Novel Biomarkers in Cats with Congestive Heart Failure Due to Primary Cardiomyopathy

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Conflict of Interest

The authors declare that there are no conflicts of interest.

Ethics

This study has obtained ethical approval from Veterinary Research Ethics Committee in the University of Glasgow, UK.

CRediT Author Statement

Mengmeng Liu: Conceptualization, Methodology, Investigation, Formal Analysis, Writing-Original Draft, Visualization. **P. David Eckersall**: Conceptualization, Methodology, Writing-Review & Editing, Supervision, Project administration. **Vladimir Mrljakc**: Resources, Writing-Review & Editing, Supervision, Project administration. **Anita Horvatić**: Investigation, Formal Analysis, Data Curation, Resources, Writing-Review & Editing. **Nicolas Guillemin**: Software, Formal Analysis, Data Curation, Resources, Writing-Review & Editing, Visualization. **Asier Galan**: Validation, Resources, Writing-Review & Editing. **Liza Koster**: Resources, Writing-Review & Editing, Supervision, Funding acquisition. **Anne French**: Conceptualization, Methodology, Resources, Writing-Review & Editing, Supervision, Project administration, Funding acquisition

Abbreviations

A1A	α-1 antitrypsin
A2AP	α-2 antiplasmin
ACTB	actin beta
APOA1	apolipoprotein A-I
APOA2	apolipoprotein A-II
APOC1	apolipoprotein C-I
APOM	apolipoprotein M
ATIII	antithrombin III
ATE	arterial thromboembolism
BCA	bicinchroninic acid
C8G	complement C8 gamma chain
CHF	congestive heart failure
CKD	chronic kidney disease
CPN1	carboxypeptidase N subunit 1
ECM	Extracellular matrix
FDR	false discovery rate
GO	Gene Ontology
HCM	hypertrophic cardiomyopathy
HF	heart failure
HFpEF	heart failure with preserved ejection fraction
IAA	idoacetamide
IgHC	immunoglobulin heavy chain
IgL	immunoglobin lambda
IgK	immunoglobin kappa
IgG2	immunoglobin gamma-2
IQR	interquartile range
IRIS	International Renal Interest Society
ISACHC	International Small Animal Cardiac Health Council
ITIH1	inter-alpha-trypsin inhibitor heavy chain 4
ITIH4	inter-alpha-trypsin inhibitor heavy chain 4
IVSd	interventricular septum thickness at end diastole
JCHAIN	immunoglobin J chain
LA	left atrial
LA/Ao	LA-to-aortic root ratio
LC-MS	liquid chromatography-mass spectrometry
LV FS	left ventricular fractional shortening
LVFWd	left ventricular free wall thickness at end diastole
MMP	matrix metalloproteinase
MRM	multiple reaction monitoring
NCBI	National Center for Biotechnology Information
NT-proBNP	N-terminal pro-brain natriuretic peptide
PF4	platelet factor 4
PVDF	Polyvinylidene Flouride
RCM	restrictive cardiomyopathy
SEC	spontaneous echo contrast
SERPINs	serine protease inhibitors
SRM	selected reaction monitoring

TBST	TBS containing 0.1 % Tween-20
TEAB	triethyl ammonium bicarbonate
TGF-β2	transforming growth factor beta 2
THBS1	thrombospondin 1
TIMP	tissue inhibitor of matrix metalloproteinase
TMSB4X	thymosin β-4
TMT	tandem mass tag
UCM	unclassified cardiomyopathy

Significance

Cardiomyopathies affect both cats and humans, and they can cause serious consequence such as congestive heart failure (CHF). To date, the pathophysiological mechanism of CHF is not fully understood. In this study, for the first time, we used a proteomic approach combined with bioinformatic analysis to evaluate serum protein change in cats with CHF. Results indicate systemic inflammation, coagulation protein changes, innate immunity and extracellular matrix remodeling are involved in feline CHF, which are largely comparable with findings in previous human studies. Our study provides new insights into CHF and cardiomyopathy in cats, and the identified novel biomarkers and pathophysiological pathways provide valuable information for future studies.

Highlights

- First proteomics study evaluating circulating protein change in feline CHF
- Systemic inflammation and ECM remodeling occur in feline CHF
- Coagulation proteins and innate immunity play a role in feline CHF
- A list of novel biomarkers is identified and predicted

Abstract

The pathogenesis of feline cardiomyopathy and congestive heart failure (CHF) requires further understanding. In this study, we assessed serum proteome change in feline CHF, aiming to identify novel biomarker for both research and clinical use. The study comprised 15 cats in CHF, 5 cats in preclinical cardiomyopathy and 15 cats as healthy controls. Serum proteome profiles were obtained by tandem mass tag labelling followed by mass spectrometry. Protein concentrations in CHF cats were compared with healthy controls. Western blot was performed for proteomic validation. Correlations were assessed between the altered proteins in CHF and clinical variables in cats with cardiomyopathy to evaluate protein-cardiac association. Bioinformatic analysis was employed to identify pathophysiological pathways involved in feline CHF. Sixteen serum proteins were significantly different between CHF and healthy control cats ($P < 0.05$). These included serine protease inhibitors, apolipoproteins and other proteins associated with inflammation and coagulation. Clinical parameters from cats with cardiomyopathy significantly correlated with the altered proteins ($P < 0.05$). Bioinformatic analysis identified 13 most relevant functional profiles in feline CHF, which mostly associated with extracellular matrix organization and metabolism.

Data are available via ProteomeXchange with identifier PXD017761.

Introduction

Cardiomyopathy is a common cardiac condition in both cats and humans. Apart from known genetic mutation factors responsible for certain breed disposition and familial inheritance, the aetiology remains unclear for the majority of idiopathic cardiomyopathy cases which usually occur in a later stage of life [1, 2]. While some affected cats develop terminal conditions such as congestive heart failure (CHF) and arterial thromboembolism (ATE) soon after diagnosis, others may remain asymptomatic for life [3]. The need for improved understanding in the

disease pathogenesis is a driving force for exploring novel biomarkers for research and clinical use.

Feline cardiomyopathy has been proposed as an animal disease model particularly for human heart failure with preserved ejection fraction (HFpEF) [4]. Both conditions are found to be associated with diastolic dysfunction, with decreased ventricular compliance due to myocardial fibrosis [5, 6]. In humans, HFpEF has been associated with systemic inflammation [7]. A recent theory postulated that a systemic pro-inflammatory state induced by other disorders can contribute to cardinal pathology in HFpEF [8]. In feline CHF, inflammatory cell infiltration and myocardial remodeling has consistently been seen in cardiac pathology [6, 9, 10]. One previous study reported circulating tumor necrosis factor-α was increased in 40% of CHF cats with various phenotypes of cardiomyopathy, suggesting a link between systemic inflammation and feline CHF [11]. Coagulation disorders have also been documented in both human and feline cardiomyopathy [12, 13, 15, 16]. In feline cardiomyopathy, platelet activation and hypercoagulation has been found more prevalent in those with advanced disease or having thrombotic risk/event [13, 16]. However, the pathophysiology of coagulation activation in CHF and cardiomyopathy in cats is far from clear. As few studies have investigated systemic changes in feline CHF, a global detection of circulating protein change would be useful to provide additional information on disease pathogenesis.

Although proteomics is being increasingly used in veterinary and animal sciences [17, 18] there have been few reports of the use of this advanced protein analysis technology to study feline disease. In this study, we aimed to evaluate the serum proteome change in feline CHF by using shotgun proteomics combined with bioinformatic analysis. We hypothesized that the serum proteome would change in feline CHF and that novel biomarkers might be identified based on the differentially regulated proteins.

Materials and Methods

Animals and Sample Collection

Twenty cats diagnosed with primary cardiomyopathy were enrolled in the study with full owner consent and ethical approval in a referral cardiology service. At admission, 15 cats were in CHF and 5 cats were preclinical. Cardiomyopathy and CHF diagnoses were based on echocardiography, thoracic radiographs and/or thoracic ultrasonographic findings. Diagnostic criteria for cardiomyopathies were based on previous publications [2, 19-22]. Before enrolment, cats with any of the following conditions were excluded; (1) secondary cardiomyopathy due to hyperthyroidism, acromegaly or systemic hypertension; (2) primary chronic kidney disease (CKD) at International Renal Interest Society (IRIS) stage II or above at the time of admission; (3) pre-existing inflammatory comorbidities including any active infectious disease, recent inflammatory events (surgery or trauma) or systemic diseases (pancreatitis, neoplasia and anaemia secondary to chronic inflammatory disease). A clinical record was collected for each cat with cardiomyopathy at the entry of the study including age, sex, breed, cardiomyopathy and CHF diagnoses, CHF severity, body weight, left atrial (LA) diameter, LA-to-aortic root (LA/Ao) ratio, left ventricular free wall thickness at end diastole (LVFWd), interventricular septum thickness at end diastole (IVSd), left ventricular fractional shortening (LV FS), spontaneous echo contrast (SEC), intra-cardiac thrombus, ATE, arrhythmia and comorbidities. Blood was sampled via venepuncture, followed by centrifugation at 5433g for 3 min, serum was separated, collected into a collection tube and stored at -20°C temporarily prior to transferring to -80°C for storage.

Commercial serum samples (Biobest, UK) from 15 clinically healthy cats were used as normal controls. As echocardiographic exams were not performed in those cats, N-terminal pro-brain natriuretic peptide (NT-proBNP) assay (Cardiopet, IDEXX) was used for screening for occult

cardiomyopathy in the healthy control population [23, 24]. All the enrolled healthy controls had an NT-proBNP under the detectable range (< 24 pmol/l).

Tandem Mass Tag (TMT)-Based Quantitative Proteomics

Protein Sample Preparation

Total serum protein concentration of each sample was determined by using a Bicinchroninic Acid (BCA) protein Assay (Thermo Scientific, Rockford, USA). An internal standard control was prepared by pooling equal amount of protein from all samples used in the study. Prior to peptide labelling, protein reduction, alkylation, precipitation and digestion were performed as previously described [25]. In brief, for each sample and the standard control, 35 µg serum protein was diluted with 0.1 M triethyl ammonium bicarbonate (TEAB, pH 7.8) to a final volume of 50 µl. A volume of 2.5 µl of 200 mM DTT was added for protein reduction, followed by 1 h incubation at 55°C. Samples were alkylated by adding 2.5 µl of 375 mM idoacetamide (IAA) and incubation for 30 min at room temperature in the dark. Subsequently, samples were precipitated by adding 300 µl pre-chilled acetone at -20°C overnight. After centrifugation at 8000 x g for 10 min at 4°C, 35 µg protein pellets were reconstituted in 50 µl of 0.1 M TEAB and digested with trypsin (1:40, w/w) at 37°C overnight.

Peptide Labelling

Tryptic peptides were labelled with TMT (Thermo Scientific), by adding 18 µl of the TMT labelling reagent to each digested sample and incubating for 1 h at room temperature. The internal standard was labelled with the TMT *m/z* 126 isobaric tag while the remaining 5 tags were allocated randomly to the samples. Labelling reaction was quenched by adding 8 µl of 5% hydroxylamine HCl solution (w/w) to the mixture to react for 15 min. For each experiment, equal amounts of 6 different TMT-modified samples (including an internal standard) were combined into the new tube as a final sample. The final samples were aliquoted, dried and stored at -80°C for further analysis.

Liquid Chromatography-Mass Spectrometry (LC-MS)/MS

LC-MS/MS analysis of TMT-labelled peptides was performed for protein identification and quantification. Dried TMT-labelled peptides were reconstituted in buffer A (2% ACN in 0.1 % FA) and an amount of 1 µg was loaded on the trapping column (C18 PepMap100, 5 µm, 100 A, 300 µm x 5 mm) using Dionex Ultimate 3000 RSLS nano flow system (Dionex). All peptides were separated using PepMap™ RSLC C18 50 cm x 75 µm ID column with linear gradient 5-35% buffer B (0.1% FA in 80% ACN) over 120 min at a flow rate of 300 nl/min. Eluate from the column was introduced in Thermo Scientific Q Exactive Plus mass spectrometer by Nanospray Flex ion source and SilicaTip emitter (New Objective). The ionisation voltage was set to 1.9 kV and the ion transfer tube temperature at 250 °C. MS was operating in positive ion mode using FT HCD MS2. Full scan FTMS spectra were acquired in range from *m/z* 350.0 to 1900.0 with a resolution of 70000, AGC target value of 1 x 106 and the maximum injection time 110 ms. Eight most intense peaks from MS spectrum were selected for fragmentation, excluding the ions with the unassigned charge state, as well as +1 and more than +7. The HCD MS/MS scan was fixed to start from *m/z* 100.00 with a resolution of 17500 using MS2 AGC target of 5 x 104. The step collision energy was set to 25, 35 and 40% NCE, respectively. An isolation window of ± 1.6 Da was applied to isolate precursor ions with dynamic exclusion of 30 s.

MS Data Analysis

Thermo raw files were used for SEQUEST search against National Centre for Biotechnology Information (NCBI) database mining *Felis catus* fasta files (downloaded from NCBI database on 15/09/2017, 44239 sequences), using Proteome Discoverer v2.0. HCD tandem mass spectra were used for protein identification, and TMT-sixplex relative quantification was performed based on reporter ion intensity ratio. The following setting was used: carbamidomethylation (C) peptide modification, oxidation (M) and TMT sixplex (peptide N-terminus, K) dynamic

modification; one trypsin missed cleavage site; precursor tolerance and ion fragment tolerance were set at 10 ppm and 0.05 Da, respectively. At least two unique peptides and false discovery rate (FDR) less than 5% were required for reporting identified proteins.

Proteomic Data Validation Using Western Blot

Prothrombin and tetranectin were selected for the validation based on biological significance, research interest and antibody availability. Feline serum samples were diluted 5-fold with Tris-HCl 50 mM pH 7.5/0.05% SDS and supplemented with 5 X Laemmli buffer {87.5mM Tris-HCl pH 6.8, 45% Glycerol (v/v), SDS 0.5% (m/v), 0.01 % Bromophenol blue (m/v) and 12.5% β-mercaptoethanol (v/v)}, then heated for 10 min at 70°C. Samples were subsequently loaded onto an SDS PAGE gel (4% acrylamide-bisacrylamide stacking gel and 10% acrylamide-bisacrylamide resolving gel), subjected to electrophoresis for 10 min at 90 V followed by 60 min at 120 V. When electrophoresis stopped, wet blotting was performed on Polyvinylidene Flouride (PVDF) membranes (GE-Healthcare, IL, USA) for 2 h at 4 °C and 150 mA intensity. After blotting, membranes were blocked for 1 h using 0.2% I-block^{TM} Protein-Based Blocking Reagent (Thermo Fisher Scientific, Waltham, MA, USA) prepared in TBST {TBS containing 0.1 % Tween-20 (Sigma-Aldrich, Taufkirchen, Germany)} at room temperature on a shaker.

Anti-rat prothrombin and anti-human tetranectin rabbit antibodies (ab113431 and ab202134, Abcam, Cambridge, UK) were diluted into 1:1000 and 1:600 respectively with 0.2% I-block^{TM} Protein-Based Blocking Reagent/TBST buffer. After 1 h incubation at room temperature on a shaker, membranes were washed 3 times with TBST for 5 min then incubated with a secondary antibody (mouse anti-rabbit 1:5000 dilution, Santa Cruz Biotechnology, TX, USA) in 0.2% i-block/TBST for 1 h at room temperature on a shaker. Subsequently, membranes were washed 3 times with TBST for 5 min, then dried and covered with luminol reagent (Santa Cruz Biotechnology, TX, USA) to react for 5 min at room temperature. Chemiluminescence imaging was performed in an Odyssey chemiluminescence and fluorescence imager (LI-COR

Biosciences, Lincoln, NE, USA). Protein blots were stained with Ponceau S solution (Sigma-Aldrich, Taufkirchen, Germany) for obtaining total proteins in each sample, protein band intensities were subsequently quantified using Image J software (National Institutes of Health, USA). For comparing the target protein concentrations in CHF and in the healthy control group, semi-quantification was performed by normalizing the target proteins to total proteins in each sample, then mean values of CHF/healthy control group were calculated.

Statistical Analysis

Prior to statistical analysis, peptides were filtered out if not detected in more than 75% of samples. Subsequently the remaining peptides were grouped at the protein level. Outliers were detected by using boxplots and were excluded for statistical analysis. Due to non-normal distribution of data, differentially regulated proteins between CHF and healthy controls were analysed with Wilcoxon test, followed by Bonferroni test to adjust p-value. Correlations between relative intensity ratios (i.e. original MS intensities normalized to the internal standard controls) of the differential proteins and clinical variables were determined using Spearman rank test. Following using Shapiro-Wilk tests confirmed data normality distribution, Student t-tests were performed to compare age, weight, echocardiography parameters, NT-proBNP data and protein validation data among the CHF, preclinical cardiomyopathy and the healthy control groups. R Software (version 3.3.1) (R core team) and Microsoft Office Excel (Microsoft, USA) was used for above analyses. Statistical significance was defined as $P < 0.05$.

Bioinformatic Analysis

Followed the statistical analyses, from those proteins differentially regulated, Gene Ontology (GO) analyses were performed using a previously published approach [26] to determine the GO terms for proteins over or under-expressed in CHF (Figure 1). For each GO terms, the full list of proteins/genes/chemicals and their interactions were downloaded from Reactome.org. Following initial analyses, a further GO enrichment was performed to generate a better

interactome for a deeper view of GO terms based on the initial set of markers and their first interactors. Clusterization was performed to group proteins sharing strongest interactions. GO analyses were performed in each protein cluster as well as in the unclustered proteins. Based on similarity of GO terms and their nodes, positions of GO terms were calculated by Cytoscape algorithms. Once the most global overview was obtained, a list of best candidate markers was proposed based on protein interactions with different GO terms.

Results

Study population

Clinical information of the study populations is summarized in Table 1 and Figure 2. There was no significant difference in age among the three cat groups. There were more male cats in all three groups. Serum NT-proBNP clearly differentiated the three groups. Median NT-proBNP concentration was 1500 pmol/l {interquartile range (IQR) 1180-> 1500} in CHF cats; 119 pmol/l (IQR 69-254) in preclinical cats and < 24 pmol/l in the healthy controls. There was no further clinical data available for the healthy controls. Body weight was not significantly different between the CHF and preclinical cats. LA diameter and LA/Ao ratios were higher in the CHF cats compared to the preclinical cats ($P < 0.001$). There no differences observed in left ventricle echo variables i.e. LVFWd, IVSd and LV FS between the CHF and preclinical groups. Comorbidities recorded in CHF cats were: stable diabetes mellitus (n = 1), suspected abdominal carcinomatosis (n = 1), mild to moderate anaemia (n = 2); and in the preclinical cats were: chronic cervical mass (n = 1) and eosinophilia from unknown origin (n = 1).

All CHF cats were in International Small Animal Cardiac Health Council (ISACHC) Classification IIIa (n = 7) or IIIb (n = 8), with various clinical presentations including pleural effusion (n = 11), pulmonary oedema (n = 8), pericardial effusion (n = 3) and ascites (n = 3). They received different combinations of the following cardiac medications: furosemide, torasemide, pimobendan, benazepril, spironolactone, clopidogrel, aspirin and diltiazem. Eight

cats in CHF were at risk of thrombotic events, 7 had SEC and 1 had an intra-cardiac thrombus. The following arrhythmias were identified in the CHF population: ventricular premature complex (n = 9); atrial fibrillation (n = 3); anterior fascicular block (n = 2); atrial premature complex (n = 1) and ventricular tachycardia (n = 1). There were no arrhythmias noted in the preclinical group.

Serum proteome profile comparison in CHF, healthy control and preclinical cats

Initially 439 peptides were detected by the TMT mass spectrometry and the LC-MS/MS spectra from the healthy and CHF feline groups have been archived by submitting to PRIDE archive via ProteomeXchange Consortium via the PRIDE [27] partner repository with the dataset identifier PXD017761. After eliminating those peptides with substantial undetected data, 271 peptides remained. After applying the filtering criteria i.e. unique peptide number ≥ 2 and FDR $< 5\%$, 116 different proteins were identified (Supplementary Table 1) and analysed statistically.

In total 16 serum proteins were found significantly altered ($P < 0.05$) in the feline CHF group compared to the healthy controls. Among them, 11 proteins were increased in the CHF group (Table 2), including serine protease inhibitors (SERPINs) {i.e. α-1 antitrypsin (A1A), antithrombin III (ATIII), α-2 antiplasmin (A2AP) and C1 inhibitor}, apolipoproteins {i.e. apolipoprotein A-II (APOA2) and apolipoprotein M (APOM)}, carboxypeptidase N subunit 1 (CPN1), ceruloplasmin, platelet factor 4 (PF4), prothrombin and thymosin β-4 (TMSB4X). Five protein concentrations were significantly lower in the feline CHF group compared to the healthy controls (Table 3), these were immunoglobins {i.e. immunoglobulin heavy chain (IgHC), immunoglobulin kappa (IgK) and immunoglobulin lambda (IgL)}, complement C8 gamma chain (C8G) and tetranectin. Protein relative intensities from individual cats are summarized in Supplementary Table 2.

Although not statistically significant, another 11 proteins showed trends of increase or decrease in the CHF group compared to healthy controls ($P < 0.1$). These were used in later bioinformatic pathway analysis in addition to the significantly regulated proteins. There were 6 proteins showed an up-regulated trend, including inter-alpha-trypsin inhibitor heavy chain 4 (ITIH4) ($P = 0.092$), complement C4-A ($P = 0.07$), vitamin D-binding protein ($P = 0.062$), apolipoprotein A-I (APOA1) ($P = 0.074$), apolipoprotein C-I (APOC1) ($P = 0.054$) and thrombospondin 1 (THBS1) ($P = 0.052$). Five proteins showed a decrease trend, including actin beta (ACTB) ($P = 0.086$), immunoglobin gamma-2 (IgG2) heavy chain ($P = 0.098$), inter-alpha-trypsin inhibitor heavy chain 1 H (ITIH1) ($P = 0.060$), immunoglobulin J chain (JCHAIN) ($P = 0.097$) and vitronectin ($P = 0.081$).

Among all the altered proteins, tetranectin was significantly higher in preclinical cardiomyopathy compared to healthy controls and CHF cats ($P < 0.05$). For the rest of the proteins, the concentrations in the preclinical group were either similar to healthy control or CHF, or in between the two groups.

Validation of proteomic results by Western Blot

Serum prothrombin was detected by Western blot in CHF and healthy control cats ($n = 10$ respectively) (Figure 3). Two distinct protein bands were detected at 72 kDa and 24 kDa respectively. Semi-quantification results showed prothrombin level was higher in CHF group compared to healthy controls, although the change was not statistically significant. Variable prothrombin levels were seen in each group. On tetranectin immunoblots, weak distinct bands between 15 kDa and 24 kDa were detected, similar to prothrombin, overall tetranectin concentrations were higher in the healthy control samples than in the CHF group, however, the concentrations varied within each group (data not shown).

Correlations of the differentially regulated proteins and clinical variables in cats with cardiomyopathy

In the CHF population, proteins that were altered in CHF showed significant correlations with echo parameters ($P < 0.05$) (Table 4-A). The SERPINs ATIII, A2AP and C1 inhibitor were found positively correlated with LV FS, and ATIII showed a moderate positive correlation with LVFWd. APOA2 also showed a moderate positive correlation with LVFWd. The presence of SEC was found positively correlated with IGHC.

When the cats with preclinical cardiomyopathy were included (Table 4-B), ATIII and APOA2 remained positively correlated with LVFWd ($P < 0.05$). SERPIN A1A showed significant positive correlation with NT-proBNP in the combined groups cardiomyopathy population ($r = 0.69$, $P < 0.01$). Positive correlations were also identified between APOM and LA/Ao, PF4 and LV FS, A2AP, C8G and LVFWd ($P < 0.05$). ATIII and A2AP showed negative correlations with body weight ($P < 0.05$).

GO pathway analysis

Due to the lack of available database in feline species, *Homo sapiens* ortholog was used for bioinformatics analysis. From those proteins which showed differential serum concentrations in feline CHF compared to normal cats ($P < 0.1$), initial GO analysis revealed 4 over-expressed pathways (GO terms) based on the Reactome pathway database (Reactome.org). These were platelet degranulation, complement cascade, formation of fibrin clot and plasma lipoprotein assembly. The terms were all identified from the up-regulated proteins in CHF, no term was detected in the down-regulated proteins due to insufficient protein number. Subsequent enrichment generated a network which included 473 protein nodes and 5433 interactions. Ten clusters and one unclustered protein group were identified in the enriched network, GO analysis of the clusters revealed 19 GO terms. For each of the 19 GO terms, a full list of proteins/genes/chemicals and their interactions were identified in Reactome database, which

generated a complete network. The final GO analysis on the global network detected 23 GO terms (Figure 4). Cytoscape calculation revealed three most dominant GO terms groups (Figure 5), which were (1) extracellular matrix (ECM) organization and metabolism; (2) plasma vesicles and transport and (3) immune/wound system against altered cells. For the proteins showing differential concentrations in CHF and the proteins interacting with GO term in the complete network, protein cell location was examined, and most proteins were in the extracellular space. Additionally, 264 genes had at least 2 degree of interaction with the GO terms, 21 genes had at least 4 degree of GO term interaction and they were considered as best potential candidate gene markers based on prediction. They were FURIN, ELANE, FN1, VTN, A2M, COL18A, COL7A1, FGA, FGB, FGG, HSPG2, ITGAV, ITGB1, ITGB3, PLG, PROS1, SERPING1, TGFβ-1, TGFβ-2, TGFβ-3 and VWF.

Discussion

Current results showed that serum protein profile alters in cats with CHF due to primary cardiomyopathy compared to healthy controls. By using proteomic techniques, we detected 27 proteins differentially regulated in feline CHF. They were associated with inflammation, coagulation, lipid metabolism and immune system. Some of these proteins such as the SERPINs were significantly correlated with clinical cardiac variables in cats with CHF and cardiomyopathy, supporting their involvement in the disease. Others such as TMSB4X and tetranectin may be involved in regulating myocardial remodeling features in feline cardiomyopathy including myocardial disarray, interstitial fibrosis and angiogenesis [9, 28], based on previous studies of these markers in other species. Bioinformatic analysis provided a global view of the deregulated proteins and relevant biological pathways were primarily associated with ECM remodeling and metabolism, innate immunity and plasma vesicles and transport.

The complex relationship between heart failure (HF) and inflammation has been postulated to be bidirectional in humans. Systemic or local inflammation can trigger pathological remodeling in HF including cardiomyocyte apoptosis, maladaptive hypertrophy, endothelial dysfunction and ECM remodeling, whilst a decompensated heart in return can cause inflammation by locally releasing pro-inflammatory cytokines or through haemodynamic response [7, 29]. Coagulation, fibrinolysis and complement cascades play a key role in circulatory inflammatory response in cardiovascular disease [30, 31]. Coagulation and fibrinolytic activation has been reported both in feline and human cardiomyopathy [12-15], while complement activation has been observed in human HF and canine dilated cardiomyopathy [32-34] but not be confirmed in feline CHF or cardiomyopathy.

In this study, most of the differentially regulated proteins in CHF cats have either pro- or anti-inflammatory properties, which are mainly involved in coagulation, fibrinolytic and complement pathways. Most of the proteins showed small changes in the mean value comparison, which is likely affected by small sample size and presence of abundant proteins. However, the results still supported that some of those proteins carry biomarker potential for feline CHF and cardiomyopathy. Validation of the proteomic results is an important part of the interpretation of results with Western blotting being a useful method for this purpose. However for a species such as the cat, where antibodies to feline antigens are not generally available, this can be problematic. In this study the Western blot using antibody to rat prothrombin was able to demonstrate the validity of the proteomic result for this protein. The Western blot using antibody to human tetranectin gave only faint banding and a feline specific antibody would be needed for clearer results. Similarly, a human A1A antibody (Cat No. 702047, Invitrogen) has also been tested in the Western blot, but was demonstrated not suitable for feline samples.

In this study over 50% of recruited CHF cats were in a pro-thrombotic state which was demonstrated by echocardiography i.e. SEC. Although in the correlation analysis, none of the

upregulated coagulation proteins identified were associated with the incidence of SEC, interestingly, ATIII, A2AP and PF4 showed significant positive correlations with LVFWd and/or LV FS, suggesting these markers might be related to left ventricular myocardial remodeling and function in feline cardiomyopathy. The roles of these coagulation proteins in feline CHF are yet to be defined, but they could represent interesting candidates for future research.

C1-inhibitor is an extracellular protease inhibitor known to have anti-inflammatory effect by preventing the initial step of complement activation [35]. In this study, it had 1.4 fold increase in CHF cats compared to healthy controls. Its coding gene SERPING1 was also identified as a candidate biomarker with high probability in the bioinformatics prediction. Similar to ATIII and A2AP, C1 inhibitor was also positively correlated with LV FS in CHF cats, which indicated the SERPINs may be associated with cardiac systolic function in CHF cats. Previously, C1-inhibitor was found to have a cardioprotective effect in a feline myocardial ischemia and reperfusion model [36]. By locally inhibiting neutrophil-endothelial interaction, it preserved endothelial function. In humans, localized increase of C1 inhibitor was observed in damaged cardiomyocyte following acute myocardial injury, which suggested its endogenous regulation of myocardial inflammation [37]. C1 inhibitor increase was also seen in acute HF in humans, the authors postulated it worked as an acute phase protein reflecting the inflammatory response [38].

Alpha-1 antitrypsin has an anti-inflammatory effect in lung tissue by inhibiting neutrophil elastase and plasminogen activators to avoid excessive tissue damage [39]. Serum A1A was significantly higher in CHF cats, and this finding was consistent with a published human study in which circulating A1A was found increased in HF patients [40]. Interestingly, this proteinase inhibitor also showed close positive correlation to serum NT-proBNP level in cats with cardiomyopathy. Similarly, in human HF, A1A was found significantly correlated with BNP level and its increase was correlated with HF progression [41]. It is known A1A oxidation

occurs in human HF regardless of primary aetiology, and due to the loss of its elastase inhibition ability, the oxidized A1A can cause endothelial dysfunction and thus cell death [42]. It was proposed to be an oxidative-stress related biomarker in HF and also a potential therapeutic target for the condition [43].

Ceruloplasmin is a copper binding protein which increases in the acute phase response [44]. In the current study, it increased in CHF cats compared to healthy controls. This finding was consistent with the results from our previous study using a different methodology and a larger study population [45]. In human HF patients, higher ceruloplasmin level is associated with poorer clinical outcomes [46]. It was found to affect ferroxidase I activity and nitric oxide plasma concentration, and this may explain its pathogenetic role in HF [47, 48].

Thymosin β-4 had a 2.8-fold increase in CHF cats compared to the healthy control, although it was only detectable in 33% CHF cats and in 46% healthy controls. TMSB4X is known to be anti-inflammatory and it promotes tissue regeneration in the wound healing process [49]. In human fetal heart, TMSB4X was detected in vascular endothelium, endocardium and epicardium, suggesting its involvement in cardiac tissue development [50]. Other evidence has suggested TMSB4X has a cardiac-protective property and is involved in cardiac repair via interaction with vascular endothelium [51]. Clinical trials with TMSB4X have been conducted for treating human myocardial infarction [52]. The role of TMSB4X in feline CHF and cardiomyopathy is not clear yet, however, given the fact that adverse myocardial remodeling is common in feline cardiomyopathy and CHF, TMSB4X may be a potential therapeutic candidate for repairing myocardial damage in cats.

Tetranectin is a glycoprotein binding to the kringle 4 part of plasminogen. It was postulated to be a regulator of fibrinolysis and proteolysis in connective tissue [53, 54]. In this study, tetranectin was a distinct marker which was highest in preclinical cats compared to the other

groups, but lowest in the CHF cats. The role of tetranectin in cardiovascular disease is not clear yet. In a coronary artery disease study, while serum tetranectin decreased, a local increase of tetranectin was found in the atherosclerotic arteries and correlated to the disease severity [55]. The systemic reduction of tetranectin might have been a result of endogenous recruitment to atherosclerosis lesions. It could be hypothesized that tetranectin and plasminogen activation may have a direct impact on myocardial remodeling during cardiomyopathy and CHF. In a multi-biomarker risk stratification study, lower circulating tetranectin was found to be associated with adverse events in human cardiovascular disease [56]. This marker has excellent potential as a prognostic marker for feline cardiomyopathy cases and warrants further investigation.

In previous studies, leptin and fatty acid profile has been found altered in feline hypertrophic cardiomyopathy (HCM) [57, 58]. In our study, apolipoproteins APOA2, APOM, APOA1 and APOC1 were found increased in CHF cats compared to healthy controls, which supports altered lipid metabolism in CHF in feline cardiac disease. Generally, the role of apolipoproteins in cardiovascular disease has been considered beneficial. This is possibly due to their association with high density lipoprotein or inflammation based on previous *in vitro* and *in vivo* studies in humans [59-62]. More recently, increased APOM was found to be associated with better survival in human CHF, which was postulated possibly due to its cardiomyocyte protection effect through the Akt pathway [63]. In the future, a more comprehensive profile of lipoprotein metabolism could be evaluated in feline cardiomyopathy, such a profile would include lipoprotein-cholesterol concentration, apolipoprotein concentration and the activity of the key enzymes involved in lipoprotein metabolism [64].

The role of innate immunity in HF has attracted considerable attention recently, and immunomodulation has been considered as a therapeutic approach in regulating inflammatory cytokines in HF [65]. In a human study using intravenous immunoglobin in HF management,

a positive outcome was achieved for patients on long term supplement [66]. In our study, lower immunoglobulins were observed in the CHF group compared to the healthy controls, which may indicate an 'overuse' of circulating immunoglobin in the disease process. It should be noted that the commercial healthy control serums were collected for antibody titre purpose post rabies vaccination, which might be another explanation for the higher immunoglobin levels in healthy controls compared to the CHF group.

Bioinformatics analysis is powerful for biological pathway identification from proteomic data. In the current study, the final GO pathway analysis suggests ECM remodeling is a major event in feline CHF due to cardiomyopathy, which is consistent with previous studies from others [10, 57]. Aupperle and colleagues detected significantly increased concentrations of transforming growth factor beta 2 (TGF-β2), matrix metalloproteinase (MMP)-2 and tissue inhibitor of matrix metalloproteinase (TIMP)-2 in diseased feline myocardium [10]. In another study, TIMP-1 and MMP-3 transcripts were found significantly higher in hearts of HCM cats compared to the controls [28]. The same markers were also identified in our biomarker prediction list. Together with the two other significant aspects identified: 'immune/wound against altered cells' and 'plasma vesicle and transport', it could be postulated that an injury-repair process may occur in in cats with advanced cardiomyopathy. Recently, inflammatory cell infiltration has been associated with ECM remodeling in feline cardiomyopathy [9, 28], for example, local macrophages have been proposed to be a driving factor for myocardial remodeling in feline HCM, and together with damaged cardiomyocytes, they may contribute to myocardial interstitial fibrosis by releasing inflammatory and profibrotic cytokines [28]. In this study, however, it remains unknown whether the systemic increase of ECM remodeling profile is a cause or consequence of local cardiac changes.

There are several limitations of this study, but it does highlight the potential for use of proteomics to study feline disease. Firstly, this is a pilot biomarker study at discovery phase,

future work would be necessary for further verification and validation. Current Western blot validation was limited due to antibody availability and optimization, more optimal validation methods could be explored in future studies. Using a recombinant feline protein as a positive control in future antibody-based validation would be helpful to establish the cross-reaction information when using antibodies for other species. To by-pass the use of antibodies, bioanalytical validation using selected reaction monitoring (SRM)/multiple reaction monitoring (MRM) targeted proteomic approaches could be used. However, unlike antibody-based methods, it has not been robustly applied for clinical application due to sensitivity issues [67, 68]. In this study, validation tests were focused on the protein markers derived from the initial proteomic results, biomarkers with high degree prediction from the GO analysis would also be interesting to investigate and validate in future work. Regarding the study populations, only a small number of cats were recruited, particularly the preclinical group. For the clinically recruited cats with cardiomyopathy, comorbidities and therapeutic effects were not assessed, which were possible confounders of current results; LV FS was assessed in the current study as a left ventricular systolic function marker, however, it should be noted that it is not ideal especially if left ventricular hypertrophy is present. For the healthy controls, clinical information was limited, and the occult cardiac disease was screened by cardiac biomarker NT-proBNP rather than echocardiography. For the proteomic techniques, current setting of FDR was 5%, which may compromise the numbers of biomarker detected in the study. The dynamic protein range could be an issue as we did not deplete the serum abundant proteins such as albumin in this study, which would lose low abundance proteins such as classic cardiac biomarkers NT-proBNP and troponin [69]. Future work combined with high abundance protein depletion would be necessary for detecting low abundance protein targets. Survival analysis was not performed in the current study, it would be interesting to investigate correlations

between the identified biomarkers and event of death in future larger scale study, to determine whether these biomarkers can predict the risk of death in feline CHF and cardiomyopathy.

Conclusion

Overall, the results suggest systemic inflammation, coagulation protein changes, innate immunity and ECM remodeling occur in feline CHF and there are complex links between them. These findings bridged the gap between current understanding of pathogenesis in human and feline HF due to cardiomyopathy. The novel biomarkers identified in this study warrant further investigation for establishing their roles in disease pathogenesis and clinical use.

Supplementary data

The LC-MS/MS spectra from the healthy and CHF feline groups have been archived by submitting to PRIDE archive via ProteomeXchange with the dataset identifier PXD017761. Additionally, the full list of proteins identified in the feline plasma samples are provided in the supplementary data.

PRIDE repository Reviewer account details:

Username: reviewer25571@ebi.ac.uk

Password: uU7Gtrd6

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Table 1: Clinical information of the study population.

	CHF (n = 15)	**PrC(n = 5)**	**Nor (n = 15)**
Age (years)	6.1 (IQR 4.9-11.2)	8 (IQR 4.6-11.5)	5.3 (IQR 3.5-5.9)
Sex	Female (n = 4) Male (n = 11)	Female (n = 1) Male (n = 4)	Female (n = 7) Male (n = 8)
Breed	DSH (n = 13); Ragdoll (n = 1); Sphinx variant (n = 1)	DSH (n = 4); BSH (n = 1)	
CM Diagnosis	HCM (n = 5); RCM (n = 3); UCM (n = 7)	HCM (n = 5)	
Weight (kg)	4.5 (IQR 3.6-4.8)	5.2 (IQR 5.2-7.2)	
LA Diameter (mm)	20 (IQR 17-22.5)	13 (IQR 12-15)	
LA/AO ratio	2.5 (IQR 1.9-2.6)	1.34 (IQR 1.27-1.41)	
LVFWd (mm)	6.5 (IQR 5.3-7.4)	6.21 (IQR 4.34-6.29)	
IVSd (mm)	5.7 (IQR 4.7-7.5)	5.32 (IQR 5.14-8.1)	
LV FS%	27 (IQR 22-40)	52 (IQR 34-54)	

For healthy controls, only age and sex information were available. CHF, congestive heart failure; PrC, preclinical; Nor, normal healthy controls; IQR, interquartile range; DSH, domestic short hair; BSH, British short hair; CM, cardiomyopathy; RCM, restricted cardiomyopathy; UCM, unclassified

cardiomyopathy; LA, left atrial; LA/AO, left atrial to aortic root ratio; LVFWd, left ventricular free wall thickness at end diastole; IVSd, interventricular septum thickness at end diastole; LV FS, left ventricular fraction shortening.

Table 2: Significantly up-regulated proteins in CHF cats compared to healthy controls (n = 15 in each group)

Accession	Protein Description	Gene ID	Protein Sequence Coverage	Unique Peptide Number	Fold Change	Detectable Samples	P-value
755822186	A1A	SERPIN A1	41.1	16	1.1	30	0.024
410958740	APOM	APOM	23.4	5	1.1	30	0.038
755776692	CPN1	CPN1	7.2	3	1.3	22	0.048
410985907	AT III	SERPIN C1	56.3	23	1.1	30	0.028
410973663	prothrombin	F2	48.2	21	1.1	30	0.002
1126453607	Ceruloplasmin	CP	41.4	36	1.1	30	0.042
1126474650	A2AP	SERPIN F2	21.7	9	1.1	30	0.042
755812005	TMSB 4X	TMSB 4X	29.5	2	2.8	12	0.008
410986627	APOA2	APOA2	60	6	1.3	30	0.007
755722705	PF4	PF4	16.3	2	1.3	30	0.012
587002983	C1 inhibitor	SERPING1	17.6	8	1.4	30	0.001

CHF, congestive heart failure; A1A, α-1 antitrypsin; SERPIN A1, serine protease inhibitor family A member 1; APOM, apolipoprotein M; CPN1, carboxypeptidase N subunit 1; AT III, antithrombin III; SERPIN C1, serine protease inhibitor family C member 1; F2, coagulation factor II; CP, ceruloplasmin; A2AP, α-2 antiplasmin; SERPIN F2, serine protease inhibitor family F member 2; TMSB 4X, thymosin β-4; APOA2, apolipoprotein A-II; PF4, platelet factor 4; SERPIN G1, serine protease inhibitor family G member 1.

Table 3: Significantly down-regulated proteins in CHF cats compared to healthy controls (n = 15 in each group)

Accession	Protein Description	Gene ID	Protein Sequence Coverage	Unique Peptide Number	Fold Change	Detectable Samples	P-value
410950994	tetranectin	CLEC3B	28.2	5	0.9	26	0.045
325651605	IgHC variable region subgroup 3	IGHC	42.6	4	0.8	30	0.032
6456731	IgK light chain	IGK	44.2	8	0.8	30	0.010
1126466567	IgL-like polypeptide 5	IGL	49.8	8	0.8	30	0.012
587009725	C8G	C8G	36.3	6	0.9	30	0.021

CHF, congestive heart failure; CLEC3B, C-Type Lectin Domain Family 3 Member B; IGHC, immunoglobulin heavy chain; IGK, immunoglobulin kappa; IGL, immunoglobulin lambda; C8G, complement C8 gamma chain.

Table 4-A Significant correlations between clinical variables and protein biomarkers in CHF population only (n = 15).

	ATIII	A2AP	APOA2	C1 inhibitor	IGHC
LVFWd	0.56*		0.63*		
LV_FS%	0.67***	0.55*		0.65**	
SEC					0.65**

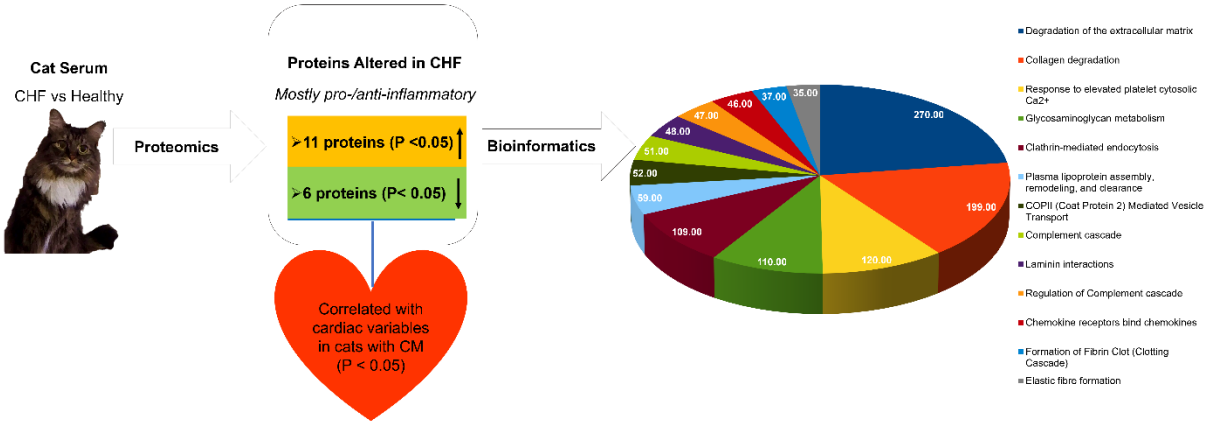
ATIII, antithrombin III; A2AP, α-2 antiplasmin; APOA2, apolipoprotein A-II; IGHC, immunoglobulin heavy chain variable region subgroup 3; LVFWd, left ventricular free wall thickness at end diastole; LV FS, left ventricular fraction shortening; SEC, spontaneous echo contrast. *indicates statistical significance with P value <0.05; ** indicates P value <0.01. Spearman test was used to evaluate statistical significance.

Table 4-B Significant correlations between clinical variables and protein biomarkers in combined CHF and preclinical cardiomyopathy groups (N = 20).

	A1A	APOM	ATIII	A2AP	APOA2	PF4	C8G
BW			-0.48*	-0.45*			
NT-proBNP	0.69**						
LA/Ao		0.49*					
LVFWd			0.47*	0.47*	0.61**		-0.51*
LV_FS%						0.49*	

A1A, α-1 antitrypsin; APOM, apolipoprotein M; ATIII, antithrombin III; A2AP, α-2 antiplasmin; PF4, platelet factor 4; C8G, complement C8 gamma chain; BW, body weight; NT-proBNP, N-terminal-pro brain natriuretic peptide; LA/Ao, left atrial to aortic root ratio; LVFWd, left ventricular free wall thickness at end diastole; LV FS, left ventricular fraction shortening.*indicates statistical significance with P value <0.05; ** indicates P value<0.01. Spearman test was used to evaluate statistical significance.

Graphic Abstract



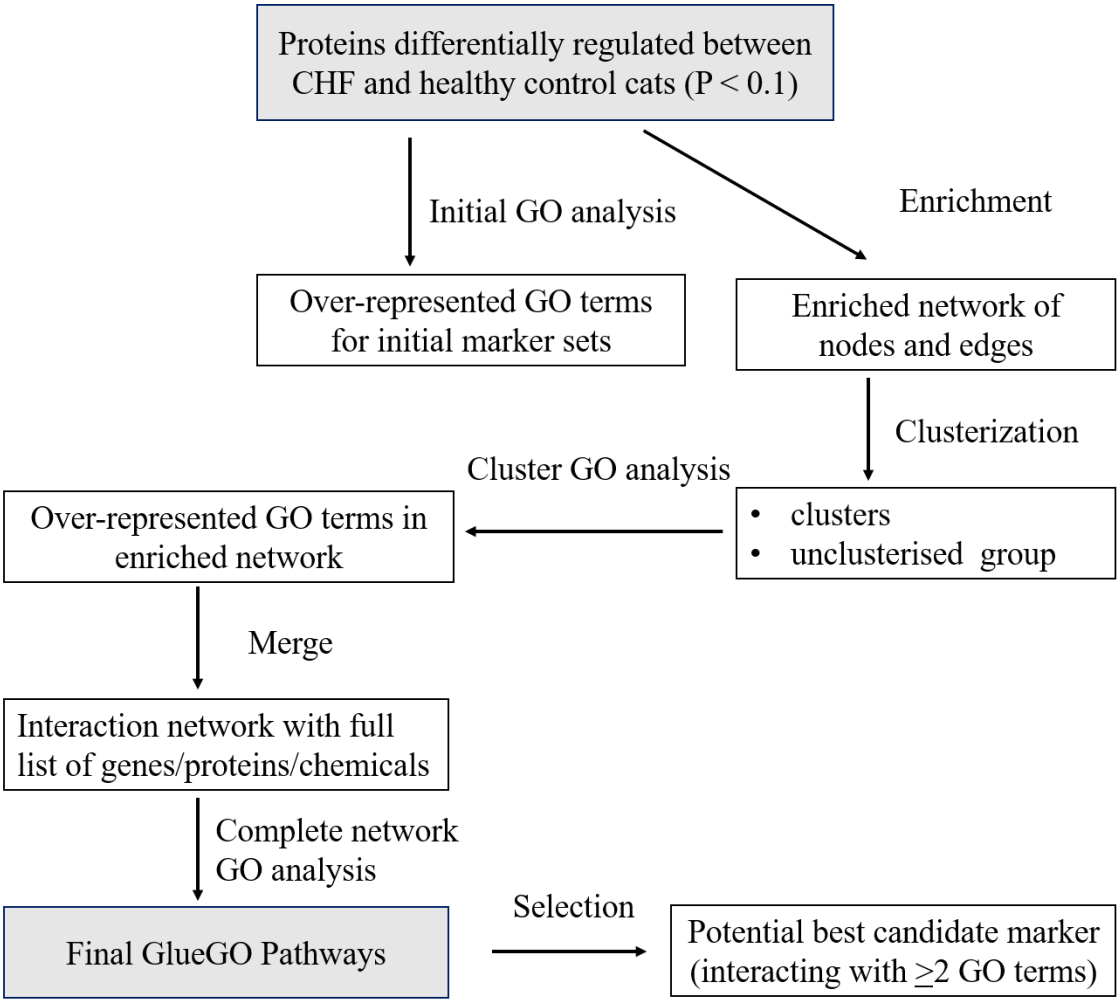


Figure 1. Schematic diagram of gene ontology (GO) analyses.

GO analyses were performed based on the differentially regulated proteins in CHF. Subsequent enrichments were performed to get the most global view of the network, additional candidate biomarkers were further predicted based on the final network.

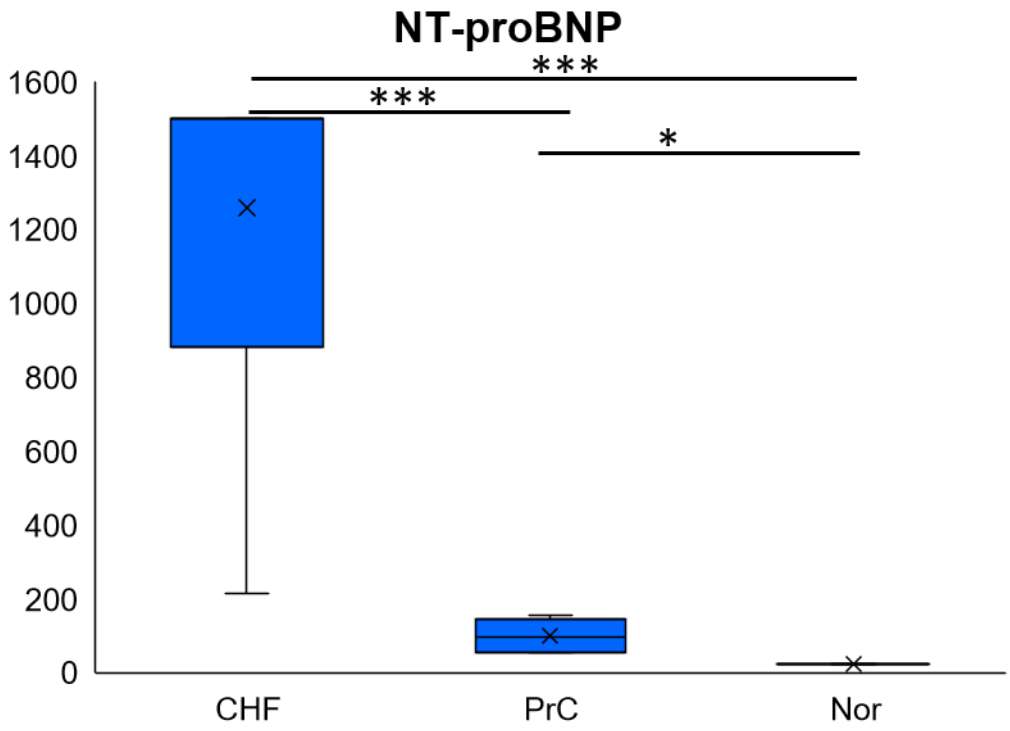


Figure 2. Serum NT-proBNP in three cat groups.

NT-proBNP clearly differentiated the CHF cats (n = 11) from preclinical cats (PrC) (n = 5) and healthy controls (Nor) (n = 15). Four CHF cats had no NT-proBNP measurements due to a sample limitation. *** indicates a statistical significance $P < 0.001$; * indicates a statistical significance $P < 0.05$.

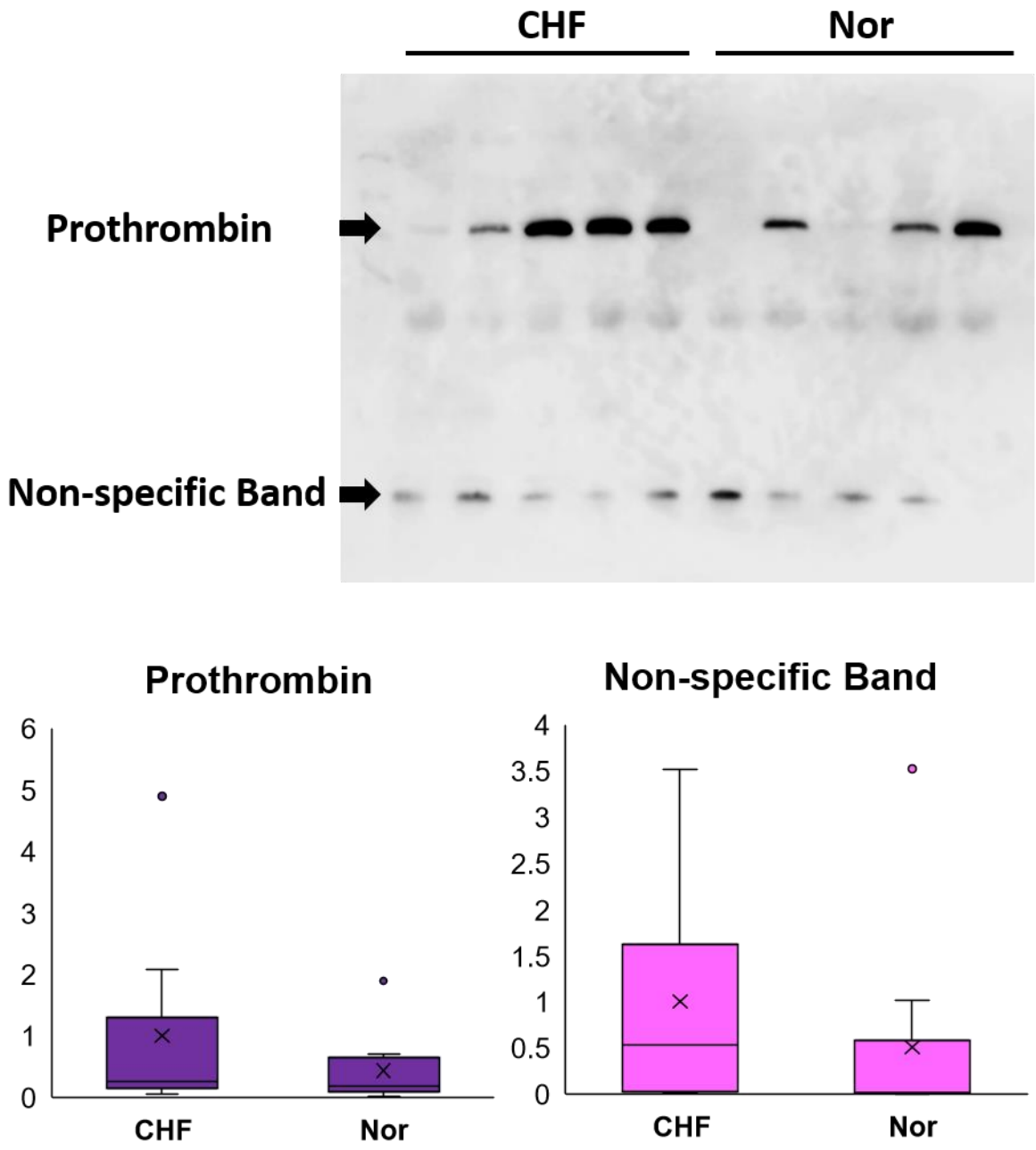


Figure 3. Serum prothrombin detection in CHF and healthy controls by using Western blot. Prothrombin was detected in CHF and healthy control (Nor) feline serum samples (n=10 respectively), examples of protein band pattern (n=5 for each group respectively) were demonstrated in protein blot. The blot showed distinct prothrombin band (size around 72kD, consistent with predicted band on datasheet) and a non-specific band (size around 24kD, suspected to be a cleaved protein product). After normalized with Ponceau S total protein staining, the semi-quantification results showed no statistical significance in prothrombin

concentrations between the two groups (P>0.01). Although median levels of prothrombin and its possible cleaved product were higher in CHF group than in the control group (box plots).

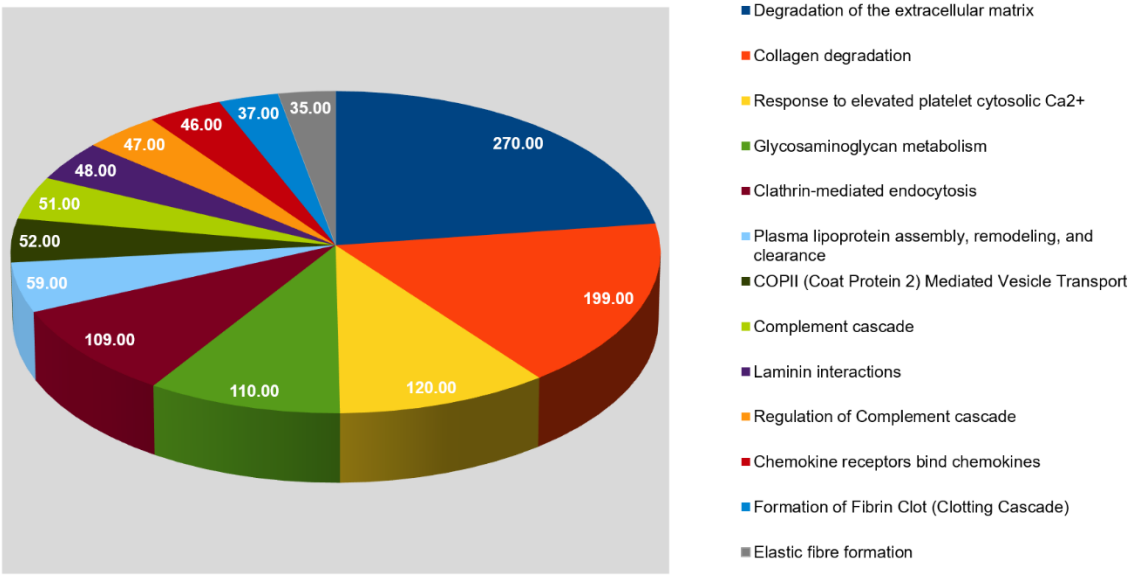


Figure 4. Final GO Enrichment analysis.

The complete network analysis identified 13 most relevant functional profiles in CHF cats, mostly associated with extracellular matrix remodeling. Based on GO results, 246 potential candidate biomarkers were predicted with 2-6 degree (Supplementary Excel File 1).

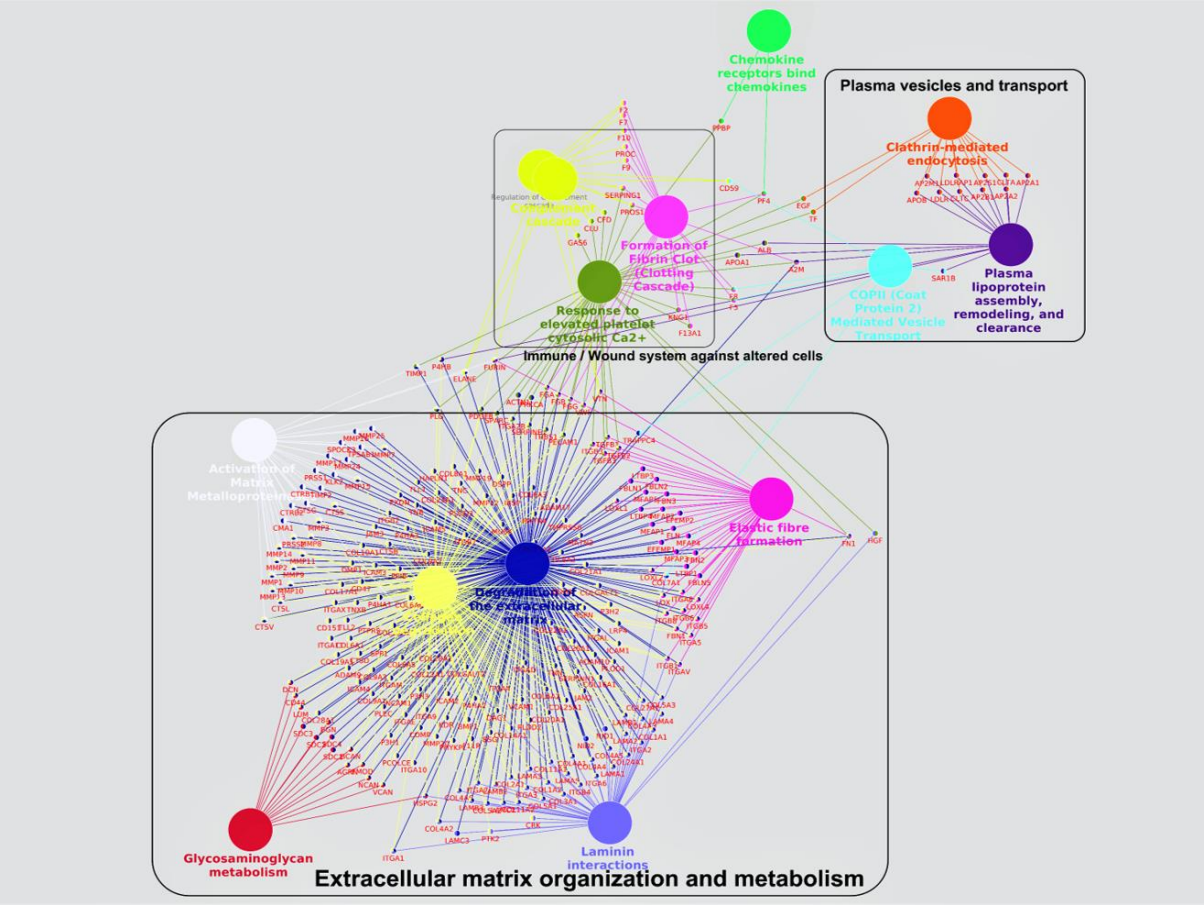


Figure 5. Complete GO network involved in feline CHF.

Supplementary Table 1. Full list of 116 identified proteins in cat serum

Accession	Protein Description	Peptide Number	Unique Peptide Number	Protein Sequence Coverage
586997898	PREDICTED: fibronectin isoform X7 [*Felis catus*]	70	70	42.9264
586986149	PREDICTED: complement C4-A [*Felis catus*]	67	67	53.70052
1126436109	PREDICTED: LOW QUALITY PROTEIN: pregnancy zone protein-like [*Felis catus*]	67	67	57.77778
586991840	PREDICTED: alpha-2-macroglobulin [*Felis catus*]	66	61	65.22034
1126406397	PREDICTED: complement C3-like [*Felis catus*]	85	56	67.52513
1126453965	PREDICTED: serotransferrin [*Felis catus*]	53	51	69.20904
410955758	PREDICTED: apolipoprotein B-100 [*Felis catus*]	44	44	11.14614
1126453607	PREDICTED: ceruloplasmin isoform X2 [*Felis catus*]	36	36	41.40481
586987778	PREDICTED: plasminogen [*Felis catus*]	36	36	58.00493
1126406395	PREDICTED: complement C3-like [*Felis catus*]	76	35	62.02532
1126420349	PREDICTED: afamin [*Felis catus*]	31	31	55.57404
586984640	PREDICTED: vitamin D-binding protein [*Felis catus*]	28	28	67.08861
410963161	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H2 [*Felis catus*]	28	28	30.23256
755765069	PREDICTED: inhibitor of carbonic anhydrase-like [*Felis catus*]	30	28	54.44126
755767178	PREDICTED: apolipoprotein A-I [*Felis catus*]	25	25	76.69173

755784610	PREDICTED: complement C5 isoform X1 [*Felis catus*]	25	25	16.07143
587018338	PREDICTED: C4b-binding protein alpha chain [*Felis catus*]	23	23	51.08514
410985907	PREDICTED: antithrombin-III [*Felis catus*]	23	23	56.25
410978975	PREDICTED: LOW QUALITY PROTEIN: gelsolin [*Felis catus*]	23	23	34.14322
755767173	PREDICTED: apolipoprotein A-IV [*Felis catus*]	22	22	66.75393
1126471858	PREDICTED: complement C5-like isoform X1 [*Felis catus*]	22	22	32.89474
312147375	haptoglobin precursor [*Felis catus*]	21	21	45.82133
410973663	PREDICTED: prothrombin [*Felis catus*]	21	21	48.23151
1126489264	PREDICTED: complement factor H isoform X1 [*Felis catus*]	21	21	32.44681
755747228	PREDICTED: pregnancy zone protein [*Felis catus*]	26	21	22.66576
1126467206	PREDICTED: heparin cofactor 2 [*Felis catus*]	21	21	48.58871
410951365	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H1 [*Felis catus*]	21	21	25.82418
410958796	PREDICTED: complement factor B [*Felis catus*]	20	20	33.50717
1126402137	PREDICTED: complement component C7 isoform X3 [*Felis catus*]	19	19	26.90864
410970797	PREDICTED: kininogen-1 isoform X1 [*Felis catus*]	19	19	45.90164

410956438	PREDICTED: clusterin [*Felis catus*]	18	18	43.62416
1126452740	PREDICTED: histidine-rich glycoprotein isoform X2 [*Felis catus*]	17	17	32.28782
586978046	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H3 [*Felis catus*]	17	17	29.62963
755822186	PREDICTED: alpha-1-antitrypsin-like [*Felis catus*]	16	16	41.05505
410973069	PREDICTED: hemopexin [*Felis catus*]	16	16	37.71552
755722459	PREDICTED: complement factor I isoform X3 [*Felis catus*]	15	15	32.89037
1126402180	PREDICTED: complement component C9 [*Felis catus*]	15	15	31.31673
3402547	IgM heavy chain, partial [*Felis catus*]	15	14	46.71202
1126412335	PREDICTED: attractin isoform X1 [*Felis catus*]	14	14	9.567682
1126479244	PREDICTED: alpha-1B-glycoprotein [*Felis catus*]	13	13	45.01845
1126477106	PREDICTED: beta-2-glycoprotein 1 [*Felis catus*]	13	13	46.66667
1351908	RecName: Full=Serum albumin; AltName: Allergen=Fel d 2; Flags: Precursor	68	12	88.48684
1126402123	PREDICTED: complement component C6 [*Felis catus*]	12	12	12.64737
755731552	PREDICTED: vascular non-inflammatory molecule 3 isoform X1 [*Felis catus*]	12	12	30.70866
586999703	PREDICTED: alpha-2-HS-glycoprotein [*Felis catus*]	12	12	51.80055

755796073	PREDICTED: LOW QUALITY PROTEIN: apolipoprotein E [*Felis catus*]	11	11	44.65649
755774684	PREDICTED: angiotensinogen [*Felis catus*]	11	11	26.77824
410980197	PREDICTED: pigment epithelium-derived factor [*Felis catus*]	11	11	32.61391
1126483442	PREDICTED: insulin-like growth factor-binding protein complex acid labile subunit [*Felis catus*]	11	11	18.52941
410952310	PREDICTED: serum paraoxonase/arylesterase 1 [*Felis catus*]	10	10	34.64789
410980361	PREDICTED: vitronectin [*Felis catus*]	10	10	27.99145
1126474650	PREDICTED: alpha-2-antiplasmin isoform X2 [*Felis catus*]	9	9	21.74797
755801251	PREDICTED: zinc-alpha-2-glycoprotein [*Felis catus*]	9	9	36.64384
1126452747	PREDICTED: fetuin-B isoform X2 [*Felis catus*]	9	9	27.82152
1126444019	PREDICTED: complement component C8 alpha chain [*Felis catus*]	9	9	17.28814
290560143	Chain G, Crystal Structure Determination Of Cat (*Felis Silvestris Catus*) Hemoglobin At 2.4 Angstrom Resolution	9	9	88.65248
1126401809	PREDICTED: glutathione peroxidase 3 [*Felis catus*]	9	9	41.15044
1101972906	TPA: globin C1 [*Felis catus*]	8	8	83.09859

587002983	PREDICTED: plasma protease C1 inhibitor [Felis catus]	8	8	17.61252
309951108	protein AMBP precursor [*Felis catus*]	8	8	30.11364
1126441122	PREDICTED: GDH/6PGL endoplasmic bifunctional protein isoform X5 [*Felis catus*]	8	8	10.66499
410970733	PREDICTED: carboxypeptidase N subunit 2 [*Felis catus*]	8	8	16.45338
6456731	immunoglobulin kappa light chain [*Felis catus*]	8	8	44.21488
1126466567	PREDICTED: immunoglobulin lambda-like polypeptide 5 [*Felis catus*]	11	8	49.79253
1126436027	PREDICTED: complement C1r subcomponent isoform X1 [*Felis catus*]	7	7	10.61453
755757807	PREDICTED: extracellular matrix protein 1 isoform X1 [*Felis catus*]	7	7	14.76868
576864479	immunoglobulin G2 heavy chain consant region, partial [*Felis catus*]	11	6	49.85075
1126463686	PREDICTED: hyaluronan-binding protein 2 [*Felis catus*]	6	6	10.79137
1126451434	PREDICTED: vitamin K-dependent protein S isoform X1 [*Felis catus*]	6	6	10.97923
1126444017	PREDICTED: complement component C8 beta chain isoform X2 [*Felis catus*]	6	6	10.49724
410986627	PREDICTED: apolipoprotein A-II isoform X3 [*Felis catus*]	6	6	60

410982722	PREDICTED: apolipoprotein C-II [*Felis catus*]	6	6	70.29703
586976411	PREDICTED: leucine-rich alpha-2-glycoprotein isoform X1 [*Felis catus*]	6	6	17.85714
587009725	PREDICTED: complement component C8 gamma chain isoform X1 [*Felis catus*]	6	6	36.63366
475392719	retinol binding protein 4 [*Felis catus*]	6	6	45.27363
60392244	RecName: Full=Coagulation factor IX; AltName: Full=Christmas factor; Contains: RecName: Full=Coagulation factor IXa light chain; Contains: RecName: Full=Coagulation factor IXa heavy chain; Flags: Precursor	5	5	11.58798
410957470	PREDICTED: immunoglobulin J chain [*Felis catus*]	5	5	36.70886
347666817	apolipoprotein B, partial [*Felis catus*]	5	5	7.359307
3402543	IgG1 heavy chain, partial [*Felis catus*]	17	5	66.26866
1126422662	PREDICTED: phosphatidylinositol-glycan-specific phospholipase D isoform X2 [*Felis catus*]	5	5	6.785714
2914011	IgM constant chain, partial [*Felis catus*]	6	5	43.7788
410958740	PREDICTED: apolipoprotein M isoform X1 [*Felis catus*]	5	5	23.40426
755821540	PREDICTED: complement C3-like, partial [*Felis catus*]	25	5	41.56545
1080114020	CD5 antigen-like [*Felis catus*]	17	5	46.08696
755813091	PREDICTED: properdin [*Felis catus*]	5	5	11.70213

410950994	PREDICTED: tetranectin [*Felis catus*]	5	5	28.21782
410987405	PREDICTED: carbonic anhydrase 2 [*Felis catus*]	5	5	22.69231
325651605	immunoglobulin heavy chain variable region subgroup 3, partial [*Felis catus*]	7	4	42.63158
1126423477	PREDICTED: complement C2 isoform X2 [*Felis catus*]	4	4	5.125
755741265	PREDICTED: thrombospondin-1 [*Felis catus*]	4	4	3.675214
410957528	PREDICTED: platelet basic protein [*Felis catus*]	4	4	44.34783
1126493169	PREDICTED: uncharacterized protein LOC109496317 [*Felis catus*]	4	4	9.649123
566559804	transthyretin precursor [*Felis catus*]	4	4	51.36986
587011734	PREDICTED: membrane primary amine oxidase [*Felis catus*]	4	4	5.526316
1126395104	PREDICTED: apolipoprotein C-II-like, partial [*Felis catus*]	4	4	53.16456
1126395712	PREDICTED: odorant-binding protein-like [*Felis catus*]	4	4	30.84112
1126462331	PREDICTED: mannose-binding protein C [*Felis catus*]	4	4	21.8107
755808301	PREDICTED: C-reactive protein [*Felis catus*]	4	4	25.56054
1126480759	PREDICTED: apolipoprotein C-I [*Felis catus*]	3	3	35.89744
325651527	immunoglobulin heavy chain variable region subgroup 1, partial [*Felis catus*]	4	3	29.89691

755776692	PREDICTED: carboxypeptidase N catalytic chain [*Felis catus*]	3	3	7.158837
544581253	mutant coagulation factor XII [*Felis catus*]	3	3	8.214286
755789862	PREDICTED: LOW QUALITY PROTEIN: membrane primary amine oxidase [*Felis catus*]	3	3	4.325033
1126455929	PREDICTED: apolipoprotein C-III [*Felis catus*]	3	3	28
586991757	PREDICTED: complement C1s subcomponent [*Felis catus*]	3	3	5.179856
586994526	PREDICTED: complement C1q subcomponent subunit A [*Felis catus*]	2	2	13.46939
755801558	PREDICTED: actin, cytoplasmic 1 [*Felis catus*]	7	2	23.2
325651674	immunoglobulin heavy chain variable region subgroup 3, partial [*Felis catus*]	3	2	16.66667
410981199	PREDICTED: retina-specific copper amine oxidase [*Felis catus*]	2	2	2.380952
1080114022	CD5 antigen-like [*Felis catus*]	14	2	37.44589
1126405556	PREDICTED: inter-alpha-trypsin inhibitor heavy chain H4 isoform X6 [*Felis catus*]	43	2	58.68852
587000359	PREDICTED: biotinidase isoform X2 [*Felis catus*]	2	2	5.075188
755812005	PREDICTED: thymosin beta-4 [*Felis catus*]	2	2	29.54545
755722705	PREDICTED: platelet factor 4-like [*Felis catus*]	2	2	16.34615

47825211	alpha1-glycoprotein acid, partial [*Felis catus*]	10	2	41.91919
1126440492	PREDICTED: fibulin-1 [*Felis catus*]	2	2	4.225352

Supplementary Table 2. Relative intensity ratios (normalized to the internal standards) of each deregulated protein in the whole study population.

Animal ID	A1A	APOM	CPN1	ATIII	Prothrombin	CP	A2AP	TMSB4X	APOA2	PF4	C1 inhibitor	Tetranectin	IgHC	IgK	IgL	C8G
CHF1	0.94	1.27	0.88	0.99	0.99	0.67	0.98	1.51	1.36	1.16	1.01	NA	0.78	0.78	0.8	0.97
CHF2	1.25	1.2	NA	1.03	1.04	1.06	1.05	1.51	1.19	0.91	0.96	0.77	0.88	0.92	1	1.04
CHF3	1.14	1.26	0.82	1.16	1.03	1.19	1.15	0.84	1.05	0.95	1.34	0.85	1.09	0.7	0.83	0.79
CHF4	1.14	1.04	0.69	1.25	1.03	1.37	1.26	NA	0.97	0.79	1.51	0.91	0.86	0.95	0.97	1.11
CHF5	0.82	0.95	NA	1.17	1.5	0.62	1.49	NA	1.36	2.18	1.06	1.2	0.69	1.79	0.78	1.17
CHF6	1.12	1.25	1.25	1.21	1.1	1.23	1.27	NA	1.28	0.88	1.22	0.76	0.95	1	1.02	0.95
CHF7	1.05	1.14	NA	1.25	1.06	1.1	1.1	1.2	0.94	1.18	1.33	0.84	1.07	0.71	0.84	0.44
CHF8	0.93	0.88	0.94	0.92	0.93	0.98	1.31	0.8	0.8	0.9	0.89	1.64	1.04	0.87	1.14	1.08
CHF9	0.91	0.87	1.43	0.84	0.99	0.93	0.96	NA	0.95	0.6	0.71	1.08	1.09	0.87	1.24	1.35
CHF10	0.95	0.95	1.05	0.92	1.12	1.18	0.89	NA	0.91	1.28	1.05	0.81	0.89	0.87	0.91	1.08
CHF11	0.96	1	NA	0.97	1.02	0.95	1.13	NA	1.08	0.69	1.04	1.09	0.87	0.93	0.81	1.06
CHF12	0.98	0.91	1.17	1.16	1.05	1.12	1.15	NA	0.97	1.14	1.63	0.79	0.79	0.96	0.88	0.86
CHF13	1.14	1.25	1.58	1.37	1.2	1.11	1.31	NA	2.11	1.36	1.58	0.89	0.73	0.6	0.52	0.82
CHF14	1.07	1.07	0.99	0.96	1.03	1.19	0.95	NA	0.93	1.15	1.2	0.94	0.94	1.01	0.95	1.08
CHF15	1.08	1.34	1.14	1.14	0.89	0.95	0.99	NA	0.77	1.71	1.49	0.6	0.69	0.65	0.64	0.96
PrC1	1.24	1.37	0.92	1.14	1.16	1.1	1.13	0.46	1.68	0.87	1.24	NA	0.52	0.52	0.62	0.87
PrC2	0.98	0.92	NA	1.04	0.86	1.05	1.01	0.71	0.76	0.63	1.26	1.2	1.1	1.07	0.99	1.08
PrC3	0.91	0.93	0.84	1	1.01	1.03	1.11	1.03	1.02	1.49	1.15	1.31	1.14	0.84	1.02	0.96
PrC4	0.86	1.01	1.03	0.87	0.96	0.8	1.03	NA	1.01	1.18	0.93	0.88	0.92	1.01	1.45	1.06
PrC5	0.9	0.77	NA	0.88	1.03	0.75	0.96	NA	1.04	1.38	0.85	1.14	0.71	1.23	0.82	1.03
Nor1	0.93	0.99	1.05	0.94	0.95	1.13	0.96	0.61	0.66	0.9	0.79	NA	1.62	1.1	1.35	1.1
Nor2	1.07	1.17	NA	1.14	1.03	0.91	0.99	0.73	1.16	1	1.02	0.94	0.85	1.04	0.77	1.08
Nor3	0.95	1.01	1.08	1.04	0.95	1.07	1.06	0.44	1.68	0.88	1.09	0.83	0.94	0.7	1.22	1.24
Nor4	0.97	0.97	0.97	0.96	0.97	0.88	1.05	NA	0.87	0.76	0.79	0.94	1.09	1.11	1.02	1.07
Nor5	0.95	1.01	0.76	0.93	1.01	0.84	1.02	NA	0.97	0.88	0.81	1.08	0.98	0.73	1.07	1.12

Continued…

Nor6	0.93	0.98	NA	0.99	0.98	0.97	0.96	NA	1.29	0.91	0.71	1.02	1.19	0.94	1.11	1.07
Nor7	0.89	0.89	0.88	0.98	0.98	0.79	1.02	NA	0.65	0.78	0.87	1.34	1.19	1.32	1.04	1.14
Nor8	0.85	0.78	0.76	0.75	0.98	0.88	0.94	NA	0.83	0.56	0.88	1.09	1.42	1.67	1.59	1.1
Nor9	0.82	0.83	NA	0.86	0.94	0.91	1.02	NA	0.85	0.93	0.66	1.05	1.37	1.43	1.3	1.19
Nor10	0.85	1.05	0.88	0.87	1.02	1.01	1	0.3	0.58	0.48	0.68	NA	1.09	1.13	1.21	1.06
Nor11	1.11	1.09	0.89	1.16	0.92	0.98	0.92	0.2	0.69	0.52	0.83	NA	0.95	0.96	1.33	1
Nor12	0.7	0.81	0.95	0.77	0.85	1	0.65	0.21	0.39	0.33	0.71	0.86	1.02	0.67	1.14	1.15
Nor13	0.8	0.84	NA	0.84	1.16	0.88	1	1.38	0.44	1.57	0.99	0.91	0.86	1.38	1.19	0.55
Nor14	0.99	0.9	0.47	1.04	0.93	0.95	1.18	NA	0.84	0.75	1.03	1.05	0.92	1.13	0.87	0.93
Nor14	1.07	1.05	0.69	1.02	0.94	0.98	1.25	NA	0.86	0.87	1.25	0.89	0.81	0.9	0.71	1.05

CHF, congestive heart failure; PrC, preclinical; Nor, healthy control; A1A, α-1 antitrypsin; APOM, apolipoprotein M; CPN1, carboxypeptidase N subunit 1; AT III, antithrombin III; CP, ceruloplasmin; A2AP, α-2 antiplasmin; TMSB 4X, thymosin β-4; APOA2, apolipoprotein A-II; PF4, platelet factor 4; IgHC, immunoglobulin heavy chain; IgK, immunoglobulin kappa; IgL, immunoglobulin lambda; C8G, complement C8 gamma chain; NA, not available.