Serum proteome profiling in canine chronic valve disease using a TMT-based quantitative proteomics approach

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Abstract

Chronic valve disease (CVD) is the most common clinically significant heart disease of dogs, affecting 20 to 40% of dogs. The aim of this study was to evaluate the serum protein profile of healthy and CVD affected dogs, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. Additionally, conventional cardiac biomarkers were measured in the serum, functional bioinformatics analysis was employed for elucidating molecular mechanisms and pathways associated with CVD, and validation of proteomic results was performed by immunoassays and Western blotting. Of 290 identified and quantified proteins, 15 proteins showed significantly different abundances ($p<0.05$), including antithrombin-III, alpha-2-antiplasmin, tetranectin, apolipoprotein M, adiponectin, inter-alpha-trypsin inhibitor heavy chain H1, gelsolin and apolipoprotein B-100. The identified proteins with differently abundances are involved in a number of pathways, such as complement and coagulation cascades, haemostasis, regulation of actin cytoskeleton, lipid metabolism and transport. We found comparative similarities with human disease in terms of identified proteins and GO pathways, which confirmed similar pathophysiology of this disease, but also differences, mainly in lipid metabolism.

Keywords: chronic valve disease, dog, serum, TMT-based quantitative proteomics, biomarker

Significance

There have been few investigations of canine serum proteome despite the potential for biomarker discovery and comparative disease analysis. Establishing serum proteomic signatures in healthy dogs and dogs with CVD will benefit for understanding the aetiology of disease in dogs, identify putative biomarkers and provide models of comparative human disease. Circulating biomarkers are important for understanding of the mechanisms of cardiovascular disease and high incidence of CVD in dogs prioritizes the search for novel biomarkers.

Highlights

- Establishing serum proteomic signatures in healthy dogs and dogs with CVD
- TMT-based relative quantification revealed 15 proteins with significantly lower differential abundances in dogs with CVD
- Proteins validated with immunoassays confirmed consistence with proteomic results
- Serum proteins involved in extracellular structure organization, haemostasis pathway and lipoprotein metabolism identified as potential markers in canine CVD

1. Introduction

Veterinary proteomics is an evolving field which holds great promise not only for fundamental and applied discoveries regarding biology and pathology of domestic species, but can also be implemented in comparative applications of human diseases research [1]. The heart in dogs and humans is similar in many characteristics on both the organ and cellular levels. Canine heart rate, body weight, and heart weight are more comparable to humans than the other animals such as mice, rabbits, and rats. The opportunity to use dog as model animal for comparative disease studies is based on the sequencing of the canine genome and the increasing availability of canine specific biological tools and reagents [2]. Chronic valve disease (CVD) is the most common heart disease in dogs and the most frequent cause of congestive heart failure in this species, representing approximately 75% of all heart disease in the dog [3]. CVD is pathologically identical in humans and dogs, suggesting a common pathogenesis in these species, and creating an increasing interest in the canine CVD as a model for the human medicine. Various other names for the disease are used and include endocardiosis, valvular regurgitation, valvular insufficiency, mitral regurgitation, myxomatous degeneration of the valve, degenerative mitral valve disease, senile nodular sclerosis, mucoid degeneration, chronic mitral valve fibrosis [4].

Disease is characterised by a chronic progression, from mild, clinically silent disease to severe disease with signs of congestive heart failure. Myxomatous valvular degeneration most often leads to lesions of the mitral valve (62% of cases), sometimes both atrioventricular valves (33% of cases) and infrequently the tricuspid valve (1% of cases) [5] .

Reported risk factors associated with progression of disease or death in dogs with CVD include age, gender, intensity of heart murmur, degree of valve prolapse, severity of valve lesions, the degree of mitral valve regurgitation, degree of left atrial enlargement, severity of eccentric hypertrophy, rupture of chordae tendinae and increased concentration of natriuretic peptides. The vast majority of the breeds at elevated risk of CVD are small or toy breeds, males are 1.5 times more represented than females, and the disease is rare before the age of 4 years [3, 4, 6].

The aim of this study was to evaluate the serum protein profile of healthy and CVD affected dogs, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. Additionally, conventional cardiac biomarkers were measured in the serum, functional bioinformatics analysis was employed for elucidating molecular mechanisms and pathways associated with CVD, and validation of proteomic results was performed by immunoassays and Western blotting. Only one previous study has been performed to identify serum proteins that were differentially expressed in healthy Cavalier King Charles Spaniel and those affected by CVD in mild to severe stages, using two-dimensional gel electrophoresis separation and analysis by MALDI-TOF-MS for protein identification [7]. To the authors´ knowledge, this is the first proteomic study of serum of dogs with CVD, where label-based quantitative LC-MS/MS approach was used.

2. Materials and methods

2.1 Animals

Two groups of dogs were enrolled in the study in the period between June 2015 and May 2017: 8 clinically healthy dogs (used as controls) and 8 dogs diagnosed with CVD. The study was approved by the Committee on the Ethics of the University of Zagreb, Faculty of Veterinary Medicine (Permit Number: 640–01/14–305/16, 251–61-01/139–14-28). Healthy dogs were admitted to the Clinic for Internal Diseases, Faculty of Veterinary Medicine, University of Zagreb, Croatia, while diseased dogs were admitted to the Small Animals Clinic, Department of Clinical Veterinary Science, Vetsuisse Faculty, University of Bern, Switzerland. Serum of dogs with CVD was collected at the time of initial diagnosis and before any treatment.

All dogs of the control group underwent clinical examination, haematological and biochemical serum analyses, as well as cardiac function evaluation performed in unsedated dogs, which included a 1-min 6 lead ECG (ASPEL, AsCard Mr. Silver) and transthoracic echocardiography using Esaote MyLab40 Vet machine and a 5 MHz sector transducer. Dogs were diagnosed with CVD-MV if the history, clinical exam results and the results of an ultrasound imaging were confirmative and other acquired heart disease so as congenital heart diseases were ruled out [8]. The cardiac evaluation of dogs included physical examination,

thoracic radiographs evaluated by a board-certified radiologist, a 1-min 6 lead ECG (Schiller AT 101) and transthoracic echocardiography performed by a board certified cardiologist (AK). Echocardiography was performed using an Aloka ProSound Alpha 5SV machine and a 5-MHz sector transducer in unsedated dogs. Echocardiography was performed in a standard manner [8]. Diseased dogs were classified according to the American College of Veterinary Internal Medicine (ACVIM) consensus guidelines for the diagnosis and treatment of myxomatous mitral valve disease in dogs [9].

All procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments, as well as subject to informed owner consent.

Serum was obtained from all dogs by centrifugation of completely clotted blood at 3500g for 10 min at room temperature. Supernatants were collected, aliquoted and stored at −80 °C until analyses. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing.

2.2 Serum biochemistry and cardiac biomarker analyses

One serum aliquot was used for measurement of biochemical parameters using commercial reagents (Beckman Coulter) per manufacturer's instructions in an automatic analyser (Olympus AU 640, Hamburg, Germany). The following parameters were measured: serum urea, creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transferase (GGT), alkaline phosphatase (AP), creatine kinase (CPK), lactate dehydrogenase (LDH), C-reactive protein (CRP), alpha-amylase, lipase, cholesterol, triglycerides, calcium, phosphates.

Cardiac troponin I (cTnI) was measured using a commercially available ADVIA Centaur TnI-Ultra assay, which is a high-sensitivity immunoassay validated for use in both humans and dogs [10]. Analysis was performed in Dubrava Clinical Hospital, Department of clinical diagnostics (Zagreb, Croatia) using Siemens Advia Centaur XP according to manufacturer's instructions. Samples which had serum concentration of cTnI below the lower level of detection of the assay were allocated a value of 0.01 μg/L. Analysis of N-terminal pro b-type natriuretic

peptide (NT-proBNP) concentration in all samples was performed in Vet Med Labor GmbH, reference IDEXX Laboratory (Germany) using IDEXX Cardiopet® proBNP test.

2.3 Proteomic analysis by LC-MS/MS

Proteomic analysis of canine serum samples was performed by TMT-based quantitative approach as described previously [11]. In brief, after total protein concentration determination using Bradford assay (Thermo Scientific, Rockford, USA), 35 μg of total proteins from samples and internal standard (a pool of equal protein amount from all samples used as a reference for normalization) were diluted to a volume of 50 μL using 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA), reduced by adding 2.5 μL of 200 mM DTT (60 min, 55 °C) (Sigma Aldrich, St. Louis, MO, USA), alkylated by adding 2.5 μL of 375 mM IAA (30 min, room temperature in the dark) (Sigma Aldrich, St. Lois, MO, USA) and acetone-precipitated (addition of 300 μL, overnight, −20 °C). Protein pellets were collected subsequently by centrifugation (9000g, 4 °C), dissolved in 50 μL of 0.1 M TEAB and digested using 1 μL of trypsin (1 mg/mL, Promega; trypsin-to-protein ratio 1:35, at 37 °C overnight).

TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA) were prepared according to the manufacturer's procedure and 19 μL of the appropriate TMT label was added to each sample used for the labelling reaction (60 min, room temperature) which was quenched by 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Five TMT-modified peptide samples were combined with the internal standard (labeled with TMT *m/z* 126) into the new tube, aliquoted, dried and stored at −20 °C for further analysis. A total of 16 samples led to 4 individual TMT experiments with the inclusion of internal standards in each experiment but 2 samples were repeated using the same internal standard because of low labelling efficiency.

High resolution LC-MS/MS analysis of TMT-labelled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were dissolved in loading solvent (1% ACN, 0.1% formic acid) and loaded onto the trap column (C18 PepMap100, 5 μm, 100A, 300 μm×5 mm), desalted for 12 min at the flow rate of 15 μL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm×75 μm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min, 45% to 90% for 2 min, held at

80% for 2 min and re-equilibrated at 5% B for 20 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific, Bremen, Germany) containing a 10 μm-inner diameter SilicaTip emitter (New Objective, USA). The MS operated in positive ion mode using DDA Top8 method. Full scan MS spectra were acquired in range from *m/z* 350.0 to *m/z* 1800.0 with a resolution of 70,000, 110 ms injection time, AGC target 1×10^6, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17,500 and AGC target of 2×10^5. Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analysed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Canis lupus* FASTA files (downloaded from NCBI database on 14/10/2016, 41787 sequences) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow based on the search results against a decoy database and was set at 1% FDR. At least two unique peptides and 5% FDR were required for reporting confidently identified proteins.

Protein quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standard was used to compare relative quantification results for each protein between the experiments (sixplexes).

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017129.

2.4 Bioinformatic analysis

Proteins with significantly differential abundances observed by proteomic analyses were further functionally analysed using bioinformatics tools. As the human database is more

complete than the canine one (reviewed proteins in UniprotKB/Swiss-prot release 2018_01: humans 20,259, dogs 817), dog's proteins were converted to converted to human gene official names by the database bioDBnet. The 10 unique proteins have been enriched for their best interacting proteins according to Intact and Reactome databases, using the tool CluePedia (v1.5.2) from Cytoscape (v3.6.1). Maximum number of 10 best interactors has been set.

The resulting interactome was used to determine the enriched GO terms, using ClueGO (v2.5.2) from cytoscape with GO_biologicalprocess (04.09.2018), GO levels from 3 to 12, minimal number of gene = 2, mimimum percentage = 3.0, Kappa score theshold = 0.4, evidence codes used All_without_IEA.

List of GO terms was then submitted to analysis by ReviGO to remove redundant terms and define GO groups based on similarity, with the SimRel semantic similarity measure. Redundant GO terms were then removed from the interactome, realized with Cytoscape. GOterms with a minimum of 2 initial proteins have been considered for the analysis of GO terms related with CVD. Interactome have been designed using the radial layout of Cytoscape.

2.5 Validation of proteomic results

Validation of proteomics results was performed by ELISA and Western blotting using the serum samples of the same patients as in proteomic analysis. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid possible changes caused by repetitive thawing and freezing.

Canine specific ELISA kits were used for apoB-100 (BlueGene Biotech, Shanghai, China), apoD (ABclonal, Woburn, USA) and adiponectin (Wuhan Fine Biotech Co., LTD., Wuhan, China) according to manufacturer’s instructions. For analytical performance of the ELISA assays, assay precision and accuracy were calculated [12, 13]. For intra-assay precision, pool of samples with different concentrations of analytes were prepared from serum, while for inter-assay precision, pool was divided into aliquots and stored in plastic vials at −20 °C until analysis. Intra-assay coefficient of variation (CV) was calculated after analysis of pool six times in a single assay run. Inter-assay CV was determined by analysing the same samples in five separate runs carried out on different days. The accuracy of the assays was evaluated indirectly by

linearity under dilution. Briefly, serum pool was serially diluted with diluent provided with the kit and analysed.

For immunoblotting, the samples (30 µg of total protein) were boiled for 3 min at 95 ^{0}C in Laemmli SDS loading buffer and loaded on 10% SDS polyacrylamide gel and after electrophoresis (1 h at 110 V) transferred to a PVDF membrane (Amersham Hybond, 0.45 PVDF, GE Healthcare Lifescience) (2 h at 65 V, 150 mA) in 20% methanol (Sigma) transfer buffer at 4 °C using Biostep electro blotting module. The membranes were blocked for 1 h at room temperature with shaking in blocking buffer (1xTris buffered saline (TBS)/0,1% Tween 20/0,2% I-Block reagent). Subsequently, the membrane was incubated at 4 ºC with primary antibody for adiponectin (1:500 in blocking buffer; from Santa Cruz Biotechnology, Heildeberg, Germany) and kininogen-1 (1:500 in blocking buffer; Antibodies-online GmbH, Achen, Germany). Membranes were then washed three times with TBST buffer and incubated with secondary antibody (rabbit anti-mouse from Santa Cruz Biotechnology, 1:2000). Proteins were visualized by chemiluminescence using HRP chemiluminescence blotting substrate (Radiance Plus, Azure Biosystems, USA) on Odyssey Fc (LI-COR, Bad Homburg, Germany). The abundance of the protein of interest was normalized to the total amount of protein in each lane after staining with Ponceau S. Western blots were quantified using ImageJ software (National Institutes of Health). Statistical validation of the data was achieved by Mann-Whitney test, with P value < 0.05 considered statistically significant.

2.6 Statistical analysis

For proteomics, statistical analysis was performed using using R (v3.4.3) under the RStudio environment (v1.0.143) [14, 15]. Paired t-test was applied to calculate p-values to determine statistical significance among healthy dogs and dogs with CVD. For immunoblotting, statistical analysis was performed using the statistical software, GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). Differences between healthy and diseased dogs were assessed by Mann-Whitney test. Non-parametric statistics was chosen due to the small sample size. P-value < 0.05 were considered statistically significant for all performed tests.

3. Results

3.1 Animals and heart function

The control group consisted of 6 females (1 spayed) and 2 males, aged between 3 and 10 years, of following breeds: 2 Miniature Schnauzers, Dachshund, Maltese, Border collie and 3 mixed-breed dogs. In the group of dogs diagnosed with CVD there were 3 females (all spayed) and 5 males (of which 3 castrated), aged between 8 and 12 years. These were dogs of 7 different breeds (2 Cavalier King Charles Spaniels, Australian Kelpie, Coton de Tuléar, Borzoi, Miniature Schnauzer, Toy Australian Shepherd and Doberman). At initial physical examination all dogs of CVD group were considered in heart failure based on elevated respiratory rate and effort, signs of interstitial or interstitial-alveolar lung pattern and absolutely dilated pulmonic veins on the thoracic radiographs. All were classified as ACVIM class C. Six dogs were in sinus rhythm. Two of those dogs had rare ventricular premature complexes and one dog rare supraventricular premature beats. Two dogs showed atrial fibrillation with a ventricular rate 190-250/min. Seven of the eight dogs also had a tricuspid endocardiosis, four of which had signs of mild to moderate pulmonary hypertension. Two out of eight dogs showed a slight regurgitation on the aortic valve and pulmonary valves (Supplemental data 1).

3.2 Serum biochemistry and cardiac biomarkers

Values of serum biochemical parameters are presented in Table 1. as median and interquartile range, together with P value for comparisons of groups. Concentrations of urea, creatinine, total proteins, CRP, Ca, phosphates and cholesterol, as well as activities of ALT, AP and LDH were significantly higher in serum of dogs with CVD compared to healthy dogs. Cardiac biomarkers, cTnI and NT-proBNP, also had significantly higher concentrations in dogs with CVD compared to healthy dogs.

Table 1. Serum biochemical parameters measured in serum of dogs with CVD and healthy dogs.

AST - aspartate aminotransferase, ALT - alanine aminotransferase, GGT - gamma-glutamyl transferase, AP - alkaline phosphatase, CPK - creatine kinase, LDH - lactate dehydrogenase, CRP C-reactive protein, cTnI - cardiac troponin I, N-terminal pro b-type natriuretic peptide - NT-proBNP.

Parameter (unit)	**Healthy dogs (median(Q1-Q3)**	**Dogs with CVD (median(Q1-Q3)**	**P value**
Urea (mmol/L)	5.55 (4.98 - 7.40)	17.05 (12.68 - 34.78)	0.003
Creatinine (µmol/L)	82 (61.5 - 85.75)	113.5 (86.25 - 144)	0.008
Total protein (g/L)	59.5 (58.25 - 65.75)	74 (66 - 91)	0.007
Albumin (g/L)	32.5 (29.75 - 35)	34.50 (34 - 43.5)	0.112
Bilirubin (µmol/L)	3.05 (2.93 - 3.55)	3.3 (1.95 - 5.28)	0.650
Glucose (mmol/L)	5.65 (5.33 - 5.8)	6.65 (4.98 - 8.18)	0.372
AST (U/L)	23 (21.5 - 32.25)	41.50 (25.5 - 76.75)	0.091
ALT (U/L)	42.5 (22 - 54.75)	84 (54.5 - 129.5)	0.031
GGT (U/L)	3.5 (3 - 4.75)	2.5 (1.75 - 7.25)	0.694
AP (U/L)	29.5 (24.5 - 46)	198.5 (74.25 - 320)	< 0.001
CPK (U/L)	104 (81.75 - 134.3)	164.5 (99.5 - 391.5)	0.161
Alpha amylase (U/L)	599 (362.3 - 691)	614.5 (440.3 - 1079)	0.279
Lipase (U/L)	272.5 (146.5 - 534.8)	480 (389.3 - 631.8)	0.065
LDH (U/L)	59 (47 - 74.75)	217.5 (161.8 - 361.8)	0.001
CRP (mg/L)	0.6 (0.05 - 6.1)	23.2 (9.15 - 44.05)	0.006
Calcium (mmol/L)	2.55 (2.5 - 2.6)	2.84 (2.66 - 3.15)	0.007
Phosphates (mmol/L)	1.4 (1.2 - 1.48)	2.1 (1.72 - 2.45)	0.013
Triglycerides (mmol/L)	0.65 (0.53 - 0.78)	1.3 (1.03 - 1.65)	0.005
Cholesterol (mmol/L)	6.85 (5.38 - 8.65)	10.6 (9.75 - 12.8)	0.021
cTnI (µg/L)	0.01 (0.01 - 0.028)	1.32 (0.11 - 4.52)	< 0.001
NT-proBNP (pmol/L)	421 (265.5 - 651.3)	2919 (1574 - 6620)	0.009

315

316 **3.3 Proteomics**

317 In this study, 290 quantifiable proteins (171 of which were master proteins and 47 master
318 protein candidates, respectively) were identified by label-based quantitative proteomic approach
319 according to set criteria (2 unique peptides and 5% FDR) (Supplemental data 2). In total, there
320 were 15 proteins with significantly differential abundances between healthy and dogs with CVD,
321 and all of them were downregulated in diseased dogs (Table 2).

322 Table 2. Proteins with significantly differential abundances between healthy and dogs with CVD
323 identified and quantified using TMT approach.

Accession number[a]	**Protein**	**P-value**	**Fold change**
545528321	**apolipoprotein B-100**[b]	0.03	-0.72
359320010	**antithrombin-III**	0.04	-0.56
545518174	**gelsolin**	0.04	-0.56
57109938	**kininogen-1 isoform X2**	0.04	-0.55
345796419	**kininogen-1 isoform X1**	0.04	-0.55
73967363	**alpha-2-antiplasmin isoform X2**[b]	0.03	-0.61
545512145	**alpha-2-antiplasmin isoform X1**[b]	0.03	-0.61
928162811	**tetranectin**[b]	0.02	-0.73
928151046	**apolipoprotein M isoform X2**[b]	0.02	-0.65
928180090	**apolipoprotein D**[b]	0.04	-0.64
15825495	**adiponectin, partial**	0.04	-0.59
54792748	**adiponectin precursor**	0.04	-0.59
545553489	**adiponectin isoform X1**[b]	0.04	-0.59
218051927	**adiponectin**	0.04	-0.59

928186333	**inter-alpha-trypsin inhibitor heavy chain H1-like isoform X1**[b]	0.02	-0.62

[a]Accession number from NCBI protein database for *Canis lupus familiaris*.

[b]proteins predicted in *Canis lupus familiaris*, with no evidence of existence to date at protein, transcript or homology levels

3.4 Bioinformatics

Proteins with significantly differential abundances were used as a starting point for creating protein interacting networks using different softwares. An additional 77 associated proteins have been added to the initial proteins by the enrichment step. From the 87 genes (initial proteins + enriched), 46 (54.8%) were associated to a GO term. Nine of the 10 (90%) initial proteins have been associated with a GO term. Only ITIH1 was not associated with a GO term. In total, 32 GO terms have been identified, forming 13 groups.

Table 3. GO terms selected to be representative of canine CVD.

GOterm	Total genes	Associated proteins	-log10(p-value)
wound healing	17	4	6.94
extracellular structure organization	16	3	8.25
negative regulation of response to external stimulus	12	4	6.26
organic hydroxy compound transport	9	3	3.74
regulation of plasma lipoprotein particle levels	8	3	5.19
positive regulation of lipid metabolic process	8	2	4.30
lipid homeostasis	7	2	3.73
retinoid metabolic process	6	2	3.21
plasminogen activation	5	3	4.96
cholesterol homeostasis	5	2	2.58
regulation of smooth muscle cell proliferation	5	3	2.14
renal protein absorption	2	2	2.12

After refinement, 12 representative GO terms have been selected (Table 3, Figure 1). Adiponectin (identified initially by proteomics) was associated with multiple GO terms. GO terms extracellular structure organization and wound healing seems to be essential in this

disease. Lipid metabolism and transport are also important, as they are present with different related GO terms like lipid homeostasis, cholesterol homeostasis, regulation of plasma lipoprotein particle levels, retinoid metabolic process, and different apolipoproteins.

3.5 Validation of proteomic results

In order to verify differences in serum protein abundances observed by proteomic analysis, selected proteins were validated by ELISA assays and Western blotting. All ELISA assays evaluated in the present study showed adequate precision with intra- and inter-assay CVs lower than 15%, the limit of the objective analytic performance standard for precision [16]. For accuracy of the assays linearity under dilution was accomplished by ordinary linear regression analysis comparing the measured concentrations of analyte with the expected levels. Dilution of canine serum samples with different analyte concentrations resulted in linear regression equations with correlation coefficient close to 1.0 ($r > 0.98$ for all assays).

All tested markers, apolipoprotein B-100, apolipoprotein D and adiponectin, showed significant differences between dogs with CVD and healthy dogs (Figure 2). Adiponectin concentration was significantly lower in serum of dogs with CVD (median, interquartile range: 489.4 ng/mL, 311.5 – 859.1 ng/mL) compared to healthy dogs (1887 ng/mL, 769.9 – 2179 ng/mL; $P = 0.002$), as well as apoB-100 concentration in dogs with CVD (2.9 μg/mL, 2.3 – 5.3 μg/mL) compared to healthy dogs (5.6 μg/mL, 5.2 – 6.8 μg/mL; $P = 0.026$) and apoD concentration in dogs with CVD (608.1 pg/mL, 487 – 978.1 pg/mL) compared to healthy dogs (1108 pg/mL, 822.9 – 2257 pg/mL; $P = 0.050$). In total, all proteins validated with immunoassays confirmed consistence with proteomic results.

Two selected proteins, adiponectin and kininogen-1, were validated by Western blotting using total protein load after Ponceau S staining as a normalisation reference. The relative abundance of ADPN was significantly lower in serum of dogs with CVD compared to healthy dogs ($P = 0.005$), while for kininogen-1 there was no statistical significance (data not shown). We were not able to confirm kininogen-1 consistence with proteomic data, possibly due to the existence of different KIN1 isoforms in serum.

4. Discussion

Using TMT label-based relative quantification proteomics approach, we have found 15 differentially abundant serum proteins between dogs with CVD and healthy dogs. The identified proteins with differently abundances are involved in a number of pathways, such as complement and coagulation cascades, haemostasis, regulation of actin cytoskeleton, lipid metabolism and transport.

One previous study in Cavalier King Charles Spaniel using two-dimensional gel electrophoresis separation and analysis by MALDI-TOF-MS for protein identification identified eight proteins differentially expressed among healthy and dogs with CVD [7]. None of those proteins were identified as differentially abundant in this study, probably due to difference in proteomic approach. However, there are similarities with research in humans, with special emphasis to tetranectin and gelsolin as novel biomarkers of cardiovascular disease. Other identified proteins are also consistent to similar studies in human medicine, with exception of apolipoproteins which showed different trend.

One of the proteins we found differentially expressed in CVD dogs was adiponectin, a major adipocyte-secreted protein (adipokine), a key component that mediates the cross-talk between adipose tissue, cardiac cells and the vasculature. Several studies in humans and rodents showed that adipokines affect cardiovascular functions, as well as many other physiological processes including regulation of energy metabolism, immune function, and inflammation [17]. In a recent study adiponectin concentrations were found significantly lower in dogs with CVD [18], while in another study increased adiponectin concentration was found in dogs with dilated cardiomyopathy compared to healthy dogs and to dogs with CVD in dogs [19]. Identification of mRNAs encoding both adiponectin receptors in cardiac tissues of dogs confirms that circulating adiponectin directly affects cardiomyocytes in dogs with CVD [19].

High adiponectin concentrations in healthy humans are associated with low cardiovascular risk and adiponectin concentration is associated with slowing the progression of cardiovascular diseases such as cardiac hypertrophy, ischemic injury, and atherosclerosis in humans [20, 21].

In our study we found lower abundances of this protein in dogs with CVD compared to controls, confirmed with both, immunoassay and immunoblotting, validation method, suggesting

its role in modulating cardiovascular function by acting with an anti-inflammmatory effect, reducing oxidative stress and promoting endothelial repair during vascular dysfunction.

Another identified protein is part of the inter-alpha-trypsin inhibitors (ITI) family of plasma serine protease inhibitors, composed of a light chain – bikunin, and homologous heavy chains, contributing to extracellular matrix stability by covalent linkage to hyaluronan. So far, inter-alpha-trypsin inhibitor heavy chain H1 (ITIH) molecules have been shown to play a particularly important role in inflammation and carcinogenesis, and have been demonstrated to be both positive and negative acute phase proteins under various conditions [22]. In our study, lower protein abundances were found in CVD group. The most consistent histopathologic finding in CVD is accumulation of glycosaminoglycans, mainly hyaluronic acid, in the extracellular matrix of the mitral valve [23, 24]. Decrease of ITIH1 could be due to the impairment of extracellular matrix stabilization in cardiac tissue of diseased dogs. Additionally, reduced ITIH1 levels in the serum may contribute to reduced protease inhibitor activity and excess protease-mediated tissue injury in CVD group. This is also suggestive of an inflammatory component underlying this disease, as rapid consumption of protease inhibitors, such as ITIH1, are evidenced in pathophysiology of inflammation in order to prevent excess activation of proteases and limit the potential injurious actions of protease activation on endothelial and epithelial tissues [25].

Gelsolin, the actin-scavenging protein, had lower protein abundances in CVD group compared to controls. Actins are released into the systemic circulation after disruption of the cell membrane as a result of necrosis. The release of actin into the systemic circulation in response to injury or illness-associated necrosis results in adverse pathophysiologic consequences including increase of blood viscosity and disturbances in microvascular flow, activation of platelets with resulting platelet aggregation, and microvascular thrombosis, all contributing to tissue injury due to the high toxicity of actin [26]. In a majority of diseases, decline of gelsolin precedes, and therefore might predict, tissue and organ injury, and can be a predictor of critical care complications; and these alterations are primarily associated with actin scavenging and anti-inflammatory features of gelsolin. A compelling number of animal studies also demonstrate a broad spectrum of beneficial effects mediated by gelsolin, suggesting therapeutic utility for extracellular recombinant gelsolin [26]. Similar mechanism of this actin-scavenging protein could be proposed in canine CVD, due to lower protein abundances found in dogs with CVD.

Cardiovascular diseases are associated with alteration of haemostasis. We found lower protein abundances of antithrombin III (AT III), alpha-2-antiplasmin, tetranectin and kininogen-1 in dogs affected with CVD compared to healthy dogs.

In humans, low antithrombin is established risk factor for thrombosis and the risk of cardiac events was positively correlated to fibrinogen and negatively correlated to antithrombin III activity measurements [27]. In previous studies of canine CVD leading to congestive heart failure (CHF), AT III were decreased in dogs with CVD [28]. Natural anticoagulant mechanisms are amplified to prevent excessive thrombin generation. AT III, as part of the most important anticoagulant pathway, inhibit fibrinogen conversion into plasmin by creating thrombin-antithrombin complexes. Low AT III levels are caused by excessive thrombin generation and thereby increased consumption of the inhibitor leads to hypercoagulability in CVD.

Alpha-2-antiplasmin is a major inhibitor and regulator of fibrinolysis and one of the essential factors involved in haemostasis. It is a member of the serine proteinase inhibitor (serpin) family and inhibits proteases in general, including trypsin, chymotrypsin, plasma kallikrein, but its main physiological activity is very rapid inhibition of plasmin by forming a stable complex with this proteinase [29]. Lower protein abundances of alpha-2-antiplasmin found in CVD group may suggest fibrinolysis inhibitors consumption and increased fibrinolytic activity due to hypercoagulable state present in disease. Reported alterations in haematologic parameters would shift an overall haemostatic balance toward a more hypercoagulable state in the dogs with CVD.

Another regulator of fibrinolysis, tetranectin, was found to be decreased in our study in dogs with CVD compared to controls. Tetranectin is a C-type lectin and an adhesion molecule found on endothelial cells and platelets that specifically binds to the plasminogen kringle 4 domain, thereby enhancing plasminogen activation and inhibits the proliferation of endothelial cells [30]. A recent proteomics study discovered that the serum level of tetranectin was among the predictors of atherosclerotic cardiovascular disease after adjusting for established risk factors, with tetranectin levels inversely correlated with the risk of atherosclerotic cardiovascular disease [31]. Population studies have shown that decreased plasma tetranectin levels are also associated with coronary artery disease [32] and acute myocardial infarction [33]. These studies in humans are in agreement with the result obtained in this research, suggesting that changes of coagulation and fibrinolysis system play a vital role in pathophysiology of CVD. It is possible

that the downregulation of tetranectin is due to its fibrinolytic property in thrombus breakdown.

Kininogen-1, component of a coagulation system, is the precursor protein to high-molecular-weight kininogen (HMWK), low-molecular-weight kininogen (LMWK), and bradykinin. Kinins are generated from HMWK and LMWK by kininogenases such as plasma and tissue kallikrein. A local kallikein-kinin system exists in the heart, which enables it to synthesize and release kinins [34]. Kinins released locally may act as autocrine/paracrine hormones, regulating cardiac function. The contact activation system of the intrinsic pathway of coagulation consists of four plasma proteins: factor XII (FXII), factor XI (FXI), prekallikrein (PK) and HMWK. Previous study showed increased concentrations of FXI, HMWK and PK in patients with a history of myocardial infarction as compared to controls, suggesting that high PK plasma levels may favor contact activation resulting in increased generation of activated FXII and FXI, leading to enhanced activation of the intrinsic pathway of coagulation (FIX) and subsequent thrombin formation [35]. These data indicate that possibly due to increased cleavage of kininogen-1 in activated coagulation system during progression of CVD, we found decreased levels of this precursor.

Lipoproteins in blood play an important physiological role transporting cholesterol, lipids and lipid-soluble substances to the different organs of the body. Diseases such as atherosclerosis, cardiovascular disease and stroke are associated with defects in lipoprotein metabolism. Apolipoproteins are the best lipid-related predictors to cardiovascular diseases [36]. Measurements of apolipoproteins are internationally standardized, automated, cost-effective and more convenient and precise than those for LDL cholesterol. Apolipoproteins, especially apoB, could also replace the standard ‘lipid profile’ as a target for therapy in at-risk patients [36].

Apolipoprotein B-100 (apoB) is the chief protein component constituent of the atherogenic very-low-density lipoprotein (VLDL), of intermediate-density lipoprotein (IDL) and of LDL particles, each particle including one apoB molecule [37]. ApoM is predominantly found in the HDL fraction and to a smaller extent in LDL, VLDL and chylomicrons. ApoM levels were shown to be dramatically reduced in patients with sepsis and systemic inflammatory response syndromes (SIRS) acting as a negative acute phase protein [38]. Apolipoprotein D (apoD) is a component of HDL.

In our study, protein abundances of all 3 apolipoproteins found (apoB-100, apoM and apoD) were lower in CVD group compared to healthy dogs, which was further validated by independent immunoassay for apoB-100 and apoD. These data were not consistent with findings in human medicine. Possible explanation for this is significant variations of plasma lipoprotein profiles among different animal species. In dogs and cats, HDL is the predominant lipoprotein and major cholesterol-carrying particle, with additionally quite different distribution than in humans. Dogs have five to six times as much HDL as LDL, whereas in humans LDL is 2 to 3 times as much as HDL [39, 40]. In case of apoM, which has been suggested as novel negative acute phase protein, its decrease could be contributed to possible underlying inflammatory process in CVD. In affected canine valves several inflammatory cytokine genes were up-regulated suggesting valve endothelium as a source of inflammatory mediators [24].

To review the current status of proteomic biomarkers associated with cardiovascular diseases in humans, comprehensive meta-analysis was recently conducted, summarizing original research articles using proteomics technologies [41]. Identified proteins associated with cardiovascular disease represented pathways in inflammation, wound healing and coagulation, proteolysis and extracellular matrix organization, handling of cholesterol and LDL. Our proteomic and bioinformatics analysis resulted in interactome with enriched GO terms showing high consistency with results of this meta-analysis. Also, a prospective study in cardiovascular disease initiative using discovery and targeted proteomic studies, identified single protein biomarkers and panel of proteins that were associated with risk of myocardial infarction or atherosclerotic cardiovascular diseases ASCVD [31]. Two proteins from our list, tetranectin and gelsolin, were also highlighted as novel biomarkers of new-onset cardiovascular disease. Furthermore, another large study using a proteomic platform identified tetranectin as protein biomarker that predicts cardiovascular outcomes and all-cause mortality [42].

In addition to the proteomic analysis, two potential biomarkers of cardiac function in dogs (cTnI and NT-proBNP) were tested herein. Both cTnI and NT-proBNP were found to be prognostic markers for dogs with CVD at the highest risk of death [43, 44]. Cardiac troponin I is a protein specifically produced in the cardiomyocytes which is being rapidly released from the injured heart cells into the bloodstream. Since it is heart-specific, has low basal plasma concentration and persists in the circulation during the injury, it is commonly used as a

biomarker of myocardial injury in humans [45]. Serum cTnI levels were found to be significantly increased in dogs with CVD in our study, which was already demonstrated before [46, 47]. Limitations of cTnI as a biomarker for CVD include its lack of specificity for the cardiac disease, elevation if kidney damage is present and possibly normal levels when the disease is mild [48]. As well as cTnI, NT-proBNP is gaining interest as a biomarker of canine heart diseases over the past few years. B-type natriuretic peptide (BNP) is a hormone secreted from cardiomyocytes due to myocardial overload and strain of the cells, in order to promote natriuresis, diuresis and vasodilatation. Once secreted, BNP is cleaved by serum proteases and gives rise to NT-proBNP, a stable biomarker of myocardium overload [49]. In several studies of dogs with CVD, there were increased levels of NT-proBNP compared to controls [50-52], which was also confirmed in our study. Nevertheless, new studies show that biologic variability should be taken into account when interpreting changes in both cTnI and NT-proBNP values in dogs with CVD [52, 53].

Proteins validated with immunoassays and Western blotting showed results consistent to proteomics. Differences in values of fold changes between the immuno-based measurements and the fold changes found by proteomics could be related to the sensitivity of TMT-based LC-MS technology. Numerous studies have been conducted in order to compare the performance of immunoassays and LC-MS methodology, favoring the LC-MS in terms of accuracy and less false results. However, the results of those methodologies are reported as positively correlated [11]. As there are commercially available ELISA kits for some of the proteins listed as potential biomarkers for CVD, further evaluation of their prognostic value would be of interest. As limitations of the study, we can address limited number of cases and differences regarding sex, breed and age between healthy dogs and dogs with CVD. Low number of animals could be justified by demand for complete diagnostic investigation, while inadequate crossmatching is also a result of disease occurring in dogs at an advanced age.

5. Conclusions

Label-based high-resolution quantitative proteomics analysis and bioinformatics approach used herein represent a valid tool for elucidating complex pathophysiology of canine CVD and unveiling disease relevant proteins with biomarker potential. Early diagnosis and therapeutic intervention may prevent severe complications in dogs with CVD. Therefore, it is crucial to identify biomarkers that can be used in clinical practice. We found comparative similarities with

human disease in terms of identified proteins and GO pathways, which confirmed similar pathophysiology of this disease, but also differences, mainly in lipid metabolism. Proteins associated with extracellular structure organization, haemostasis pathway and lipoprotein metabolism were identified as potential markers in canine CVD.

Acknowledgements

This work was supported by the Croatian Science Foundation "BioDog" project (Grant Agreement 4135) and European Structural and Investment Funds (Grant Agreement KK.01.1.16.0004).

Competing interests

The authors declare that they have no competing interests.

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Figure legends

Figure 1. *In silico* inferred interactome network of identified GO terms over-represented in canine CVD (healthy versus dogs with CVD). Differentially expressed proteins interacting with at least 1 term were added. Radial layout was applied and the GO group leader terms are in black text.

Figure 2. Concentrations of adiponectin (ADPN), apolipoprotein B-100 and apolipoprotein D in serum of dogs with CVD and healthy dogs in a box and whisker plot (median is marked with a vertical line inside the box, the box spans the interquartile range, whiskers min-max).

Figure 3. Western blotting and relative density comparison of serum adiponectin from healthy dogs and dogs with CVD (figures of individual membranes were cropped to show the band of interest; data are shown as median with range, $P = 0.005$).

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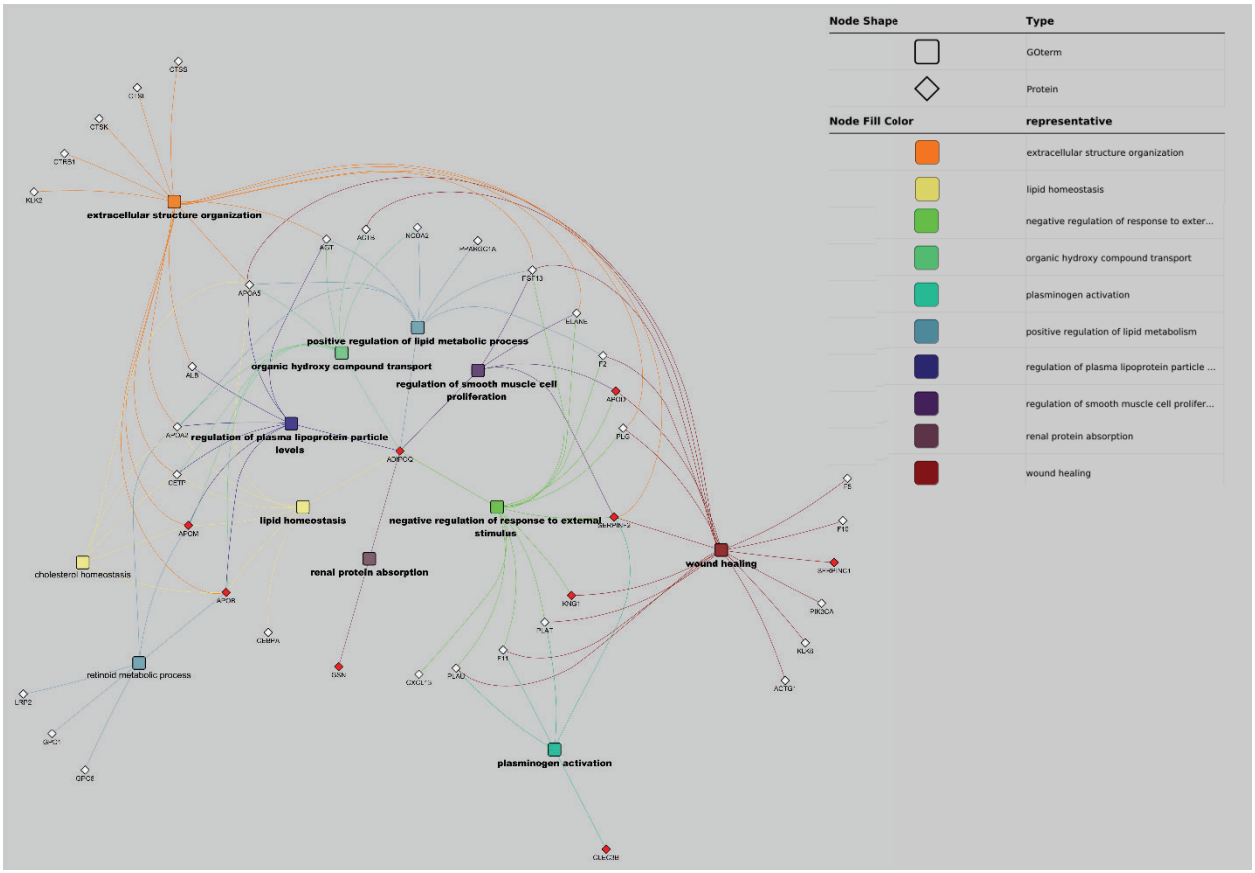
748 Figure 1



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752 Figure 2

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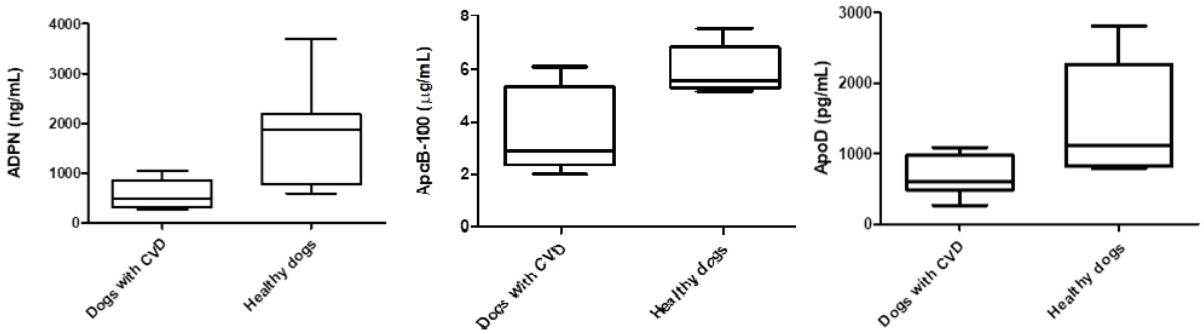

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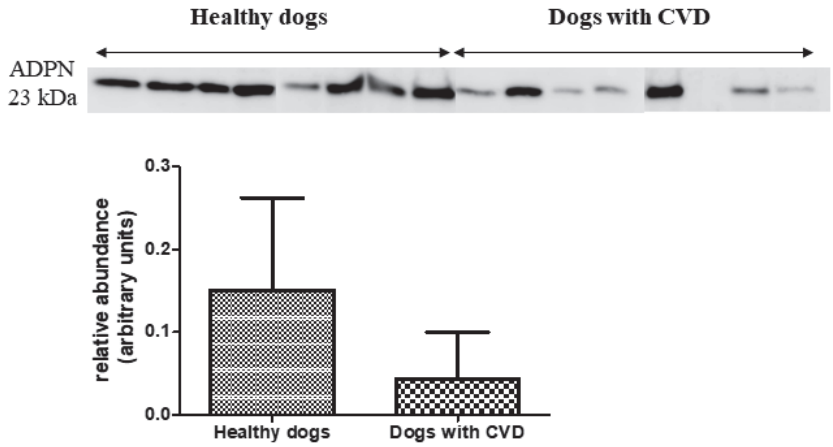
Figure 3


Healthy dogs
Dogs with CVD
ADPN
23 kDa
relative abundance
(arbitrary units)
0.3
0.2
0.1
0.0
Healthy dogs
Dogs with CVD

Graphical abstract