Quantitative Proteomics of Cerebrospinal Fluid using Tandem Mass Tags in dogs with recurrent epileptic seizures

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Conflicts of interest

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

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Abstract

This prospective study included four dog groups (group A: healthy dogs, groups B: dogs with idiopathic epilepsy under antiepileptic medication (AEM), C: idiopathic epilepsy dogs without AEM administration, D: dogs with structural epilepsy). The purpose of the study was to compare the proteomic profile among the four groups. Samples were analyzed by a quantitative Tandem Mass Tags approach using a Q-Exactive-Plus mass-spectrometer. Identification and relative quantification were performed using Proteome Discoverer, and data were analyzed using R. Gene ontology terms were analyzed based on *Canis lupus familiaris* database. Data are available via ProteomeXchange with identifier PXD018893. Eighteen proteins were statistically significant among the four groups ($P < 0.05$). MMP2 and EFEMP2 appeared down-regulated whereas HP and APO-A1 were up-regulated (groups B, D). CLEC3B and PEBP4 were up-regulated whereas APO-A1 was down-regulated (group C). IGLL1 was down-regulated (groups B, C) and up-regulated (group D). EFEMP2 was the only protein detected among the four groups and PEBP4 was significantly different among the epileptic dogs. Western blot and SPARCL immunoassay were used to quantify HP abundance change, validating proteomic analysis. Both, showed good correlation with HP levels identified through proteomic analysis ($r = 0.712$ and $r = 0.703$, respectively).

Significance: The proteomic analysis from CSF of dogs with epileptic seizures could reflect that MMP2, HP and APO-A1 may contribute to a blood-brain barrier disruption through the seizure-induced inflammatory process in the brain. MMP2 change may indicate the activation of protective mechanisms within the brain tissue. Antiepileptic medication could influence several cellular responses and alter the CSF proteome composition.

Keywords: dogs, idiopathic epilepsy, SPARCL immunoassay, TMT-based proteomics, Western blot

1. Introduction

Epilepsy has been defined as a brain disorder characterized by recurrent seizures in dogs and humans [1, 2]. Epilepsy is not a disease but a disorder of aberrant neuronal connections [3]. The most recent International League against Epilepsy (ILAE) classification in dogs was based on the etiology of seizures, which is idiopathic, structural and unknown (cryptogenic) [4]. Idiopathic or primary epilepsy refers to recurrent seizures with no underlying cause other than a strongly suspected or confirmed genetic or familial basis [5, 6, 8]. Idiopathic epilepsy is the most common cause of seizures in dogs with an estimated prevalence of 1-2% in a referral hospital population and 0.6% in first opinion practice [7-9]. Cryptogenic epilepsy or probable symptomatic epilepsy refers to recurrent seizures caused by an underlying brain disease that is strongly suspected but cannot be identified despite extensive investigations [5]. The term "epilepsy of unknown etiology" has been proposed to replace the term cryptogenic epilepsy in humans, it may be of value in veterinary medicine due to its more immediate meaning [10]. The term structural epilepsy refers to recurrent epileptic seizures caused by a known and identifiable structural forebrain disorder such as vascular, inflammatory/infectious, traumatic, anomalous/developmental, neoplastic and degenerative diseases [5].

Diagnosis of idiopathic epilepsy is a diagnosis of exclusion. Therefore, after careful history-taking (young age and early seizure onset), physical and neurological examinations, blood chemistry tests, brain imaging and cerebrospinal fluid analysis have ruled out other causes of recurrent seizure activity, the diagnosis of idiopathic epilepsy is strongly suspected [11].

Many genes have been incriminated for idiopathic epilepsy, however no definite proof is available [12-15]. Therefore it is necessary to identify additional diagnostic tools in order to facilitate correct clinical diagnosis and to better understand the molecular mechanisms of epilepsy and generate therapies to cure or prevent epilepsy [16]. Since cerebrospinal fluid (CSF) is in direct contact with the central nervous system (CNS), it could be a promising source for identifying biomarkers for epilepsy [17]. Proteomic analysis is an advanced method to detect proteins in various biological materials (serum, urine, saliva, seminal plasma, tears, cerebrospinal fluid, bronchoalveolar lavage fluid, or tissues) from diseased and healthy animals. All the data are stored within databases in which proteome profiles from the same biological material between healthy and diseased animals can be compared [18].

Both dogs and humans suffer from naturally occurring epilepsies that share many clinical characteristics [14]. There are several studies reporting proteomic analyses in brain tissue of humans and animals suffering from central nervous system diseases [16, 19]. Regarding epilepsy, there are previous studies using hippocampi from rats as a model to study human temporal lobe epilepsy [20-25], however the usage of human CSF from proteomic analysis in epileptic patients is limited. In human patients suffering from mesial temporal lobe epilepsy, proteomic analysis obtained in hippocampal specimens revealed increased apolipoprotein AI (APO-AI) levels, showing that APO-AI is a marker of the extend and the severity of central nervous system injury [21]. On the other hand, another study in CSF of humans suffering from temporal lobe epilepsy showed elevated expression of apolipoprotein J, and some

proteins were identified only in the CSF of epileptic patients as tetranectin, apolipoprotein E, immunoglobulins [16].

Although canine idiopathic epilepsy shares more similarities with human epilepsy [14, 26], there are only few studies on CSF proteomic analysis in dogs with idiopathic epilepsy [27, 28]. The identification of biomarkers related to disease provides diagnostic tools as well as potential therapeutic and prognostic targets [29, 30]. The advancement of proteomic technologies has added new dimensions to the analyses of clinically relevant samples and promises to enhance the way diseases will be managed in the future. The ability to obtain a profile of the biochemical responses at the protein level could provide a more comprehensive view of cellular control mechanisms, which may have direct outcomes in improving our understanding of animal and human disease states [26].

In this initial study, CSF proteomic analysis was performed in dogs suffering from recurrent epileptic seizures (of acquired etiology or idiopathic causes). The purpose of the study was to compare identified proteins among the epileptic groups of dogs to healthy dogs, in an attempt to investigate proteomic profile differences in dogs with epileptic seizures of various etiologies.

The proteomic results, especially those related to acute phase protein haptoglobin (HP), suggested further investigation by using established methods (western blot and immunoassay) to quantify the change and to validate results from the proteomic analysis.

2. Materials and Methods

2.1. Clinical material

This was a prospective study involving canine patients divided into four groups. All dogs were treated according to European legislation on animal handling and experiments (86/609/EU). The study was approved by the Ethical Committee of the School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece (Prot. No. 567/13/03/2018). The owners of the epileptic dogs were briefed about the proposed diagnostic plan (clinicopathological and diagnostic imaging testing) and signed a statement of informed consent for participation in the study. The first group (group A) consisted of healthy dogs (control group) with no history of seizures or any other disease. The other three groups, after a detailed diagnostic investigation, consisted of dogs with recurrent seizures. Group B included dogs with idiopathic epilepsy receiving antiepileptic medication and group C those with idiopathic epilepsy without antiepileptic medication. Group D was consisted of dogs with structural epilepsy.

The study population included canine patients that suffered from recurrent epileptic seizures. The age of seizure onset should range from 6 months to 5 years in group B and in group C dogs. There was no limitation on age for group D dogs. Prior administration of antiepileptic medication (AEM) was not an exclusion criterion for the study population. The antiepileptic medication and the duration of therapy were recorded. Epidemiological data, age of seizure onset, frequency and the type of seizures were also recorded. For dogs receiving AEM, the response to therapy, the frequency and the type of seizures were included in the database. Dogs that weighed less than 2kg and dogs with reactive seizures

(seizures that are caused due to systemic metabolic or exogenous toxic disorder detected either during history taking or during clinicopathological testing), acute/history of head trauma and congenital diseases (hydrocephalus) were excluded from the study. A detailed history (age of seizure onset, frequency, type and duration of seizures, onset of antiepileptic medication, previous laboratory investigation, previous brain diagnostic imaging) was taken, combined with visual proof of the episode using video-footage brought by the owner of the epileptic dog in order to distinguish epileptic seizure from other paroxysms that can mimic epileptic seizure. Laboratory testing included complete blood counts (CBC), serum biochemistry profile and urinalysis. Complete blood counts were performed using ADVIA 120 Hematology System (Bayer Diagnostics, Dublin, Ireland) and serum biochemistry was done using Viatal Lab Flexor E (Spankeren, The Netherlands). Diagnostic imaging investigation included thoracic radiographs and abdominal ultrasound. Dogs with any concurrent systemic disease, revealed during diagnostic investigation, were excluded from the study. Brain diagnostic imaging involved computed tomography (CT) or/and magnetic resonance imaging (MRI). When the diagnostic investigation did not reveal any structural abnormality and the dog had compatible age (>6 months and <5 years old) and recurrent epileptic seizures, diagnosis of idiopathic epilepsy was strongly suggestive. Patients receiving AEM on admission were allocated in the "idiopathic epilepsy with AEM" group (Group B). Some dogs that belong in group D underwent AEM on admission as well. Not only the onset of AEM but also the duration of the therapy was crucial and thus it was set as an exclusion criterion. Therefore, dogs that were on AEM on admission were included in the study if the AEM was used in appropriate dose regimen and for a prolonged period to ensure adequate therapeutic serum concentrations. For AEM that were used in the study population (phenobarbital (PB), levetiracetam (LEV), bromide (Br)) the treatment duration should have been at least 1 month (for phenobarbital and levetiracetam), except for bromide which should have been at least 3 months. Serum drug concentrations were monitored in group B and D dogs in order to assess therapeutic efficacy. Cerebrospinal fluid (CSF) samples were collected *via* cisternal tap under general anesthesia and after the confirmation from CT or/and MRI brain imaging for the safety of the procedure. In group D dogs, where brain structural lesions had been detected (and could potentially increase intracranial pressure) CSF collection was performed *via* lumbar tap.

The collected amount of CSF was 1mL/5 kg of body weight. CSF samples with iatrogenic blood contamination were excluded from the study. CSF analysis was performed within 30 min after collection and included total cell counts, measurements of total protein and cytological examination. The CSF cytological examination was performed in stained slides (Aerospray Pro slide stainer/ cytocentrifuge ELI Tech Droup WESCOR) and the total cell counts were performed microscopically using a haemocytometer (BLAUBRAND Neubauer improved). CSF total proteins were measured in an automated biochemistry analyzer (FLEXOR Vitalab, The Netherlands) using the pyrrogalol red method (Dia Sys Diagnostic Systems, France). The remained CSF samples were centrifugated to remove cells and they were frozen at -80^{o} C for forthcoming proteomic analysis.

The control (healthy) study group (group A) consisted of clinically healthy dogs, with no history of seizures and normal laboratory and brain imaging tests. The dogs were recruited from the stray animals spraying/neutering program, run at the School of Veterinary

Medicine in cooperation with the local municipality, following a written agreement. Brain imaging scans and CSF sample collection was done at the time of spraying/neutering surgery. All dogs were subsequently vaccinated, micro chipped and adopted.

2.2. Proteomic investigation of canine cerebrospinal fluid

Relative quantification of proteins in canine cerebrospinal fluid samples was performed using Tandem Mass Tag (TMT)-based shotgun methods previously described [31]. In brief, total protein concentration was determined using BCA assay. An amount of 35 µg of total CSF proteins from all individual samples and internal standard (pool of all samples) was diluted using 0.1 M triethyl ammonium bicarbonate (TEAB) and subsequently reduced (20 mM DTT, 60 min, 55°C), alkylated (30 mM IAA, 30 min, room temperature in the dark) and acetone-precipitated (6 volumes, overnight,-20°C). Protein pellets were collected by centrifugation, dissolved in 0.1 M TEAB and digested using trypsin Gold (Promega; 1:35 w/w, at 37°C overnight). Peptides were labelled with freshly prepared TMT sixplex reagents (Thermo Scientific). Differentially TMT-modified samples were combined with the internal standard into the new tube, aliquoted, dried and stored at -20°C for further analysis.

LC-MS/MS analysis was performed using the Ultimate 3000 RSLCnano system (Dionex) and Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) as described elsewhere [31]. After desalting on the trap column, TMT-labelled peptides were separated on the analytical column (PepMap™ RSLC C18, 50 cm x 75 µm) using linear gradient 5-45% mobile phase B (0.1% formic acid in 80% ACN) over 120 min at the flow rate of 300 nL/min. Ionisation was achieved using nanospray Flex ion source (Thermo Fisher Scientific). MS was operating in positive ion mode using DDA Top8 method with parameters set as follows: full scan MS spectra range from *m/z* 350.0 to *m/z* 1800.0, resolution of 70000, injection time 120 ms, AGC target $1x10^6$,isolation window± 2.0 Da and the dynamic exclusion 30s. For HCD fragmentation, resolution was set to 17500 and AGC target to $2x10^5$.

Raw data were analyzed using Proteome Discoverer software (version 2.3., Thermo Fisher Scientific) with SEQUEST algorithm implemented. Database search against *Canis lupus familiaris* NCBInr FASTA files (downloaded 13/10/2016, 41787 entries) was performed according the following parameters: two trypsin missed cleavage sites, precursor and fragment mass tolerances of 10 ppm and 0.05 Da, respectively; carbamidomethyl (C) fixed peptide modification, oxidation (M) and TMT sixplex (K, peptide N-terminus) dynamic modifications. Proteins with at least two unique peptides and 5% FDR were selected as reliably identified. Internal standard was used to compare data between TMT experiments. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018893 [32].

2.3. Immuno detection and assay of specific protein in canine cerebrospinal fluid

Haptoglobin (HP) by western blot: Western blot analysis was performed as previously described [27]. Briefly, 2 µg of CSF total protein was denatured in Laemmli Buffer at 90°C for 4 min and electrophoresis performed using 26 well 4-12% Bis-Tris precast Criterion XT gels (Bio-Rad, USA). Separated proteins were transferred to a nitrocellulose membrane using the

iBlotsystem (Invitrogen), blocked with 5% milk powder in 0.1% Tween 20 in Tris-buffered saline (T-TBS) incubated overnight at 4^{o}C with anti haptoglobin antibody at 1/25,000 dilution (Reactive Lab Ltd, Singapore), washed, then incubated with HRP conjugated secondary antibody (Abcam Ltd. UK) in 5% milk powder in T-TBS for 2 hours at room temperature. Complexes were detected using the ECL reaction (Thermo Fisher Scientific) and visualized using radiographic film (Hyperfilm ECL, Amersham Biosciences). The intensity of the protein bands was quantified using Image J NIH software. The samples generated for each treatment were analyzed over two gels with group A and C samples common to both gels. The optical density intensity of these groups was used to generate a between gel conversion factor for the treated epilepsy samples to permit a statistical comparison of the values across all four groups. The protein profile of 0.2 μg of each sample was visualized by silver staining (SilverXpress, Invitrogen Ltd) as detailed in the manufacturer's handbook to provide a visual confirmation of equal protein loading for all samples. In addition, following transfer to nitrocellulose, the blots were stained with Ponceau S to confirm equal loading.

Haptoglobin (HP) by quantitative immunoassay: SPARCL™ (Spatial Proximity Analyte Reagent Capture Luminescence) technology, developed by Lumigen (Michigan USA), allows development of rapid, homogeneous, chemiluminescent immunoassays [33]. The use of SPARCL ^{TM} for measurement of HP in canine plasma has been previously described and validated [34]. The concentration of HP in CSF was also determined by SPARCL™ immunoassay (HAPT-4-SP, Life Diagnostics Inc, Pennsylvania USA), using a LUMIstar Omega plate luminometer (BMG Labtech Inc, North Carolina USA). CSF samples were tested at dilutions of 1:50 or 1:1000 as appropriate. Values were converted from μg/ml to μg/mg of total protein to allow comparison with proteomic and Western blot results.

2.4. Statistical and bioinformatic analysis

All statistics were performed using R v3.2.2 [35]. Sample outliers were detected and removed from further analysis per each group, for each of the protein using the Dixon's test from R package *outliers* v0.14 [36]. As the majority of analyzed proteins did not follow the normal distribution, tested by Shapiro-Wilk test, the difference in protein abundance between groups was tested using Kruskal-Wallis test. For proteins showed to be significantly differentially abundant ($P< 0.05$), Conover *post-hoc* test was performed for pairwise multiple comparisons using the R package *PMCMR* v4.3 [37]. Fold change between two groups was calculated as mean (Epileptic)/ mean (Healthy dogs) and expressed on log2 scale.

Volcano plots and validation plots were designed using R package *ggplot2* v3.1.1, heatmap was designed using R package *ggplot2* v3.1.1 and *ggdendro* 0.1-20 and Venn diagrams were designed using web tool Interacti Venn [38-40].
Proteins Gen Info identifier (GI) accession numbers were converted into official gene symbol either by DAVID conversion tool, UniProt KB ID mapping or from Mascot search engine implemented into Proteome Discoverer. Pathway enrichment analysis was performed using STRING db v11.0, with default settings [41]. Network of relationship between desired Reactome pathway and proteins with significantly different abundances between groups were designed using Cytoscape v3.7.1 [42].

The results from proteomic analysis for HP were compared to Western blot and SPARCL immunoassay in order to validate obtained proteomic results. After removal of possible outliers (defined with common 1.5 x IQR rule), difference between groups was determined using Kruskal-Wallis test with Conover *post*-hoc test for pairwise comparisons with FDR corrections, separately for Western blot and SPARCL immunoassay. To compare results of HP levels obtained with three methods, Spearman correlation was used.

3. Results

Thirty-four dogs met the inclusion criteria. Ten dogs belonged to group A (healthy dogs); nine dogs to group B (idiopathic epilepsy receiving AEM), eight dogs to group C (idiopathic epilepsy without AEM) and seven dogs to group D (structural epilepsy).

3.1. Study population

Mixed breed dogs predominated in the study population (19/34). The descriptive statistics of the study population dogs are summarized in Table 1.

3.2. History data of Epileptic dogs

The cause of admission was history of epileptic seizures in 22/24 dogs (Groups B, C and D). One dog appeared cluster seizures and one dog *status epilepticus* on admission.

The median age for seizure onset was 39 months for group B, 27 months for group C and 96 months for group D. One dog (group C) had a history of increasing duration of epileptic seizures and six dogs of increasing seizure frequency. Four dogs (group D) had an increasing seizure frequency.

The type of seizures and the seizure frequency of the study population dogs are summarized in Table 1. Some group-B dogs presented multiple seizure types. The clinical manifestations of the post-ictal period are summarized in Table 1.

Group B dogs received AEM on admission. Five dogs underwent only one AEM and 4 dogs underwent combination of two AEM. The duration of AEM treatment ranged from 1 month to 12 months (median 6 months). Three dogs (group D) were under AEM on admission (1 PB, 1 LEV and 1 PB+Br).

3.3. Diagnostic investigation

Complete blood counts and urinalysis were normal in all dogs. Serum biochemistry was normal, with the exception of 6 dogs (4 in group B and 2 in group D) that had mildly increased liver enzyme (ALP and ALT) activities, most likely due to PB treatment.

Thoracic radiographs and abdominal ultrasonography were normal in all dogs. Diagnostic imaging examination of the brain (CT) was normal in all dogs of groups A, B, C. Two dogs (group B) underwent MRI along with CT with no abnormalities detected. Group D cases underwent brain CT and 2 dogs also had a brain MRI. CT/MRI investigation revealed forebrain neoplasia (2 dogs), ischemic encephalopathy (3 dogs), cryptogenic epilepsy (2 dogs).

CSF cell count was within normal limits in all dogs. The total number of nucleated cells were <5/µl in all CSF samples. In 3 dogs (group A), 5 dogs (group B), 8 dogs (group C) and 3 dogs (group D), there were no detectable cells during microscopic evaluation of the CSF. One dog (group B), one dog (group C) and 2 dogs (group D) had an increased CSF total protein concentration.

3.4. Proteomic analysis

After exclusion of proteins with less than two unique peptides, proteomic analysis identified 888 proteins for further analysis (Table S1) (supplementary material). Out of those, fifty proteins were statistically significant ($P<0.05$) among the four groups of dogs, leading to eighteen unique proteins (Table 2). The fifty statistically significant proteins are shown in Table S2 of the supplementary material.

The results from proteomic analysis of CSF samples between four groups of dogs are summarized in Table 2 and are additionally depicted using Volcano plots (Fig. S1A-F, supplementary material), heatmap (Fig. 1), Venn diagrams to show their overlap (Fig. 2A-B). Results of pathway analysis are shown in Table 3 and Figure 3.

Results showed that, when comparing group B with group A, apolipoprotein (APOA1) was mildly up-regulated, immunoglobulin lambda-1 (IGLL1) was strongly down-regulated, haptoglobin (HP) was strongly up-regulated, matrix metallopeptidase 2 (MMP2) was mildly down-regulated, EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2) was mildly down-regulated. Group C compared to group A showed that IGLL1 was down-regulated, tetranectin (CLEC3B) was mildly up-regulated, EFEMP2 was mildly down-regulated, and phosphatidylethanolamine binding protein 4 (PEBP4) was mildly up-regulated. Group D compared to group A showed that APOA1 was mildly up-regulated, HP was strongly up-regulated, MMP2 and EFEMP2 were slightly down-regulated and PEBP4 was moderately down-regulated. Group C compared to group B showed that APOA1 was mildly down-regulated, CLEC3B was mildly up-regulated and PEBP4 was moderately up-regulated. Group D compared to group B showed that IGLL1 was up-regulated and PEBP4 was slightly down-regulated. Group D compared to group C showed that APOA1 was slightly up-regulated; IGLL1 and HP were moderately up-regulated, CLEC3B was mildly down-regulated and PEBP4 was down-regulated.

When looking at the overlap between significant abundant proteins, EFEMP2 was the only protein detected when comparing the three groups of epileptic dogs (groups B, C and D) with healthy dogs (group A).The only protein that was detected among the epileptic dogs was PEBP4.

Reactome pathway analysis indicated that MMP2 mediated extracellular matrix organization. Additionally, it revealed the contribution of MMP2 in the immune system and particularly through its interaction with interleukin-4 and interleukin-13 signaling. It showed an interaction with EFEMP2, HP and APO-A1. Haptoglobin (HP) contributed to immune system processes, scavenged heme from plasma and interacted with CLEC3B. Apolipoprotein A1 (APO-A1) contributed to protein's metabolism, post-translational protein modification, hemostasis, platelet degranulation and worked as an anti-oxidant.

Furthermore, an interaction among APO-A1, HP and CLEC3B was revealed. Tetranectin (CLEC3B) contributed in platelet degranulation and hemostasis and it reacted with EFEMP2. Pathway analysis revealed an interaction between PEBP4 and PTGDS (prostaglandin D2 synthase). Immunoglobulin lambda-1 (IGLL1) contributed to the cell surface interactions at the vascular level and to hemostasis. EGF-containing fibulin-like extracellular matrix protein 2 (EFEMP2) contributed to the organization of the extracellular matrix and interacted with MMP2 and CLEC3B.

3.5. Western blot of haptoglobin in cerebrospinal fluid

Western blots of CSF from four groups of dogs are shown in Figure 4A-B and Figure S2A-D (supplementary material). Not all samples could be analyzed due to low volumes. Clear bands corresponding to the β-chain of canine HP (Mw 40kDa) were observed in 2/9 of the controls (Group A), 8/8 of Group B (IE receiving AEM), 5/8 of Group C (IE not receiving AEM) and 6/7 of Group D (structural epilepsy). Quantification of the Western blots by scanning and determination of the OD units was achieved and after outlier removal there were statistical differences among groups (P=0.011) (Fig. S3A). There were highly significant differences between groups A and D (P=0.006), there was a significant difference between groups A and B (P=0.032), but there was not significant differences between groups C and D (Table 4, P=0.06). Overall assessment of the results found there was a high correlation between HP results determined after outlier removal by Western blot and the SPARCL immunoassay (Fig. S4A, r=0.873) and between the Western blot result and HP levels determined by proteomics (Fig. S4B, r=0.712). Results prior to outlier removal are shown in supplementary material (Fig. S5A-C).

3.6. SPARCL immunoassay of haptoglobin in cerebrospinal fluid

Quantification of HP concentration in CSF showed (Fig. S3B) that after outlier removal there were statistical differences among groups (P=0.001). There were highly significant differences between groups A and B, groups A and D, groups B and C and groups C and D (Table 4, P<0.001). Overall assessment of the results found there was a good correlation between the HP results determined after outlier removal by the SPARCL immunoassay and HP levels determined by proteomics (Fig. S4C, r=0.703). Results prior to outlier removal are shown in supplementary material (Fig. S5A-C).

4. Discussion

In this study, it was observed that epilepsy altered the CSF levels of several proteins with physiologically relevant functions identified as differentially abundant. Matrix metallopeptidase 2 (MMP2) appeared to be down-regulated in dogs receiving AEM (group B and group D). In contrast, group C dogs were not under antiepileptic treatment and did not show any statistical significance regarding MMP2. When comparing the behavior of MMP2 between group B and group D, group B showed more severe down-regulation of MMP2 compared to group D. The changes of MMP2 in these two groups (B and D) could be associated to administered medications. Epileptic seizures are the result of an excessive synchronized discharge of a network of neurons and the subsequent elicited inflammatory process within the brain that may be an important component of seizure pathophysiology

[43]. This seizure-induced inflammation can contribute to neurodegeneration and cell death [44]. Matrix metalloproteinases are a family of enzymes that degrade components of the extracellular matrix, which is important for normal blood-brain barrier (BBB) function [45]. In particular, MMP2 and MMP9 belong to gelatinase group of enzymes that degrade the basic membrane leading to BBB disruption and recruitment of inflammatory cells [46]. These finding were supported from Reactome pathway results regarding MMP2's contribution to extracellular matrix organization and immune system processes. MMP2 has been reported to increase in various neurological disorders, infectious/inflammatory [46-50], or non-infectious but with an inflammatory component [45, 51-56]. MMP9 is the predominant metalloproteinase that has been investigated for its role in epileptogenesis, while there is limited information of MMP2 [57]. It is known that both MMPs (MMP2 and MMP9) contribute to epileptogenesis, neuronal network remodeling, neuronal cell death and BBB leakage after seizures [58]. As MMP2 has a major role in BBB disruption and epileptic seizure is a potentially inflammatory process, it would be expected to appear increased in group B and D dogs [57-61]. However, MMP2 was down-regulated in these two groups of dogs in the current study. The role of MMP2 on BBB disruption, the antiepileptic medication with assumed protective role against seizures and the long interval that elapsed from the last seizure to CSF tap (at least 7 days) could be some possible explanations for the down-regulation of MMP2 in the current study [53]. CSF MMP2 levels were decreased in patients with Alzheimer's disease justifying this speculation by the protective role of MMP2 in the brain tissue [62]. Moreover, MMP2 has showed a slow increasing response pattern in experimental animals [59, 63, 64]. As it is revealed through the pathway analysis, the interaction between MMP2 and interleukin-4 and interleukin-13, which both work as anti-inflammatory agents following an inflammatory stimulus in the brain, confirmed the protective role of MMP2 [65]. The slow increase of MMP2 and the usage of MMP2 by the brain tissue for its protective role in it; this could be another possible explanation for the down-regulation of MMP2 in epileptic patients in the current study.

Epileptic seizures have been associated with cerebral blood flow and oxygenation changes [66]. Haptoglobin (HP) is a α2 glycoprotein functioning as an antioxidant by binding hemoglobin and preventing hemoglobin induced oxidative tissue damage [67]. Experimental evidence supports the hypothesis that decreased HP is associated with poor clearance of free hemoglobin in the central nervous system and may lead to seizure disorders [68]. Furthermore, increased HP in CSF is indicative of blood-brain barrier dysfunction [69]. Combining its function with the BBB dysfunction, HP has been investigated in many neurological diseases either in plasma or in CSF [70-74]. Especially, in epilepsy cases findings are controversial. Previous studies demonstrated an association between decreased plasma HP and the occurrence of seizures in idiopathic epilepsy cases [68, 75, 76]. An association between the phenotype of HP (HP 2-2) and seizure frequency was found [76]. However, another study failed to recognize HP as a risk factor for the occurrence of acute epileptic seizures [77]. There is an intriguing possibility that the protein detected in the CSF is analogous to human zonulin, which shares sequence with human HP 2-2 and antibody responses with anti-human HP. Though most relevant to intestinal diseases, it has been suggested that zonulin is also associated with diseases of the nervous system in humans [78]. Its role has been investigated and proved in many autoimmune diseases including

neurological diseases indicating the contribution of serum zonulin in BBB dysfunction and the following activation of HP within the nervous system [78].

In the current study, HP was up-regulated in group B (idiopathic epilepsy under AEM) and group D dogs (structural epilepsy) compared to healthy dogs. These changes were confirmed by alternative methodology, both with the Western blot investigation and the SPARCL immunoassay, which both correlated to the changes in abundance, found by proteomic analysis. With the use of these methods, there were further statistical differences identified between groups with lower p-values. These immunoassays, based on antibodies to canine HP currently are more sensitive, having a greater ability to quantify specific protein, however with further advances in proteomics especially in targeted approaches could improve ability of quantitative proteomics to rival that of these immunoassay procedures.

The findings on CSF HP are also supported by previous reports in humans with structural brain disorder [79, 80]. In human idiopathic epilepsy, an association was found between the phenotype of HP and the presence of epileptic seizures [77]. An older study indicated the association between hypohaptoglobineamia and familial epilepsy [68]. Regarding canine idiopathic epilepsy, there is no information concerning the significance of HP changes and the potential association to antiepileptic therapy. In the current study, HP was up-regulated in group B (dogs receiving AEM); however it was not statistically significant in group C (dogs not receiving AEM). Thus, dogs with epileptic seizures receiving AEM were found with up-regulated levels of CSF HP indicating that therapy might potentially impact HP. Furthermore, the epileptic seizures appeared in both group B and D dogs may reflect a BBB dysfunction and an increase need for brain protection from further damage caused by the seizures, can be a possible explanation for the up-regulation of HP in these two groups of dogs in the current study. As it is reflected through pathway analysis and confirmed by previous literature, HP regulated the immune system, and had anti-oxidant and anti-inflammatory properties indicating its complex and protective role in patients presented with epileptic seizures [81].

Apolipoprotein A1 (APO-A1) is a multi-factorial protein that has a pivotal role in cholesterol transport and regulation of inflammation [82]. While its role in reversing cholesterol transport in the periphery is known, its role in the central nervous system is not fully elucidated [83]. APO-A1 is not synthesized in the brain but may cross the BBB by trancytosis [84]. Although the biological function of apolipoproteins in the brain is not clear, APO-A1 and APO-E are the major transport pathway of lipids in the brain [85]. In human medicine the role of APO-A1 changes in serum/plasma or CSF in people with neurological disorders has been investigated. In some studies APO-A1 decreased [86-89]; whereas in others it increased [83, 90-95]. However, it can be either increased or decreased in the course of the same disease, indicating a change of the protein structure from the early to the late stages of the disease [94]. Regarding its role in epilepsy cases, previous reports had focused on APO's metabolic lipid pathway in epileptic people under AEM therapy. Changes in APO (up- or down- regulation) depended on the type of antiepileptic medication [96-100]. In this study, APO-A1 was up-regulated in the idiopathic epilepsy group under AEM (group B) and in the structural epilepsy group (group D) compared to healthy dogs. Especially, in group D, up-regulation was more prominent than the up-regulation in group B. Both groups were treated

with AEM and suffered from epileptic seizures, which could mean a BBB dysfunction (as it was mentioned previously), associated with APO-A1 up-regulation. Furthermore, APO-A1 was down-regulated in idiopathic epilepsy group without AEM (group C) when compared to idiopathic epilepsy group under AEM (group B). Its role in modifying proteins (as it was revealed through pathway analysis) was reflected through its different biological behavior between epileptic dogs that received and those that did not receive AEM. This could also indicate the potential AEM involvement in the increase of APO-A1 in CSF of epileptic dogs. The hypothesis regarding the protective role of APO-A1 in severely injured nervous tissue was supported by previous reports, in which APO-A1 increased in CSF of patients with subarachnoid hemorrhage [91]. As it is shown through pathway analysis in the current study, this protective role of APO-A1 in the brain tissue was proved through its anti-oxidant role (reducing free hemoglobin) and by preventing further damage caused by micro-hemorrhage or BBB disruption. Regarding idiopathic epilepsy, increased APO-A1 was found in brain tissue of patients suffering from mesial temporal lobe epilepsy [101]. The increased APO-A1 is caused by extravasation, bleeding or proliferation of microvascular endothelial cells known to synthesize APO-A1 [101].

Tetranectin (CLEC3B) is produced in the brain and its major role is the regulation of proteolytic process via its binding to plasminogen. It is also involved in the process of neuronal degradation, remodeling and regulated the hemostatic system [102-104]. This suggests a potential role in epileptogenesis since pathological changes including structural and cellular reorganization of the hippocampal formation and neocortex in human temporal lobe epilepsy have been established [105]. Tetranectin has been previously isolated from CSF of epileptic patients and reported to increase in CSF in people with epilepsy compared to healthy controls [16, 90]. In this study tetranectin was up-regulated in untreated epileptic dogs (group C) compared to healthy dogs. There was no statistically significant difference among early onset, drug-refractory and drug-effective epileptic patients [90]. However, tetranectin was not statistically significant in dogs receiving AEM (group B) compared to healthy dogs (group A). Antiepileptic medication may have an impact in the processes regulated by tetranectin and the difference with the humans is the different kind of antiepileptics that were used in the study population.

PEBP4 is a protein with multiple functions, involved in lipid transfer, membrane biogenesis or binding various nucleotides which are associated with signaling mechanisms between cell membrane and cytoplasm [106-108]. Its trophic role in the central nervous system was reflected through its interaction with the prostaglandin D2 synthase (PTGDS) as it was shown in the reactome pathway analysis. The particular mechanism of PEBP4 action in epileptic dogs is unknown. PEBP4 was up-regulated in idiopathic epilepsy dogs that were not under AEM (group C). Immunoglobulin lambda light chain (IGLL1) has been previously isolated from CSF of humans with idiopathic temporal lobe epilepsy compared to healthy controls [16]. This finding is in contrast with the results of the current study. IGLL1 was down-regulated when comparing idiopathic epilepsy cases with healthy dogs, but up-regulated when comparing structural epilepsy cases with the two groups of idiopathic epilepsy. The different AEM and the underlying structural abnormalities in group D dogs could be a possible explanation for the finding regarding IGLL1's biological behavior. The impact of the AEM in IGLL1's behavior is shown through pathway analysis which revealed

the cell surface interactions at the vascular wall. Moreover, intravenous immunoglobulin has been previously administered in humans to control epileptic seizures that did not respond to AEM, such data in veterinary medicine are lacking and it should be further investigated [109, 110]. EFEMP-2 gene encodes the EGF-containing fibulin-like extracellular matrix protein-2 or fibulin-4. This protein is responsible, as verified by the reactome pathway analysis, for assembling the elastic fibers providing strength and flexibility to connective tissue in the extracellular matrix [111]. EFEMP-2 is found in organs and tissues that are rich in elastic fibers like blood vessels, lungs, heart valves, skin [111]. It has been over-expressed in gliomas/glioblastomas, but has not been previously investigated in epilepsy cases [112, 113]. EFEMP2 was found to contribute to proliferation, apoptosis and invasion of the glioma cells [112]. In the current study EFEMP2 was down-regulated in all the three groups with epileptic seizures (groups B, C and D) compared to group A (healthy dogs). Furthermore, it was not statistically significant in any of the comparisons among the epileptic groups.

As shown through reactome pathway analysis, there was a complex interaction among the proteins MMP2, HP, APO-A1, CLEC3B and EFEMP2. Briefly, the interpretation of the interaction among the five proteins identified in the CSF of epileptic dogs revealed that there may be a BBB disruption in the brain suffering from epileptic seizures. There was also indication of a seizure-induced, inflammatory process, however simultaneously the brain activated protective mechanisms to eliminate this inflammatory process (through anti-inflammatory and anti-oxidant agents). Furthermore, AEM administration contributed in this protective process, which is reflected by modulating the biological behavior of the proteins.

It was a limitation of the study that the serum concentration of HP was not determined as it could have revealed any association between serum and CSF levels of this protein. This should be addressed in future studies. The diversity of AEM that were used in the study population was an additional limitation of the current study.

The dog groups were heterogenous regarding their breed, age, seizure onset and frequency, CSF site collection, and administered AEM. Further studies in dogs diagnosed with idiopathic or acquired epilepsy are needed in order to investigate whether proteomic profile changes are associated with seizure pathogenesis, which could likely have diagnostic or prognostic impact, especially in patients with refractory epilepsy or structural brain abnormalities. Besides, providing a panel of biomarkers could be a future research field as it can improve the sensitivity and the specificity of the isolated biomarkers.

5. Conclusions

The current study provides information addressing the CSF proteomes in dogs with recurrent epileptic seizures. The proteomic analysis from CSF of dogs with epileptic seizures could reflect that MMP2, HP and APO-A1 may contribute to a blood-brain barrier disruption involving the seizure-induced inflammatory process in the brain. MMP2 change may indicate the activation of protective mechanisms within the brain tissue. Antiepileptic medication may influence the biological behavior of the proteins isolated from CSF proteomic analysis.

Conflicts of interest

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

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Appendix A. Supplementary information

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD018893.
The supplementary information includes Figures S1, S2, S3, S4 and S5 and Table S1 and S2.

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899 glioblastoma. Onco Targets Ther 2017; 10: 387-95.

900 **Figure captions**

901 **Figure 1.** Heatmap of the 18 significant differentially abundant proteins among four groups
902 of dogs.

903 **Figure 2.**Overlap of the number of significant differentially abundant proteins among four
904 groups of dogs; (A) in comparison to healthy dogs, and (B) among various groups of epileptic
905 dogs.

906 **Figure 3.** Network of the 18 significant differentially abundant proteins among four groups
907 of dogs with significantly enriched Reactome pathway terms.

908 **Figure 4.** Results of validation analysis. (A) Assessment of CSF haptoglobin levels by Western
909 blot (HP detected as a prominent band at Mw 40 kDa corresponding to the β-chain of canine
910 HP), (B) The samples generated for each treatment were analyzed over two gels with group
911 A and C samples common to both gels. The optical density intensity of these groups was
912 used to generate a between-gel conversion factor for the treated epilepsy samples to permit
913 a statistical comparison of the values across all four groups. (C) The relative fold change in
914 HP levels recorded by Western blot, SPARCL immunoassay and proteomic analysis. The data
915 obtained by Western blot and SPARCL immunoassay are shown in Figure S3. Statistical
916 analysis comparing the HP levels obtained by different methods is summarized in Table 4.

917 **Tables**

918 **Table 1.** Descriptive characteristics of the epileptic dogs.

	Group A	Group B	Group C	Group D
Median age on	24	48	45	96

admission (months)				
Median weight (Kg)	26.5	10	28.55	21
Type of epileptic seizures				
Generalized tonic-clonic		5	7	6
Generalized tonic		1		1
Focal		2		
Focal evolved to generalized		2		
Complex partial		2	1	
Frequency of epileptic seizures				
Single seizures		4/month	3/3 months	
Cluster seizures		1/month	1/3 months**	At least 1/month*
Status epilepticus		1/ month		
Post-ictal clinical manifestation				
Aggressiveness			1	
Ataxia			4	2
Cognitive dysfunction		2		
Depression			2	2
Disorientation		4	1	
Head pressing				2
Increased appetite			1	
Pacing		2		1
Temporary blindness		2	1	
Vomiting		1		
Walking disorders		1		
No detectable signs		3	3	1

*six dogs

** three dogs

Table 2. Significant differentially abundant proteins among four groups of dogs.

Gene	Description	log2 fold change					
		B vs	C vs	D vs	C vs	D vs	D vs

		A	A	A	B	B	C
AHSG	Alpha-2-HS-glycoprotein	/	/	0.57	/	0.57	0.3
APOA1	Apolipoprotein A-I	0.49	/	0.61	-0.46	/	0.58
C7	Complement component C7	-0.56	/	-0.4	0.53	/	-0.37
CLEC3B	Tetranectin	/	0.37	/	0.45	/	-0.71
CP	Ceruloplasmin	-0.63	-0.27	/	/	0.51	/
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	-0.75	-0.31	-0.3	/	/	/
HP	Haptoglobin heavy chain	1.84	/	1.74	/	/	1.07
IGFBP2	Insulin-like growth factor-binding protein 2	-0.46	/	-0.57	0.31	/	-0.43
IGLL1	Immunoglobulin lambda 1 light chain	-1.44	-1.22	/	/	1.26	1.05
IL13RA2	Interleukin 13 receptor subunit alpha 2	-0.98	/	/	0.96	0.75	/
MMP2	Matrix metalloproteinase 2	-0.6	/	-0.32	/	/	/
OGN	Mimecan	-0.65	/	-0.69	0.52	/	-0.56
PCOLCE	Procollagen C-endopeptidase enhancer 1	/	0.18	-0.49	0.53	/	-0.67
PEBP4	Phosphatidylethanolamine-binding protein 4	/	0.44	-1.02	0.76	-0.7	-1.46
PROS1	Vitamin K-dependent protein S	/	/	-0.28	/	-0.26	-0.35
PTGDS	Prostaglandin D synthase	-0.71	/	-0.58	0.71	/	-0.59
SERPINF1	Pigment epithelium-derived factor	/	/	-0.41	0.42	/	-0.56
VGF	Neurosecretory protein VGF	/	/	-0.44	/	-0.31	-0.49

Table 3. Significantly enriched Reactome pathways from the 18 differentially abundant proteins among four groups of dogs.

ID	Description	FDR	Identified proteins
HSA-381426	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	1.13E-07	AHSG, APOA1, CP, IGFBP2, MMP2, VGF

HSA-8957275	Post-translational protein phosphorylation	1.20E-04	AHSG, APOA1, CP, VGF
HSA-114608	Platelet degranulation	1.50E-04	AHSG, APOA1, CLEC3B, PROS1
HSA-2168880	Scavenging of heme from plasma	1.40E-03	APOA1, HP
HSA-109582	Hemostasis	2.20E-03	AHSG, APOA1, CLEC3B, IGLL1, PROS1
HSA-977606	Regulation of Complement cascade	9.00E-03	C7, PROS1
HSA-392499	Metabolism of proteins	1.03E-02	AHSG, APOA1, CP, IGFBP2, MMP2, PROS1, VGF
HSA-1474244	Extracellular matrix organization	1.83E-02	EFEMP2, MMP2, PCOLCE
HSA-6785807	Interleukin-4 and Interleukin-13 signaling	2.98E-02	IL13RA2, MMP2
HSA-168256	Immune System	3.79E-02	AHSG, C7, HP, IL13RA2, MMP2, PROS1
HSA-202733	Cell surface interactions at the vascular wall	3.93E-02	IGLL1, PROS1
HSA-597592	Post-translational protein modification	3.93E-02	AHSG, APOA1, CP, PROS1, VGF

Table 4. Western blot OD, SPARCL immunoassay and Proteomic changes of HP in the study population.

	Group A			Group B			Group C		
	Western Blot	SPARCL assay	Proteomic analysis	Western Blot	SPARCL assay	Proteomic analysis	Western Blot	SPARCL assay	Proteomic analysis
Group B	**0.032**	**<0.001**	0.119	-	-	-	0.234	-	-
Group C	0.234	0.319	0.262	-	**0.002**	0.468	-	-	-
Group D	**0.006**	**0.001**	**0.015**	0.322	0.832	0.262	0.06	**0.008**	0.131

*FDR adjusted P-value after *post hoc* Kruskal-Wallis test (Conover test)

Supplementary material

Figure S1. Volcano plots showing the biological behavior of the proteins between (A) groups B and A, (B) groups C and A, (C) groups D and A, (D) groups C and B, (E) groups D and B, and (F) groups D and C.

Figure S2. (A-D) Full length scans of the Western blot x-ray films showing a prominent band corresponding to HP β-chain with minimal detection of nonspecific bands. All available samples were separated by SDS-PAGE and the gels silver stained to demonstrate comparable protein loading across almost all samples.

Figure S3. Quantification of HP levels recorded by (A) Western blot, (B) SPARCL immunoassay and (C) proteomic analysis.

Figure S4. Correlation of HP between (A) Western blot and SPARCL immunoassay, (B) Western blot and proteomics, (C) proteomics and SPARCL immunoassay after outliers removal.

Figure S5. Correlation of HP between (A) Western blot and SPARCL immunoassay, (B) Western blot and proteomics, (C) proteomics and SPARCL immunoassays before the removal of outliers.

Table S1. Eight hundred eighty-eight proteins identified from proteomic analysis.

Table S2. Proteins (all) with significantly differential abundances between pairwise group comparison (A –Healthy dogs, B – Idiopathic Epilepsy receiving antiepileptic medication, C – Idiopathic Epilepsy without antiepileptic medication, D –Structural Epilepsy).

GI accession	log_2(fold change)						Gene	Description
	AB	AC	AD	BC	BD	CD		
1418506317	-0.6	NS	-0.32	NS	NS	NS	MMP2	72 kDa type IV collagenase [Canis lupus dingo]
928128352	-0.6	NS	-0.32	NS	NS	NS	MMP2	72 kDa type IV collagenase [Canis lupus familiaris]
1418515495	NS	NS	0.57	NS	0.57	0.3	AHSG	alpha-2-HS-glycoprotein [Canis lupus dingo]
928167527	-0.63	-0.27	NS	NS	0.51	NS	CP	ceruloplasmin isoform X1 [Canis lupus familiaris]
1239957836	-0.63	-0.27	NS	NS	0.51	NS	CP	ceruloplasmin isoform X2 [Canis lupus familiaris]
73990367	-0.63	-0.27	NS	NS	0.51	NS	CP	ceruloplasmin isoform X3 [Canis lupus familiaris]
1239957839	-0.63	-0.27	NS	NS	0.51	NS	CP	ceruloplasmin isoform X4 [Canis lupus familiaris]
1418329739	-0.56	NS	-0.4	0.53	NS	-0.37	C7	complement component C7 isoform X1 [Canis lupus dingo]
73953824	-0.56	NS	-0.4	0.53	NS	-0.37	C7	complement component C7 isoform X1 [Canis lupus familiaris]
1418329741	-0.65	NS	-0.43	0.59	NS	-0.36	C7	complement component C7 isoform X2 [Canis lupus dingo]
1239899370	-0.65	NS	-0.43	0.59	NS	-0.36	C7	complement component C7 isoform X2 [Canis lupus familiaris]
1418329743	-0.48	NS	-0.44	0.41	NS	-0.37	C7	complement component C7 isoform X3 [Canis lupus dingo]
928160462	-0.75	-0.31	-0.3	NS	NS	NS	EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2 [Canis lupus familiaris]
258499	1.84	NS	1.74	NS	NS	1.07	HP	haptoglobin heavy chain, HpH chain [dogs, Peptide, 245 aa]
73957095	1.73	NS	1.64	NS	NS	1.01	HP	haptoglobin-like [Canis lupus familiaris]
1239964848	-1.44	-1.22	NS	NS	1.26	1.05	IGLL1	immunoglobulin lambda-1 light chain isoform X16 [Canis lupus familiaris]
1239964850	-1.44	-1.22	NS	NS	1.26	1.05	IGLL1	immunoglobulin lambda-1 light chain isoform X17 [Canis lupus familiaris]
1239964866	-1.44	-1.22	NS	NS	1.26	1.05	IGLL1	immunoglobulin lambda-1 light chain isoform X26 [Canis lupus familiaris]
1239964868	-1.44	-1.22	NS	NS	1.26	1.05	IGLL1	immunoglobulin lambda-1 light chain isoform X27 [Canis lupus familiaris]
1239964896	-1.42	-1.17	NS	NS	1.14	0.89	IGLL1	immunoglobulin lambda-1 light chain isoform X44 [Canis lupus familiaris]
1418342184	-0.32	NS	-0.55	0.29	NS	-0.52	IGFBP2	insulin-like growth factor-binding protein 2 isoform X1 [Canis lupus dingo]
1418342186	-0.46	NS	-0.57	0.31	NS	-0.43	IGFBP2	insulin-like growth factor-binding protein 2 isoform X2 [Canis lupus dingo]
1239981567	-0.46	NS	-0.57	0.31	NS	-0.43	IGFBP2	insulin-like growth factor-binding protein 2 isoform X2 [Canis lupus familiaris]
1418342188	-0.46	NS	-0.57	0.31	NS	-0.43	IGFBP2	insulin-like growth factor-binding protein 2 isoform X3 [Canis lupus dingo]

GI accession	log_2(fold change)						Gene	Description
	AB	AC	AD	BC	BD	CD		
1239981569	-0.46	NS	-0.57	0.31	NS	-0.43	IGFBP2	insulin-like growth factor-binding protein 2 isoform X3 [Canis lupus familiaris]
7688731	-0.6	NS	-0.32	NS	NS	NS	MMP2	matrix metalloproteinase-2, partial [Canis lupus familiaris]
1418302656	-0.65	NS	-0.69	0.52	NS	-0.56	OGN	mimecan isoform X1 [Canis lupus dingo]
1418302658	-0.65	NS	-0.69	0.52	NS	-0.56	OGN	mimecan isoform X2 [Canis lupus dingo]
1418307311	NS	NS	-0.44	NS	-0.3	-0.5	VGF	neurosecretory protein VGF [Canis lupus dingo]
928136400	NS	NS	-0.44	NS	-0.31	-0.49	VGF	neurosecretory protein VGF [Canis lupus familiaris]
1418230432	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X1 [Canis lupus dingo]
345790561	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X1 [Canis lupus familiaris]
1418230422	NS	0.44	-1.02	0.76	-0.7	-1.46	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X2 [Canis lupus dingo]
1239961995	NS	0.44	-1.02	0.76	-0.7	-1.46	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X2 [Canis lupus familiaris]
1418230426	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X4 [Canis lupus dingo]
1239961999	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X4 [Canis lupus familiaris]
1418230428	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X5 [Canis lupus dingo]
1239962001	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X5 [Canis lupus familiaris]
1418230430	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X6 [Canis lupus dingo]
345790559	NS	0.58	-0.7	0.73	-0.56	-1.28	PEBP4	phosphatidylethanolamine-binding protein 4 isoform X6 [Canis lupus familiaris]
119637837	NS	NS	-0.41	0.42	NS	-0.56	SERPINF1	pigment epithelium-derived factor [Canis lupus familiaris]
545510243	NS	NS	-0.41	0.42	NS	-0.56	SERPINF1	pigment epithelium-derived factor isoform X1 [Canis lupus familiaris]
1418307444	NS	0.18	-0.49	0.53	NS	-0.67	PCOLCE	procollagen C-endopeptidase enhancer 1 [Canis lupus dingo]
4850328	-0.71	NS	-0.58	0.71	NS	-0.59	PTGDS	prostaglandin D synthase [Canis lupus familiaris]
3915607	0.49	NS	0.61	-0.46	NS	0.58	APOA1	RecName: Full=Apolipoprotein A-I; Short=Apo-AI; Short=ApoA-I; AltName: Full=Apolipoprotein A1; Contains: RecName: Full=Proapolipoprotein A-I; Short=ProapoA-I; Contains: RecName: Full=Truncated apolipoprotein A-I; Flags: Precursor

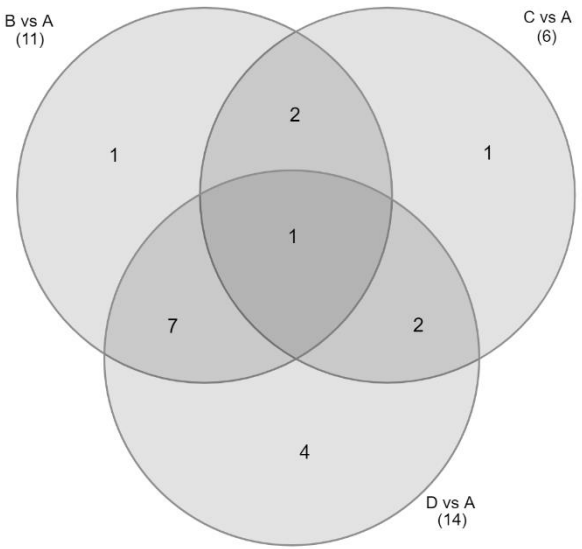
GI accession	log_2(fold change)						Gene	Description
	AB	AC	AD	BC	BD	CD		
123511	1.73	NS	1.64	NS	NS	1.01	HP	RecName: Full=Haptoglobin; Contains: RecName: Full=Haptoglobin alpha chain; Contains: RecName: Full=Haptoglobin beta chain
1418220357	NS	0.37	NS	0.45	NS	-0.71	CLEC3B	tetranectin [Canis lupus dingo]
16607718	-0.98	NS	NS	0.96	0.75	NS		unnamed protein product [Canis lupus familiaris]
1418199974	NS	NS	-0.28	NS	-0.26	-0.35	PROS1	vitamin K-dependent protein S [Canis lupus dingo]
1239976411	NS	NS	-0.28	NS	-0.26	-0.35	PROS1	vitamin K-dependent protein S [Canis lupus familiaris]

Figure 1


IL13RA2
IGLL1
APOA1
AHSG
MMP2
EFEMP2
CP
PCOLCE
CLEC3B
VGF
PROS1
IGFBP2
C7
SERPINF1
PTGDS
OGN
PEBP4
HP
B vs A
C vs A
D vs A
C vs B
D vs B
D vs C
log2(FC)
1
0
−1
Not significant

Figure 2

A



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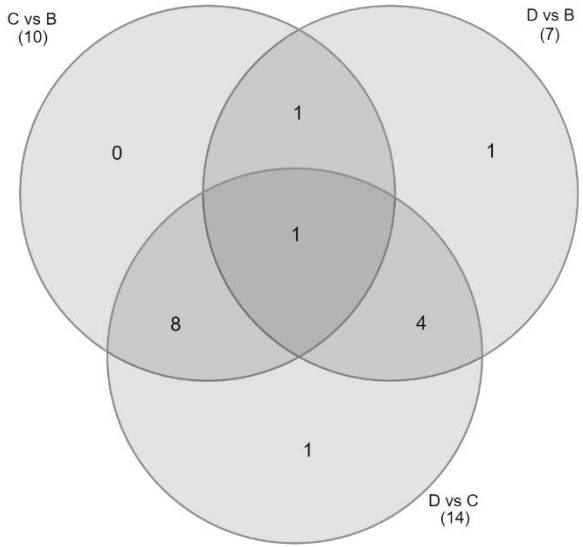


Figure 3

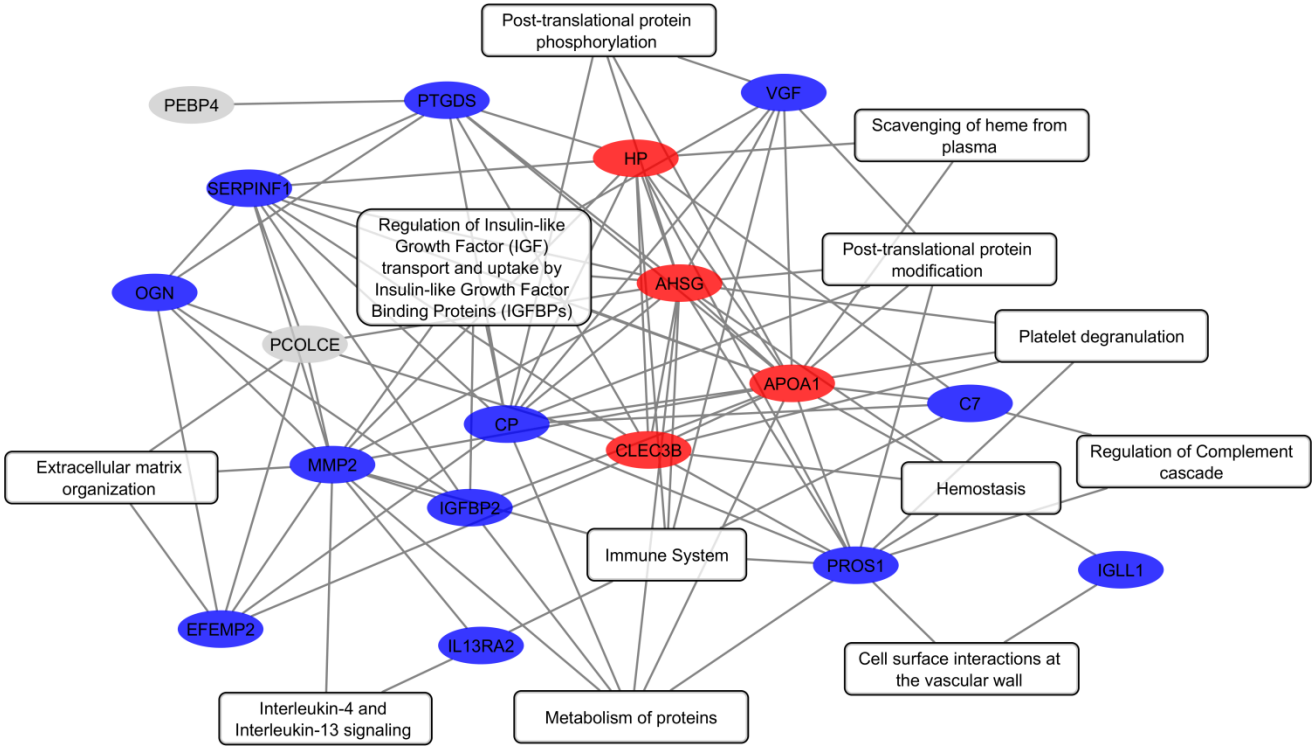

Post-translational protein phosphorylation
PEBP4
PTGDS
VGF
Scavenging of heme from plasma
HP
SERPINF1
Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)
Post-translational protein modification
AHSG
OGN
PCOLCE
Platelet degranulation
APOA1
C7
CP
CLEC3B
Regulation of Complement cascade
Extracellular matrix organization
MMP2
Hemostasis
IGFBP2
Immune System
PROS1
IGLL1
EFEMP2
IL13RA2
Cell surface interactions at the vascular wall
Interleukin-4 and Interleukin-13 signaling
Metabolism of proteins

Figure 4

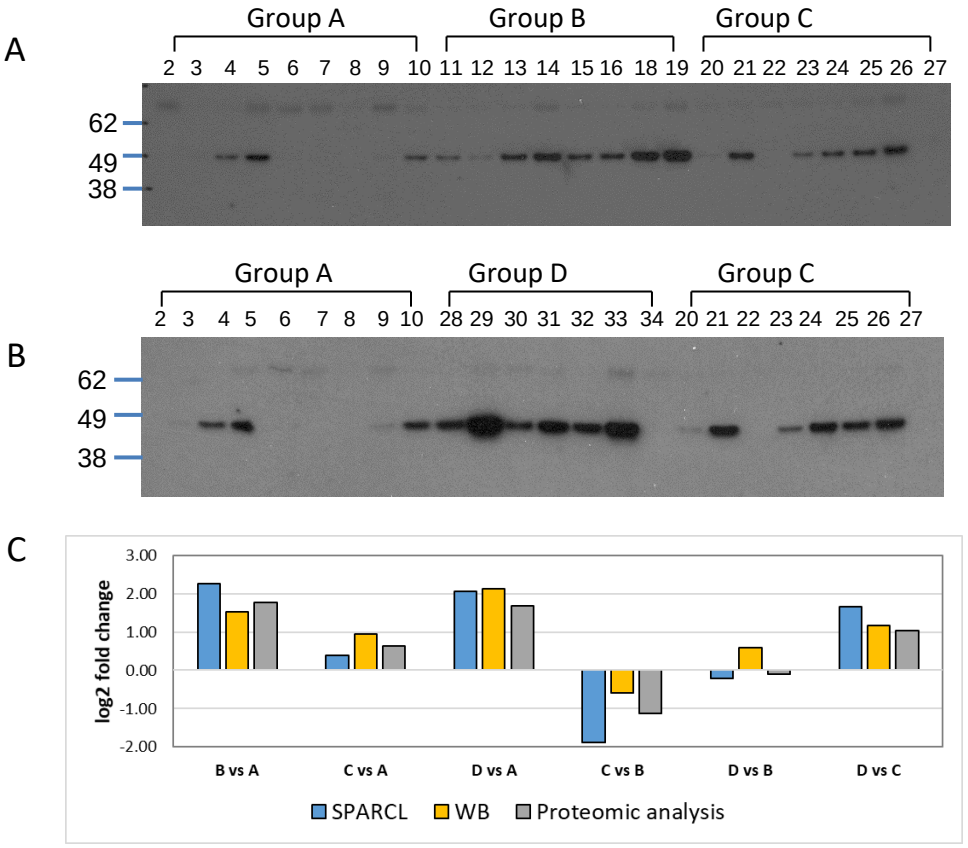
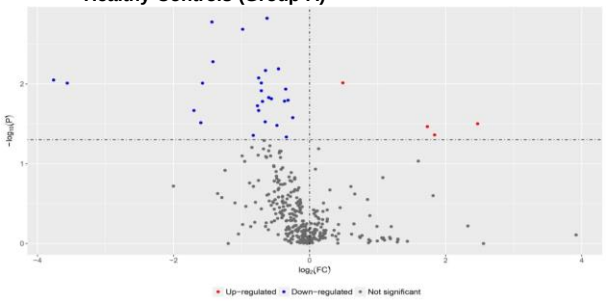
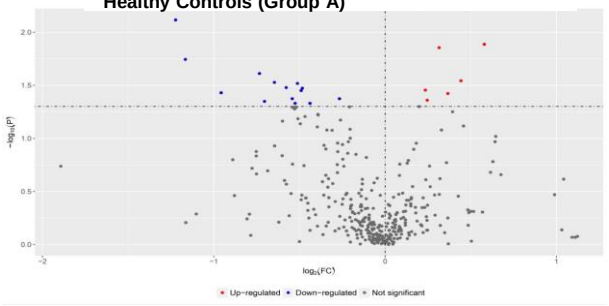

A
Group A
Group B
Group C
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 18 19 20 21 22 23 24 25 26 27
62
49
38
B
Group A
Group D
Group C
2 3 4 5 6 7 8 9 10 28 29 30 31 32 33 34 20 21 22 23 24 25 26 27
62
49
38
C
log2 fold change
3.00
2.00
1.00
0.00
-1.00
-2.00
B vs A
C vs A
D vs A
C vs B
D vs B
D vs C
SPARCL
WB
Proteomic analysis

Figure S1

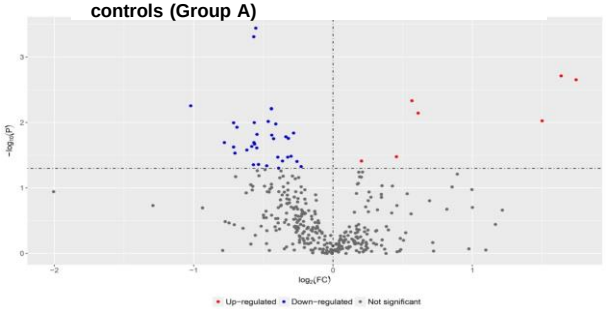
(A) Idiopathic epilepsy receiving AEM (Group B) VS Healthy Controls (Group A)



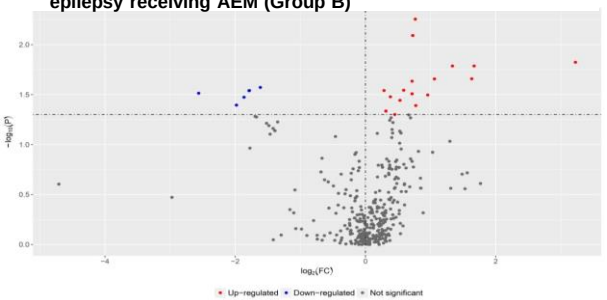
(B) Idiopathic epilepsy without AEM (Group C) VS Healthy Controls (Group A)



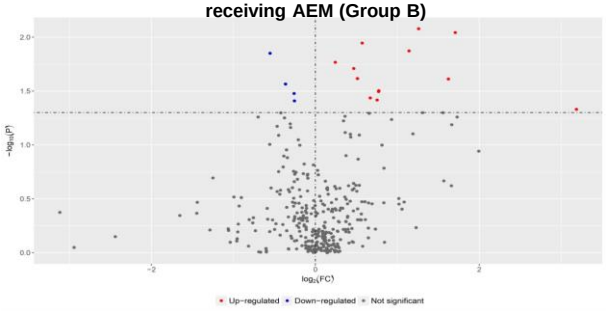
(C) Structural epilepsy (Group D) VS Healthy controls (Group A)



(D) Idiopathic epilepsy without AEM (Group C) VS Idiopathic epilepsy receiving AEM (Group B)



(E) Structural epilepsy (Group D) VS Idiopathic epilepsy receiving AEM (Group B)



(F) Structural epilepsy (Group D) VS Idiopathic epilepsy without AEM (Group C)

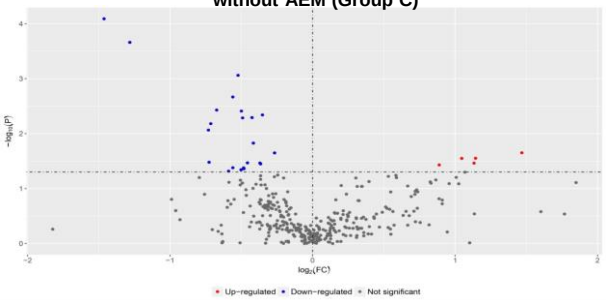


Figure S2

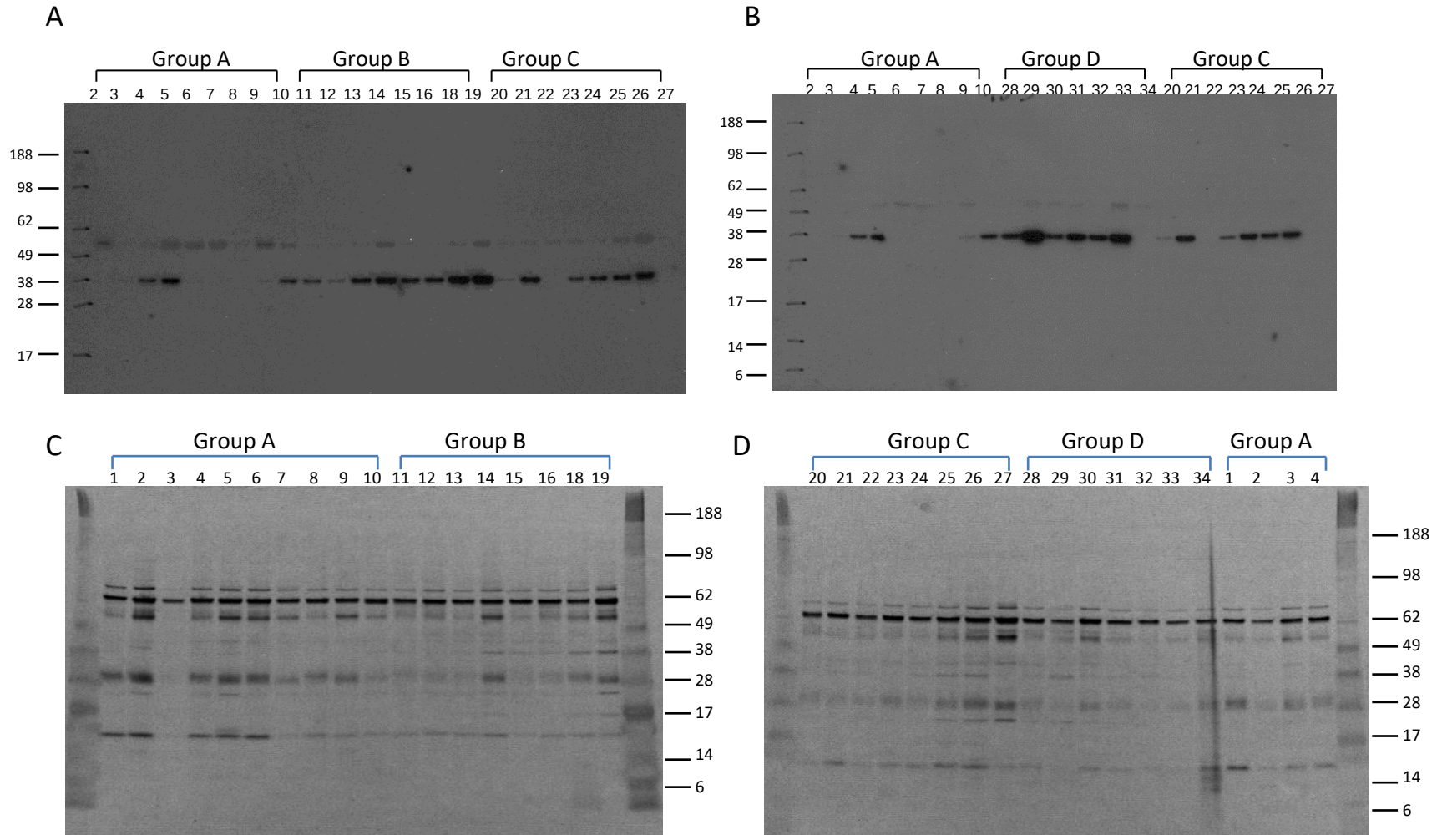

A
Group A
Group B
Group C
2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 18 19 20 21 22 23 24 25 26 27
188
98
62
49
38
28
17
B
Group A
Group D
Group C
2 3 4 5 6 7 8 9 10 28 29 30 31 32 33 34 20 21 22 23 24 25 26 27
188
98
62
49
38
28
17
14
6
C
Group A
Group B
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 18 19
188
98
62
49
38
28
17
14
6
D
Group C
Group D
Group A
20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 1 2 3 4
188
98
62
49
38
28
17
14
6

Figure S3

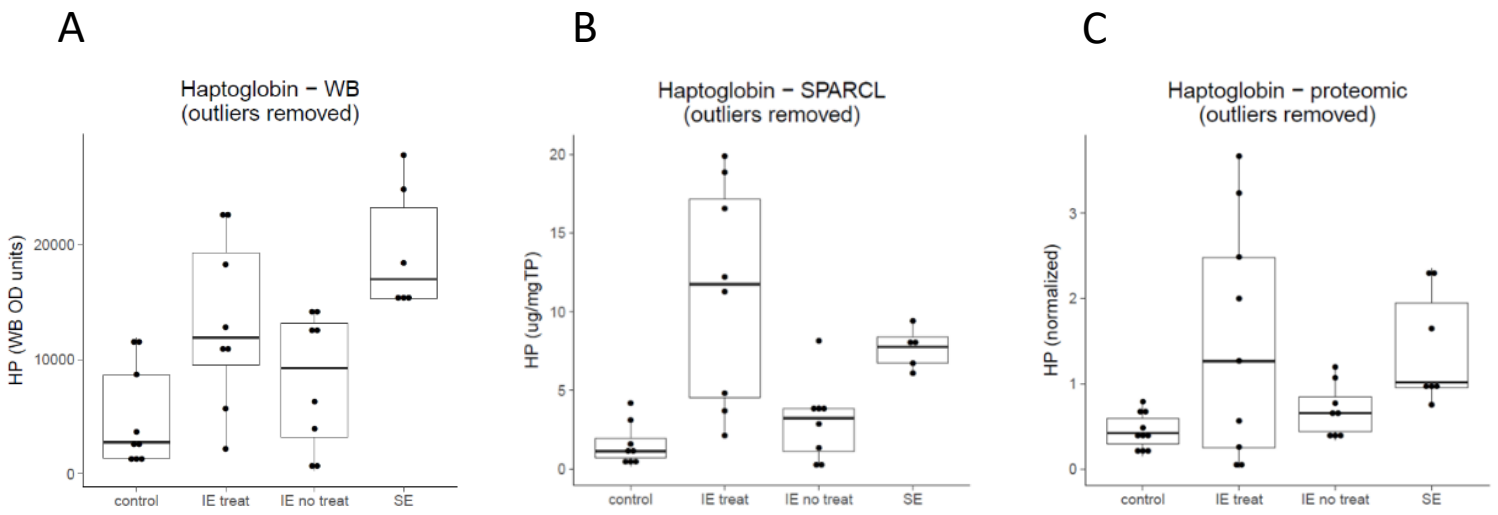

A
Haptoglobin − WB
(outliers removed)
HP (WB OD units)
0
10000
20000
control
IE treat
IE no treat
SE
B
Haptoglobin − SPARCL
(outliers removed)
HP (ug/mgTP)
0
5
10
15
20
control
IE treat
IE no treat
SE
C
Haptoglobin − proteomic
(outliers removed)
HP (normalized)
0
1
2
3
control
IE treat
IE no treat
SE

Figure S4

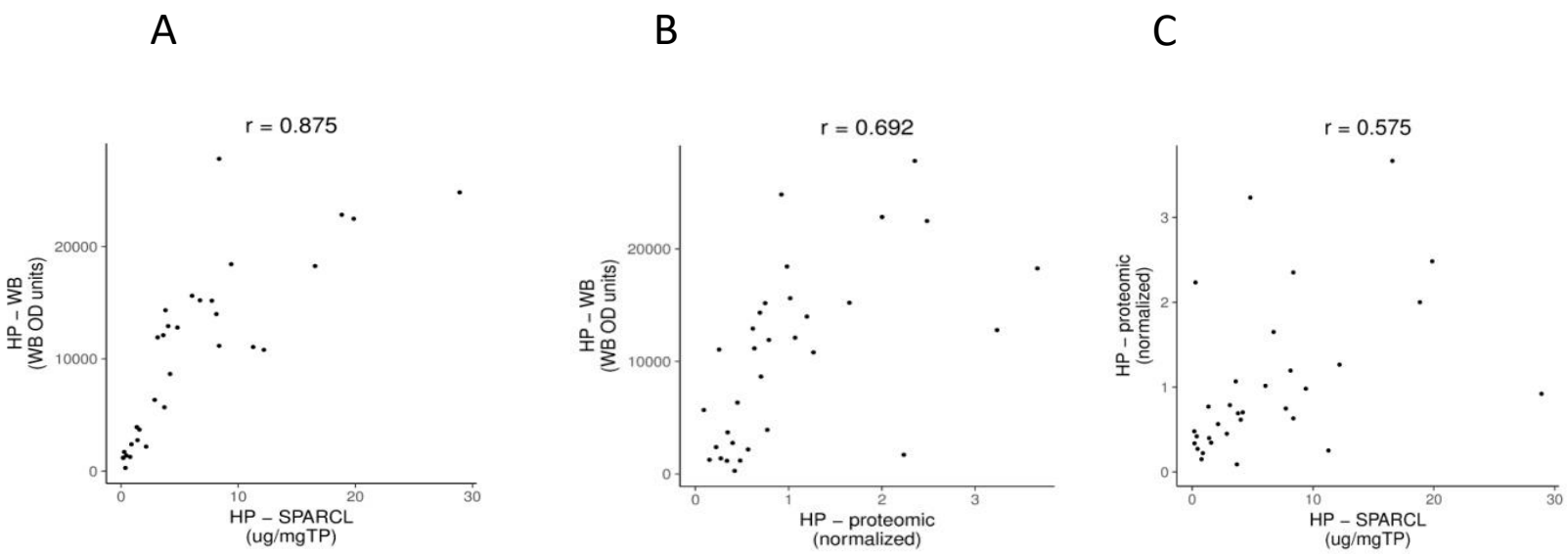

A
r = 0.875
HP – WB
(WB OD units)
HP – SPARCL
(ug/mgTP)
B
r = 0.692
HP – WB
(WB OD units)
HP – proteomic
(normalized)
C
r = 0.575
HP – proteomic
(normalized)
HP – SPARCL
(ug/mgTP)

Table S2

Gene	Description	log2 fold change					
		B vs A	C vs A	D vs A	C vs B	D vs B	D vs C
AHSG	Alpha-2-HS-glycoprotein	/	/	0.57	/	0.57	0.3
APOA1	Apolipoprotein A-I	0.49	/	0.61	-0.46	/	0.58
C7	Complement component C7	-0.56	/	-0.4	0.53	/	-0.37
CLEC3B	Tetranectin	/	0.37	/	0.45	/	-0.71
CP	Ceruloplasmin	-0.63	-0.27	/	/	0.51	/
EFEMP2	EGF-containing fibulin-like extracellular matrix protein 2	-0.75	-0.31	-0.3	/	/	/
HP	Haptoglobin heavy chain	1.84	/	1.74	/	/	1.07
IGFBP2	Insulin-like growth factor-binding protein 2	-0.46	/	-0.57	0.31	/	-0.43
IGLL1	Immunoglobulin lambda 1 light chain	-1.44	-1.22	/	/	1.26	1.05
IL13RA2	Interleukin 13 receptor subunit alpha 2	-0.98	/	/	0.96	0.75	/
MMP2	Matrix metalloproteinase 2	-0.6	/	-0.32	/	/	/
OGN	Mimecan	-0.65	/	-0.69	0.52	/	-0.56
PCOLCE	Procollagen C-endopeptidase enhancer 1	/	0.18	-0.49	0.53	/	-0.67
PEBP4	Phosphatidylethanolamine-binding protein 4	/	0.44	-1.02	0.76	-0.7	-1.46
PROS1	Vitamin K-dependent protein S	/	/	-0.28	/	-0.26	-0.35
PTGDS	Prostaglandin D synthase	-0.71	/	-0.58	0.71	/	-0.59
SERPINF1	Pigment epithelium-derived factor	/	/	-0.41	0.42	/	-0.56
VGF	Neurosecretory protein VGF	/	/	-0.44	/	-0.31	-0.49