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Research Article

Evaluation of Serum and Ascitic Fluid Proteomes in Dogs with Dilated Cardiomyopathy [1]

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Abstract

The aim of the study was to investigate serum global proteomes in dogs with overt dilated cardiomyopathy (DCM) and to evaluate protein expression in serum with that in ascitic fluid. Eight healthy dogs (control group) and 8 dogs with DCM were included in the study. DCM was diagnosed based on echocardiographic evidence including increased left ventricular dimension at diastole and systole, increased E point to septal separation, and decreased fractional shortening. Serum and ascitic fluid samples were analyzed for proteomes using a label-free LC-MS/MS method. Proteome analyses revealed significantly different expressions of eight proteins in all samples. Expressions in serum of apolipoprotein (Apo) A1, Ig heavy chain V, superoxide dismutase and plasminogen were higher (P<0.001), while expressions of clusterin, hemoglobin subunit ß, Apo-CII, and β2 glycoprotein I (ß2GPI) were lower (P<0.001) in dogs with DCM than in control dogs. In addition, Apo-A1, clusterin, hemoglobin subunit ß, Ig heavy chain V, plasminogen and ß2GPI were down-regulated whereas Apo-CII and superoxide dismutase were up-regulated in ascitic fluid compared with serum in dogs with DCM. Data obtained in the present study suggest that serum and/or ascitic fluid proteomes may explain some of the pathophysiological mechanisms involved in the progression of DCM.

Keywords: *Dilated cardiomyopathy, DCM, Congestive heart failure, Proteomics, Dog*

Dilate Kardiyomiyopatili Köpeklerde Serum ve Asites Sıvısı Proteomlarının Araştırılması

Özet

Çalışmamızın amacı ileri düzeyde dilate kardiyomiyopati (DCM) tanısı konulan köpeklerde serum global proteomların araştırılması ve aynı hastaların asites sıvısı proteomları ile ilişkilerinin değerlendirilmesidir. Sekiz sağlıklı (kontrol grubu) ve 8 DCM'li köpek çalışmaya dahil edildi. DCM tanımlaması ekokardiografik olarak sistol ve diyastolde artmış sol ventriküler çap, artmış E point to septal separasyon değeri ve azalmış fraksiyonel kasılma verileri temelinde yapıldı. Serum ve asites sıvı örnekleri label-free LC-MS/MS metoduna göre analiz edilmiştir. Proteom analizi ile tüm örneklerde toplam sekiz adet proteom ekspresyonu belirlendi. DCM'li köpeklerde kontrol grubuna göre serum apolipoprotein (Apo) A1, Ig heavy chain V, superoksit dizmutaz ve plazminojen ekspresyonlarında artış (P<0.001), klasterin, hemoglobin subunit ß, Apo-CII, ve β2 glikoprotein I (ß2GPI) ekspresyonlarında ise azalma (p<0.001) belirlendi. Buna ek olarak; asites sıvısı serum örnekleri ile karşılaştırıldığında Apo-A1, klasterin, hemoglobin subunit ß, Ig heavy chain V, plazminojen ve ß2GPI azalırken, Apo-CII ve superoksit dizmutaz artış gösterdi. Çalışmada elde edilen bu verilerin; serum ve/veya asites sıvısı proteomlarının DCM gelişiminde rolü alan bazı patofizyolojik mekanizmaların açıklanmasına katkı verebileceği kanısındayız.

Anahtar sözcükler: *Dilate kardiyomiyopati, DCM, Konjestif kalp yetmezliği, Proteomik, Köpek*

INTRODUCTION

Dilated cardiomyopathy (DCM) sis one of the most common organic heart defects in dogs [1]. Echocardiographic features include ventricular dilation, atrial dilation, normal or thin wall and septal thickness, depressed systolic thickening of the free wall and septum, poor fractional shortening (FS), large E point to septal separation (EPSS), reduced aortic wall motion and global hypokinesis [1,2]. Ventricular dilation including generally the left side of the

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heart and poor myocardial function are the main findings of DCM [2]. Such abnormalities of the heart muscle result in chemical and organic reactions cause biomarker release such as natriuretic peptides and cardiac troponins [3]. Many researchers have focused on specific biomarker indicating myocardial injury in DCM [3]; however, new trend of the science is interested in such small peptides, rather than the whole proteins, called proteomics [6].

Proteomics is the large-scale study of protein expression, protein-protein interactions, or post-translational modifications [6-8]. Use of proteomics technology in veterinary medicine is presently under development. Samples and study designs are discussed due to the limitations of mass spectrometry [9]. In veterinary medicine proteomics were performed in such conditions as canine lymphoma [10] and heartworm disease [11] while canine DCM has not yet been studied using proteomics. In addition, although proteomics were performed in several canine body fluids such as cerebrospinal fluid [12], urine [13], bronchoalveolar lavage fluid [14] and follicular fluid [15], no data have yet been presented on ascitic fluid in dogs with DCM. In the present study, we hypothesized that many of the serum proteomes with low abundance and low molecular weight may have roles in the development of DCM in dogs. Besides, proteomic analyses of ascitic fluid may provide further details to explain some patho-physiologic mechanism and/or some clinical complications such as abdominal distention and pleural effusion in dogs with DCM.

Therefore, we aimed to analyze expressional proteomics in both serum and ascitic fluid samples, using label-free LC-MS/MS method, in order to present novel data that may help improve our understanding of the pathophysiology of canine DCM.

MATERIAL and METHODS

Animals

Eight dogs with DCM (5 females, 3 males) with different breed (Mixed breed n=4, Terrier n=2, Pointer n=1, Anatolian Sheepdog n=1) and an average age of 54.8±30.8 months (range: 10-96 months), and 8 clinically healthy dogs (4 females, 4 males) with different breed (Mixed breed n=5, Terrier n=2, Anatolian sheepdog n=1) and an average age of 50.2±16.4 months (range: 2 to 8 years) were included in the study. All dogs with DCM were suffering from congestive heart failure (CHF) findings such as cough, lethargy, anorexia, dyspnea, exercise intolerance, abdominal distension and/or pleural effusion.

Electrocardiographic and Echocardiographic Evaluations

After routine clinical examination, electrocardiography (ECG, Esaote, P200®- Florence, Italy), thoracic radiography and echocardiographic examinations were performed. Two-dimensional (2D) echocardiography, M-mode, color flow imaging and spectral Doppler examinations were performed using a CarisPlus® (Esoate, Florence, Italy) with a 2.5-5 MHz phased-array transducer using standard techniques [2]. The dogs were not sedated throughout the ultrasound examination, and were gently restrained in right lateral recumbency. DCM was diagnosed based on the echocardiographic findings such as increased chamber size, increased EPSS and poor FS along with ECG and thoracic radiographic findings. Diagnosis was confirmed using a scoring system for DCM proposed by European Society for Veterinary Cardiology [2]. Patients presented with all of the criteria for DCM but did not have any other cardiac pathology including congenital malformations or tumors. Dogs with primary congenital heart disease, mitral valve disease or endocrine disorders such as hypothyroidism were excluded.

Radiologic Evaluation

Assessment of cardiomegaly on thoracic radiography was based on a combination of subjective experience and the use of the vertebral heart scale system [16].

Sample Collection and LC-MS/MS Analysis

Serum and ascitic fluid samples were obtained from patients before the treatment. Samples were kept in -80ºC freezer until being sent in cold chain to the laboratory for analyses [TUBITAK, Genetical Engineering and Bio-technology Institute (GEBI), Gebze, Kocaeli, Turkey].

Protein expression analyses in serum and ascetic fluid samples were performed using a label-free nano liquid chromatography - mass spectrometry method. Extracted proteins from the samples were reduced with dithriothreitol (DTT; 5 mM, 15 min) and alkylated with iodoacetic acid (IAA; 10 mM, 30 min at dark). Tryptic peptides were generated by incubating the protein mixture at 37ºC with sequencing grade trypsin (1:50 ratio, Pierce). The peptide mixture was loaded on a nanoACQUITY UPLC Symmetry C18 Trap column (5 µm particle size, 180 µm i.d. x 20 mm length) at 5 µl/min flow rate for 5 min. Peptides were eluted from the trap column by gradient elution onto an analytical column (nanoACQUITY UPLC BEH C18 Column, 1.7 µm particle size, 75 µm i.d. x 250 mm length, Waters), at 300 nl/min flow rate with a linear gradient from 5 to 40% acetonitrile over 90 min. Data independent acquisition mode (MS^E) was carried out by operating the instrument at positive ion V mode. Mass drift was corrected by the internal mass calibrantglu-fibrinopeptide infused every 45 sec through the nanolockspray ion source at 300 nl/min flow rate. Peptide signal data between 50-1.600 m/z values were collected. Data processing parameters were set to standard operating values [17]. The Apex3D parameters were set to 0.2 min chromatographic peak width and 10.000 TOF resolution. 150, 50 and 1.200 counts were set

for low energy, elevated energy and intensity threshold, respectively. Tandem mass spectra extraction, charge state deconvolution and deisotoping were processed with ProteinLynx Global Server v2.3 software (PLGS) (Waters Corp., Milford, MA). Protein sequence database from Uniprot was used. Databank search query was set to minimum 3 fragment ion matches per peptide, minimum 7 fragment ion matches per protein, minimum 1 peptide matches per protein and 1 missed cleavage. Carbamidomethyl-cysteine fixed modification and Acetyl N-terminal, deamidation of asparagine and glutamine, oxidation of methionine variable modifications were set. Normalization of the proteins was achieved against the digest of the internal calibrant P00330.

Statistical Analyses

Data were analyzed by Student's t test using SPSS 10.0 Statistical Software (SPSS Inc), and the results were expressed as Mean ± Standard error of means (SEM). The intensity % coefficient of variation (%CV Int) were calculated to be around 14% across the identified proteins so three times the %CV Int value is set for the cut-off for statistical significance. Only proteins expressional changes showing more than 40% up-regulation or down-regulation were reported. For all comparisons, values of $P<0.05$ were considered significant.

RESULTS

Clinical Findings

Clinical charts of dogs with DCM included dyspnea (6/8), lethargy (4/8), exercise intolerance (8/8), and anorexia (5/8). Clinical examination revealed weak femoral artery pulse, distension of the jugular vein, abdominal distension, increased cardiac auscultation area and mitral and/or tricuspidal murmurs with different severities in all patients with DCM.

Electrocardiographic (ECG) Findings

ECG analysis revealed some cardiac rhythms abnormalities in dogs with DCM in which atrial fibrillation (5/8) *(Fig. 1)*, ventricular extra systoles (2/8), atrioventricular block (1/8) and sinus tachycardia (2/8) were the most common.

Radiological Findings

Enlarged heart size, deviation of the trachea, mild to severe pulmonary edema, increased vertebral heart scale (14.2±1.3), pulmonary pattern, caudal vena cava distension, and pleural and peritoneal effusions were observed on thoracic radiography in dogs with DCM *(Fig. 2)*.

Two-dimensional Echocardiographic Findings

All dogs with DCM had geometric and functional cardiac abnormalities. LA diameter (4.62±0.4 cm), LA/Ao ratio (2.08±0.4), left ventricular diastole diameter (6.3±0.7 cm) and EPSS value (1.1±0.3 cm) were above the reference limits. Poor FS (15.8±4.8%) was observed in dogs with DCM, as well *(Table 1, Fig. 3)*. Pulmonary artery flow velocity was higher ($P<0.001$) but aortic flow velocity was lower ($P<0.001$) than those of healthy controls *(Table 1)*.

Serum and Ascitic Fluids Proteomics

Expressions of 8 proteins (Apolipoprotein[Apo]A1, hemoglobin subunit ß, clusterin, Ig heavy chain V region, Apo-CII, plasminogen, b 2 glycoprotein I [ß2GPI] and superoxide dismutase) differed significantly in blood and ascitic fluid samples *(Table 2)*. Apo-A1, Ig heavy chain V, superoxide dismutase and plasminogen expressions in serum samples were higher ($P<0.05$ - 0.01), while clusterin, hemoglobin subunit ß, Apo-CII and β2GPI expressions were lower ($P<0.05$ - 0.001) in dogs with DCM than in healthy controls. Expressions of Apo-A1, clusterin, hemoglobin subunit ß, Ig heavy chain V, plasminogen, and ß2GPI were

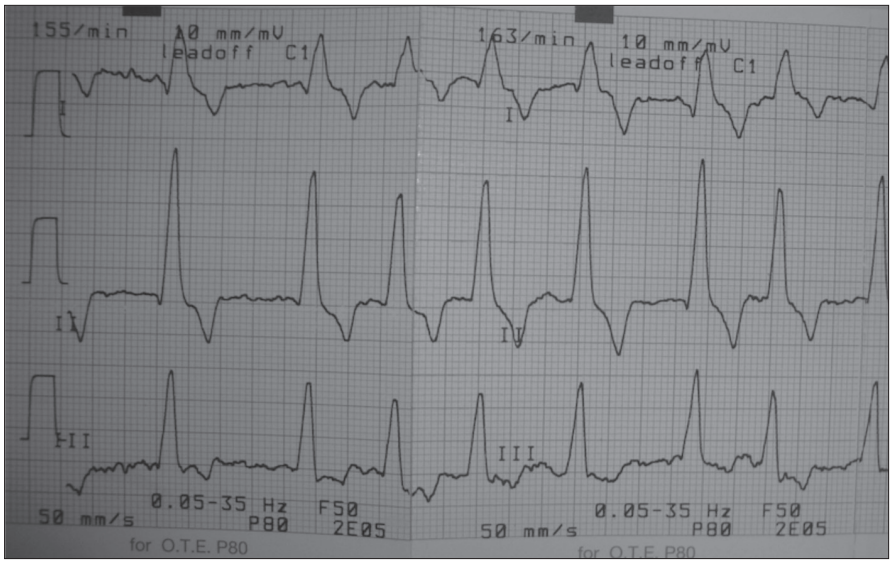

Fig 1. ECG from a dog with DCM (7 year-old, male, Turkish Shepherd Dog) revealed an atrial fibrillation due to absence of "P waves" and increased heart frequency (Calibration: 50 mm/second, 10 mm/1 mV)

Şekil 1. DCM'li bir köpek EKG'sinde (7 yaşlı, erkek, Kangal) 'P dalgalarının' olmaması ve artmış kalp frekası nedeniyle atriyal fibrilasyon belirlenmiştir (Kalibrasyon: 50 mm/saniye, 10 mm/1 mV)

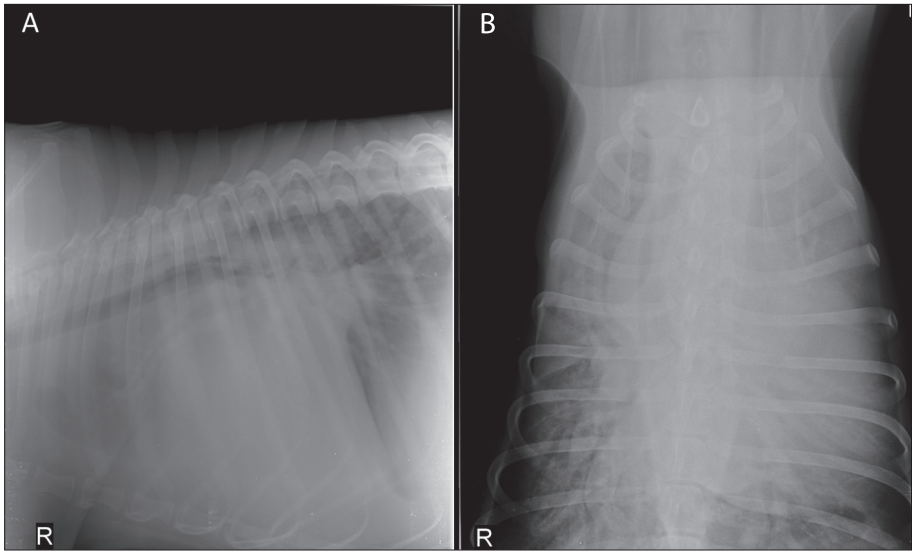

Fig 2. Radiologic evaluation of the thorax in a 8-year-old male Turkish Shepherd Dog. A: The right lateral radiograph points out the general lung tissue opacity, dorsal deviation of the trachea and the vascular hilus, bronchiectasis of the cranial lobe bronchus and enlargement of the heart borders in the thoracic cavity, B: The ventrodorsal radiograph shows general opacity, alveolar model influence of the lung lobes and unclear hearth silhouette as well as left side shift of the hearth suspected due to cardiomegaly

Şekil 2. 8 yaşlı erkek Kangal bir köpekte toraksın radyolojik olarak değerlendirilmesi. A: Sağ lateral radyografi genel akciğer dokusu opasitesindeki artışı, trakeanın dorsale deviyasyonu ve hilus vaskülarizasyonunu, kraniyal lob bronşunda bronşiyektaziyi ve torasik boşlukta kalbin sınırlarının genişlemiş olduğunu vurgulamaktadır, B: Ventrodorsal grafi genel opasite, akciğer loblarında alveoler etkilenim ve şüphelenilen kardiyomiyopati nedenli sol tarafa kaymaya ek olarak belirsiz kalp siluyeti belirtmektedir

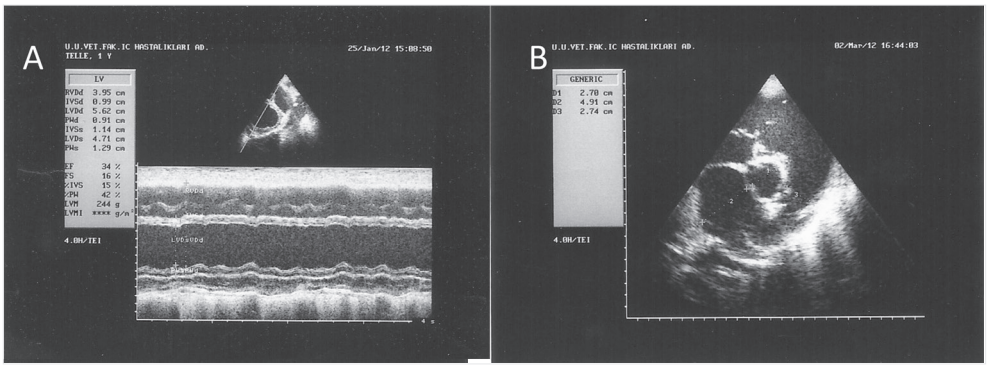

Fig 3. M-mode measurement of the left ventricle at right parasternal short axis view showed increased chamber size, poor fractional shortening and interventricular septal akinesia. Hyperechoic line in the pericardial sac indicated mild pericardial effusion (A). Left atrial dilation (left atrium/aorta ratio: 1.7) was observed on right parasternal short axis view - aortic level (B)

Şekil 3. Sol ventrikülün sağ parasternal kısa eksen M-mod görüntüsü artmış odacık büyüklüğü, zayıf fraksiyonel kısalma ve interventriküler septal akinezi olduğunu göstermiştir. Perikardiyal kesedeki hiperekoik çizgi hafif bir parikasrdiyal efüzyonu işaret etmektedir (A). Sağ parasternal kısa eksen görüntü- ortik düzeyde sol atriyal genişleme (Sol atriyum /aorta oranı: 1.7) belirlenmiştir (B)

down-regulated whereas expressions of Apo-CII and superoxide dismutase were up-regulated in ascitic fluid compared with serum in dogs with DCM.

DISCUSSION

The present study reported, for the first time, evaluation of protein expression changes in blood and ascitic fluid of dogs with DCM and made comparisons with regard to serum proteomes between dogs with DCM and healthy controls. Significant differences were detected in expressions of a total of 8 proteins (Apo-A1, hemoglobin subunit ß, clusterin, Ig heavy chain V region, Apo-CII, plasminogen, ß2GPI and superoxide dismutase) in both serum and ascitic fluid of dogs with DCM. In the study, DCM was diagnosed based on the echocardiographic

Tablo 1. *DCM'li ve sağlıklı köpeklerin ekokardiografik parametreleri (Mean ± SE)*

Table 1. *Echocardiographic parameters of the dogs with DCM and healthy dogs (Mean ± SE)*

Parameter	Dogs with DCM n=8	Healthy dogs n=8	P value
Body weight (Kg)	32.2 ±8.5	28.4±4.3	ns
IVSD (cm)	1.1±0.5	0.82±0.06	<0.01
IVSs (cm)	2.53±1.17	1.63±0.41	ns
LVDd (cm)	6.37 ±0.7	4.54±0.33	<0.001
LVDs (cm)	5.4±0.7	3.20±0.25	<0.001
PWd (cm)	0.88±0.37	0.94±0.11	<0.01
PWs (cm)	1.03±0.34	1.05±0.12	ns
Ao diameter (cm)	2.22 ±0.4	2.0±0.1	ns
LA diameter (cm)	4.62±0.4	2.3±0.0	<0.001
LA/Ao ratio	2.08±0.4	1.2±0.0	<0.001
EPSS (cm)	1.1±0.3	0.3±0.1	<0.001
FS %	15.8±4.8	30.20±2.41	<0.001
PA Vmax m/s	0.6±0.09	0.33±0.03	<0.001
AoVmax m/s	0.7±0.14	1.3±0.1	<0.001

BW: *body weight;* ***IVSD:*** *interventricular septum diastole;* ***IVSs:*** *interventricular septum systole;* ***LVDd:*** *left ventricular diameter diastole;* ***LVDs:*** *left ventricular diameter systole;* ***PWd:*** *post wall diastole;* ***PWs:*** *post wall systole;* ***Ao:*** *aorta;* ***LA:*** *left atrium;* ***LA/Ao ratio:*** *left atrium/ aorta ratio;* ***EPSS:*** *E-point to septal separation;* ***FS:*** *fractional shortening;* ***PA:*** *pulmonary artery;* **ns:** *not significant*

Table 2. *List of the protein expression results and fold changes (+ increase; - decrease) between serum from dogs with DCM (DCM) and healthy controls (H) or ascitic fluid (A)*

Tablo 2. *DCM'li (DCM) ve sağlıklı (H) köpeklerin serumları veya asites sıvıları (A) arasındaki protein salınım sonuçlarının ve son değerlerin başlangıç değerlerine oranlarının listesi (+ artış; - azalış)*

Accession #	Description	Fold Change	
		DCM *vs* H	DCM *vs* A
P02648	Apolipoprotein A 1	1.42 (+)**	1.41 (+)**
P25473	Clusterin	1.40 (-)*	1.21 (+)*
P60524	Hemoglobin subunit ß	1.40 (-)*	3.65 (+)**
P01784	Ig heavy chain V region GOM	1.41 (+)**	1.77 (+)**
P12278	Apolipiprotein C II	354.8 (-)***	299.7 (-)***
P80009	Plasminogen Fragment	1.44 (+)**	1.36 (+)**
P33703	ß 2 Glycoprotein 1	2.31 (-)**	47.6 (+)***
O54210	Superoxide dismutase Mn Fe fragment	1.65 (+)*	1.66 (-)*

vs - versus, * P<0.05, ** P<0.01, s*** P<0.001

findings along with ancillary diagnostic tests including ECG and thoracic radiography, as suggested [2].

Clusterin has a protective role on stress-mediated apoptosis, oxidative stress and protein aggregation inhibition [18]. Cardiorenal syndrome may be another reason for low serum clusterin level, since clusterin is also a renal damage biomarker in dogs [19,20]. Increased vascular permeability due to serum protein escape from the blood stream and the venous return loss in response to heart failure might be responsible of low clusterin levels in dogs with DCM studied. In the present study, the possible reason for decreased level of serum clusterin in dogs with DCM may be related with the result of excessive use of the protein in situations such as increased cell death and oxidative stress status.

Previous studies suggested that Apo-A1is responsible of cholesterol transport from tissues to the liver [11]. However *vena cava caudalis* pressure overload as a result of decreased venous return to the heart may play a role on liver-ischemia-induced loss of free cholesterol esterification. In this pathophysiology, high blood cholesterol plays a role on erythrolysis and hemoglobin release [11]. The mild increase in hemoglobin subunit ß protein detected in the present study might have resulted from increased demand of O_2 in the body in response to decreased cardiac output (lower aortic flow velocity), the myocardial contractility loss (lower FS), and decreased venous return (Jugular distention and higher pulmonary artery flow velocity) in dogs with DCM. An increased O_2 demand may be explained by superoxide anion radical formation in conjunction with increased superoxide dismutase, an anti-oxidant protein. β_2GPI molecule was classified as an Apo, and it was initially termed Apo-H [21]. Among connected results, decreased level of serum Apo-H might be a result of protection mechanism against oxidative stress induced by apoptotic ischemic cells. Consistent with this hypothesis, Apo-H was shown to be present in atherosclerotic plaques [22] and involved in apoptosis process at surfaces of cells undergoing apoptosis [21,23].

In our study, Apo-CII was low (354.8 fold change) in serum samples of dogs with DCM. This might be the result of increased permeability of veins and impaired circulation ended with Apo-CII escape from circulation to ascites fluid. Apo-CII was reported to be a cofactor for lipoprotein lipase and identified as a putative substrate of matrix metalloproteinases (MMP-7, MMP-14) in humans, and a deficiency in Apo-CII was associated with atherosclerosis [24]. In good accordance, Kawano *et al.*[25] showed that a mutation in the Apo-II gene caused coronary atherosclerosis. Since canine Apo-CII has not been studied yet, we can only speculate that it might be involved in pathogenesis of atherosclerosis as in humans.

Venous return loss, impaired circulation and pleural and peritoneal effusion, as observed in the present study, are the signs of congestive heart failure (CHF) in dogs with DCM [1,2]. A previous study [26] reported high plasma fibrinogen, D-dimer, thrombin-antithrombin complex and low anti-thrombin activity in dogs with chronic CHF. We showed, in the present study, changes in expressions of

proteins related with coagulation in dogs with DCM, based on serum proteomics data including increased expression of plasminogen and decreased expression of β_2GPI. It is well known that plasminogen has a fibrinolytic activity, and β_2GPI has procoagulant and anticoagulant activities in the coagulation cascade [27]. β_2GPI can also bind to plasminogen and lipoprotein A, a molecule conferring a putative risk for atherosclerotic disease in humans [21]. It may thus be suggested that plasminogen and β_2GPI act together to regulate intrinsic fibrinolytic pathways in dogs with DCM [21,27].

On the other hand, our study has several limitations. The number of the dogs is not enough to represent the whole population, because this is a pilot study in which specific breed or sex variation was not investigated. In addition, while our results seem like not related with breed type or sex, physiological states like ovulation may be involved in alterations in serum proteomics. These limitations warrant a larger-scale study.

Since hematological or endocrinological changes would change the proteomic scale, serum proteome mapping of the dogs with DCM was challenging. Despite having controlled many variables, still some other mechanisms might have affected the analysis results. Serum proteomics can easily be affected by physiological conditions therefore proteins found in this study might be altered by physiological variables [9]. In parallel with this explanation, human studies showed that patients show different proteomic patterns in pathophysiological conditions such as sepsis [28] and diabetes mellitus [29].

There are some studies on structural proteomics of the canine heart [30,31], and our study did not include brain natriuretic peptide or cardiac troponin I detection in both samples (serum and ascitic fluid). This might be associated with potential loss of high molecular bounded target proteins in protein immune-affinity depletion step of the proteomic analysis technique or binding to the depletion column [32-34]. Immune-affinity depletion is essential for eliminating most abundant proteins such as albumin, but loss of target proteins in this step narrows the data and makes more questionable [9].

In conclusion, the study presented here that DCM in dogs shows different peptide pattern in the blood stream, which are the result of increased O_2 demand, hemostatic and fibrinolytic system changes and oxygen free radicals and progressive organ damage, as well as the heart itself. In this study, observed proteomes in serum and ascitic fluid samples enhances the search for informative biomarkers in biological samples from healthy dogs or dogs with DCM, thus allowing an earlier and more precise diagnosis and a deeper comprehension of pathogenesis, development and outcome of cardiomyopathy. Proteomic analysis in dogs with DCM may be useful to evaluate dynamic pathophysiological changes of the disease process, and especially serum Apo-CII level may be accepted as a risk factor for developing DCM in dogs.

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Conflict of Interest

None of the authors of this article has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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