The Plasma Amyloid Beta 42 ($A\beta_{42}$) and Proteomics Profile Related to Canine Cognitive Dysfunction Syndrome (CCDS) in Thailand

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Abstract

Background: Canine cognitive dysfunction syndrome (CCDS) is a progressive neurodegenerative disorder found in senior dogs. Due to the lack of biological markers, CCDS is commonly underdiagnosed. The aim of this study was to identify potential plasma biomarkers using proteomics techniques and to increase our understanding of the pathogenic mechanism of the disease. Plasma amyloid beta 42 ($A\beta_{42}$) has been seen to be a controversial biomarker for CCDS. Proteomics analysis was performed for protein identification and quantification.

Results: Within CCDS, ageing, and adult dogs, 87 proteins were identified specific to *Canis* spp. in the plasma samples. Of 87 proteins, 45 and 52 proteins were changed in the ageing and adult groups, respectively. Several distinctly expressed plasma proteins identified in CCDS were involved in complement and coagulation cascades and the apolipoprotein metabolism pathway. Plasma $A\beta_{42}$ levels considerably overlapped within the CCDS and ageing groups. In the adult group, the $A\beta_{42}$ level was low compared with that in the other groups. Nevertheless, plasma $A\beta_{42}$ did not show a correlation with the Canine Cognitive Dysfunction Rating scale (CCDR) score in the CCDS group ($p = 0.125$, $R^2 = 0.27$).

Conclusions: Our present findings suggest that plasma $A\beta_{42}$ does not show potential for use as a diagnostic biomarker in CCDS. The nano-LC-MS/MS data revealed that the predictive underlying mechanism of CCDS was the co-occurrence of inflammation-mediated acute phase response proteins and complement and coagulation cascades that partly functioned by apolipoproteins and lipid metabolism. Some of the differentially expressed proteins may serve as potential predictor biomarkers along with $A\beta_{42}$ in plasma for improved CCDS diagnosis.

Background

Canine cognitive dysfunction syndrome (CCDS) or ‘canine dementia’ is a neurodegenerative disease causing behavioural changes that are characterized by gradual reductions in learning, memory, spatial awareness, social interactions and sleeping patterns [1]. The prevalence of CCDS is high and affects up to 60% of mostly dogs older than 11 years [2]. There is no breed-specific difference in the clinical signs or pathology of the disease [3]. However, clinical signs of CCDS are more often observed and reported in smaller dogs [4, 5].

Several studies of neurodegenerative diseases in animals have shown strong similarities in pathology and characteristics between cognitive dysfunction in dogs and Alzheimer’s disease (AD) in humans [6-8]. Typical pathological hallmarks of CCDS are characterized by cortical atrophy, ventricular widening, demyelination, neuronal loss and the presence of amyloid beta (Aβ) plaques in the brain parenchyma and vessels such as AD in humans [9, 10]. The inflammation cascade and oxidative stress have been proposed to be underlying mechanisms of AD. An immunohistochemical study of the CCDS brain showed Aβ accumulation on meningeal vessels [11]. Aβ accumulation in the brain shows a significant relation with cognitive decline [9, 12, 13]. In AD, Aβ plaque-activated glial cells were postulated as a putative

mechanism for chronic inflammation [14]. Aβ deposition and diffuse plaque formation lead to microglial activation and many inflammation-related proteins, such as complement factors, acute phase proteins, chemokines and cytokines, such as interleukin-1 (IL-1), interleukin-6 (IL-6), tumour necrosis factor-α (TNF-α), and transforming growth factor-β (TGF-β) [15-17]. Genetically, the *ε4* allele of the *apolipoprotein E* (*APOE*) gene has been identified as a main risk factor in late-onset AD [18]. Aβ clearance in the brain depends on the affinity between Aβ and the *APOE gene.* Moreover, the *APOEε4* allele has low affinity for Aβ, affecting the clearance mechanism in AD [19]. Nevertheless, there were no reported specific genes involved in CCDS [20]. In the human brain, there are two main forms of Aβ ($A\beta_{40}$ and $A\beta_{42}$). The accumulation of $A\beta_{42}$ is more toxic and is more related to the pathologies of AD than the accumulation of $A\beta_{40}$ [21]. A correlation was observed between the severity of cognitive deficit in dogs and the density of $A\beta_{42}$ accumulation in their brains [22].

Under normal conditions, Aβ is in equilibrium between biosynthesis and clearance. The clearance of Aβ from the brain can be completed by several mechanisms through nonenzymatic pathways, such as transport across vessel walls into blood circulation or enzymatic pathways, including neprilysin and insulin-degrading enzymes [23]. Cerebral Aβ is transported across the blood-brain vessel walls through scavenger receptors such as lipoprotein receptor-related protein 1 (LRP1) and very low-density lipoprotein receptor (VLDLR) into the blood circulation. Sequester proteins increase affinity to binding with scavenger receptors [21] and function as stabilizers of monomeric Aβ with the inhibition of Aβ aggregation [24]. Sequester proteins such as alpha-2-macroglobulin, apolipoprotein E (apo E) and transthyretin were shown to increase the capability to transport Aβ via LRP1 [25, 26]. The relation between sequester proteins and Aβ may serve as a diagnostic biomarker in human AD.

At present, CCDS diagnosis depends on observations from owners and veterinarians. No biological marker allows accurate CCDS diagnosis [20]. Screening questionnaires with a list of clinical signs have been used as diagnostic tools in the veterinary field. Several questionnaires have proposed criteria for the diagnosis and staging of CCDS [2, 22, 27-30]. The Canine Cognitive Dysfunction Rating scale (CCDR) is one of the most frequently used questionnaires with high diagnostic accuracy (98.9%) [28, 31, 32]. The CCDR includes assessment of behaviour frequency and the categorization of the score for identification as non-CCD, the risk of developing CCD, and CCD [28]. In Thailand, CCDS is also a major health problem in older dogs. The prevalence of Thai CCDS in dogs between 7 and 12 years old ranged from 43 to 68%, and the prevalence of CCDS increased with age [33]. Even though many candidate biomarkers have been identified in both blood and cerebrospinal fluid (CSF), none of these markers has been used routinely in the clinic. In previous studies, plasma $A\beta_{42}$ was evaluated as a biomarker in AD and CCDS, but it is highly variable and seems to be controversial [7, 34, 35]. Therefore, the identification and characterization of novel biomarkers are necessary for the reliable diagnosis of CCDS.

Proteomics is one of the most significant techniques that allows an extended investigation of neurodegenerative diseases. A blood-based proteomics approach was used extensively in humans with AD to study potential biomarkers or mechanisms related to this disease [36-38]. Proteomics in AD

revealed many interesting proteins or pathways from CSF and blood, such as fatty acid oxidation and the advanced glycation end products/receptors for advanced glycation end products (AGE/RAGE) pathway [39, 40]. Moreover, there are no reports of serum proteome profiles in CCDS, and blood-based proteomics approaches in dogs are limited.

In the present study, we determined the association between the plasma $A\beta_{42}$ expression level by the ELISA technique in adult, ageing and CCDS dogs and the CCDR score. Proteomics techniques were used to identify a dataset of potential plasma biomarkers and to investigate the underlying mechanisms of CCDS. These findings may provide new insights into the underlying mechanisms of CCDS. Moreover, potential plasma biomarkers from LC-MS/MS may be helpful and applied together with questionnaires in the evaluation of CCDS.

Results

Correlation between the CCDR score and plasma $A\beta_{42}$ levels

First, levels of plasma $A\beta_{42}$ in CCDS dogs were lower than those in ageing dogs but higher than those in the adult group (for $A\beta_{42}$: 75.40 pg/mL in CCDS, 179.21 pg/mL in the aging group and 5.88 pg/mL in the adult group). There were no significant differences in $A\beta_{42}$ concentrations between groups (Fig. 1a). Nevertheless, the $A\beta_{42}$ level in the adult group was low compared to that in the other groups. Furthermore, clinical diagnosis of the CCDS group was performed with the CCDR questionnaire. CCDR scores above 50 are indicative of CCDS in older companion dogs. Second, we evaluated individual plasma $A\beta_{42}$ levels in all dogs. Our study showed that plasma $A\beta_{42}$ levels in the CCDS group were not correlated with the CCDR score ($p = 0.125$, $R^2 = 0.27$) (Fig. 1b).

Proteomics profile

Plasma proteins of the CCDS group were compared with others by a proteomics approach to study differential protein expression. The proteins were separated by One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was cut into 11 pieces (Fig. 2a.) prior to in-gel trypsin digestion. The digested peptides were identified by nano-LC-MS/MS analysis, and each protein was quantified using an exponentially modified protein abundance index (emPAI) value from the label-free spectral counting technique. In total, 1037 proteins were identified in the plasma samples from domestic dogs in Thailand against a non-redundant National Center for Biotechnology Information (NCBInr) database specific to *Mammalia* spp. as a taxonomic filter.

The protein bands in SDS-PAGE of CCDS samples in rows 5, 7 and 10 were different than those of the other groups by macroscopic appearance (Fig. 2a). Data from nano-LC-MS/MS unveiled proteins in band 5 of CCDS samples that were composed of immunoglobulin gamma heavy chain B, fibrinogen beta chain, alpha-2-HS-glycoprotein, fibrinogen gamma chain, immunoglobulin gamma heavy chain C, vitamin D-binding protein, beta-2-glycoprotein 1 precursor and immunoglobulin A heavy chain constant region.

The proteins in band 7 of CCDS samples were composed of haptoglobin, complement C4-A and immunoglobulin lambda-like polypeptide 5-like. The proteins in band 10 of CCDS samples were composed of haptoglobin.

Heat map analysis, presenting differential capability of protein expression, illustrated 3 distinct groups (Fig. 2b). For species specificity, proteomic data were identified only for *Canis* spp. A total of 87 proteins were matched, among which 45 and 52 proteins showed at least 1.5-fold differences in their expression levels according to the emPAI values in the CCDS vs ageing and CCDS vs adult group comparison (Table 1).

Table 1.
Protein detection in each group (*Canis* spp.)

Group	Protein identification	Protein change	Upregulate	Downregulate
CCDS	67	-	-	-
Ageing	61	45	16	29
Adult	47	52	7	45

The most upregulated and downregulated proteins in comparisons of the CCDS group with the adult and ageing groups are shown in Fig. 3. The most upregulated proteins in the CCDS group compared with the adult group were involved with the coagulation cascade, while the most upregulated proteins in the CCDS group compared with the ageing group were involved with apolipoprotein. The most downregulated proteins in the CCDS group compared with the adult group or the ageing group were alpha-2-macroglobulin and alpha-1B-glycoprotein.

Gene Ontology (GO) and pathway enrichment analyses of proteins

We first mapped the proteins onto GO databases via the PANTHER database using 3 primary categories: molecular function and biological process. In the GO molecular function category, the upregulated proteins in comparisons between the CCDS group and both the ageing group and the adult group were similarly classified into 4 groups: binding, molecular function regulator, catalytic activity and transporter activity. In the GO biological process category, the upregulated proteins in comparisons between the CCDS group and both the ageing group and the adult group were similarly classified into 4 groups: metabolic process, cellular process, biological regulation and transporter activity (Fig. 4).

Pathway enrichment analysis of CCDS proteins of interest using STRING showed some relation between amyloid precursor protein and some proteins of interest. Proteins at the core of the traffic link have good protein-protein interactions.

The downregulated proteins in comparisons of the CCDS group with both the adult group and the ageing group included 4 proteins: alpha-2-macroglobulin, alpha-1B-glycoprotein, complement factor B and immunoglobulin lambda-like polypeptide 5-like. The downregulated proteins, involved in blood coagulation and the complement cascade, are shown in Table 2.

Table 2.
Downregulated proteins in comparisons of the CCDS with both the adult and the ageing

Accession number[a]	Protein name	Protein mass	pI	Protein score	Biological process
gi|345792424	alpha-2-macroglobulin	165114	6.27	101	negative regulation of complement activation
gi|545487024	alpha-1B-glycoprotein	61261	5.81	47	platelet degranulation
gi|345778397	complement factor B	86266	7.18	75	regulation of complement activation
gi|545544683	immunoglobulin lambda-like polypeptide 5-like	24739	6.41	1116	innate immune response

[a]Accession number from the NCBInr database for *Canis* spp.

The upregulated proteins in the comparisons of the CCDS group with both the ageing group and the adult group were specifically involved in several biological processes. The biological process of upregulated proteins linked to neurodegenerative disease was mostly blood coagulation, acute phase protein and complement cascade, as shown in Table 3.

Table 3. Upregulated proteins in comparisons of the CCDS group with both the adult and the ageing (see additional file1)

To explore the potential proteins, we performed a pathway analysis by using STRING version 11.0. Total protein changes in comparisons of the CCDS group with both the ageing group and the adult group were expanded to show the evidence of an interaction, giving a total of 24 proteins. We compared this protein set to those of the Gene Ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome pathways databases. Most proteins involved with the Gene Ontology biological process category are stress response proteins. Most proteins involved with the KEGG and Reactome pathway databases are complement and coagulation cascade and immune system proteins, respectively (Fig. 5).

Discussion

Currently, CCDS can be diagnosed by using a screening questionnaire, but no biomarkers have been identified. In our study, the plasma $A\beta_{42}$ level did not show a correlation with the questionnaire score and could not distinguish CCDS dogs from normal dogs. In AD studies, results for the use of plasma $A\beta_{42}$ as a biomarker have been controversial [35]. The expression was in contrast with that reported in a previous

study, which showed that plasma $A\beta_{42}$ was significantly gradually decreased in early CCDS and severe CCDS, while the highest $A\beta_{42}$ plasma level was observed in younger dogs [7, 34]. Some studies in human and rat models found increased $A\beta_{42}$ or cleavage at the onset of AD; conversely, in the later stage, $A\beta_{42}$ levels were decreased in the CSF and plasma, which may be caused by plaque deposition [41-43]. Next, we performed nano-LC-MS/MS to provide a dataset of potential biomarkers to improve the diagnosis of CCDS. In our study, plasma $A\beta_{42}$ could not be detected and identified by nano-LC-MS/MS, which may be because of abundant protein interference, including that from immunoglobulin and albumin [44].

Using the raw MS/MS data, a biological heat map analysis of CCDS, ageing and adult dogs was performed. The results indicated the differentiation of the 3 sample groups according to the clustered pattern of their expression. In the enrichment analysis, differentially expressed plasma proteins were involved in complement and coagulation cascades or were acute phase proteins or apolipoproteins. This finding suggested that CCDS was enhanced by the increase in inflammation in peripheral organs, leading to the activation of the acute phase response and complement and coagulation cascades that partly functioned by apolipoproteins. Nano-LC-MS/MS analysis was used to discover the underlying mechanisms of CCDS.

Aβ can trigger inflammation and activate the complement cascade classical and alternative pathways [45, 46]. Complement downstream induced a proteolytic cascade, resulting in the opsonization of Aβ from the brain to the peripheral circulation. Complement component 4 binding protein binds to $A\beta_{42}$ in the brain and is elevated in the plasma and CSF of AD samples [47]. Aβ plays a role not only in inflammation but also in the coagulation cascade. There is an association between haemostatic factors and inflammatory mechanisms in AD [37]. In our study, we found an increase in plasminogen, fibrinogen and kininogen. In AD, plasminogen was found to colocalize with Aβ plaques [37], while fibrinogen was capable of enhancing Aβ aggregation and fibrillization, causing impairment in AD [48, 49]. Patients with higher levels of plasma fibrinogen and plasminogen modulating neuroinflammation had worsening cognitive decline and Aβ deposition [37, 50, 51].

There is an interaction between the acute phase response proteins that arise in early inflammation and other inflammatory pathways. The acute phase response is part of the innate immune system that responds to systemic inflammation. In our study, we found an increase in acute phase proteins (haptoglobin and prothrombin). The increase in acute phase proteins is generally related to defence against physiological damage and the restoration of homeostasis. Haptoglobin can bind misfolded proteins to prevent Aβ aggregation [52]. Moreover, prothrombin, localized within the vascular endothelium, was upregulated to shrink at microvascular sites [53]. Our proteomic results were in accordance with human AD studies, and the comparison showed increased haptoglobin and prothrombin in the plasma of AD patients, indicating an increased risk for cognitive decline and deterioration [54, 55]. The downregulation of alpha-1B-glycoprotein in CCDS dogs was present as in the AD study; however, the exact mechanism of alpha-1B-glycoprotein is not yet known [56].

Apo E and apolipoprotein A-I (apo A-I), the major apolipoproteins present in CSF, influence neurodegeneration via cholesterol and lipid metabolism [57, 58]. Apo A-I or apo E can bind with cholesterol to form high-density lipoprotein (HDL)-like particles that are important for neurons in membrane growth and repair [59]. Apo E can be measured in both CSF and blood; however, the use of apo E as a potential biomarker in AD is inconsistent and controversial [60]. In our study, apo E, apo A-I and apo A-IV were increased in the CCDS group. In accordance with other studies, apo E colocalizes with capillary Aβ in the brains of aged dogs and humans [61]. Interestingly, the human *APOE ε4* gene has been reported as a major genetic risk factor for late-onset AD [62]. In AD studies, apo A-I has the capability to prevent the formation of $A\beta_{42}$ and reduce $A\beta_{42}$ toxicity, and immunohistochemistry revealed the colocalization of apo A-I with $A\beta_{42}$ [63].

The plasma $A\beta_{42}$ level was lower in the CCDS group, which may be due to an increase in clearance mechanisms. Our results show a high level of sequester proteins or Aβ binding proteins due to the clearance mechanism. The clearance of Aβ occurs by binding with soluble Aβ to prevent aggregation and increase degrading mechanisms [21]. Many studies have suggested that alpha-2-macroglobulin, apolipoproteins, transthyretin, clusterin and the complement system are involved in AD pathogenesis through the sequestration of Aβ, leading to increased Aβ clearance *in vivo* [64-67]. Transthyretin and clusterin are sequester proteins that function as inhibitors of Aβ fibril formation and further suppress the toxicity of oligomers. A previous study in human and transgenic mouse models indicated that the plasma clusterin concentration was significantly increased in AD patients and was associated with the level of fibrillar Aβ in the brain. Moreover, plasma transthyretin levels were also significantly increased in comparisons between patients with AD and controls [36, 68, 69]. Aβ sequester proteins may have a dual function by reducing the formation of toxic species and increasing clearance and degradation through LRP-1-mediated endocytosis [21]. However, another study showed no statistically significant expression of serum fibrinogen, lipoprotein A and plasminogen-activator-inhibitor-1 in AD patients [70]. Therefore, this set of sequester proteins needs more study for use as a blood-based biomarker of CCDS.

Conclusions

Blood biomarkers have the potential to be used as diagnostic tools for evaluating CCDS. Our study revealed evidence for the existence of a specific blood-based proteomics profile of CCDS from domestic dogs in Thailand, which may be an interesting tool for diagnostic purposes. Plasma $A\beta_{42}$ detection may be insufficient to distinguish CCDS dogs from normal ageing dogs. Our present findings suggest the predictive underlying mechanisms of cognitive dysfunction syndrome in dogs: the co-occurrence of inflammation-mediated acute phase response proteins and complement and coagulation cascades that partly function by apolipoproteins. Some of the differentially expressed proteins need validation to serve as potential predictor biomarkers along with the use of a questionnaire for improved CCDS diagnosis. Further study, our proteomic results provide a list of potential biomarkers that require validation by other techniques for assessing the progression of cognitive decline. A study of the association between plasma biomarker panels and core pathological features of CCDS is also needed.

Methods

Patient enrolment

Experiments on Thai domestic dogs were carried out at the Prasu-Arthorn Animal Hospital, Faculty of Veterinary Science, Mahidol University. Client-owned dogs were recruited: 8 ageing dogs (age > 7 years), 4 adult dogs (age range 1-7 years) and 10 dogs that were diagnosed with CCDS. The exclusion criteria were brain diseases other than CCDS or concurrent medical problems that mimic signs of cognitive impairment. CCDS was classified according to the CCDR questionnaire rating score [28]. The study protocol was approved by the Mahidol University Animal Care and Use Committee (AICUC) (UI-01287-2558).

Blood sampling

Blood samples were collected from the cephalic or jugular vein into vials containing ethylenediaminetetraacetic acid (EDTA), and the samples were centrifuged at 3000 rpm for 10 min. Plasma was divided into 2 aliquots and kept at -80 °C. The first aliquot was used for the ELISA procedure, and the second aliquot was used for the proteomics study.

ELISA for $A\beta_{42}$ detection

Plasma $A\beta_{42}$ of all dogs in each group was quantified using specific sandwich ELISA kits for humans (Elabscience, Wuhan, China) in accordance with the manufacturer's instructions as described. Briefly, plates were incubated with 100 µL of sample or standard for 90 min at 37 °C. The liquid was then removed from each well. Biotinylated antibody was added to the plates and incubated for 1 h at 37 °C. After several wash steps, 100 µL horseradish peroxidase (HRP)-conjugated working solution was added to each well and incubated for 30 min at 37 °C. After repeated wash steps, the substrate solution was then added. Positive samples developed a blue colour. The reaction was stopped by the addition of stop solution and further measured at 450 nm.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to gel-based separation, plasma protein concentrations were determined by the Bradford assay (Bio-Rad, Benicia, CA, USA) at 590 nm with bovine serum albumin (Thermo Fisher, Waltham, MA, USA) as a standard. Protein samples in each lane were pooled from 4 dogs in each group. For gel-based separation by SDS-PAGE, 30 µg of protein was loaded on 12%SDS-PAGE. After that, the gel was stained with Coomassie Brilliant Blue R-250 (Bio-Rad, Benicia, CA, USA) and de-stained with 30% ethanol (Merck, Darmstadt, Germany) in 10% acetic acid (Merck, Darmstadt, Germany). The gel was then scanned using a GS-710 scanner (Bio-Rad, Benicia, CA, USA). The protein band was divided into 11 segments per lane according to size and chopped into 1 mm^3 pieces. For protein identification, each piece was subjected to in-gel digested prior to being subjected to nano liquid chromatography tandem mass spectrometry (nano-LC-MS/MS).

In-gel digestion

Gel pieces were de-stained using 50% acetonitrile (ACN) (Thermo Fisher, Waltham, MA, USA) in 50 mM ammonium bicarbonate (Merck, Darmstadt, Germany). After that, disulfide bonds were reduced with 4 mM dithiothreitol (DTT) (Omnipur, Darmstadt, Germany) in 50 mM ammonium bicarbonate for 10 min at 60 °C. Gel pieces were alkylated in 250 mM iodoacetamide (IAA) in 50 mM ammonium bicarbonate for 30 min at room temperature in the dark. The gel pieces were dehydrated 2 times in 100% ACN for 15 min and dried at room temperature. Then, trypsin (Sigma-Aldrich, St Louis. MO, USA) in 50 mM ammonium bicarbonate was added, and the gel pieces were incubated overnight at 37 °C. The tryptic peptides were extracted from the gels using 100% ACN. Finally, peptide mixtures were dried in a vacuum centrifuge to dryness and kept at -80 °C until further nano-LC-MS/MS analysis.

Analysis of peptide patterns by nano-LC-MS/MS

The extracted peptides were dissolved in 0.1% formic acid (Merck, Darmstadt, Germany) in LC/MS-grade water. Each sample was injected into the UltiMate 3000 nano-LC system. Three biological replications were performed. Peptide separation was performed on a C18 column. The flow rate was set at 300 nL/min. The elution occurred during the 30-min gradient from the 4% mobile phase B (80% acetonitrile in 0.1% formic acid) to the 50% mobile phase A (0.1% formic acid in water), and the eluent was infused into a microTOF-Q (Bruker Daltonics, Bremen, Germany). The mass spectra from the mass spectrometry (MS) and tandem mass spectrometry (MS/MS) covered mass ranges of m/z 400–2000 and m/z 50–1500, respectively.

LC-MS/MS Data analysis

LC-MS/MS data files were converted to a mascot generic file (.mgf) format with DataAnalysis 3.4 version software. Mascot daemon version 2.3.02 (Matrix Science, London, UK) was used to merge the .mgf files and to identify the proteins against those in the NCBInr database (24 October 2019) specific to Mammalia as a taxonomic filter. Protein expression was quantified by peptide count analysis using the emPAI value provided by Mascot. Differentially expressed proteins in at least two of the biological replicates were reported as protein alterations in each group. Processed protein-level data were analysed through a range of software tools. A heat map was constructed using the R studio program. Protein-protein interaction network and functional analysis, based on GO enrichment, KEGG, and Reactome pathways, were analysed using online STRING software (https://string-db.org) at the default setting. The graphic of the proteomic workflow is shown in Fig. 6.

Statistical Analysis

Statistical analysis was performed using descriptive statistical procedures and software (GraphPad Prism, Version 5). Pearson correlation analyses were used to examine the correlations between the CCDR score and plasma $A\beta_{42}$ levels. The statistical significance of differences in plasma $A\beta_{42}$ levels between

groups was determined with a paired, non-parametric Student's t-test ($p < 0.05$ was considered statistically significant).

List Of Abbreviations

1D-SDS-PAGE**:** One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis; AD: Alzheimer's disease; $A\beta_{42}$: Amyloid beta 42; ACN: Acetonitrile; apo A-I: Apolipoprotein A-I; apo A-IV: Apolipoprotein A-IV; apo E: Apolipoprotein E**;** CCDR: Canine Cognitive Dysfunction Rating scale; CCDS: Canine cognitive dysfunction syndrome; DTT: Dithiothreitol; EDTA: Ethylenediaminetetraacetic acid; emPAI: Exponentially Modified Protein Abundance Index; GO: Gene Ontology; IAA: Iodoacetamide; KEGG: Kyoto Encyclopedia of Genes and Genomes; LC: Liquid chromatography; LRP1: Lipoprotein receptor-related protein 1; mgf: Mascot generic file; MS/MS: Tandem mass spectrometry**;** NCBInr: Non-redundant National Center Biotechnology Information

Declarations

- **Ethics approval and consent to participate**

We state that ethical approval was obtained from our institutional ethics committee (Mahidol University-Institute Animal Care and Use Committee) (UI-01287-2558). The dog owners were informed about the methods and the purpose of the study and gave their written informed consent.

- **Consent for publication**

Not applicable

- **Availability of data and materials**

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

- **Competing interests**

The authors declare that they have no competing interests.

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- **Authors' contributions**

DC: was involved in enrolment and performed the neurological examinations of the patient, planned the experiments and interpreted the data.

BC: interpreted the bioinformatic dataset and analysed the data.

SP: performed all experiments, wrote the manuscript and interpreted the data.

SP and OR: performed proteomics experiments and interpreted the proteomics data.

All authors participated in the interpretation of the results and the preparation of the manuscript. All authors read and approved the final manuscript.

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Figures

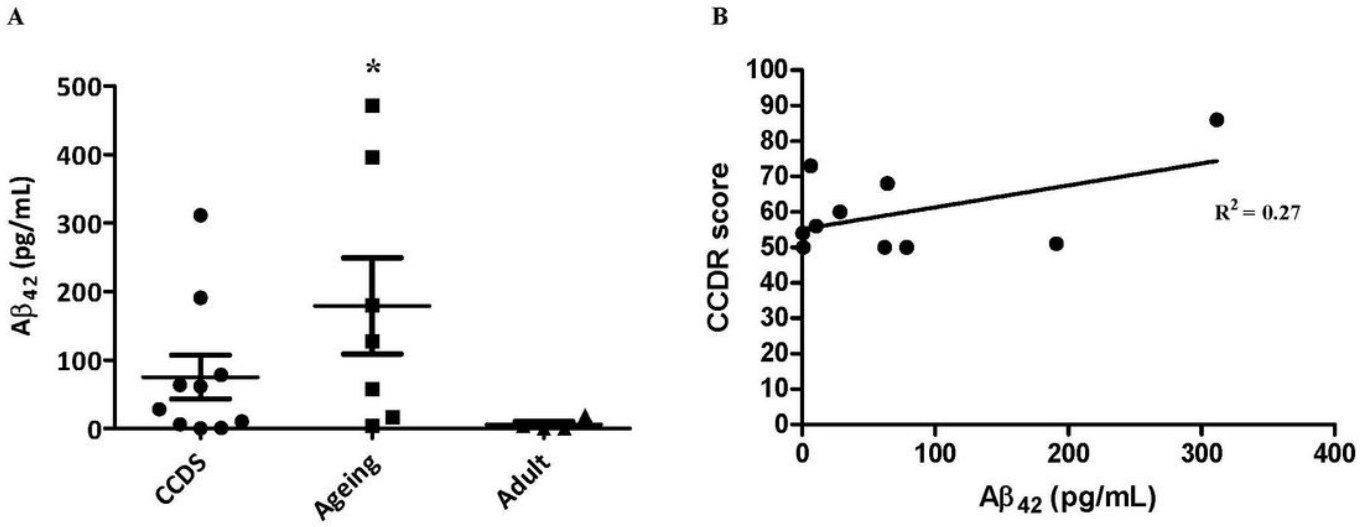

Figure 1

Plasma Aβ42 concentration (a). Plasma Aβ42 concentrations (pg/μL) in each group (* =outlier data) (b). Plasma Aβ42 concentrations and CCDR scores in the CCDS group

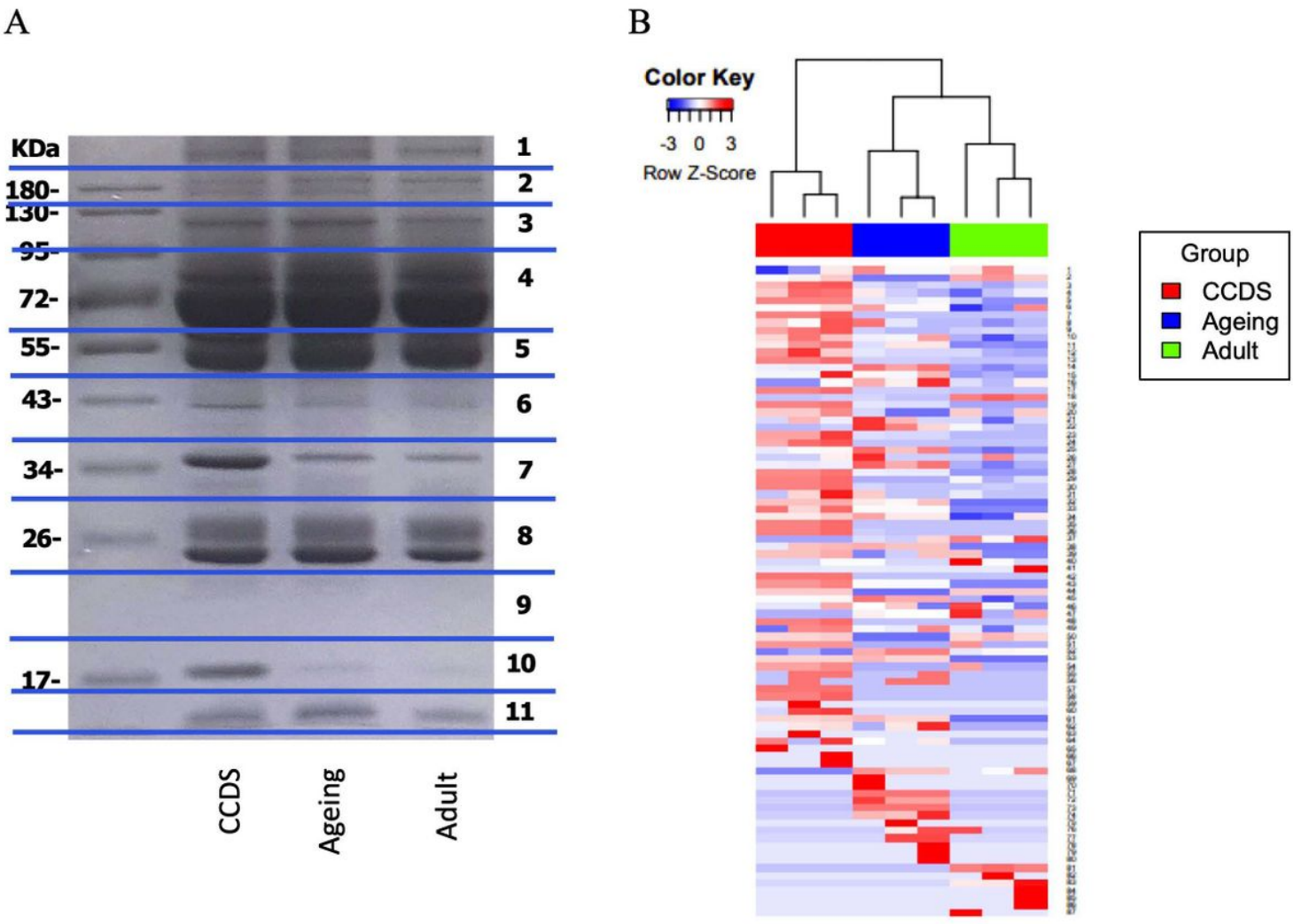

Figure 2

Protein patterns using (a). SDS-PAGE with Coomassie blue R-250 staining (b). Heat map of differentially expressed protein patterns

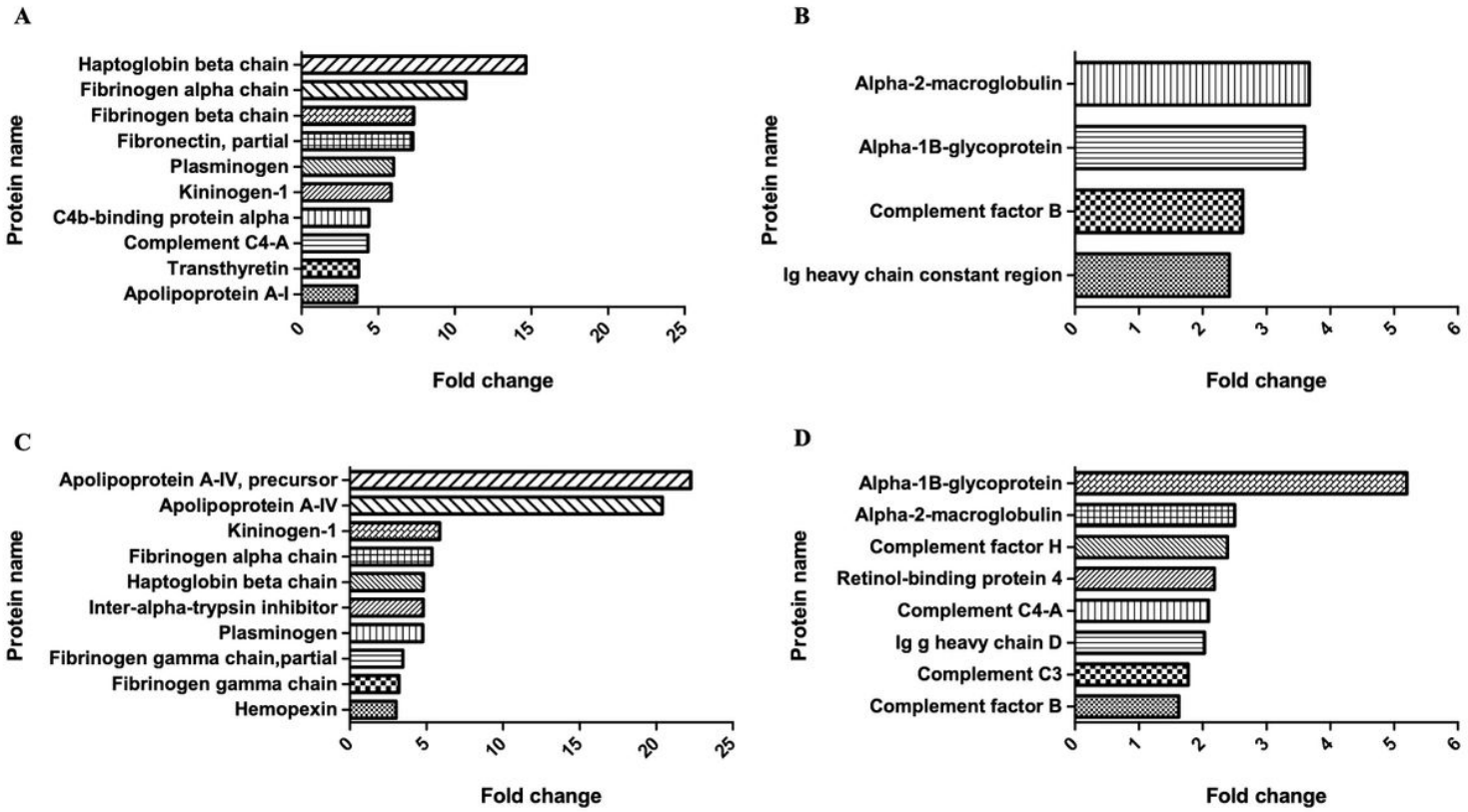

Figure 3

The changes in most proteins ranked by emPAI value: (a). Upregulated proteins in comparisons between the CCDS group and the adult group (b). Downregulated proteins in comparisons between the CCDS group and the adult group (c). Upregulated proteins in comparisons between the CCDS group and the ageing group (d). Downregulated proteins in comparisons between the CCDS group and the ageing group

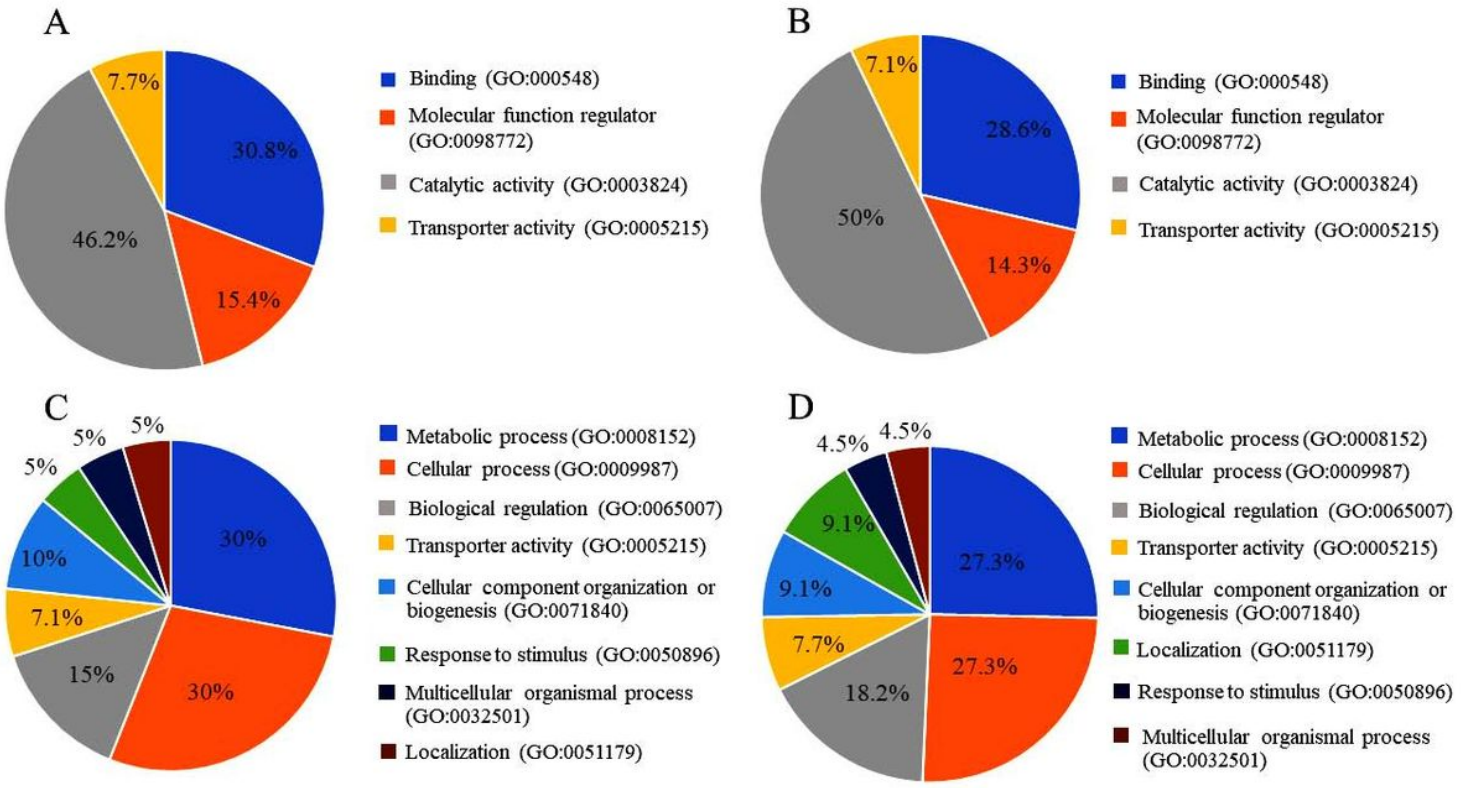

Figure 4

Gene Ontology molecular function and biological process categories for upregulated proteins in the CCDS group: (a). comparison of the CCDS group with the ageing group in molecular function; (b). comparison of the CCDS group with the adult group in molecular function; (c). comparison of the CCDS group compared with the ageing group in biological process; and (d). comparison of the CCDS group with the adult group in biological process.

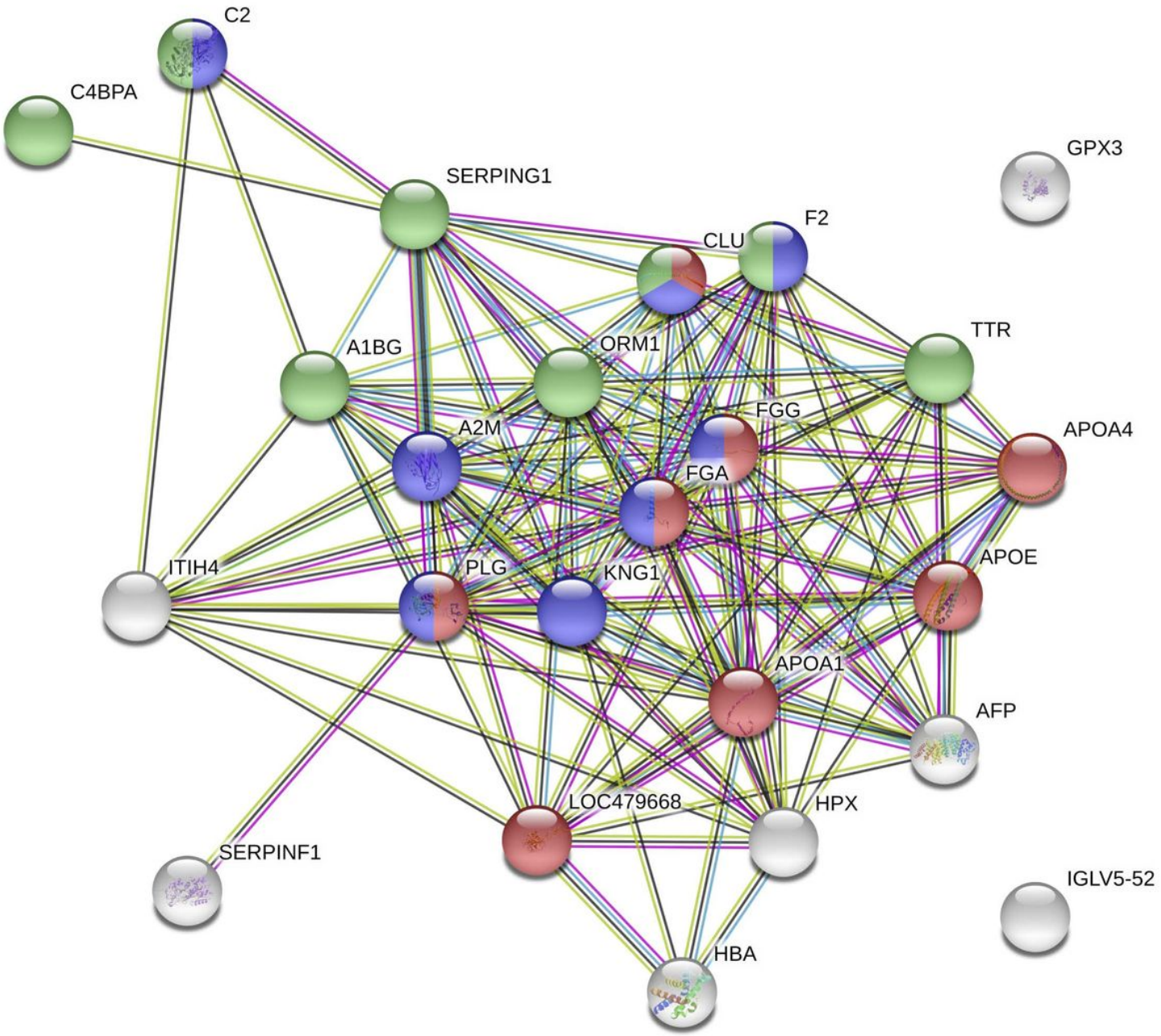

Figure 5

STRINGS protein-protein interaction: Analysis of protein changes in the CCDS group compared with both the adult group and the ageing group (total proteins = 24, red colour = response to stress, blue colour = complement and coagulation cascades and green = innate immune system)

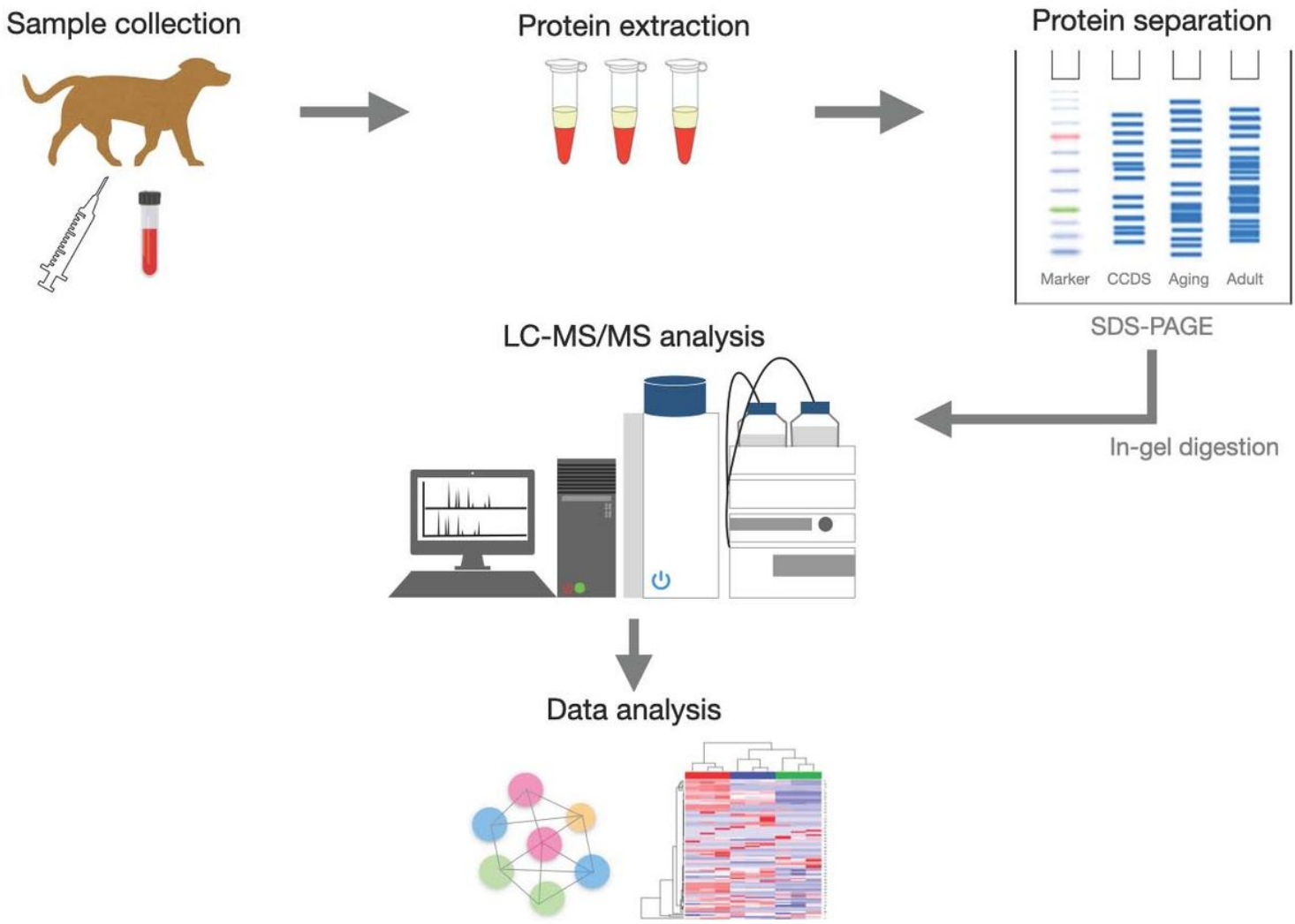

Figure 6

Proteomics workflow

Supplementary Files

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