 2 delta 3 and calcium voltage-gated channel subunit alpha1 S may lead to defects in functional Ca^{2+} currents and consequently trigger damage of the cardiac contraction process typical in DCM [40]. In addition, the downregulated gene ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2, an ATPase enzyme is involved in the regulation of Ca^{2+} level during muscle contraction and relaxation. It has been reported that the downregulation of the ATPase sarcoplasmic/endoplasmic reticulum Ca^{2+} transporting 2 gene was also correlated with heart failure [41], and was also involved in the "cyclic guanosine monophosphate activates protein kinase G signaling pathway" that plays a fundamental role in the maintenance of the heart's physiological state [42].

Another interesting aspect regards the presence of inflammatory infiltrate, observed in three dogs, and the fibrosis detected in six dogs, suggesting a possible chronic evolution of the myocardial disease. The presence of pathogens recorded in three samples (two of them associated with lymphocytic interstitial infiltrates) could suggest a possible involvement in the evolution of secondary DCM-p.

The transcriptomic profile of the dogs with inflammation showed a strong deregulation of several genes associated with myocarditis pathways, raising the hypothesis that in these three dogs, the inflammatory process could be in a developmental stage. These genes were in fact related to structural cell and tissue reorganization (e.g. cell adhesion molecules and tight junction), host response to inflammatory triggers, and pathogens' activity (e.g. cell migration, bacterial invasion of epithelial cells, viral myocarditis). Part of the differentially expressed genes regulate leukocyte transendothelial migration, a process that is fundamental in immune surveillance and inflammation in heart tissue. This process involves several molecules of diagnostic and pathogenic significance during inflammatory cardiomyopathy associated with DCM in humans [43,44]. These results strongly harmonized with the histologic diagnosis of an interstitial lymphocytic infiltration without degeneration or necrosis of cardiomyocytes observed in this study.

In this research, the mitogen-activated protein kinases pathway is activated in animals showing the presence of canine parvovirus 2 (2/3) and canine distemper virus (1/3). Although reported in other species, this pathway is a major cell signaling commonly associated with RNA viral infections such as parvovirus [45]. Several studies also reported the involvement of the mitogen-activated protein kinase pathway in human and rats with DCM, demonstrating a possible role in the pathogenesis of this cardiomyopathy [46,47]. The analysis of the samples collected showed the activation of the Rap1-enriched pathway. Rap1 is a small guanosine triphosphatases (GTPase) protein that plays a fundamental role in adenosine-cyclic monophosphate signaling in cardiac tissues. In cases in which Rap1 is suppressed, it can impair cardiac myofibrils and the conduction system [48,49]. Recently, Zhang et al. [50] reported the involvement of the Rap1 signaling pathway in viral myocarditis (parvovirus B19), suggesting a contribution to the progression of DCM-p. Although RNA-seq data seem to support the PCR results, the effects of the pathogens cannot be confirmed because of the lack of evidence of active viral replication in the cardiac tissue. Pathogen detection by qualitative PCR does not confirm causation; thus this is a limitation of this research.

Other limitations of this study relate to the different ages, breeds, treatment regimens, and the small number of dogs of the control group. However, using data retrieved from a previous approved study allowed us to make up for the absence of EMB not performed in healthy dogs for obvious ethical reasons. Furthermore, all affected dogs in this study had congestive heart failure as a result of their DCM-p. This makes it difficult to definitively determine whether the identified molecular and genetic factors relate solely to the DCM-p or could also relate to presence of congestive heart failure.

Conclusions

Next-generation sequencing technology performed *in vivo* on endomyocardial biopsies has allowed for identification of molecular and genetic factors that may play a role in the development of DCM or a DCM-p in dogs. The presence of myocarditis in combination with the activation of myocarditis-related genetic pathways observed in three of the nine dogs suggests that inflammatory processes may be an important trigger in the development and progression of a DCM-p in some dogs. This study, although it had some limitations, showed that alterations in cellular energy metabolism and cardiac structural and/or functional protein genes

were identified in dogs with DCM-p and congestive heart failure. However, further studies are needed to investigate the possible role of insulin resistance more thoroughly, as well as the potential involvement of specific viruses in the development of DCM-p in dogs.

Conflicts of Interest Statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jvc.2024.02.008.

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