The plasma proteome and the acute phase protein response in canine pyometra

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Abstract

Canine pyometra is a common inflammatory disease of uterus in sexually mature bitches caused by secondary bacterial infection, leading to change in plasma proteins associated with the innate immune system. Proteomic investigation is increasingly being applied to canine diseases in order to identify and quantify significant changes in the plasma proteome. The aim of the study was to assess and quantify changes in plasma proteome profiles of healthy and pyometra affected bitches using a TMT-based high-resolution quantitative proteomic approach. As a result, 22 proteins were significantly down-regulated including transthyretin, antithrombin III, retinol-binding protein, vitamin D binding protein, paraoxonase 1, and kallikrein, while 16 were significantly up-regulated including haptoglobin light chain, alpha-1-acid glycoprotein, C-reactive protein precursor, and lipopolysaccharide-binding protein in dogs with pyometra. Pathway analysis indicated that acute inflammatory response, regulation of body fluid levels, protein activation cascade, the humoral immune response, and phagocytosis were affected in pyometra. Validation of biological relevance of the proteomic study was evident with significant increases in the concentrations of haptoglobin, C-reactive protein, alpha 1 acid glycoprotein, and ceruloplasmin by immunoassay. Pyometra in bitches was shown to stimulate an increase in host defense system proteins in response to inflammatory disease including the acute phase proteins.

Keywords: Canine pyometra, Inflammation, TMT-based proteomics, Acute phase proteins

Significance

The label-based high-resolution quantitative proteomics analysis and bioinformatic approach used in this study provide insight into the complex pathophysiology of inflammation associated with pyometra revealing proteins with biomarker potential. Early diagnosis and therapeutic intervention may prevent severe complications associated with advancing sepsis in dogs with pyometra. Therefore the identification of diagnostic biomarkers that, after clinical validation may be used in veterinary practice and protein relevant to pathways responding to disease are important findings of the study. Data are available via ProteomeXchange with identifier PXD015951.

Highlights

- Plasma samples from dogs with pyometra were compared to plasma from healthy dogs.
- Quantitative proteomics determined increases in 16 proteins and decreases in 22 proteins during pyometra.
- Decreases were found in kallikrein, retinol binding protein and transthyretin among others.
- An acute phase protein response was measured by immunoassay in dogs with pyometra that corresponded to the relevant subset of differntially abundant proteins in the plasma proteome.
- Increases were measured in haptoglobin, C-reactive protein, alpha 1 acid glycoprotein and ceruloplasmin.

Introduction

Canine pyometra is a common inflammatory disease of the uterus in intact, sexually mature bitches, caused by a secondary bacterial infection [1]. The disease affects on average 25% of all intact bitches before 10 years of age [2]. Breed strongly influences the risk of developing pyometra, which indicates that genetic factors may contribute to an increased or decreased susceptibility [3].

Pyometra is characterized by severe endometrial inflammation and accumulation of pus in the uterine lumen, with a list of severe subsequent complications reported that includes sepsis,

systemic inflammatory response syndrome (SIRS), septic shock, peritonitis, disseminated bacterial infection and multi-organ dysfunction [1, 3]. Therefore, early identification of the disease and appropriate treatment is crucial to obtain a favourable outcome and increase chances of survival of this life threatening condition.

The diagnosis is based on case history, physical examination, and laboratory analyses, often combined with radiography and/or ultrasonography of the uterus and ovaries. Clinical signs vary depending on the severity of the disease. Leukocytosis, neutrophilia with left shift, anaemia, monocytosis, hypoalbuminemia as well as affected liver or kidney function are common findings [3, 4]. Surgical ovariohysterectomy is the safest and most effective treatment for pyometra, but new protocols for improved medical treatment alternatives have also been tested with promising results [3].

In recent years, new omics technologies have been developed, which has prompted possibilities for novel studies. In this respect, veterinary proteomics is an evolving field which holds a great promise not only for fundamental and applied discoveries regarding pathophysiological mechanisms of animal species diseases, but can also be implemented in comparative applications of relevance to human diseases research [5]. Furthermore, the application of proteomics to the study of canine disease for pathophysiological and biomarker analysis has been accelerating [6-9]. As a common and well described naturally occurring inflammatory disease, canine pyometra represents a good model to study the inflammatory response. The inflammatory response is an intricate cross-talk between cytokines, acute phase proteins (APPs) and cells [10, 11], but many of these ongoing interactions remain unclear, while its exacerbation is associated with high mortality in human and veterinary medicine. Therefore, an insight into proteomic profile of plasma in dogs

with pyometra may provide important findings into general mechanisms operating during diverse inflammatory reactions.

The aim of this study was to identify and quantify differentially abundant proteins (DAP) in plasma of bitches with pyometra compared to plasma from healthy dogs. This aim was achieved by an isobaric tandem mass tag (TMT) label-based high-resolution quantitative proteomic approach. A subsequent aim was to confirm changes in the DAP found by proteomics by using immunoassay to measure plasma concentrations of the canine plasma protein haptoglobin (Hp), C-reactive protein (CRP), α_1 acid glycoprotein (AGP) and ceruloplasmin (CP). Furthermore functional bioinformatic analysis was employed for elucidating molecular mechanisms and pathways associated with canine pyometra.

2. Materials and methods

2.1 Animals and sample collection

Six clinically healthy dogs (control group) and 6 dogs with pyometra were enrolled in the study. All dogs were admitted to the Small Animal Clinic, University of Veterinary Medicine Hannover and treated in accordance with the German Animal Welfare Law.

The experimental design was approved by the Animal Welfare Officer of the University of Veterinary Medicine and by the Ethic Committee of the responsible authority (Lower Saxony State Office for Consumer Protection and Food Safety, reference number 17A 101). Healthy dogs for blood collection were recruited by launching a call in the University of Veterinary Medicine Hannover network for students and staff members for haemostasis study, providing a free clinical and laboratory health check of their animal in combination with the blood collection. Residual sample material was used in the present study. Only animals for which the clinical examination, a

complete haematology profile and clinical chemical profile (including liver enzymes, bilirubin, urea, creatinine, cholesterol, glucose, total protein, albumin and calcium) were unremarkable, i.e. did not reveal any deviations from the reference intervals, were included in the study. Healthy dogs had a median age of 5 years (range 5 months–8 years), 4 were females and 2 males (1 castrated). Different breeds were represented (3 mixed-breed, 1 Golden Retriever, 1 Labrador, 1 Hovawart). Before taking the blood samples, all dogs were fasted for at least 12 hours with free access to water.

Blood samples from dogs with pyometra were collected at the time of initial presentation to the Small Animal Clinic for diagnostic purposes and the residual sample material was used for the present project. Diagnosis was based on clinical, ultrasonographic and laboratory findings as well as findings during laparotomy (all dogs received an ovariohysterectomy) and partly confirmed by histopathology. Five of 6 dogs had leukocytosis and 3 had increased body temperature, thereby, 3 fulfilled criteria of a systemic inflammatory response syndrome. Dogs with pyometra had a median age of 10 years (range 5–11 years). Different breeds were represented (2 Golden Retrievers, Labrador, Newfoundland, Siberian Husky, and a mixed-breed dog).

Plasma was obtained by centrifugation of blood collected in lithium-heparin tubes. Aliquoted samples were stored at −80 °C until analysed. All samples used for repetitive analysis were frozen in aliquots and only vials needed for each assay run were used, to avoid the repetitive thawing and freezing effect.

2.2 *Proteomic analysis by LC-MS/MS*

Proteomic analysis of canine plasma samples was performed by TMT-based quantitative approach as described previously [12]. In brief, after total protein concentration determination using a BCA assay (Thermo Scientific, Rockford, USA), 35 µg of total proteins from samples and internal

standard (a pool of equal protein amount from all samples as a reference for normalization) were diluted to a volume of 50 µL using 0.1 M triethyl ammonium bicarbonate (TEAB, Thermo Scientific, Rockford, USA), reduced by adding 2.5 µL of 200 mM dithiothreitol (60 min, 55 °C) (DTT, Sigma Aldrich, St. Louis, MO, USA), alkylated by adding 2.5 µL of 375 mM iodoacetamide (30 min, room temperature in the dark) (IAA, Sigma Aldrich, St. Lois, MO, USA) and acetone-precipitated (300 µL, overnight, −20 °C). Protein pellets were collected subsequently by centrifugation (9000 × *g*, 4 °C), dissolved in 50 µL of 0.1 M TEAB and digested using 1 µL of trypsin (1 mg/mL, Promega; trypsin-to-protein ratio 1:35, at 37 °C overnight).

TMT sixplex reagents (Thermo Scientific, Rockford, IL, USA) were prepared according to the manufacturer's procedure and 19 µL of the appropriate TMT label was added to each sample for the labelling reaction (60 min, room temperature) which was quenched by 5% hydroxylamine (Sigma-Aldrich, St. Louis, MO, USA). Five TMT-modified peptide samples were combined with the internal standard (labelled with TMT *m/z* 126) into the new tube, aliquoted, dried and stored at −20 °C for further analysis.

High resolution LC-MS/MS analysis of TMT-labelled peptides was carried out using an Ultimate 3000 RSLCnano system (Dionex, Germering, Germany) coupled to a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Peptides were dissolved in loading solvent (1% ACN, 0.1% formic acid) and desalted on the trap column (C18 PepMap100, 5 µm, 100A, 300 µm×5 mm) for 12 min at the flow rate of 15 µL/min and separated on the analytical column (PepMap™ RSLC C18, 50 cm×75 µm) using a linear gradient of 5–45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min at the flow rate of 300 nL/min. Mobile phase A consisted of 0.1% formic acid in water. Eluent was ionised using a 10 µm-inner diameter SilicaTip emitter (New Objective, USA) mounted within nanospray Flex ion source (Thermo Fisher

Scientific, Bremen, Germany). Data dependent acquisition in positive ion mode was performed using a DDA Top8 method. Full scan MS spectra were acquired in range from m/z 350.0 to m/z 1800.0 with a resolution of 70000, 110 ms injection time, AGC target 1×10^6, a ± 2.0 Da isolation window and the dynamic exclusion 30 s. HCD fragmentation was performed at step collision energy (29% and 35% NCE) with a resolution of 17500 and AGC target of 2×10^5. Precursor ions with unassigned charge state, as well as charge states of +1 and more than +7 were excluded from fragmentation.

Acquired MS/MS spectra were analysed for protein identification and quantification using the SEQUEST algorithm implemented into Proteome Discoverer (version 2.0., ThermoFisher Scientific). Database search against *Canis lupus* FASTA files (downloaded from NCBI database 13/10/2016, 41787 entries) was performed according to the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.02 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M), deamidation (N,Q) and TMT sixplex (K, peptide N-terminus) dynamic modifications. The false discovery rate (FDR) for peptide identification was calculated using the Percolator algorithm in the Proteome Discoverer workflow. At least two unique peptides and 5% FDR were required for reporting confidently identified proteins.

Protein relative quantification was accomplished by correlating the relative intensities of reporter ions extracted from tandem mass spectra to that of the peptides selected for MS/MS fragmentation. The internal standard was used to combine the results for each protein within the TMT experiments (sixplexes). The mass spectrometry-based proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [13] partner repository with the dataset identifier PXD015951.

2.3 *Statistical analysis*

Protein expression from pyometra and control group as determined by TMT proteomics were compared using the non-parametric Wilcoxon test, after an outlier correction by a Dixon test in each group. Proteins with a p-value < 0.05 were considered as significantly different between disease and control groups. For each protein, fold changes have been calculated as follow: -log2 ($mean_{disease}$/$mean_{control}$). Statistics have been performed using RStudio v1.1.463 and the script dedicated to LC-MS data analysis. R packages used for statistics were *readr*, *outliers*, *data.table*, *plotly*, and *xlsx* [14]. Statistical analysis for immunoassay results were performed using EZR 1.37 [15] which is a graphical interface for R commander [14]. The Pearson correlation coefficient was used to compare immunoassays results with protein abundance in proteomics (fold change) and the nonparametric Mann-Whitney test was used to assess differences between pyometra and control groups determined by immunoassay, with $p < 0.05$ considered as statistically significant.

2.4 *Bioinformatic analysis*

The list of significant proteins has been converted to a list of Gene symbols through BioDBnet (https://biodbnet-abcc.ncifcrf.gov). Then, genes from *Canis lupus familiaris* have been converted to their orthologous genes in *Homo sapiens* by the tool Biomart from Ensembl (www.ensembl.org). Using the Cytoscape (v3.6.1) application CluePedia (1.5.2), original list of significant genes have been enriched with their best interactors according to IntAct and Reactome databases (maximum 5 added interaction per gene). Then all genes (original and enriched) were submitted to a Gene Ontology (GO) analysis using the Cytoscape application ClueGO (v2.5.2) with following parameters: GO_BiologicalProcess in *Homo sapiens*, evidence codes used = All_without_IEA, GO level from 3 to 12, Kappa score threshold = 0.4, correction method = Bonferroni step down.

Generated GO terms were submitted to a refinement step by Revigo (revigo.irb.hr) to remove redundant terms, define groups of GO terms and assign a term as leading GO. All this bioinformatics analysis was represented using Cytoscape with a radial layout. Fold changes data for original nodes were included as a color gradient.

2.5 *APPs measurements*

APPs concentrations were determined using SPARCL immunoassays. SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) novel technology is a proximity-dependent, homogenous, chemiluminescent detection method [16] that allows rapid and cost effective immunoassay development, validation and sample analysis (Lumigen Inc, Michigan, USA). Canine specific SPARCL assays were used for Hp, CRP, AGP and CP. (Life Diagnostics Inc., West Chester, USA). Validation of SPARCL immunoassays was performed to assess the assays' precision, accuracy, detection limit and specificity. The precision was determined as the mean coefficient of variance (CV) with triplicate assay for four samples (n=12) with varying concentrations of the DAP repeated in a single assay for intra-assay and over repeated assays for inter-assay CV. The accuracy was determined by parallel dilution of samples with high concentrations of the DAP as the mean (+SD) of the observed divided by the expected concentration expressed as a percentage. The minimal detected concentration of the assays was determined as the lowest concentration of purified protein that could be measured in sample with minimal dilution and in plasma after adjustment for that dilution. Specificity was dependent on the nature of the antigen used to produce the antibody utilised in the assays. Nonparametric Mann-Whitney test was used to assess differences between pyometra and control group, with $p < 0.05$ considered as statistically significant.

3. Results

3.1 Proteomics

In this study, the label-based proteomic approach enabled the identification of 210 quantifiable proteins according to set criteria (2 unique peptides and 5% FDR). In total, there were 38 proteins with significantly different abundances following FDR correction, between healthy dogs and those with pyometra, 22 of them were down-regulated and 16 up-regulated in diseased dogs (Table 1). Outlier correction reduced the numbers per group for the statistical analysis of proteomic results but for the subset selected for validation of the proteomic results (Section 3.3) no samples were excluded (n=6 in all groups).

A volcano plot of fold changes (x-axis) and their associated -log10 transformed p-values (y-axis) for the proteins identified by LC-MS is given in Figure 1.

3.2 Bioinformatics

The list of final GO terms that were represented with their respective number of genes (original and enriched) inside each term and their associated p-value (expressed as -log10), is given in Table 2 and illustrated by histogram (Figure 2).

The gene ontology (GO) terms represented for pyometra, filtered by REVIGO and presented by Cytoscape with a radial layout are given in Figure 3A with the full interaction (detailed view can be found in Supplementary Information) and Figure 3B where the interactions at nodes in regulation of body fluid levels; protein activation cascade; humoral immune response; acute inflammatory response as best interacting GO terms are evident. Fold changes data for original nodes are presented as a color gradient.

3.3 *Acute Phase Protein measurements in the validation of proteomics results*

The SPARCL immunoassays used for validation of the proteomic results for DAP were themselves validated (Table 3) with intra-assay CVs ranging from 1.4% to 6.5% and inter-assay CVs ranging from 1.7% to 13.3%. The parallel dilution assessment for accuracy was between 96% to 102% for the four proteins. The minimal detected amount for the DAP ranged from 3.6 ng/ml for CRP to 50 ng/ml for α1 acid glycoprotein. The specificity of the SPARCL immunoassays depends on the nature of the antigen for antibody production, which was the native canine protein for Hp, CRP, AGP and CP. All SPARCL immunoassays assays were valid for use in comparison of the quantitative TMT proteomic results to their plasma concentrations.

Results of APPs measurements are presented in Table 4, as median and interquartile range, with corresponding p-value. All measured APPs were significantly different between pyometra and control groups. Higher plasma concentrations of APPs were found in samples from the pyometra group for Hp, CRP, AGP and CP.

The APPs measurements determined by SPARCL assays were compared to the fold change as determined by proteomic analysis of the same samples, thus providing validation of the proteomics results. Both the TMT-based quantitative proteomic approach and the SPARCL immunoassays showed independently that in pyometra the concentration of Hp, CRP, AGP and CP were higher than in healthy dogs (Fig 4 a-d). There was significant association between the immunoassay and quantitative proteomic results demonstrated by the correlations with $r > 0.85$, and $p < 0.001$ in all correlations, as presented in Figures 5 a-d.

4 Discussion

The inflammatory and coagulation changes that accompany severe infections in dogs are similar to those observed in humans, which is why spontaneous sepsis in dogs may serve as a physiologically relevant disease model for human sepsis [17]. Pyometra is a common bacterial infection of the uterus in dogs that frequently progresses into SIRS and sepsis [18, 19]. Recently, the inflammatory response during pyometra has been more closely explored, and reported uterine gene microarray expression pattern showed that up- and down-regulated genes in pyometra are associated with chemokines, cytokines, inflammatory cell extravasation, anti-bacterial action, the complement system and innate immune responses [20]. Using the high resolution label-based relative quantification proteomic approach, we have found 38 differentially abundant plasma proteins between dogs with pyometra and healthy dogs. Based on bioinformatic analyses, identified differences indicate involvement of several pathways in this disease, including the acute inflammatory response, regulation of body fluid levels, protein activation cascade, the humoral immune response and phagocytosis.

The acute phase response (APR) is a nonspecific, immediate and complex inflammatory reaction of the host that occurs shortly after any tissue injury (caused by infection, inflammation, neoplasia, trauma or other causes). During the APR, pro-inflammatory cytokines stimulate the production of positive APPs in hepatocytes leading to increase in their plasma concentration, while negative APPs decrease in concentration [10]. Most of the proteins found as significantly different abundant proteins between the pyometra and control groups are known to be APPs, such as CRP, Hp, CP, AGP, transthyretin, paraoxonase-1 (PON-1), inter-alpha-trypsin inhibitor heavy chain H1, alpha-2-HS-glycoprotein and transferrin. These findings confirm activation of the APR in canine pyometra. As APPs levels during the course of inflammation in an organism reflect the state of the innate immune system activation, and for confirmation of the biological validation of the

investigation, the panel of canine APPs proteins, Hp, CRP, AGP and CP were quantified by immunoassays. These demonstrated equivalent responses to the proteomic analysis thus validating the proteomic approach and also extended previous assessment of APR in pyometra.

Previous studies found higher plasma concentrations of CRP, Hp and AGP, as well as SAA in bitches with pyometra compared to plasma of healthy dogs [21-24]. CRP level, together with the percentage of band neutrophils, has been proposed as laboratory marker in order to differentiate pyometra and cystic endometrial hyperplasia/mucometra [25]. CRP is a well known major positive APP in both humans and dogs, used as sensitive, but nonspecific biomarker of systemic inflammation [11]. This study identified and quantified CP by proteomics and immunoassay as another positive APP which has an important role in protecting host tissues from toxic oxygen metabolites released from phagocytic cells during inflammatory states, and copper transport and antioxidant defence. Ceruloplasmin is a known APP in dogs, with higher and earlier increase in concentration during inflammation than in humans [10, 26].

Except those APPs, other proteins were shown by the proteomics investigation to have significant fold change in the pyometra group compared to the healthy group. α2-Heremans–Schmid-(α2-HS) glycoprotein (A2HSG) is a plasma protein synthesized in liver and is a homologue of bovine fetuin-A. This protein is a negative APP whereas its level declines following infection, inflammation and malignancy and here had a negative fold change of -0.55. α2-HS glycoprotein has also been identified as a negative APP in chicken plasma following experimental induction of the inflammatory response to bacterial lipopolysaccharide endotoxin [12].

Four identified proteins involved in haemoglobin and iron metabolism and transport are Hp, hemopexin with increased abundance, serotransferrin and transferrin receptor protein 1 (TfR1) with lower abundance in pyometra. The scavenger protein hemopexin, which bind extracellular

heme, provides the second line of defence preventing haemoglobin-mediated oxidative damage during the intravascular haemolysis and heme-bound iron loss [27]. While hemopexin helps in scavenging free heme, the free iron is taken up and transported by serotransferrin, a negative APP found in this study. Transferrin receptor protein 1 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. Levels of TfR1 are used as biomarker in iron deficiency and anaemia of chronic disease in humans [28].

Transthyretin (also known as prealbumin) is an important transport protein, which plays an essential role in the binding of thyroid hormones and vitamin A, the latter through interaction with retinol binding protein. Lower protein abundance found in pyometra group confirmed both transthyretin and retinol binding protein as negative APP in dogs, due to decreased synthesis in inflammation, trauma, tissue injury or stress [29].

Paraoxonase 1 (PON1) is considered as a marker of diseases involving oxidative stress, inflammation and liver disease. PON1 is a negative APP bound to high density lipoproteins (HDL) and during the APR protects HDL from peroxidation. During inflammation HDL molecules lose apolipoprotein A1, esterified cholesterol, and most of the HDL-associated enzymes, including PON1, which is replaced mainly by serum amyloid A and ceruloplasmin [30]. Decrease of PON1 has been demonstrated in dogs with acute pancreatitis, leishmaniosis and babesiosis [31-33]. It is a limitation of the study that analysis of PON1, as an example of a negative acute phase reactant was not available at the time of investigation and its assay in samples from bitches with pyometra should be considered in the future.

One of the findings in the complex pathophysiology of inflammation is evidence of excess systemic protease activity. An array of endogenous protease inhibitors have evolved whose

function is to prevent excess activation of proteases and limit the potential injurious actions of protease activation on endothelial and epithelial tissues [34]. Many of protease inhibitors, such as antithrombin, alpha-1 antitrypsin, and alpha-2-antiplasmin were detected by proteomic analysis in this study. They are rapidly consumed in sepsis, often leading to a failure to appropriately regulate protease activity.

The inter-alpha-trypsin inhibitors (ITI) are a family of plasma serine protease inhibitors, composed of a light chain – bikunin, and five homologous heavy chains, contributing to extracellular matrix stability by covalent linkage to hyaluronan playing a role in inflammation and carcinogenesis in humans [35]. In inflammationinter-alpha-trypsin inhibitors heavy chain (ITIH) family members have been demonstrated to be both positive and negative APPs under various conditions. In our study, lower protein abundances were found for both, ITIH1 and ITIH2, in the pyometra group compared to controls. Rapid depletion of ITIH levels may contribute to uncontrolled proteolytic activity in the plasma and exacerbate the systemic inflammatory response in severe sepsis. The reduced ITIH1 and ITIH2 levels in the plasma may contribute to reduced protease inhibitor activity and excess protease-mediated tissue injury in pyometra group.

Coagulation pathways were shown to be affected in pyometra by the proteomics study. Antithrombin III, as part of the most important anticoagulant pathway, inhibits fibrinogen conversion into plasmin by creating thrombin-antithrombin (TAT) complexes. Antithrombin III activity was decreased in bitches with endometritis-pyometra complex due to excessive mobilisation and in response to high levels of IL-6, which accompany various inflammations [36]. A lower protein abundance of antithrombin III was found in the pyometra group, possibly due to increased consumption as a result of ongoing thrombin generation and proteolytic degradation by elastase from activated neutrophils.

Alpha-2-antiplasmin, which was reduced in the pyometra group, is a major inhibitor and regulator of fibrinolysis and one of the essential factors involved in haemostasis. It is a member of the serine proteinase inhibitor (serpin) family and inhibits proteases in general, including trypsin, chymotrypsin, plasma kallikrein, but its main physiological activity is very rapid inhibition of plasmin by forming a stable complex with this proteinase [37] and its reduction may suggest consumption of fibrinolysis inhibitors and increased fibrinolytic activity due to hypercoagulable state present in inflammation. Excess free plasmin is bound by α2-macroglobulin, a relatively nonspecific inhibitor of fibrinolysis, and this protein was increased in the pyometra group accordingly.

Two actin-scavenging proteins, vitamin D binding protein, also known as group-specific component globulin (Gc-globulin), and gelsolin, had lower protein abundances in pyometra group compared to controls. Vitamin D binding protein binds to monomeric G-actin, which is released by the action of gelsolin. During the scavenging process, both G-actin–gelsolin and G-actin–vitamin D binding protein complexes are formed and subsequently cleared by the reticuloendothelial system, resulting in consumption of both gelsolin and vitamin D binding protein [38]. To date, vitamin D binding protein has been recognized widely as a protein with markedly decreased concentrations in inflammatory and necrotic diseases. The extent of the decrease may have prognostic significance for patient outcomes.

The analysis of interaction biological processes by GO terms has identified the processes most affected by pyometra revealing that the regulation of body fluid levels, the protein activation cascade relating to coagulation of blood proteins, humoral immune response and the acute inflammatory response were the most affected. In this representation of the pathways responding to the disease, the APPs appear at locations related to their biological activities rather than as part

of an acute phase protein response. Thus CRP is primarily linked to the phagocytosis node, haptoglobin to the nodes of response to reactive oxygen species, hydrogen peroxide catabolic process and cofactor catabolic process and AGP (gene ORM1) and lipopolysaccharide binding protein to the acute inflammatory response node. Notable among the nodes with less interaction was the retinoid metabolic process, response to reactive oxygen species, the cellular iron ion homeostasis and cholesterol transport, highlighting that these metabolic processes may have consequences for the development of pyometra and should not be overlooked in understanding the pathophysiology of this disease.

A limitation of this study is that the control group was substantially younger than the patients, i.e. not age-matched. Although, age-dependent changes of the vessel wall and/or subclinical multi-morbidity in elderly dogs may result in increasing concentrations of inflammatory reaction proteins and coagulation factors, those age-dependent changes in adult healthy dogs are actually only minimal [39, 40]. In addition, the food intake in the patient group is not exactly defined in contrast to the control group, but it can be assumed that the patients had a similarly empty stomach, because the pyometra disease is usually associated with inappetence (and food is withdrawn after hospitalization before surgery).

5 Conclusion

Label-based high-resolution quantitative proteomics analysis and bioinformatic approach used in this study has identified numerous DAP that are either increased or decreased in the plasma of bitches during pyometra unlike previously reported studies addressing pyometra being focused exclusively on several plasma proteins. These changes in DAP represent a valuable tool for elucidating the complex pathophysiology of inflammation associated with pyometra and unveiling disease relevant proteins with biomarker potential. Further quantification by immunoassay of a

subset of the DAP comprising HP, CRP, AGP and CP not only provide evidence of the stimulation of the APP response during pyometra but also validation of the proteomic results. Early diagnosis and therapeutic intervention may prevent severe complications associated with sepsis in dogs with pyometra and it is crucial to identify diagnostic or prognostic biomarkers such as the APP, that can be used in veterinary practice. APPs play an important role in modulating the inflammatory immunological response, and their blood concentrations reflect the intensity of inflammation, which makes APP determination of diagnostic and prognostic value in pyometra. Determination of haemostatic profile is also of importance in pyometra, in order to timely address haemostatic function impairment.

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Ethics

The study was approved by the Animal Welfare officer of the University of Veterinary Medicine Hanover and by the Ethic Committee of the Lower Saxony State Office for Consumer Protection and Food Safety, reference number 17A 101.

Author contributions

Conceptualization and study design: P.D.E, R.M., V.M.; investigation: R.M., J.K., R.F.F, A.H., C.C. methodology: R.M., J.K., A.H., R.F.F., N.G., C.C.; formal analysis: N.G, J.K., A.H.; data curation: A.H., P.D.E.; validation: C.C.; visualization: N.G., R.F.F., J.K.; supervision; P.D.E., R.M., V.M., funding acquisition and resources: P.D.E., V.M., R.M.; writing - original draft: J.K., P.D.E.; writing - review & editing: J.K., A.H., N.G., R.F.F., R.M., C.C., V.M., P.D.E.

Conflict of Interest

The authors declare that there are no conflicts of interest, except for C.C. of Life Diagnostics Inc, the manufacturer of the acute phase protein immunoassays.

Supplementary data

The LC-MS/MS spectra from the healthy and pyometra canine groups have been archived by submitting to PRIDE archive via ProteomeXchange with the dataset identifier PXD015951. Additionally, the full list of proteins identified in the canine plasma samples and detailed view to complete pathway interactions are provided in the supplementary data.

PRIDE repository Reviewer account details:

Username: reviewer14707@ebi.ac.uk

Password: SIrO7UP0

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534 Table 1: Proteins showing significant differences in abundance between plasma from dogs with
535 pyometra and healthy dogs.

Accession number[a]	Protein name	FDR-corrected p-value	Fold_change[b]	Sequence coverage	Number of unique peptides
258498	haptoglobin light chain, HpL chain [dogs, Peptide, 83 aa]	0.035	2.19	42.17	3
73957095	haptoglobin-like	0.035	1.83	70.89	24
123511	RecName: Full=Haptoglobin	0.035	1.83	74.77	24
258499	haptoglobin heavy chain, HpH chain [dogs, Peptide, 245 aa]	0.035	1.80	82.86	20
345777714	alpha-1-acid glycoprotein 1	0.033	1.26	42.93	9
936976329	C-reactive protein precursor	0.033	1.23	38.12	5
345789637	lipopolysaccharide-binding protein	0.033	1.16	28.27	11
16607721	interleukin-13 receptor subunit alpha-2 precursor	0.033	0.87	31.44	10
17066528	immunoglobulin gamma heavy chain C	0.033	0.81	45.78	11
345792424	alpha-2-macroglobulin	0.035	0.54	56.34	59
545504250*	C4b-binding protein alpha chain	0.035	0.42	47.69	23
73988725	hemopexin	0.035	0.29	56.55	17
73990367	ceruloplasmin isoform X2	0.035	0.28	37.18	28
545539301	ceruloplasmin isoform X1	0.035	0.28	36.20	28
45826457***	ceruloplasmin, partial	0.046	0.28	89.04	5
928179401**	vitamin K-dependent protein S	0.046	0.24	16.20	13
73985485	inter-alpha-trypsin inhibitor heavy chain H1 isoform X1	0.035	-0.32	30.11	22
73967363	alpha-2-antiplasmin isoform X2	0.033	-0.32	20.16	8
545512145	alpha-2-antiplasmin isoform X1	0.033	-0.32	17.84	8
57090343	plasma serine protease inhibitor	0.046	-0.34	22.11	7
545533419	inter-alpha-trypsin inhibitor heavy chain H1 isoform X2	0.035	-0.34	25.79	18
345777712	protein AMBP	0.035	-0.35	26.65	8
73975797	serum paraoxonase/arylesterase 1	0.046	-0.36	26.20	8
73949158	inter-alpha-trypsin inhibitor heavy chain H2	0.035	-0.38	27.80	20
359320010	antithrombin-III	0.033	-0.46	47.96	19
928167632	serotransferrin	0.035	-0.47	80.71	56
399567834*	hemoglobin subunit beta-like	0.046	-0.53	80.95	11
10946310	transferrin receptor	0.033	-0.53	12.73	10

545552733	transferrin receptor protein 1 isoform X1	0.033	-0.53	12.73	10
545521763	vitamin D-binding protein	0.033	-0.54	73.84	25
227343817*	Chain B, Crystal Structure Of Dog (Canis Familiaris) Hemoglobin	0.046	-0.54	86.99	12
545553759	alpha-2-HS-glycoprotein	0.035	-0.55	37.53	9
545518174	gelsolin	0.033	-0.57	26.59	17
57089193	transthyretin	0.033	-0.58	46.94	6
208342090**	immunoglobulin heavy chain variable region, partial	0.046	-0.59	21.28	2
928175781	retinol-binding protein 4	0.035	-0.69	50.75	7
545553762	histidine-rich glycoprotein isoform X1	0.046	-0.71	20.36	10
337298514***	plasma kallikrein	0.046	-0.72	7.39	3

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

[b]Fold changes calculated as: -log2 ($mean_{disease}$/$mean_{control}$)

The group size of the healthy dogs and those with pyometra was n=6 for all proteins listed except for proteins where outlier exclusion reduced the number per group and are labelled * where the healthy group n=5 and pyometra n=6; ** where the healthy group n=6 and pyometra n=5; *** where the healthy group n=5 and pyometra n=5

543 Table 2. Five best interacting Gene Ontology (GO) leader terms and number of genes associated
544 in this study for pyometra retained by REVIGO

545

GOTerm	Number of associated genes	- log10 p-value
regulation of body fluid levels	45	23.025
protein activation cascade	40	31.910
humoral immune response	35	16.967
acute inflammatory response	32	23.122
phagocytosis	31	14.857

546

547

548

549 Table 3 Validation of the immunoassays used for assessment of a subset of the differntially abundant proteins in plasma of dogs with
550 pyometra.

551

Protein	Precision		Accuracy: parallel dilutions	Minimal detected amount			Specificity
	Intra-assay CV (%)	Inter-assay CV (%)	Mean (+SD) of O/E (%)	In diluted sample ng/ml	In plasma ng/ml	minimum dilution	Antigen used for antibody production
Haptoglobin	5.5	6.9	96±4	1.7	34	1:20	Purified native canine haptoglobin
C-Reactive protein	5.4	13.3	99±4	0.18	3.6	1:20	Purified native canine C-reactive protein
α_1 acid glycoprotein	1.4	1.7	101±5	2.5	50	1:20	Purified native canine α_1 acid glycoprotein
Ceruloplasmin	5.8	17	102±5	0.34	6.8	1:20	Purified native canine ceruloplasmin

552

Table 4: Median and interquartile range of acute phase proteins in the healthy control group of dogs and in the group of dogs with pyometra.

Parameter (unit)	**Control group (N = 6)**	**Pyometra group (N = 6)**	**P value**
Hp (mg/ml)	0 (0 – 0.34)	6.81 (2.39 - 10.48)	0.002
CRP (μg/ml)	1.23 (0 – 5.66)	466 (386.1 - 488)	0.002
AGP (μg/ml)	151.9 (117.1 – 249)	1663 (819 – 2568)	0.002
CP (μg/ml)	139 (125.8 – 182.4)	248.3 (216 – 280.7)	0.009

559

560 **Legend to Figures**

561 **Figure 1.** Volcano plot for plasma from dogs with pyometra vs plasma from healthy dogs (control
562 group). Volcano plot of fold changes (x-axis) and their associated log10 transformed p-values (y-
563 axis) for the proteins identified by LC-MS. Proteins with significant different abundance between
564 pyometra and control groups (-log10 $p > 1.3$) are in green, non-significant proteins (-log10 $p <$
565 1.3) are in red.

566 **Figure 2:** The final GO terms that were represented by the plasma protein changes occurring in
567 canine pyometra.

568 **Figure 3.** Gene ontology (GO) terms represented for pyometra, filtered by REVIGO and detected
569 by Cytoscape, (A) complete pathway interactions available in supplementary data for examination
570 of detail; (B) enlargement of pathway interactions to show links between proteins showing change
571 in quantitative proteomics and GO nodes in regulation of body fluid levels; protein activation
572 cascade; humoral immune response; acute inflammatory response. Blue diamond is not detected
573 protein; red diamond is detected protein, red diamond green fill is a protein increased in pyometra;
574 red diamond with red fill is a protein decreased in pyometra. Minutely detailed view is available
575 in Supplementary data.

576 **Figure 4.** Comparison of APPs measurements by SPARCL (μg/mL or mg/mL) and by TMT label-
577 based quantitative proteomics (fold change): a) haptoglobin; b) C-reactive protein; c) alpha-1-acid
578 glycoprotein; d) ceruloplasmin. Difference between healthy and pyometra groups was analysed by
579 Mann Whitney U Test and with n=6 in all groups.

580 **Figure 5.** Correlation of APPs measurements by SPARCL (μg/mL or mg/mL) and by TMT
581 label-based quantitative proteomics (fold change): a) Haptoglobin ($R = 0.992$; $p=3.25e-10$), b)

C-reactive protein (R = 0.996; p=3.27e-7); c) Alpha-1-acid glycoprotein (R = 0.95; p = 2.21e-6);

d) Ceruloplasmin (R = 0:857; p= 0.000371)

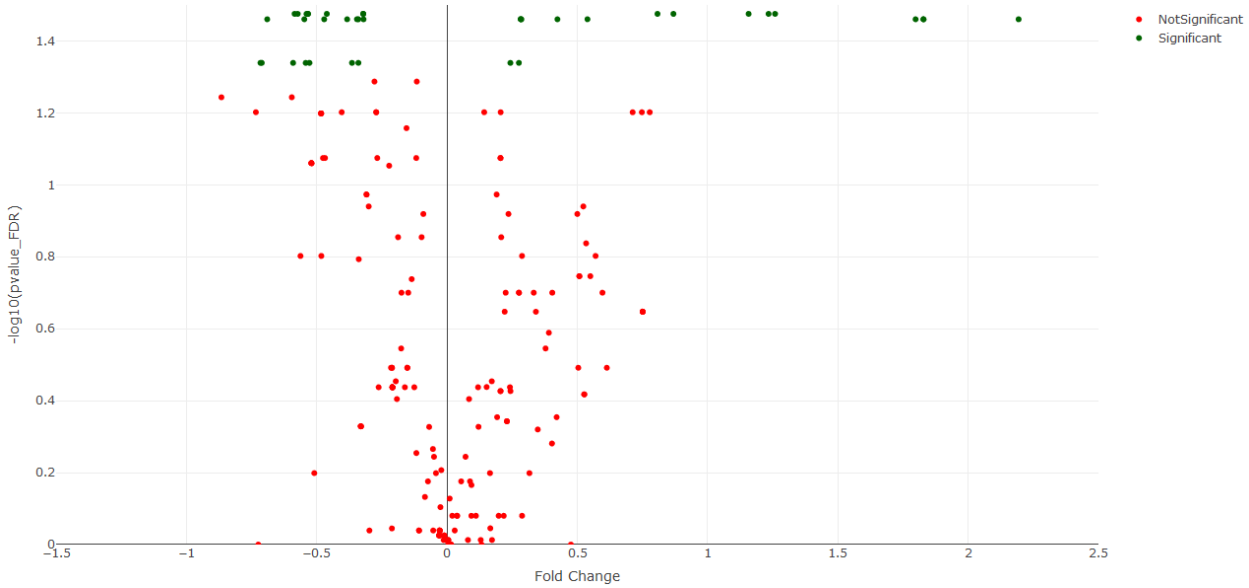


Figure 1.

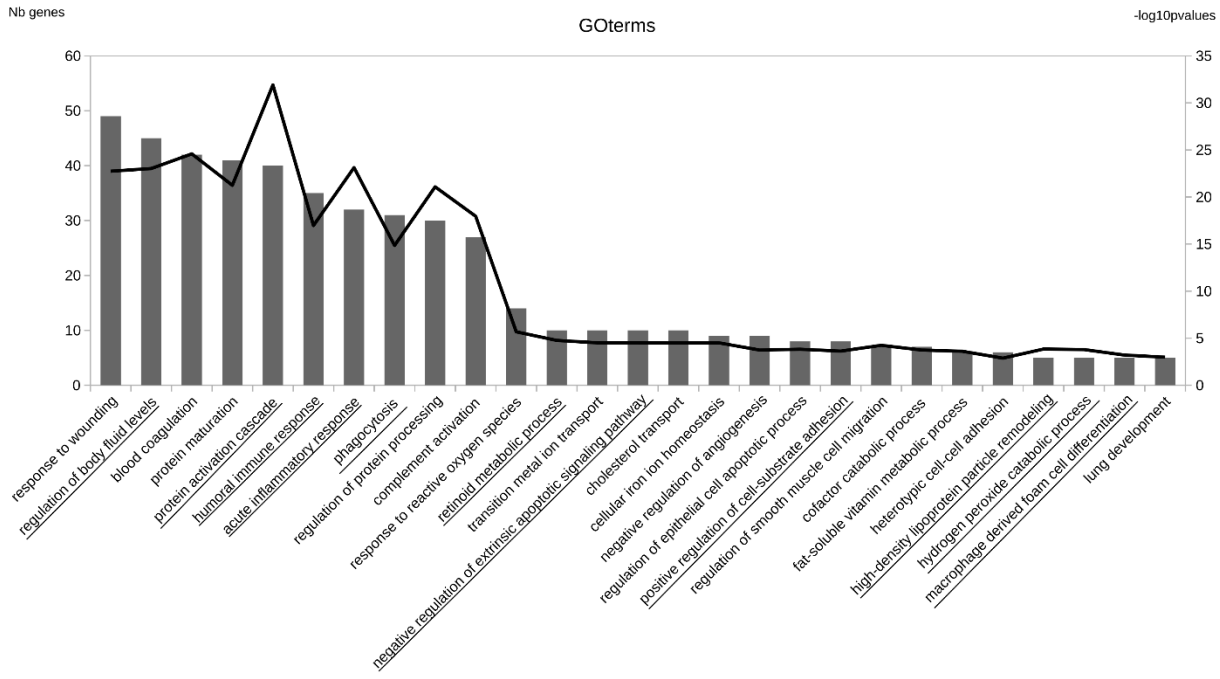
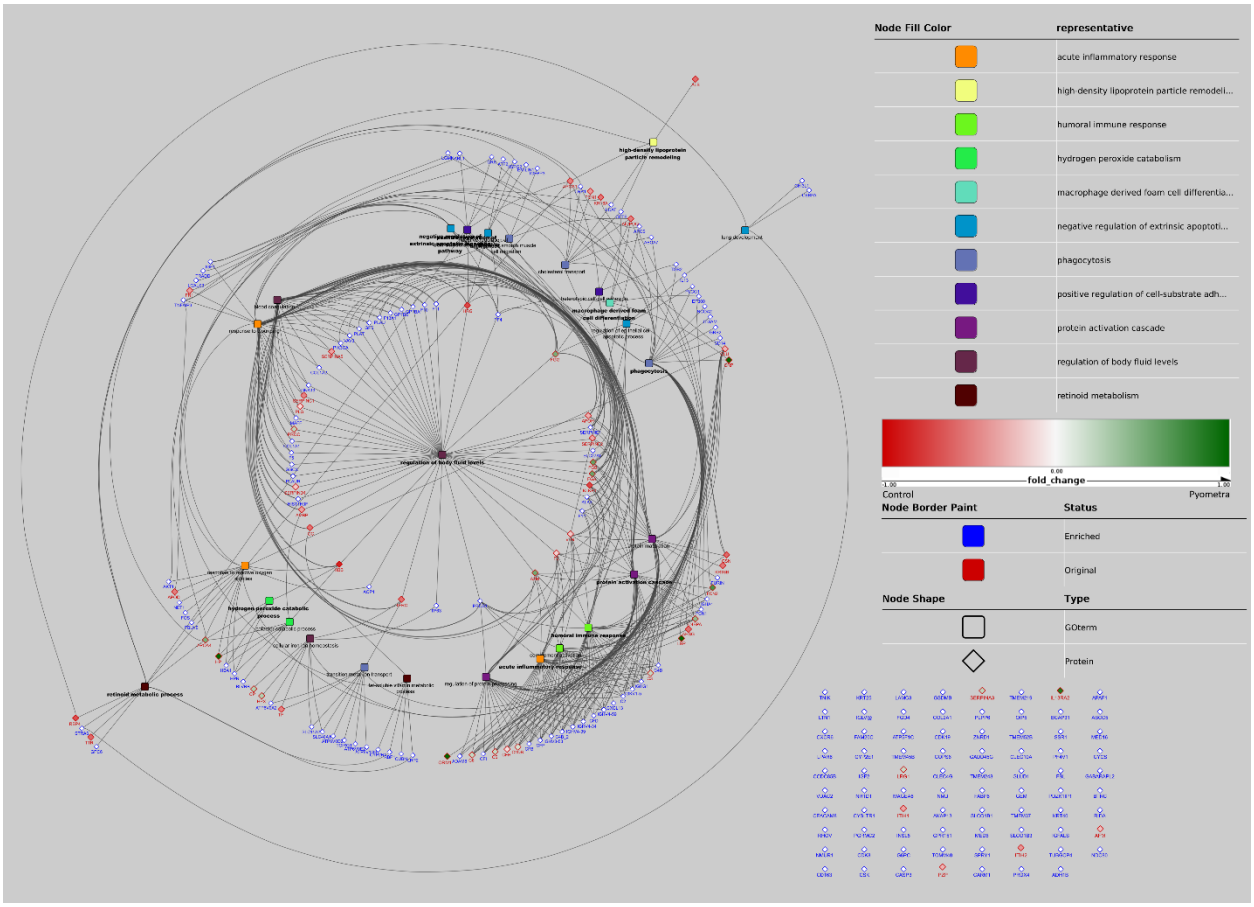


Figure 2.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high density lipoprotein particle remodeli...
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phagocytosis
positive regulation of cell-substrate adh...
protein activation cascade
regulation of body fluid levels
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Pyometra
Node Border Paint
Status
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Node Shape
Type
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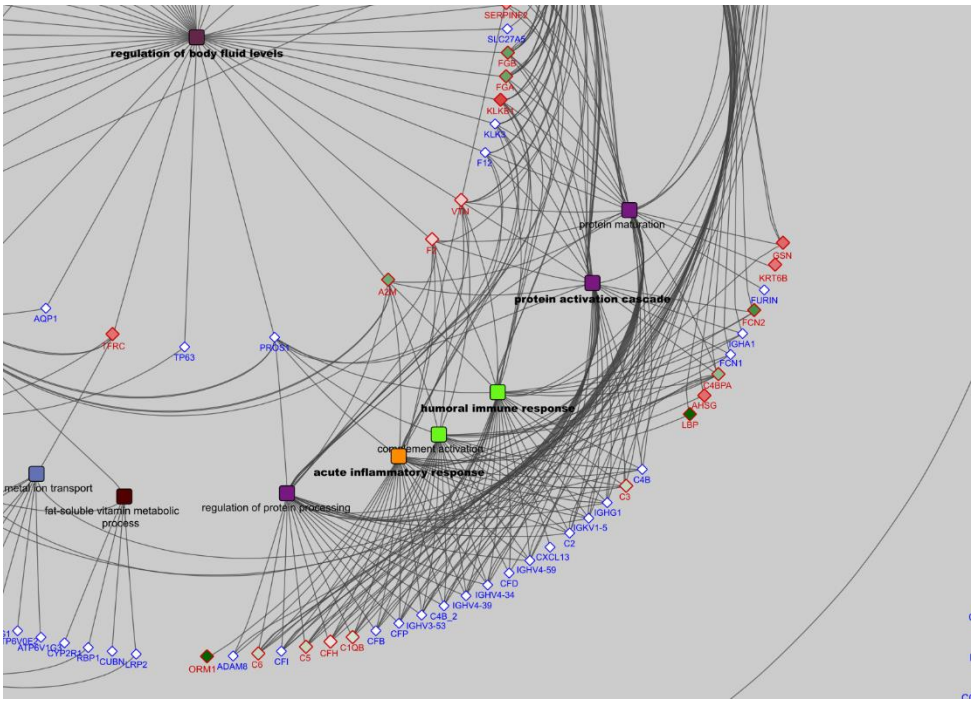
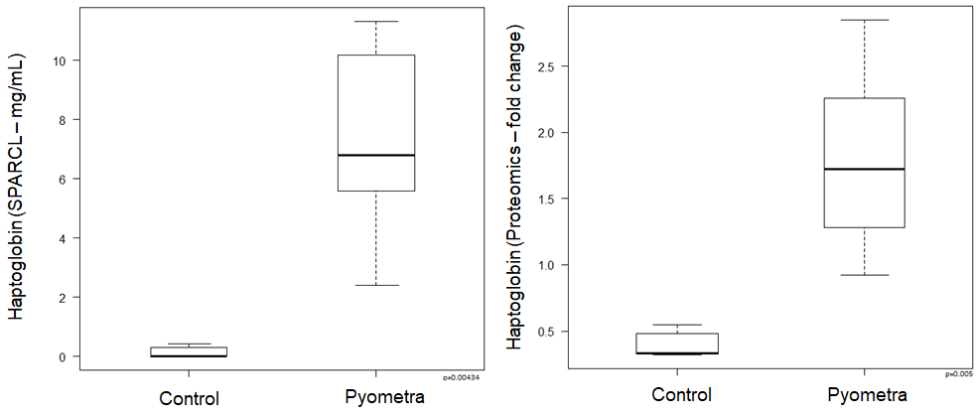
Protein

Figure 3a.



590

591 **Figure 3b.**



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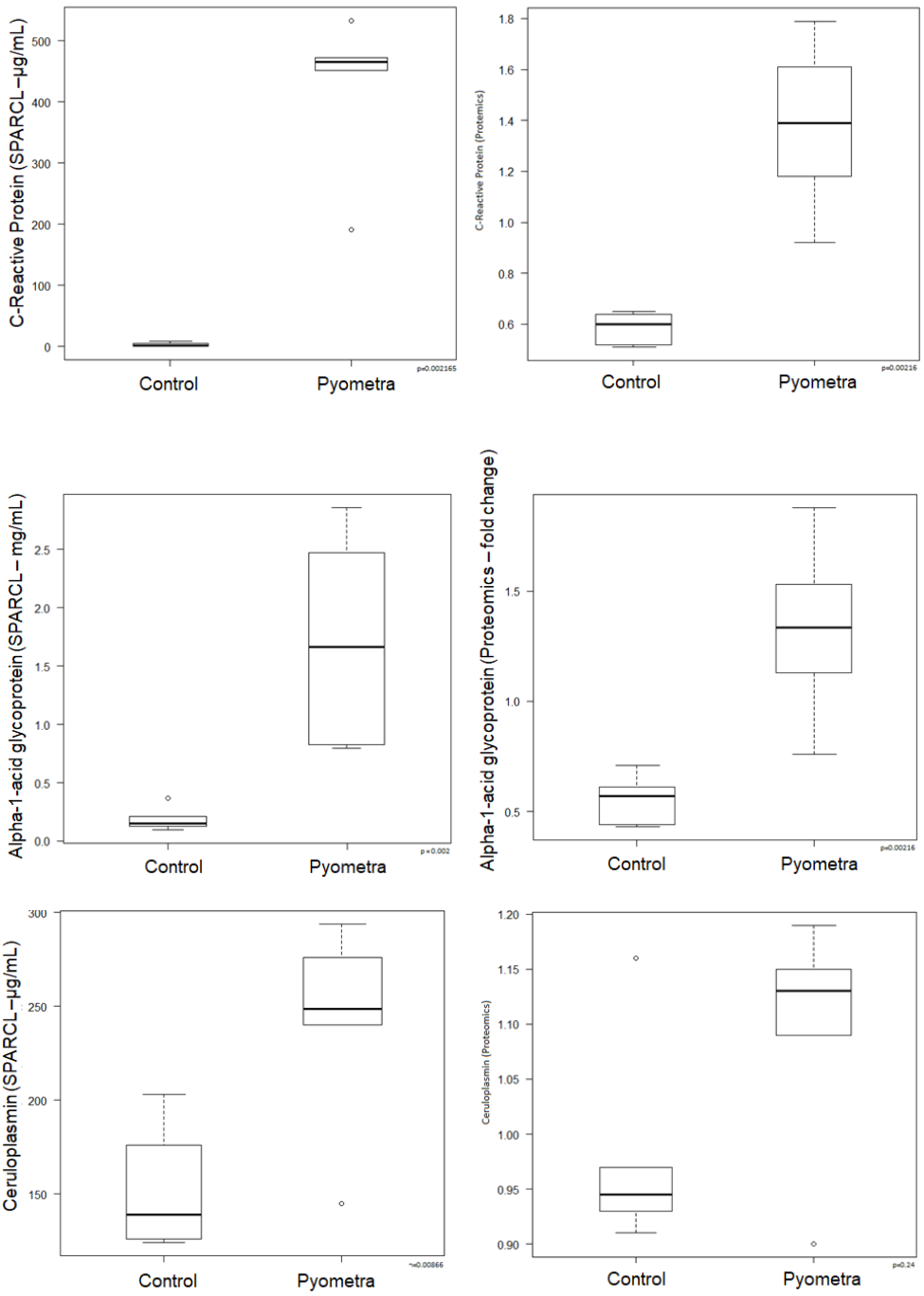
C-Reactive Protein (SPARCL–µg/mL)
0
100
200
300
400
500
Control
Pyometra
p=0.002165
C-Reactive Protein (Proteomics)
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Control
Pyometra
p=0.00216
Alpha-1-acid glycoprotein (SPARCL – mg/mL)
0.0
0.5
1.0
1.5
2.0
2.5
Control
Pyometra
p = 0.002
Alpha-1-acid glycoprotein (Proteomics – fold change)
0.5
1.0
1.5
Control
Pyometra
p=0.00216
Ceruloplasmin (SPARCL–µg/mL)
150
200
250
300
Control
Pyometra
Ceruloplasmin (Proteomics)
0.90
0.95
1.00
1.05
1.10
1.15
1.20
Control
Pyometra
p=0.24

Figure 4 a-d

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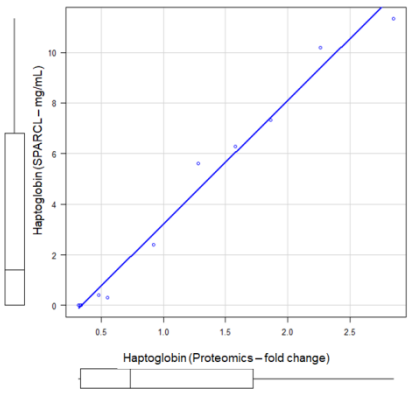

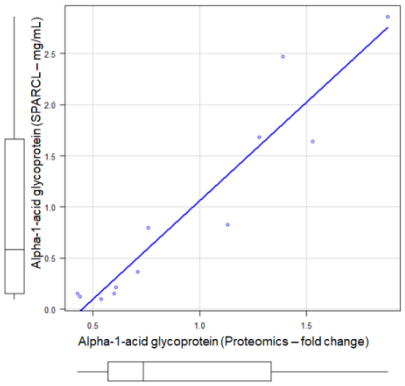

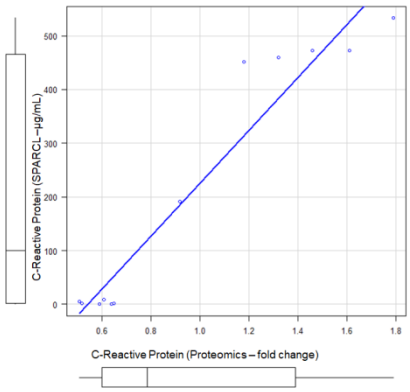

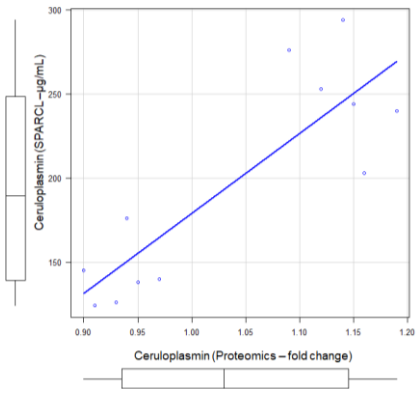

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601 **Figure 5 a-d**

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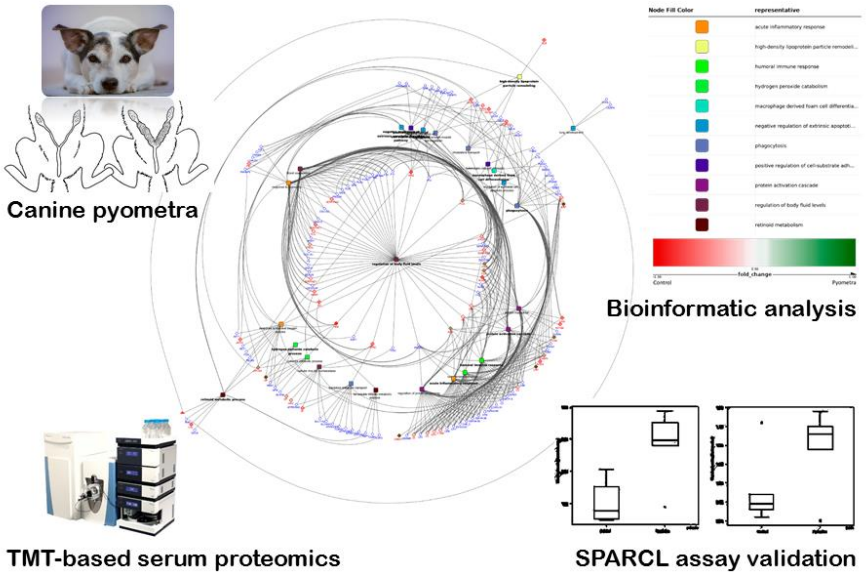
603 **GRAPHICAL ABSTRACT**



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