Serum proteome profiling in canine idiopathic dilated cardiomyopathy using TMT-based quantitative proteomics approach

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ABSTRACT

Idiopathic dilated cardiomyopathy (iDCM) is a primary myocardial disorder with an unknown aetiology, characterised by reduced contractility and ventricular dilation of the left or both ventricles. Naturally occurring canine iDCM was used herein to identify serum proteomic signature of the disease compared to the healthy state, providing an insight into underlying mechanisms and revealing proteins with biomarker potential. To achieve this, we used high-throughput label-based quantitative LC-MS/MS proteomics approach and bioinformatics analysis of the *in silico* inferred interactome protein network created from the initial list of differential proteins. To complement the proteomic analysis, serum biochemical parameters and levels of know biomarkers of cardiac function were measured. Several proteins with biomarker potential were identified, such as inter-alpha-trypsin inhibitor heavy chain H4, microfibril-associated glycoprotein 4 and apolipoprotein A-IV, which were validated using an independent method (Western blotting) and showed high specificity and sensitivity according to the receiver operating characteristic curve analysis. Bioinformatics analysis revealed involvement of different pathways in iDCM, such as complement cascade activation, lipoprotein particles dynamics, elastic fibre formation, GPCR signalling and respiratory electron transport chain.

KEYWORDS - idiopathic dilated cardiomyopathy, dog, serum, label-based proteomics, biomarker, bioinformatics

SIGNIFICANCE

Idiopathic dilated cardiomyopathy is a severe primary myocardial disease of unknown cause, affecting both humans and dogs. This study is a contribution to the canine heart disease research by means of proteomic and bioinformatic state of the art analyses, following similar approach in human iDCM research. Importantly, we used serum as non-invasive and easily accessible biological source of information and contributed to the scarce data on biofluid proteome research on this topic. Bioinformatics analysis revealed biological pathways modulated in canine iDCM with potential of further targeted research. Also, several proteins with biomarker potential have been identified and successfully validated.

1. INTRODUCTION

Dilated cardiomyopathy (DCM) is a myocardial disorder affecting 1 in 2500 human individuals and represents the most frequent cause of heart transplantation [1]. It is characterized by dilation and impaired contraction of the left or both ventricles and carries a poor prognosis with progression to congestive heart failure or fatal arrhythmias [2]. Pathologic mechanisms underlying DCM are not understood, especially regarding the idiopathic form (iDCM), for which the primary cause is unknown. If there is some evidence/suspicion of the aetiology, DCM is not considered idiopathic, but can be caused by different contributing factors, such as genetics, nutritional deficiencies, metabolic disorders, immune system abnormalities, infectious diseases or intoxication [1,3]. Taking into account DCM is the second most common heart disease of dogs, understanding its mechanisms can lead to the advances in companion animal veterinary medicine. Also, due to the similarities between the dog and human cardiovascular system on anatomical, physiological and molecular level [4], naturally occurring canine iDCM is a suitable model for studying the human counterpart.

Diagnosis of iDCM is based on echocardiography [5]. Radiography and clinical examination findings, ECG examination, genetic tests and certain biomarkers circulating in blood, such as cardiac troponin I (cTnI) and N-terminal pro-B-type natriuretic peptide (NT-proBNP), have an added value in both human and canine iDCM diagnostics. Although serum cTnI and NT-proBNP concentrations are increased in humans and dogs with iDCM, they are not exclusive biomarkers of this disease and therefore have limited clinical utility [6].

With the development of new high-throughput proteomic technologies, there is a growing interest in study of new protein biomarkers which could help to diagnose and treat iDCM, but also unravel underlying pathologic mechanisms. Plasma or serum are especially promising sources of new easily accessible protein biomarkers since the blood proteome reflects systemic changes that happen upon organ dysfunction. Proteomic research on both blood and heart tissue can reveal different aspects of the same condition, encompassing systemic and local changes taking place during the course of the disease. While there are some recent studies on myocardial tissue transcriptome and proteome in human iDCM [7,8], large-scale proteomic analyses of blood which could identify proteins with biomarker potential in both human and canine iDCM are lacking. To the authors´ knowledge, there is

only one proteomic study of serum of dogs with DCM, where label-free quantitative LC-MS/MS approach was used [9].

Therefore, we have performed a study of serum proteome changes in iDCM compared to the healthy state, using naturally occurring canine iDCM as a model, by means of an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. This was followed by validation of several biomarker candidates using Western blot. The TMT label-based approach enables multiplex identification and relative quantification of proteins between samples in an experimental set by LC-MS/MS. Functional bioinformatics analysis was also conducted on the acquired dataset in the interest of deeper understanding of mechanisms involved in iDCM. To complement these analyses, serum biochemical parameters and serum cTnI and NT-proBNP levels were measured.

2. MATERIALS AND METHODS

2.1. Animals and heart function examination

Two groups of dogs were enrolled in the study in the period between March 2015 and March 2016: 8 clinically healthy dogs (used as controls) and 8 dogs diagnosed with iDCM. 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 to the Small Animals Clinic, Department of Clinical Veterinary Science, Vetsuisse Faculty, University of Bern, Switzerland. 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-minute 6 lead ECG (ASPEL, AsCard Mr. Silver) and transthoracic echocardiography using Esaote MyLab40 Vet machine and a 5 MHz sector transducer. Dogs diagnosed with iDCM were enrolled based on the concomitant presence of the two major criteria for DCM [5]: a) enlarged left ventricular M-mode systolic (LVDs) and diastolic (LVDd) dimensions defined according to weight-adjusted values [10] and b) left ventricular M-mode fractional shortening of <20%. Exclusion criteria were evidence of any other disease than iDCM based on history, clinical examination, laboratory results or imaging. The cardiac evaluation of dogs with iDCM included physical examination, thoracic radiographs evaluated

by a board-certified radiologist, a 1-minute 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 [11]. Diseased dogs were classified according to the International Small Animal Cardiac Health Council (ISACHC) classification system [12]. All procedures were conducted in accordance with EU Directive 2010/63/EU for animal experiments, as well as subject to informed owner consent.

2.2. TMT study

Scheme of the TMT study design is shown in Figure 1. Serum samples collected from 8 dogs with iDCM and 8 healthy dogs (controls) were all processed and analysed at the same time in order to reduce inconsistencies. Highly abundant albumin was depleted from all 16 samples followed by protein concentration determination. An internal standard (IS) was made as a pool of equal protein amount from all 16 albumin-depleted samples as a reference for normalization. Equal amount of each protein sample and IS was reduced, alkylated, digested with trypsin and labelled with TMT reagents. Samples were combined at equal amounts into 4 sets (each consisting of 1 IS, 2 healthy and 2 diseased canine samples) and each set was analysed by LC-MS/MS. Acquired data was processed statistically to find differentially abundant proteins, some of which were validated as potential biomarkers of iDCM using immunoblotting. Also, bioinformatics analysis was performed in order to find pathways modulated in canine iDCM.

2.3. Serum samples and albumin depletion

Serum was obtained from all 16 dogs by centrifugation of completely clotted blood at 3500 g for 10 minutes at room temperature. Samples were aliquoted and stored at -80 °C until analysed. All samples were collected during a one-year period and thawed just once upon completion of collection, immediately before any analysis. Serum of dogs with iDCM was collected at the time of initial diagnosis and before any treatment. Before proteomic analysis, serum samples were depleted by removal of highly abundant albumin using a salt-ethanol precipitation protocol, described by Colantonio et al. [13]. Briefly, 100 µL of each serum sample was processed by adding 10 µL of 1 M sodium chloride solution (VWR, Pennsylvania, USA) to yield a final concentration of 0.1 M and incubated with rotation for 1 h

at 4 °C. Cold ethanol absolute (≥99.8%, VWR, Pennsylvania, USA) was then added to yield a final concentration of 42% and again incubated for 1 h at 4 °C. Samples were centrifuged at 16000 g for 45 min at 4 °C. First pellets were retained and supernatants were further processed. The pH of the supernatants was lowered to 5.7 by adding cold 0.6 M sodium acetate buffer (Sigma-Aldrich) of pH 5.6, and incubated for 1 h at 4 °C. Supernatants were then centrifuged as described above to yield the second pellet. Supernatants (containing albumin) were removed and the first and second pellets were combined to yield albumin-depleted samples. Pellets were resuspended in 100 mM triethylammonium bicarbonate buffer (TEAB) (Sigma-Aldrich) containing 1% SDS (Sigma-Aldrich), compatible with further proteomic analysis. Treated samples were resolved by 1-D SDS PAGE in order to test the efficiency of albumin depletion procedure for each sample. First, protein concentration was measured in duplicate using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) per manufacturer's guidelines. Samples were then mixed with Laemmli Sample Buffer containing 355 mM 2-mercaptoethanol (Bio-rad) and heated on a heating block for 5 min at 95 °C. The first well of gel was loaded with 8 µL of PageRuler Plus Prestained Protein Ladder (Thermo Fisher Scientific) and others with 10 µg of protein per sample. Proteins were separated by 1-D electrophoresis using Criterion TGX precast gels 4–15% (Bio-rad). Gels were run at 300 V for 20 min in Tris-Glycine-SDS running buffer (Bio-rad). In order to visualise protein bands, gels were stained with Coomassie Brilliant Blue R-250 solution (Bio-rad), destained in the destaining solution over night and scanned using UMAX PowerLook III scanner.

2.4. TMT labelling

Amine-reactive Tandem Mass Tags (TMT) isobaric reagents (Thermo Scientific) were used to label samples in order to multiplex quantification of serum proteins by mass spectrometry (MS). Samples were grouped into 4 experimental sets in a way that each set contained albumin-depleted serum sample of 2 healthy dogs, 2 diseased dogs and an internal standard sample. Internal standard (IS) was used to allow for normalization of data and comparison of biological replicates between 4 individual pentaplex TMT experiments. One hundred micrograms of each protein sample and IS samples was prepared and labelled at peptide level with TMT reagents according to manufacturer's instructions. In brief, samples were reduced with 200 mM DTT (Sigma-Aldrich), alkylated with 375 mM iodoacetamide (Sigma-Aldrich) and precipitated with ice-cold acetone (VWR, Pennsylvania, USA) for 4 hours.

Samples were then centrifuged at 8000 g for 10 minutes and acetone was decanted. Pellets were resuspended with 100 µL of 100 mM TEAB buffer and digested with trypsin (Promega) overnight at 37 °C (2.5 µg of trypsin per 100 µg of protein). Thereafter, IS peptide samples were labelled with TMT-126 reagent, while other 16 peptide samples were labelled randomly with TMT-127, TMT-128, TMT-129 and TMT-130 reagents to avoid possible labelling preference. TMT label reagents were equilibrated to room temperature, dissolved in anhydrous acetonitrile, LC-MS grade (Thermo Scientific) for 5 minutes with occasional vortexing and added to each sample (41 µL of the reagent to 100 µg sample). Labelling reaction was incubated for 1 hour at room temperature and then quenched by adding 5% hydroxylamine (Thermo Scientific) for 15 minutes. Samples were then combined at equal amounts into 4 pentaplex sets (as described above) and 6 µg of each mixed sample set was placed in a well of a microplate. Samples were vacuum-dried for 15 minutes and stored at -20 °C before further LC-MS/MS analysis.

2.5. LC-MS/MS analysis

The LC–MS/MS analysis was performed on Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberley, UK) and Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). TMT-labelled peptide mixtures were reconstituted in buffer A (2% acetonitrile in 0.1% formic acid). An amount of 3 µg was loaded on the trapping column C18 PepMap100 (5 µm, 100 A, 300 µm x 5 mm) and then separated using C18 RSLC PepMap ID column (15 cm x 75 µm) with linear gradient 5-35% buffer B (0.1% formic acid in 80% acetonitrile) over 135 min at a flow rate of 300 nL/min. Eluate from the column was introduced to the Orbitrap Elite MS. The ionisation voltage was set to 1.7 kV and the ion transfer tube temperature to 220 °C. MS was operating in positive ion mode using collision-induced dissociation/higher energy collisional dissociation CID/HCD fragmentation methods for MS2. Full scan Fourier transform-based mass spectrometry (FTMS) spectra were acquired in range from m/z 380.0 to 1800.0 with resolution of 60000. The maximum injection time for FTMS full scan was set as 200 ms reaching an automatic gain control (AGC) target value of $1x10^6$. Three most intense peaks from MS spectrum were selected for each fragmentation mode. Ions with the charge state 1^+ were excluded from the fragmentation list. The HCD MS/MS scan was fixed to start from m/z 100.00 with resolution of 15000 using MS2 AGC target of $5x10^4$. The collision energy was set as 40% normalized collision energy (NCE). Isolation window of ± 1.5

Da was applied to isolate precursor ions with dynamic exclusion of 20 s. Every precursor ion was repeated twice within duration time of 30 s and was excluded for 20 s. Ion trap mass spectrometry CID MS/MS scan spectra were acquired with 35% NCE and an AGC target of $1x10^4$.

2.6. MS/MS data analysis

Acquired MS/MS spectra were analyzed for protein identification and quantification using Proteome Discoverer software 2.1 (Thermo Fisher Scientific). Protein identification was performed using the Mascot algorithm against the *Canis lupus familiaris* protein database from NCBInr (version 04/05/2016; 41195 sequences), with a precursor mass tolerance of 10 ppm and fragment ion mass tolerance of 0.8 Da. Set modifications were addition of TMT 6-plex labels to lysines and N-termini, carbamidomethylation of cysteine as fixed modification and methionine oxidation as variable modification. Two missed cleavages for the trypsin digestion were permitted. Identified peptides were filtered with a cut-off criterion of a q-value of 0.01, corresponding to a 1% false-discovery rate (FDR) for highly confident peptide hits and a q-value of 0.05 (5% FDR) for peptide hits with moderate confidence. Quantification was performed using abundances of reporter ions based on signal to noise ratio values or intensity. Normalization was carried out based on total peptide amount and scaling on channels average. Abundances of reporter ions from only unique and razor peptides were used to estimate the abundances of proteins. Identification and quantification data were exported from Proteome Discoverer to Microsoft Excel software. Abundances ratios were obtained for each protein by comparing with values of corresponding internal standard and then used to calculate average fold change ratio between healthy and iDCM groups. Internal standard-normalized protein abundances ratios were used in statistical analysis to detect significant differences between the studied groups.

2.7. Validation of proteomics results

Validation of proteomics results was performed by Western blotting using the non-depleted serum samples of the same patients as in proteomic analysis. In brief, 25 µg of proteins of each serum sample (8 healthy controls and 8 iDCM) was boiled at 95 °C for 5 minutes in SDS-loading buffer and separated over 4-10% polyacrylamide gel. Proteins were then transferred to nitrocellulose membranes (Amersham Protran, GE Healthcare) for 2 h at 50 mA in 20%

methanol (Sigma) transfer buffer at 4 °C using Biostep electro blotting module. After the transfer, gels were stained with Coomassie Brilliant Blue G-250 (Amresco) in order to verify equal transfer and to use protein load as a reference for protein quantity normalization. The membranes were blocked for 1 h with 5% skim milk (VWR, Pennsylvania, USA) in Tris buffered saline (pH=7.6) containing 0.05% Tween20 (Sigma) at room temperature. The membranes were then incubated overnight at 4 °C with primary antibodies to ITIH4 (dilution 1:5000, kindly provided by F. Lampreave group, University of Zaragoza), ITIH3 (1:200, Santa Cruz Biotech, sc-21979), MFAP4 (1:500, Aviva Systems Biology, ABIN2776850), TfR1 (1:500, Covalab, pab75255), APOA4 (1:250, Biorbyt, orb5708) and AGT (1:500, Aviva Systems Biology, ABIN2781494). Specific polyclonal rabbit antiserum against the purified canine ITIH4 was raised by F. Lampreave group [14]. The ITIH3, MFAP4, TfR1, APOA4 and AGT antisera were not raised towards corresponding canine proteins, but had predicted cross-reactivity based on the immunogen amino acid sequence homology (as stated by the manufacturers). After washing, membranes were incubated for 1h at room temperature with appropriate secondary antibody (dilution 1:5000, donkey anti-goat IgG, sc-2020 and goat anti-rabbit sc-2004, Santa Cruz Biotech) conjugated with horseradish peroxidase. Immunostained proteins were detected by incubation with Western blotting luminol reagent (Santa Cruz Biotech) for 8 min and recorded in chemiluminesence mode using Li-Cor Odyssey Fc (Li-Cor, Inc). Acquired figures were analysed using ImageJ software (US National Institutes of Health, Bethesda, Maryland, USA) and differences between healthy and diseased dogs determined using unpaired t test, with p value < 0.05 considered statistically significant.

2.8. Serum biochemical parameters

One serum aliquot was used for measurement of biochemical parameters using commercial reagents (Beckman Coulter) per manufacturer´s instructions in an automatic analyser (Olympus AU640, Japan). The following parameters were measured: serum urea, creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transferase, alkaline phosphatase, creatine kinase, lactate dehydrogenase, C-reactive protein, alpha-amylase, lipase, cholesterol, triglycerides, calcium, magnesium, phosphates.

2.9. Measurement of cTnI and NT-proBNP blood concentration

Cardiac troponin I (cTnI) was measured in serum samples of control (N=8) and iDCM (N=8) group using commercially available ADVIA Centaur TnI-Ultra assay, which is a high-sensitivity immunoassay validated for use in both humans and dogs [15]. 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 NT-proBNP concentration in all 16 samples was performed in Vet Med Labor GmbH, reference IDEXX Laboratory (Germany) using IDEXX Cardiopet® proBNP test.

2.10. Statistics

Statistical analyses were performed using the R software version 3.3.1 [16]. Statistical differences in the age, sex, protein abundances ratios acquired in proteomic analysis, serum biochemical parameters and cTnI and NT-proBNP levels between healthy and iDCM groups were determined using nonparametric Mann-Whitney test. In order to compare healthy group, iDCM ISACHC class II and iDCM ISACHC class IIIA, the nonparametric Kruskal-Wallis and Dunn post hoc test were used. For all statistical comparisons, $p < 0.05$ was considered statistically significant. In order to test sensitivity and specificity of potential protein biomarkers according to proteomic analysis, receiver operating characteristic (ROC) curve analysis was performed and area under the curve (AUC) computed using MedCalc for Windows, version 18.0 (MedCalc Software, Ostend, Belgium). The required sample size for the comparison of the area under a ROC curve with a null hypothesis value was calculated using following parameters: null hypothesis value = 0.5, type I error (alpha, significance) = 0.05, type II error (beta, 1-power) = 0.2.

2.11. Bioinformatics analysis

Proteins with significantly differential abundances observed by proteomic and serum biochemical analyses (in total N=15) were further functionally analyzed using bioinformatics tools. As human database is more complete than canine one (reviewed proteins in UniprotKB/Swiss-prot release 2018_01: humans 20259, dogs 817), dog's proteins were converted to human proteins by performing BLAST (protein-protein BLAST) analysis of 15

identified canine proteins using human protein database (UniprotKB/Swiss-prot release 2017_10). The best matching protein (ID score, query coverage and E-value) was considered as ortholog for each canine protein in the initial list. Then, an enriched network of proteins was built based on the list of the initial 15 proteins, adding a maximum of 40 best interactors (using the following combination of *Homo sapiens* database: IntAct, Reactome, and String-database) with the Cytoscape (v3.5.1) application Cluepedia (v1.3.5). All types of interactions were considered. Clusters of interacting proteins were calculated by the Cytoscape application MCODE (v1.4.2), with node cutoff at 0.3 and K-core at 4. Thereafter, pathway analysis of each cluster was performed using the Cytoscape (v3.5.1) plugin ClueGO (v2.3.5). Ontology used was Reactome-Pathways (21/10/2017). P-value of each term was corrected with Bonferroni step-down. Pathway terms groups were defined according to the kappa score (threshold at 0.6) and sharing group percentage at 50%. Pathways leading term of each group was determined by its p-value. After determining Pathway terms for each cluster, terms were merged if they were identical or one term had a higher hierarchical position than another term. For the latter, the upper hierarchical Pathway term was considered as the leader term. Hierarchical orders were accessed using the Reactome.org database. Complete networks of each identified Pathway terms (available on Reactome.org with all proteins/genes and chemicals) were merged to constitute the *in silico* inferred interactome network of molecular processes from the initial 15 proteins. Using this *in silico* inferred interactome network, pathways and cellular compartment analyses were performed. Pathways analyses used the Reactome-Pathways (21/10/2017) with following parameters: evidence codes used “All_Experimental”, Kappa score threshold 0.4, number of genes = 30, minimum percentage = 70, p-value correction used = Bonferroni step down. Cellular compartment analyses used the GO-CellularComponent-EBI (27/10/2017), with following parameters: evidence codes used “All_withoutIEA”, Kappa score threshold 0.5, number of genes = 20, minimum percentage = 15, minimum GO level = 3, Maximum GO level = 10, p-value correction method used = Bonferroni step down.

3. RESULTS

3.1. Animals and heart function

The control group consisted of 8 healthy dogs with normal cardiac function, aged from 5 to 12.5 years of following breeds: 2 Belgian shepherds, 1 Border collie, 1 Beauceron, 1 Flat-coated retriever, 1 Labrador retriever and two mixed-breed dogs (25 and 38 kg). Three dogs in the control group were male, 1 was male castrated and 4 were female spayed. During the 13 months of the study, 8 adult dogs were diagnosed with iDCM at the co-authors´ (AK) institution. In the diseased group there were dogs of either sex, aged between 3 and 7 years. These were 1 mixed breed dog (40 kg) and 7 dogs from 5 different breeds (2 German shepherds, 1 Doberman, 1 Cane Corso Italiano, 1 Leonberger, 1 Bouvier des Flandres and 1 Great Dane). Two dogs were female, 2 female spayed, 1 male und 3 male castrated. There was no statistical difference in the age between the control (median; interquartile range: 8.5 years; 5.5-10.75) and iDCM (7 years; 7-7.75) group. If the neutering is disregarded, there was also no difference in the sex between the groups (each group consisted of 4 males and 4 females). At initial physical examination all dogs of iDCM 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. Four dogs were classified as ISACHC class II and four dogs as ISACHC class IIIA. In accordance with the ISACHC classification system, dogs in class II group presented with a mild to moderate heart failure, while dogs in class IIIA presented with an advanced heart failure. A grade II-VI left-sided systolic heart murmur was identified in all diseased dogs. Six dogs were in atrial fibrillation with a ventricular heart rate 150-240/min. Two of those dogs had rare ventricular premature complexes (VPC) and one dog a left bundle branch block (LBBB). Two dogs showed a sinus rhythm 150/min respec. 160/min. According to the inclusion criteria, echocardiographic examination showed abnormal left ventricular end diastolic (Figure 2a) and end systolic diameter (Figure 2b) and reduced fraction shortening (8-16%). The left atrial/aorta ratio (LA:Ao) was enlarged (1.8-3) in all dogs and all showed some functional mitral regurgitation, while 5 dogs an additional functional tricuspidal regurgitation.

3.2. Albumin depletion

The most abundant serum protein albumin, which could mask identification and quantification of low abundant proteins in downstream proteomic analysis, was removed with good reproducibility and efficiency from all 16 canine serum samples using salt-ethanol precipitation protocol, verified by 1-D SDS PAGE. Figure 3 shows that the intensity of the dominant band corresponding to albumin (~68 kDa) in non-depleted canine serum decreased remarkably in both healthy and diseased serum samples after the depletion procedure.

3.3. Protein identification and quantification using TMT approach

In this study, 4 pentaplex experimental sets containing 16 different samples (plus IS samples) were analysed using TMT label-based quantification approach. Since an IS was used in each set, it was possible to use it as a normalization reference and compare protein quantities detected in more than one set across different TMT runs. In total, 358 proteins were identified with high and medium confidence by combining all data from 4 sets. These proteins were grouped by the Proteome Discoverer software into 134 groups, with the top-ranking protein of the group listed as the master protein. Of 134 master proteins, 129 were identified with high (1% FDR) and 5 with medium (5% FDR) confidence, while quantification values were obtained for 131 proteins. Only highly confident master proteins (1% FDR), identified and quantified by 2 or more unique peptides and detected in at least 4 biological replicates were selected for the quantification analysis, which included 76 proteins (list provided as supplemental Table 1). For these proteins the average abundance fold change (iDCM/healthy ratio) ranged from 0.5 to 2. There was one protein (microfibril-associated glycoprotein 4, MFAP4) where exception was made in terms of criterion for the number of unique peptides. Although MFAP4 was identified and quantified by only one unique peptide, there was a remarkable fold change (1.6) so it was included in the statistical analysis, and its differential abundance between the groups was later tested by Western blotting. When nonparametric Mann-Whitney test was applied, 12 proteins showed significantly different levels ($p < 0.05$) in iDCM versus control serum with the fold changes ≥1.2 or ≤0.8 (Table 1). Of those 12 proteins, microfibril-associated glycoprotein 4 (MFAP4), inter-alpha-trypsin inhibitor heavy chain H4 (ITIH4) and apolipoprotein A-IV (APOA4) were also significantly

different ($p < 0.05$) between the controls, iDCM ISACHC II and iDCM ISACHC IIIA groups (Figure 4).

Table 1. Proteins with significantly differential abundances between healthy dogs and dogs with iDCM identified in serum using TMT approach.

GI accession number*	Description	N of unique peptides	Mascot score	Fold change (iDCM/healthy)	p-value (iDCM *versus* healthy)	N of detections**
			Upregulated			
928186325	inter-alpha-trypsin inhibitor heavy chain H4	21	3170	2	0.0003	16
73956164	microfibril-associated glycoprotein 4	1	369	1.6	0.0043	12
928186331	inter-alpha-trypsin inhibitor heavy chain H3	3	126	1.6	0.0286	8
545494757	angiotensinogen	5	866	1.2	0.0207	16
928150787	complement C4-A	52	16701	1.2	0.0379	16
545496317	complement component C9	2	245	1.2	0.0379	16
928159887	plasma protease C1 inhibitor	4	2194	1.2	0.0499	16
			Downregulated			
345799905	apolipoprotein A-IV	19	3621	0.6	0.0019	16
17066524	immunoglobulin gamma heavy chain A	5	7053	0.6	0.0499	16
924859480	apolipoprotein C-III precursor	4	1521	0.7	0.0379	16
50978812	transferrin receptor protein 1	2	141	0.8	0.0379	16
928133662	apolipoprotein A-I	26	45231	0.8	0.0499	16

*accession number from NCBI protein database for *Canis lupus familiaris*
**number of samples in which the protein was detected

3.4. Validation of proteomics results

In order to verify differences in serum protein abundances observed by proteomic analysis, 5 selected proteins were validated by Western blotting using total protein load as normalization reference. Consistent with TMT based proteomics results, the relative abundances of ITIH4, ITIH3 and MFAP4 were significantly increased in iDCM group compared to controls, while those of TfR1 and APOA4 were decreased (Figure 5, $p<0.05$). Angiotensinogen (AGT) relative abundance increase according to proteomic analysis was also tested by imunoblotting, but there was no significant difference between the groups (data not shown). We were not able to confirm our observation possibly due to the existence of AGT glycoprotein forms in canine plasma [17], requiring different method of validation, such as 2-D Western blotting.

3.5. Serum biochemistry

Values of serum biochemical parameters which were significantly different between dogs with iDCM and healthy dogs are presented in Table 2. C-reactive protein (CRP), urea and triglycerides concentrations, as well as creatine kinase (CK) and lactate dehydrogenase (LDH) activities, were significantly increased in serum of dogs with iDCM compared to healthy dogs. There were no significant differences in other analysed parameters: creatinine, bilirubin, glucose, proteins, albumin, aspartate aminotransferase, alanine aminotransferase, gamma-glutamyltransferase, alkaline phosphatase, alpha-amylase, lipase, cholesterol, calcium, magnesium and phosphates.

Table 2. Serum biochemical parameters with significantly differential values between healthy dogs and dogs with iDCM.

Parameter	**Healthy group**	**iDCM group**	**Reference range**
C-reactive protein (mg/L)	0 (0 - 0.3)	9.1 (8.5 - 20.1)**	0 - 10.7
Creatine kinase (U/L)	75 (69-123)	163 (132 - 460)*	0-160
Lactate dehydrogenase (U/L)	64 (37-68)	141 (106 - 228)*	45 - 233
Urea (mmol/L)	5.2 (4.4 - 5.6)	8.4 (6.2 - 17.9)*	3.3 - 8.3
Triglycerides (mmol/L)	0.6 (0.3 - 0.7)	0.9 (0.6 - 1.5)*	0.2 - 1.3

The results are expressed as median and interquartile range.
Outliers were excluded based on box plot (CRP, LDH and CK - 1 outlier in each group).
*$p < 0.05$, **$p < 0.01$ (compared to the control group)

3.6. Measurement of cTnI and NT-proBNP blood concentration

Concentrations of serum cTnI, as well as plasma NT-proBNP, were significantly increased in dogs with iDCM when compared to the control group (Figure 6a and 6b, respectively).

3.7. Bioinformatics analysis

Figure 7 displays the workflow of the bioinformatics analysis. All 15 differentially abundant canine proteins (according to proteomic (N=12) and serum biochemistry (N=3) analyses) were successfully mapped to *Homo sapiens* protein orthologs (supplemental Table 2). Then, enrichment added 334 proteins/genes to the initial 15 proteins, with best interactors matching defined criteria. All 15 initial proteins were connected to the network. In this network, 10 different clusters were defined (supplemental Table 2). All clusters were defined by at least 1 Pathway term. A group of 135 nodes was not able to form a cluster and therefore named Non-clustered, and was characterized by 4 Pathways terms. Since different clusters exhibit same Pathways terms or terms belong to the same upper hierarchical order term, some terms were merged. After merging terms from clusters, 19 pathways were identified (supplemental Table 2). All 19 Pathways terms were merged to establish the inferred interactome network, with 1341 nodes (1230 genes/proteins and 111 chemicals). All Pathways terms were connected to the network, with the exception of some parts of the Chemokine receptors bind chemokines term. Finally, Reactome Pathways terms analysis was performed on the whole inferred network, resulting with 15 identified terms (Table 3) which were grouped according to their roles into 4 groups (Figure 8). These groups were Signal transduction GPCR signaling, Immune system/Platelet, Vesicles transport and Metabolism. Furthermore, GO Cell localization terms analysis of the whole inferred network identified 17 mostly associated cell localizations terms (Table 4).

Table 3. Pathways terms (Reactome) identified on the whole *in silico* inferred network.

Pathways term	**Term P-value**	**Network Nodes**
G alpha (i) signalling events	8.23E-196	223
The citric acid (TCA) cycle and respiratory electron transport	6.91E-95	131
Clathrin-mediated endocytosis	4.45E-103	123
Response to elevated platelet cytosolic Ca2+	5.75E-102	120
PPARA activates gene expression	3.72E-91	107
Anchoring of the basal body to the plasma membrane	6.66E-71	86
Clathrin derived vesicle budding	8.46E-66	70
Plasma lipoprotein assembly, remodeling, and clearance	2.69E-54	63
Complement cascade	6.89E-44	52
Chemokine receptors bind chemokines	2.71E-42	46
G alpha (z) signalling events	1.55E-33	41
Retinoid metabolism and transport	1.77E-33	39
Formation of Fibrin Clot (Clotting Cascade)	1.43E-33	37
Elastic fibre formation	5.29E-26	35
Class C/3 (Metabotropic glutamate/pheromone receptors)	1.36E-24	31

Table 4. GO Cell localization terms of the *in silico* inferred network.

GO Cell localization term	Term P-value	Network nodes
cytoplasmic vesicle part	7.99E-56	257
membrane protein complex	6.11E-53	167
lysosome	2.79E-20	114
endosome	1.33E-13	108
cell surface	4.18E-16	107
platelet alpha granule	9.05E-75	81
inner mitochondrial membrane protein complex	5.43E-43	67
secretory granule membrane	1.02E-07	52
plasma membrane protein complex	5.69E-13	49
trans-Golgi network	1.41E-07	38
early endosome	8.19E-05	36
microtubule organizing center part	1.95E-07	31
proton-transporting two-sector ATPase complex	1.62E-24	30
azurophil granule	9.37E-05	28
clathrin-coated pit	1.23E-23	28
lysosomal lumen	2.46E-07	25
phagocytic vesicle	5.66E-06	21

3.8. The performance of biomarkers

In order to test the discriminatory power of the 12 differentially abundant proteins between the groups, ROC curves were constructed and AUC calculated, but only 4 proteins fulfilled the criterion of the required sample size (ITIH4, ITIH3, MFAP4 and APOA4). The ROC curves, AUC values, p values, sensitivity and specificity with corresponding criterion for those 4 proteins are shown in the Figure 9. All 4 proteins yielded high AUC values (above 0.9), as well as sensitivity and specificity indicating their good performance as potential biomarkers of iDCM in dogs.

4. DISCUSSION

Using the TMT label-based relative quantification proteomics approach, we have found 12 differentially abundant serum proteins between dogs with iDCM and healthy dogs. Identified differences indicate involvement of several physiological pathways in studied disease, such as complement activation, acute phase response, lipoprotein particles dynamics and tissue remodelling. Furthermore, bioinformatics analysis revealed an impact on some metabolic and signalling processes, such as ˝The citric acid cycle and respiratory electron transport˝ and ˝Signal transduction GPCR signalling˝.

Several components of the innate immune system response were found to be up-regulated by proteomic analysis in iDCM compared to healthy patients: complement C4-A, complement component C9, plasma protease C1 inhibitor (C1-INH) and newly recognized acute-phase response proteins inter-alpha-trypsin inhibitor heavy chain H3 (ITIH3) and H4 (ITIH4). Components of the complement system C4-A and C9, as well as complement regulatory protein C1-INH, were significantly increased in dogs with iDCM, but with the average fold change (iDCM/healthy ratio) of only 1.2, which suggests the activation of the complement cascade in dogs with iDCM is present, at least in some of the patients studied. Complement protein C4 is an early component of the cascade activated through the classical or lectin pathway, while complement protein C9 is one of the components of the membrane attack complex (MAC) in the terminal pathway [18].

Up-regulation of positive acute-phase response (APR) proteins ITIH4 and ITIH3 detected in serum of dogs with iDCM suggests there is an activation of APR in the setting of heart tissue injury. Acute phase response (APR) is an immediate, nonspecific and complex defensive reaction which develops in an animal upon any tissue injury (caused by infection, inflammation, neoplasia, trauma or other causes) in order to restore homeostasis. During the APR, pro-inflammatory cytokines stimulate the production of positive acute-phase proteins (APPs) in hepatocytes leading to increase in their plasma concentration [19]. ITIH3 was shown to be up-regulated in human liver upon inflammation mediated by interleukin-1 and interleukin-6 [20]. ITIH4 is a major APP in pigs which is specifically induced in hepatocytes by interleukin-6 [21,22]. ITIH4 was also elevated in serum of cows with both experimentally induced and naturally occurring mastitis [23,24]. Recently, it was found to be

a new positive APP in dogs where major surgery was used as an inflammatory model [14]. Both ITIH3 and ITIH4 bind covalently to hyaluronan, thereby promoting its stabilization. Hyaluronan is one of the main components of extracellular matrix in vertebrates which is involved in cell migration and tissue repair upon injury [25]. Increased levels of ITIH3 and ITIH4 found in our study could be due to the need of extracellular matrix stabilization in heart tissue of diseased dogs. Additionally, ITIH4 could have anti-inflammatory effects since it may have a role in complement suppression [25].

Our finding of significantly increased concentration of C-reactive protein (CRP) in iDCM group compared to healthy (measured by serum biochemistry test) also provides evidence for activation of APR in iDCM. CRP is a well known major positive APP in both humans and dogs, used as sensitive, but nonspecific biomarker of systemic inflammation [26]. This study contributes to the rare reports of serum CRP concentration evaluation in dogs with iDCM, but with limitation of a small sample size and remark that some values in iDCM group didn´t exceed the upper limit of the normal reference range. Similar results were observed in a study of dogs with chronic valvular disease (CVD), the most common acquired heart disease of the dog, where CRP concentration was significantly higher in dogs with CVD compared to healthy dogs, but with a large degree of overlap between the two groups [27]. Increased plasma CRP concentrations were also found in dogs with congestive heart failure due to mitral valve disease or dilated cardiomyopathy compared to controls, but only 5 dogs with DCM were included [28]. In a study of human patients with iDCM, serum CRP levels were increased compared to controls [29].

While there is widening evidence of role of immune processes in a portion of human DCM cases [30], such findings are poorly documented in canine DCM research. However, in a study of Buse et al. [31] there were significantly higher levels of serum auto-antibodies against myosin heavy chain and α-cardiac actin in dogs with DCM than in controls, while Day [32] found anti-mitochondrial antibodies in one third of examined English Cocker Spaniels with DCM. Results of our study support the notion that immunological processes are also involved in the pathopshysiology of canine iDCM, although sequence of events leading to their involvement in the disease cannot be clearly elucidated.

Apolipoproteins A1 (APOA1), A4 (APOA4) and C3 (APOC3), which are lipid-binding proteins involved in the transport of lipids in plasma, were found to be down-regulated in iDCM compared to control serum in our study. Lower plasma APOA1 levels were also found in human patients with iDCM compared to controls [29,33]. APOA1 is a major constituent of high-density lipoprotein particles which was found to have anti-inflammatory properties since it may interfere with the assembly of complement C9 of the MAC of complement cascade [34]. APOA4 is a protein found free in plasma or as a component of different lipoprotein particles, which was shown to have anti-atherogenic and antioxidative properties. Lower levels of APOA4 were found in human patients with cardiovascular disease compared to controls and low APOA4 levels predicted the risk for sudden cardiac death in patients with high risk [35]. It is possible that reduced levels of APOA1 and APOA4 detected in dogs with iDCM contribute to the development or advancement of the disease and could be potentially used as a risk biomarker.

Interesting finding of increased serum microfibril-associated glycoprotein 4 (MFAP4) levels in dogs with iDCM could have origin in remodelling processes taking place in dilated myocardium. MFAP4 is an extracellular matrix (ECM) glycoprotein expressed in various elastic tissues which has a role in elastic fiber organization [36]. Increased serum levels of MFAP4 were proposed to be a potential biomarker in pathologies characterised by ECM remodelling, such as liver fibrosis in hepatitis C patients [37]. MFAP4 was also found to be elevated in plasma of human patients with congestive heart failure [38]. In a proteomic study of serum glycoproteins in canine model of dyssynchronous heart failure, MFAP4 was upregulated when compared to control dogs [39]. It is well known that, in the setting of both human and canine iDCM, heart tissue is undergoing fibrosis which involves ECM degradation and remodelling [40,41]. We show herein that serum level of MFAP4 could serve as biomarker of heart tissue remodelling in iDCM.

Transferrin receptor protein 1 (TfR1) is a membrane glycoprotein which has a role in transport of iron from plasma to cell and whose expression on the surface of cells is dependent on tissue iron status. Serum TfR1 (sTfR1) represents the soluble extracellular portion of TfR1 whose level reflects TfR1 density on cells (i.e. iron status) and the number of cells expressing TfR1 (i.e. mostly cells with erythropoietic activity). Levels of sTfR1 are used as biomarker in iron deficiency and anaemia of chronic disease in humans [42]. We detected

584 slightly decreased levels of sTfR1 in diseased dogs compared to healthy, which could suggest
585 a change of erythropoietic activity or iron status in iDCM. Although anaemia is not common
586 finding in dogs with iDCM, it is present in certain number of diseased dogs [43,44].

587 Increased serum abundances of angiotensinogen (AGT) in dogs with iDCM are not a
588 surprising finding since AGT is a part of the renin-angiotensin-aldosterone system (RAAS),
589 which is known to be activated in dogs with DCM, although only in those with clinical signs
590 (the New York Heart Association (NHYA) class III and IV) [45,46]. AGT production in dogs is in
591 some minor extent stimulated by positive feedback by its final product angiotensin II at
592 physiological conditions [47]. Dogs with iDCM included in our study were classified to the
593 NHYA class II and III, which could explain for only slight elevation of serum AGT in
594 comparison to healthy dogs. While serum AGT elevation in the diseased group in our study
595 indicates activation of the RAAS, increase of specifically measured plasma NT-proBNP herein
596 also points out to the natriuretic system stimulation in dogs with iDCM. NT-proBNP was
597 found to be elevated in both humans and dogs with DCM [48]. Elevated NT-proBNP
598 concentrations are detected in blood as a response to different factors, such as volume
599 overload, hypertrophy and hypoxia. Actions of natriuretic system lead to natriuresis and
600 vasodilation, acting in contrary to the RAAS, which induces sodium retention and
601 vasoconstriction. It is known that activities of both systems contribute to the congestive
602 heart failure development [49]. Another known biomarker of myocardium damage is cardiac
603 troponin I (cTnI), which is released from cardiac myocytes upon their injury. It is used as a
604 blood biomarker of acute myocardial infarction in humans, but it also has prognostic value in
605 patients with chronic heart disease [50]. While cTnI concentrations are increased in dogs
606 with iDCM, there are limitations in its utility since it is not specific to the myocardial injury
607 cause, can be of normal level in animals with mild disease and is falsely elevated if kidney
608 injury is present [6]. We observed significant increase of serum cTnI in dogs with iDCM
609 compared to healthy, as expected.
610
611 Bioinformatics analysis of the *in silico* inferred protein network enabled us to generate more
612 information from the experimental results, even providing data on intracellular pathways,
613 not normally captured by serum proteome analysis. The strength of the *in silico* inferred
614 network analysis is reflected in the detection of pathways known to be involved in iDCM,

615 which indicates validity of the *in silico* constructed data. Also, recognition of these pathways
616 has an importance in the possibility of further targeted research.
617
618 Based on the *in silico* network, we were able to detect ˝The citric acid cycle and respiratory
619 electron transport˝ as pathway implicated in the disease. This result is consistent with GO
620 cell localization analysis of the network, which detected ˝inner mitochondrial membrane
621 protein complex˝ and ¨proton-transporting two-sector ATPase complex¨ GO terms.
622 Mitochondria, organelles which main function is energy production, are believed to have an
623 important role in aetiology and/or progression of heart dysfunction pathologies taking into
624 account myocardium high energy demand [51]. Interestingly, in several studies on heart
625 tissue of both human and canine iDCM there was a finding of perturbations in mitochondrial
626 electron transport activity [7,52,53]. Also, when Heinke et al. [54,55] performed proteomic
627 studies on left ventricular tissue of dogs with pacing-induced heart failure, model state
628 resembling iDCM, they found impairment of mitochondrial energy production. Furthermore,
629 Lopes et al. [56] found that most of the altered mitochondrial proteins of heart tissue of
630 dogs with induced or naturally occurring iDCM, compared to controls, were involved in
631 respiratory electron transport chain.

632 Very extensive group of the *in silico* network analysis was the ˝Signal transduction GPCR
633 signalling˝, which included ˝G alpha (i) and (z) signalling events˝, ˝Chemokine receptors bind
634 chemokines˝ and ˝Class C/3 (Metabotropic glutamate/pheromone receptors)˝ pathways. G
635 protein-coupled receptors (GPCRs) signalling is involved in various physiological pathways
636 and activated by most of the known neurotransmitters, hormones and chemokines [57].
637 GPCRs in turn trigger downstream signalling events, among them G alpha (i) signalling,
638 reported to be increased in human hearts affected by iDCM, where it seems to mediate
639 myocardium contractility defects [58].

640 Two pathways implicated in iDCM based on the *in silico* protein network are related to
641 haemostasis - ˝Formation of Fibrin Clot˝ and ˝Response to elevated platelet cytosolic Ca^{2+}¨.
642 Activation of coagulation system was found present in humans and dogs with iDCM, possibly
643 resulting from changes in blood flow due to dilated cardiac chambers and low cardiac output
644 or as a consequence of activation of the RAAS system [59–61]. Our bioinformatics results are
645 in accordance with the finding of procoagulant state in dogs with iDCM.

Furthermore, bioinformatics analysis confirmed our observation of immune system involvement, plasma lipoproteins remodelling and elastic fibre formation in iDCM physiopathology. One important finding of our study is the supporting evidence of immunological processes in canine iDCM, which could target the development of new treatment strategies.

5. CONCLUSIONS

Limitations of this study are small sample size and utilization of samples of the same patients for validation of proteomics results by Western blot. Therefore, this work can be considered preliminary and our findings need further validation using different samples and larger sample size. Nevertheless, we can highlight three putative biomarkers validated herein which could be relevant for underlying iDCM processes, taking into account the fold change ratio and high specificity and sensitivity resulting from the analysis of ROC curves. We propose ITIH4 to be connected with extracellular matrix changes happening upon tissue injury, which also supports its recently identified role as acute phase protein in dogs [14]. Furthermore, serum MFAP4 could serve as a biomarker of fibrosis in dogs, whose utility should be tested in different settings of canine fibrosis in both cardiac and non-cardiac diseases. Also, low levels of serum APOA4 could be tested as a risk factor for development or worsening of canine iDCM. Since the data acquired herein could be translational to human iDCM, these biomarkers may also be of relevance to human patients. In conclusion, label-based high-resolution quantitative proteomics analysis and bioinformatics approach used herein represent a valid tool for elucidating complex iDCM pathophysiology and uncovering disease relevant proteins with biomarker potential.

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Figure 1

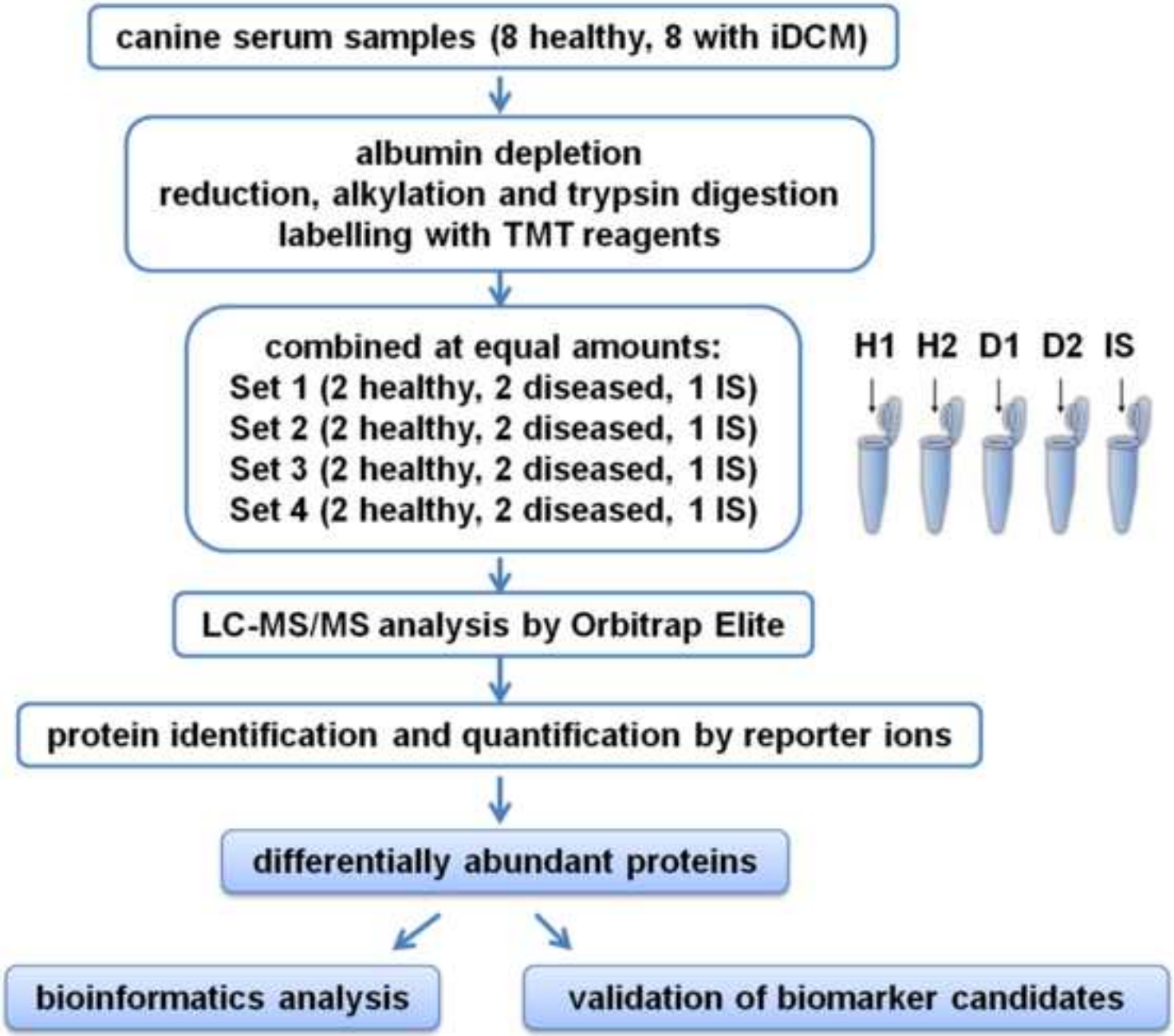
canine serum samples (8 healthy, 8 with iDCM)
albumin depletion
reduction, alkylation and trypsin digestion
labelling with TMT reagents
combined at equal amounts:
Set 1 (2 healthy, 2 diseased, 1 IS)
Set 2 (2 healthy, 2 diseased, 1 IS)
Set 3 (2 healthy, 2 diseased, 1 IS)
Set 4 (2 healthy, 2 diseased, 1 IS)
H1
H2
D1
D2
IS
LC-MS/MS analysis by Orbitrap Elite
protein identification and quantification by reporter ions
differentially abundant proteins
bioinformatics analysis
validation of biomarker candidates

Figure 1 caption

Figure 1. TMT study design for identification of proteins with biomarker potential and pathways involved in canine iDCM (H1, H2 – samples from different healthy dogs; D1, D2 - samples from different dogs with iDCM, IS – internal standard sample).

Figure 2

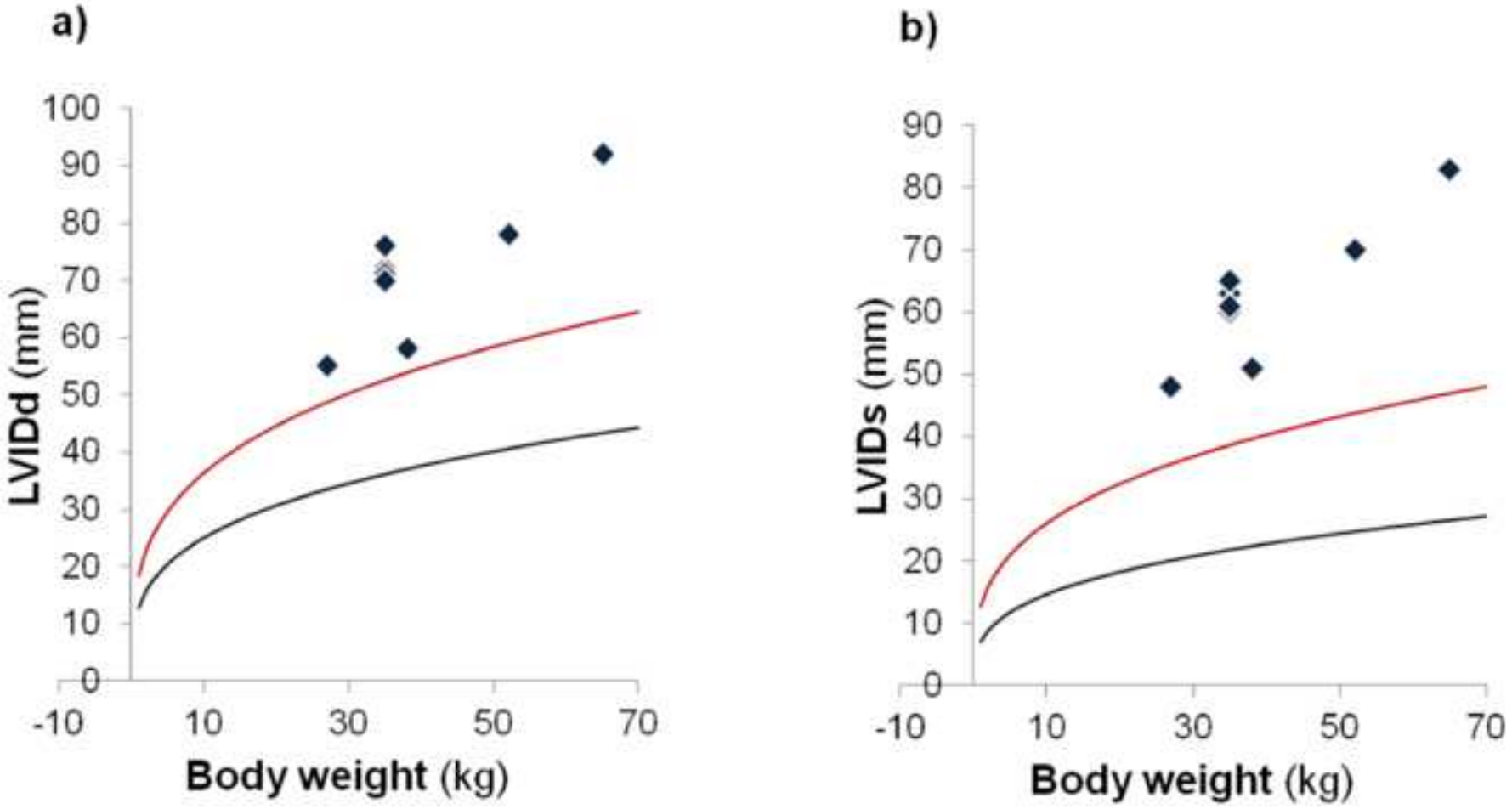
a)
LVIDd (mm)
100
90
80
70
60
50
40
30
20
10
0
-10
10
30
50
70
Body weight (kg)
b)
LVIDs (mm)
90
80
70
60
50
40
30
20
10
0
-10
10
30
50
70
Body weight (kg)

Figure 2 caption

Figure 2. Scatter plots showing the echocardiographic parameters in 8 dogs diagnosed with iDCM: a) left ventricular end diastolic diameter (LVIDd) and b) left ventricular end systolic diameter (LVIDs). All dogs are above the upper reference values (red lines).

Figure 3

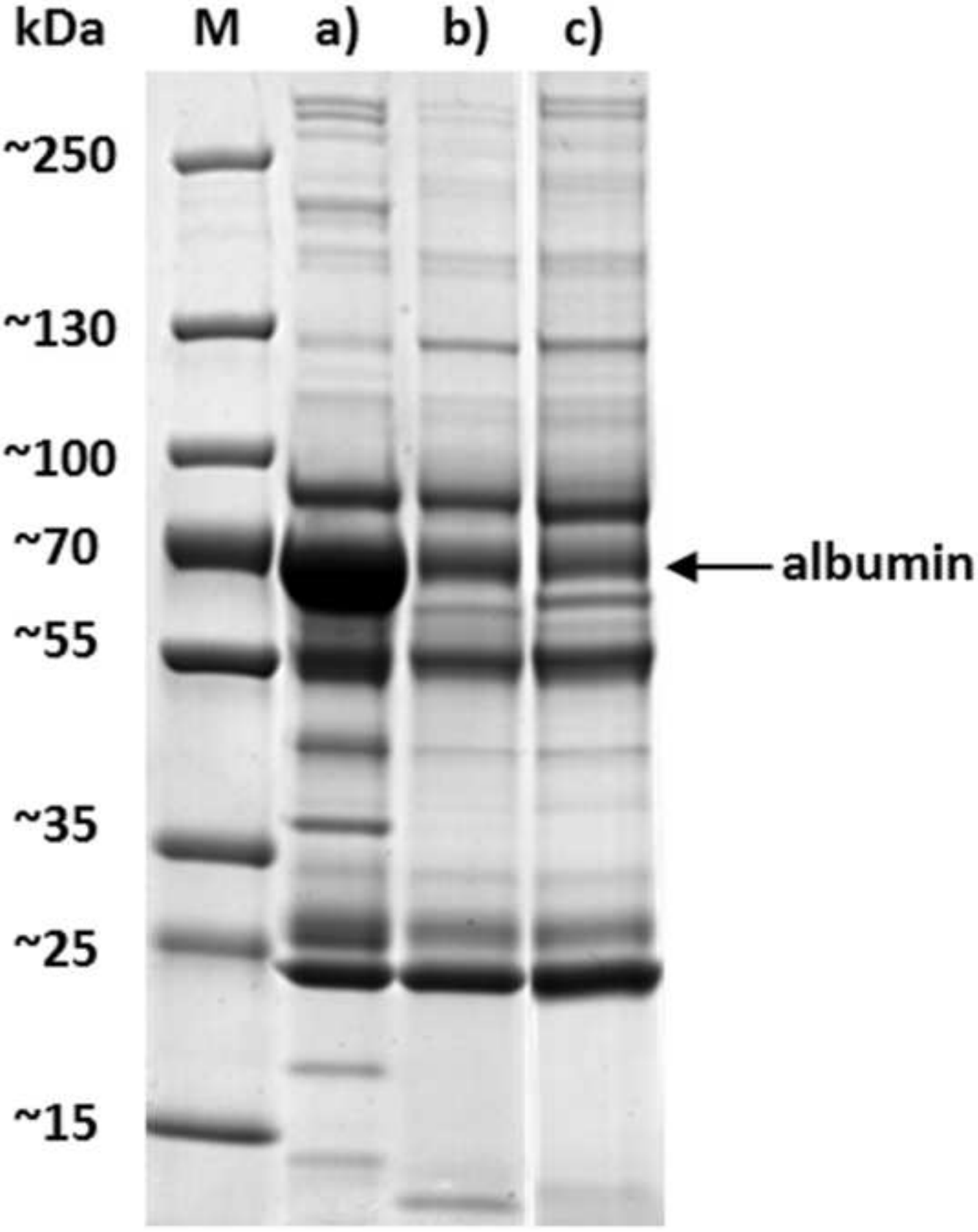

Figure 3 caption

Figure 3. Albumin depletion from canine serum using salt-ethanol procedure. 1-D SDS PAGE of a) non-depleted canine serum; b) depleted serum sample of a dog with iDCM; c) depleted serum sample of a healthy dog (Notes: a), b) and c) are sera of different dogs; marker and samples a) and b) were run on one gel and sample c) on another gel).

Figure 4

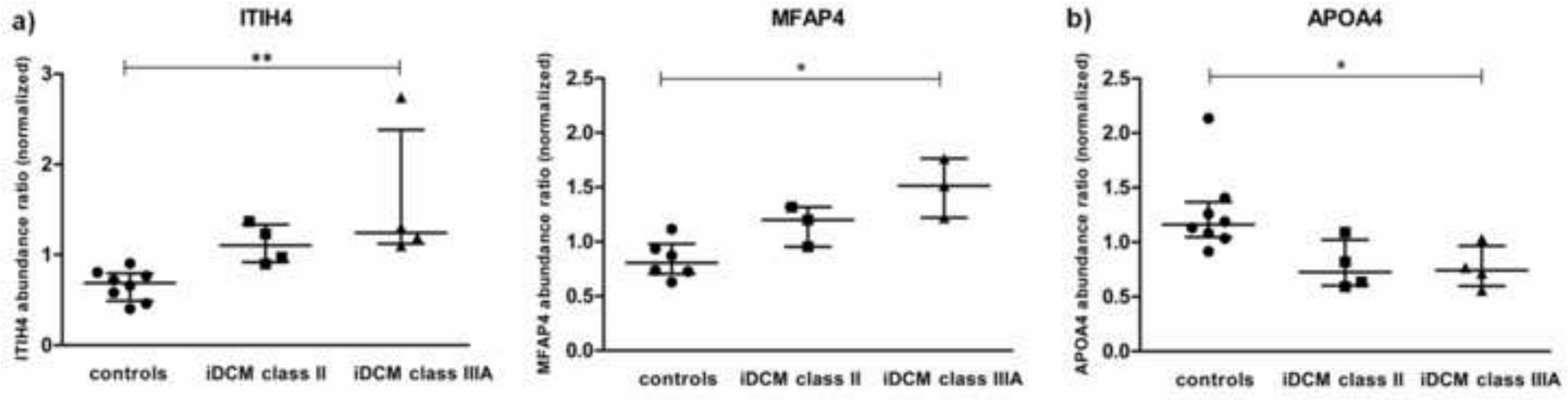
a)
ITIH4
**
ITIH4 abundance ratio (normalized)
3
2
1
0
controls
iDCM class II
iDCM class IIIA
MFAP4
*
MFAP4 abundance ratio (normalized)
2.5
2.0
1.5
1.0
0.5
0.0
controls
iDCM class II
iDCM class IIIA
b)
APOA4
*
APOA4 abundance ratio (normalized)
2.5
2.0
1.5
1.0
0.5
0.0
controls
iDCM class II
iDCM class IIIA

Figure 4 caption

Figure 4. Comparison of normalized protein abundances ratios between healthy controls, iDCM ISACHC class II and class IIIA.

a) ITIH4 and MFAP4 are up-regulated in iDCM ISACHC class IIIA compared to controls.

b) APOA4 is down-regulated in iDCM ISACHC class IIIA compared to controls.

Results are expressed as median and interquartile range, *$p < 0.05$, **$p < 0.01$.

Note that for ITIH4 and APOA4 there are N=8 controls, N=4 iDCM class II, N=4 iDCM class IIIA and for MFAP4 N=6 controls, N=3 iDCM class II and N=3 iDCM class IIIA.

Figure 5

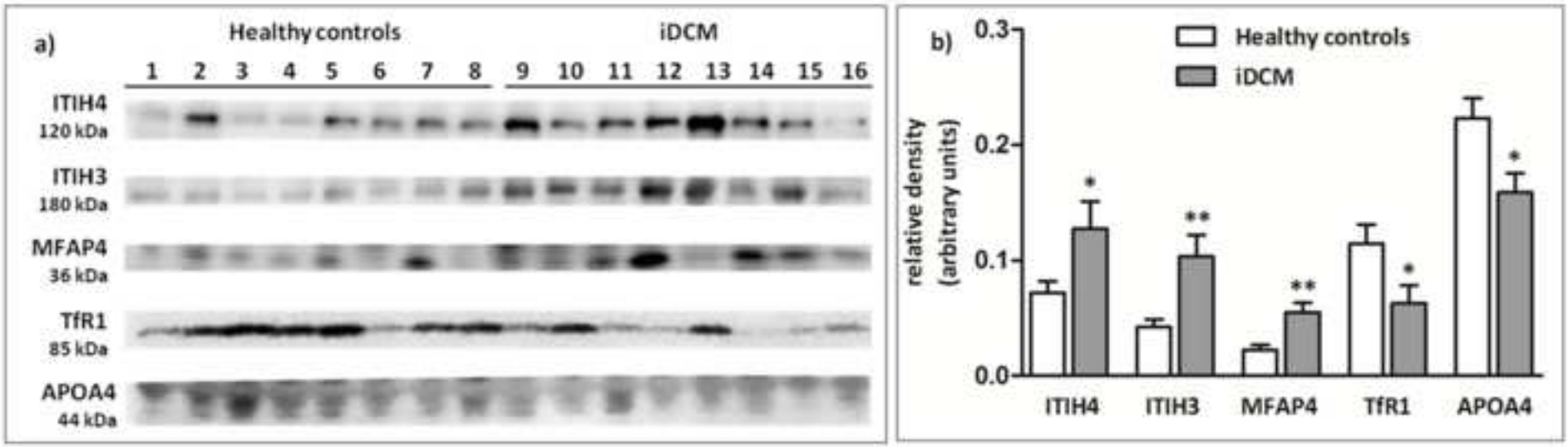
a)
Healthy controls
iDCM
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
ITIH4
120 kDa
ITIH3
180 kDa
MFAP4
36 kDa
TfR1
85 kDa
APOA4
44 kDa
b)
Healthy controls
iDCM
relative density
(arbitrary units)
0.3
0.2
0.1
0.0
*
**
**
*
*
ITIH4
ITIH3
MFAP4
TfR1
APOA4

Figure 5 caption

Figure 5. Validation of proteomics results:

a) Representative figures of Western blotting of serum ITIH4, ITIH3, MFAP4, TfR1 and APOA4 from healthy controls and dogs with iDCM (figures of individual membranes were cropped to show the band of interest).

b) Relative density comparison of the five proteins between healthy controls and iDCM; data are shown as mean with SEM, *$p < 0.05$, **$p < 0.01$.

Figure 6

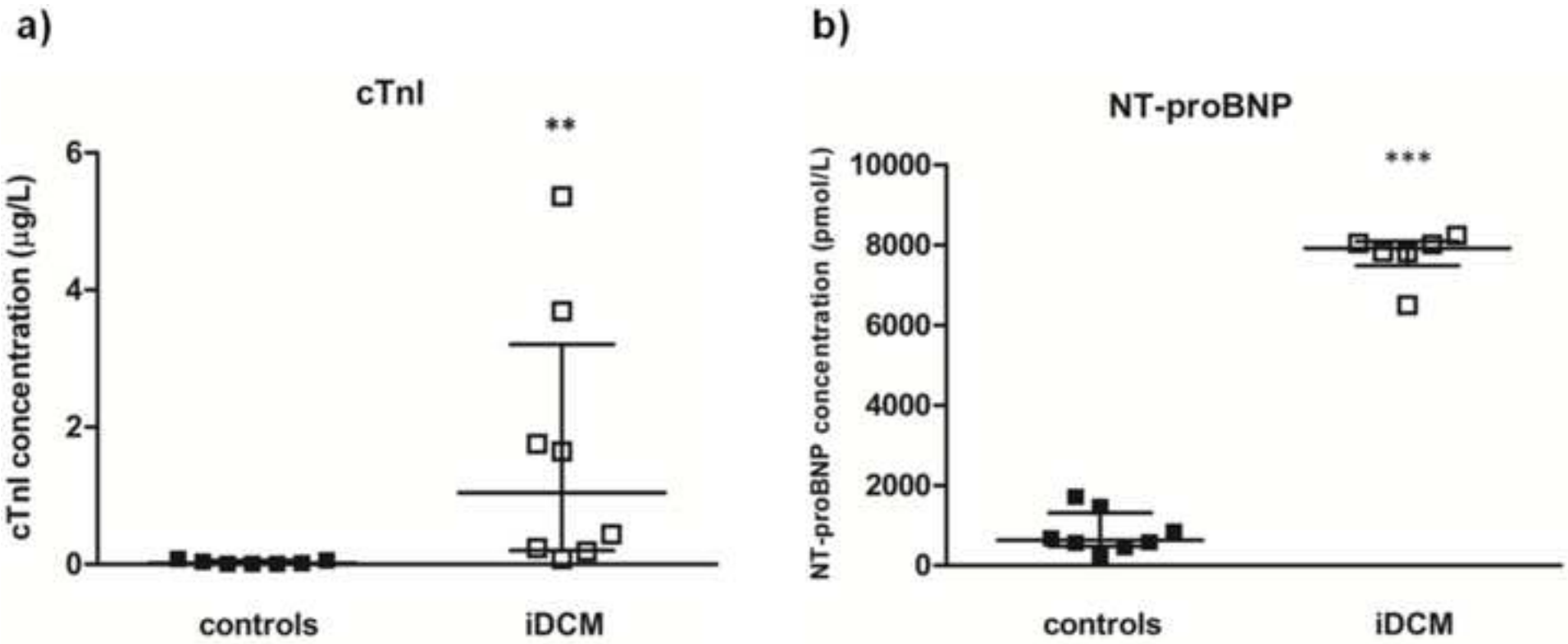
a)
cTnI
**
cTnI concentration (µg/L)
6
4
2
0
controls
iDCM
b)
NT-proBNP
***
NT-proBNP concentration (pmol/L)
10000
8000
6000
4000
2000
0
controls
iDCM

Figure 6 caption

Figure 6. Comparison of a) cTnI and b) NT-proBNP blood concentrations between healthy dogs and dogs with iDCM. The results are expressed as median and interquartile range. Outliers were excluded before statistical analysis based on grubbs' test for outliers and are not shown in the figure (cTnI – 1 outlier in the control group, no outliers in the iDCM group; NT-proBNP – no outliers in the control group, 2 outliers in the iDCM group).
** $p < 0.01$, *** $p<0.001$ (compared to the control group)

Figure 7

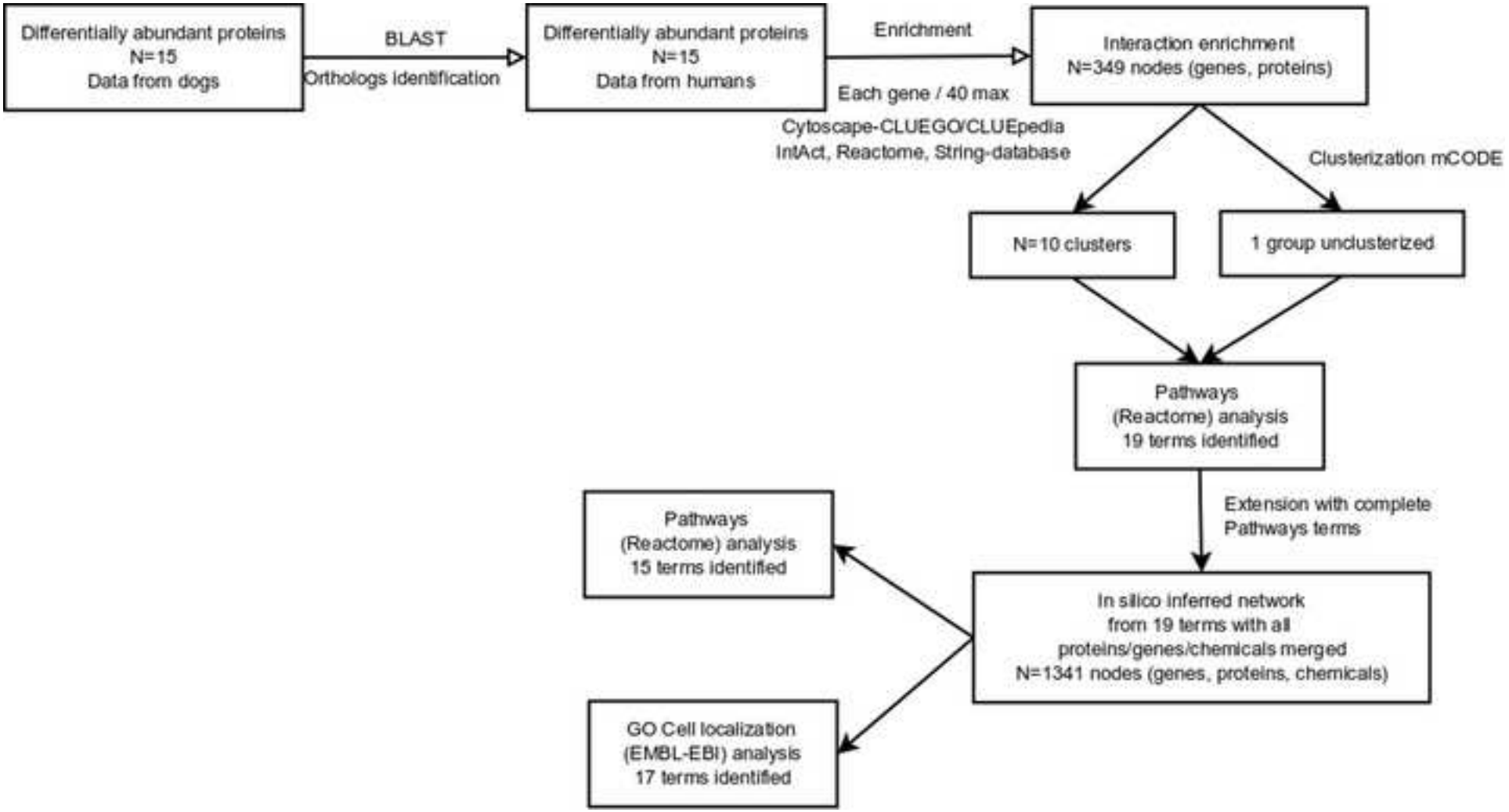
Differentially abundant proteins
N=15
Data from dogs
BLAST
Orthologs identification
Differentially abundant proteins
N=15
Data from humans
Enrichment
Each gene / 40 max
Cytoscape-CLUEGO/CLUEpedia
IntAct, Reactome, String-database
Interaction enrichment
N=349 nodes (genes, proteins)
Clusterization mCODE
N=10 clusters
1 group unclusterized
Pathways
(Reactome) analysis
19 terms identified
Extension with complete
Pathways terms
Pathways
(Reactome) analysis
15 terms identified
In silico inferred network
from 19 terms with all
proteins/genes/chemicals merged
N=1341 nodes (genes, proteins, chemicals)
GO Cell localization
(EMBL-EBI) analysis
17 terms identified

Figure 7 caption

Figure 7. Design of bioinformatics analysis based on initial list of differential proteins between healthy dogs and dogs with iDCM.

Figure 8

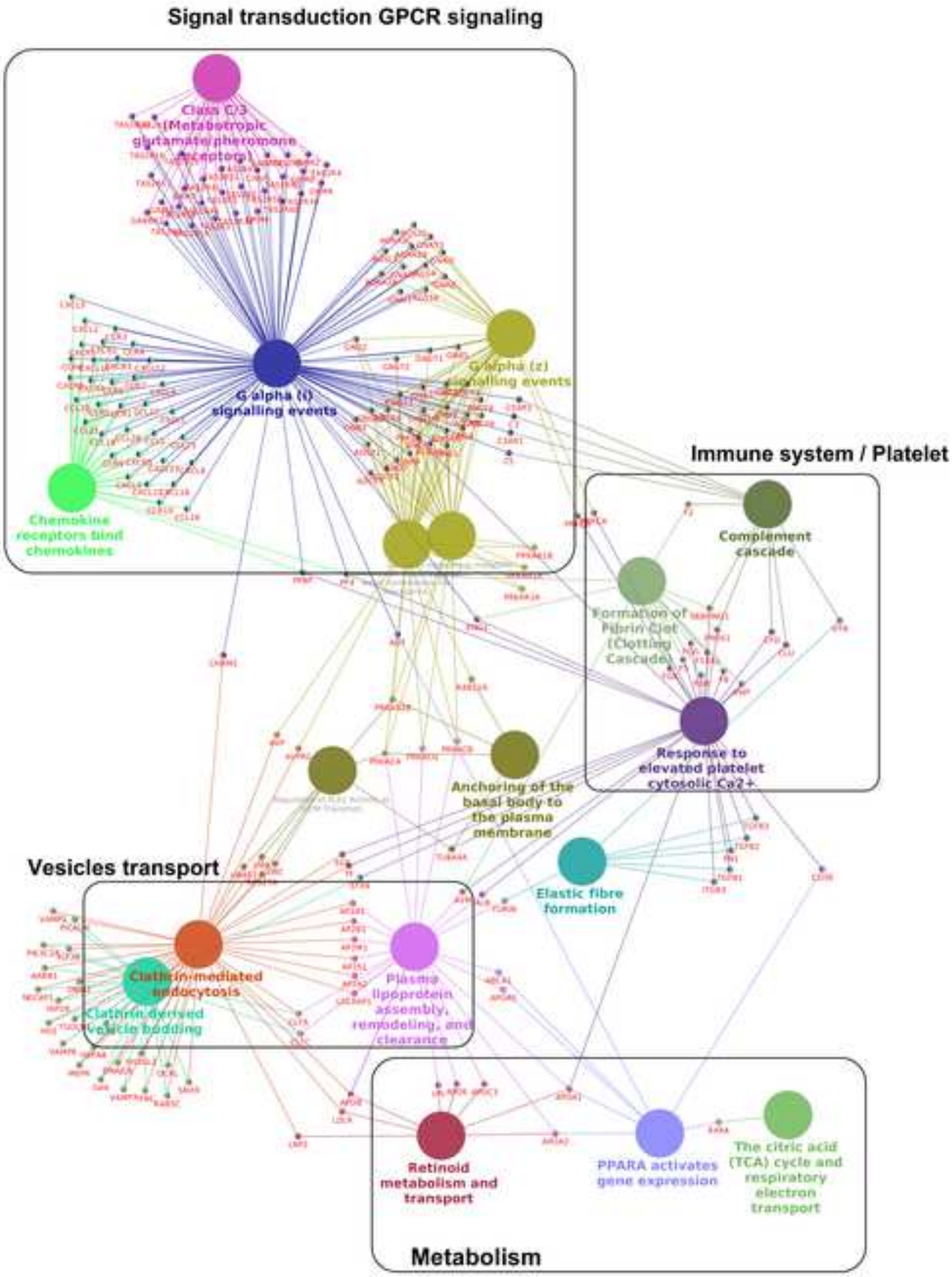
Signal transduction GPCR signaling
Class C/3 (Metabotropic glutamate/pheromone receptors)
G alpha (i) signalling events
G alpha (z) signalling events
Chemokine receptors bind chemokines
Immune system / Platelet
Complement cascade
Formation of Fibrin Clot (Clotting Cascade)
Response to elevated platelet cytosolic Ca2+
Anchoring of the basal body to the plasma membrane
Elastic fibre formation
Vesicles transport
Clathrin-mediated endocytosis
Clathrin derived Vesicle budding
Plasma lipoprotein assembly, remodeling, and clearance
Retinoid metabolism and transport
PPARA activates gene expression
The citric acid (TCA) cycle and respiratory electron transport
Metabolism

Figure 8 caption

Figure 8. *In silico* inferred interactome protein network of identified Pathways terms involved in canine iDCM. Only nodes interacting with at least 2 terms are represented. Organic layout was applied.

Figure 9

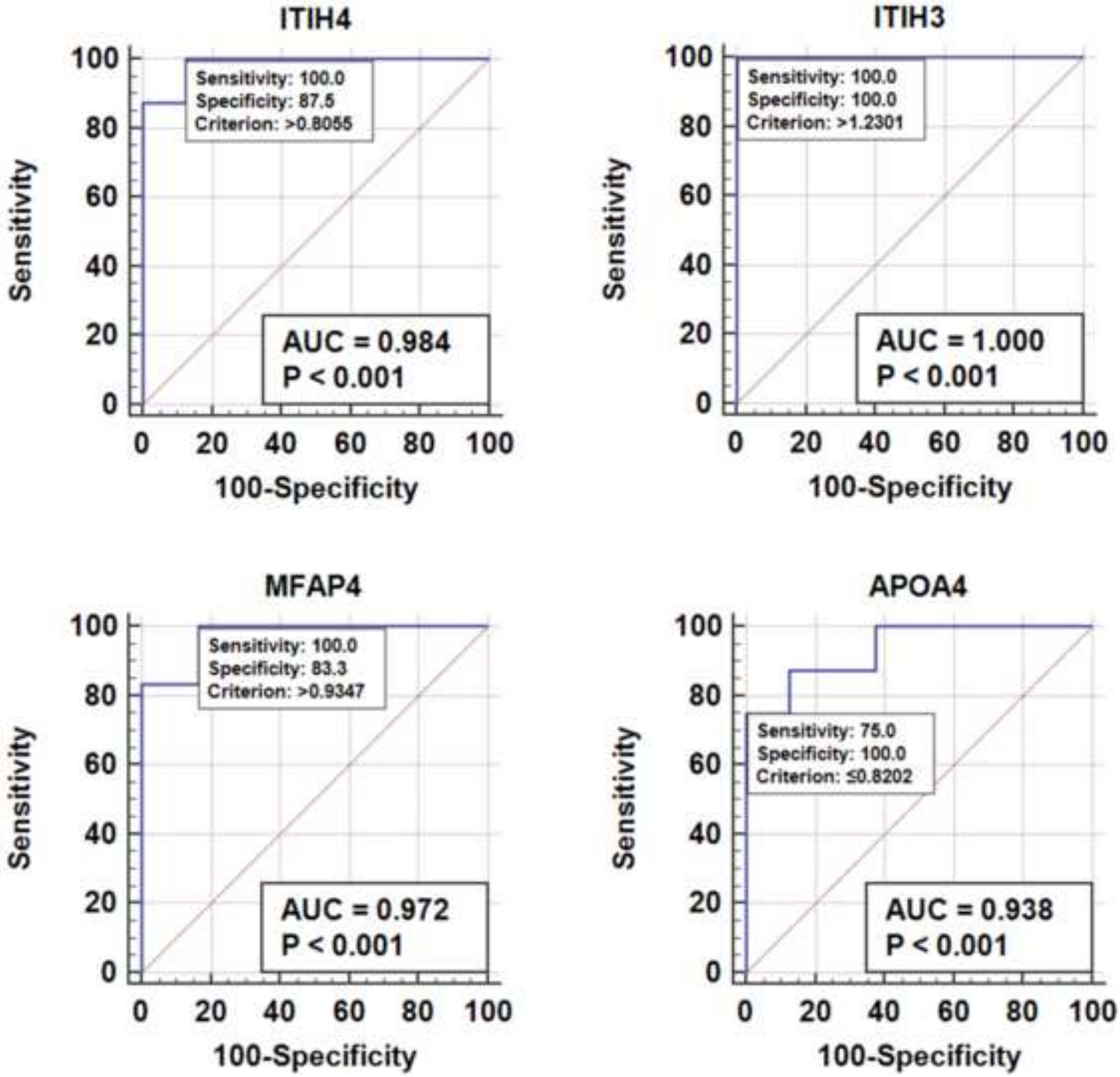
ITIH4
Sensitivity: 100.0
Specificity: 87.5
Criterion: >0.8055
AUC = 0.984
P < 0.001
ITIH3
Sensitivity: 100.0
Specificity: 100.0
Criterion: >1.2301
AUC = 1.000
P < 0.001
MFAP4
Sensitivity: 100.0
Specificity: 83.3
Criterion: >0.9347
AUC = 0.972
P < 0.001
APOA4
Sensitivity: 75.0
Specificity: 100.0
Criterion: ≤0.8202
AUC = 0.938
P < 0.001
Sensitivity
100-Specificity
0
20
40
60
80
100

Figure 9 caption

Figure 9. ROC curves of potential protein biomarkers of iDCM in dogs. Note that there were N=8 controls, N=8 iDCM values for ITIH4 and APOA4 analysis; N=6 controls, N=6 iDCM for MFAP4 analysis and N=4 controls, N=4 iDCM for ITIH3.

Supplementary material Table 1

Click here to download Supplementary material: Supplemental table 1.List of proteins included in quantification analysis.xlsx

Supplementary material Table 2

Click here to download Supplementary material: Supplemental table 2.Bioinformatic analysis.xlsx