Altered serum protein levels in frontotemporal dementia and amyotrophic lateral sclerosis indicate calcium and immunity dysregulation

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Frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS) are neurodegenerative diseases that are considered to be on the same disease spectrum because of overlapping genetic, pathological and clinical traits. Changes in serum proteins in FTD and ALS are poorly understood, and currently no definitive biomarkers exist for diagnosing or monitoring disease progression for either disease. Here we applied quantitative discovery proteomics to analyze protein changes in FTD (N = 72) and ALS (N = 28) patient serum compared to controls (N = 22). Twenty-three proteins were significantly altered in FTD compared to controls (increased—APOL1, C3, CTSH, EIF5A, MYH2, S100A8, SUSD5, WDR1; decreased—C1S, C7, CILP2, COMP, CRTAC1, EFEMP1, FBLN1, GSN, HSPG2, IGV1, ITIH2, PROS1, SHBG, UMOD, VASN) and 14 proteins were significantly altered in ALS compared to controls (increased—APOL1, CKM, CTSH, IGHG1, IGKC, MYH2; decreased—C7, COMP, CRTAC1, EFEMP1, FBLN1, GSN, HSPG2, SHBG). There was substantial overlap in the proteins that were altered in FTD and ALS. These results were validated using western blotting. Gene ontology tools were used to assess functional pathways potentially dysregulated in the two diseases, and calcium ion binding and innate immunity pathways were altered in both diseases. When put together, these results suggest significant overlap in pathophysiological peripheral changes in FTD and ALS. This study represents the first proteomics side-by-side comparison of serum changes in FTD and ALS, providing new insights into under-recognized perturbed pathways and an avenue for biomarker development for FTD and ALS.

Frontotemporal dementia (FTD) is a neurodegenerative disease and a common form of younger-onset dementia, of which the most common clinical syndrome is the behavioral variant FTD (bvFTD). Amyotrophic lateral sclerosis (ALS) is the most rapidly fatal motor neurodegenerative disease, with typical progression from symptom onset to death in 2–3 years. FTD and ALS are considered to be on the same disease spectrum because of overlapping genetic, pathological and clinical traits. The most common gene abnormality in both FTD and ALS is an expanded hexanucleotide repeat sequence in the C9ORF72 gene. Pathologically, protein aggregates of
TAR-DNA binding protein-43 (TDP-43), the microtubule associated protein tau or, less often, fused in sarcoma (FUS) are present in FTD and/or ALS brain.

Currently, there is a lack of sensitive and specific biomarkers for diagnosis and monitoring disease progression for FTD and ALS, which has hindered the capacity to develop therapies for the two diseases. Major pathological proteins, including TDP-43 and tau, have not provided accurate peripheral biomarkers (cerebrospinal fluid (CSF) or plasma/serum) for either disease\textsuperscript{6-10}. As a result, other protein markers have been explored, with neurofilament-light chain (NFL) attracting considerable interest. NFL levels are elevated in both CSF and serum in both FTD and ALS\textsuperscript{11,12}. Research into developing biomarkers specific to FTD and ALS is ongoing.

Proteomics is a technique for global quantification of protein abundance and is increasingly used to identify changes in protein levels in numerous diseases\textsuperscript{13}. Proteomics technology has been recently applied to a number of neurodegenerative diseases for the purpose of biomarker development\textsuperscript{14}. Only few proteomics studies have been carried out on ALS plasma\textsuperscript{15-17}. However, to date no work has been reported on the proteomics of FTD serum/plasma nor any side-by-side comparisons of FTD and ALS proteins in serum/plasma. Here, we used proteomics based on mass spectrometry to analyze serum proteins in FTD and ALS serum compared to controls. The primary aim was to identify altered peripheral proteins in FTD and ALS that could be exploited to develop biomarkers for these diseases. The secondary aim was to uncover and understand FTD and ALS pathophysiology associated with any protein changes.

**Materials and methods**

**Patient blood serum.** Individuals diagnosed (male/female) with sporadic bvFTD (47/25), sporadic ALS (21/7) and healthy controls (9/13) were recruited from FRONTIER, the frontotemporal dementia clinical research group now at the University of Sydney Brain and Mind Centre, from the FocFront FTD and motor neuron disease clinic at the University of Sydney Brain and Mind Centre, and from a panel of healthy study volunteers\textsuperscript{18} with no neurological or psychiatric disorders, notably no evidence of cognitive impairment. Two blood samples, taken 12 months apart (i.e. Year-1 and Year-2), were analyzed: in total, bvFTD (144 samples), ALS (56 samples) and controls (44 samples). The mean age at Year-1 was 61.5, 52.7 and 69.8 years respectively. The study was approved by the University of New South Wales (approval numbers: HC12573 and the University of Sydney (approval numbers: 2012/160, 2014/539, 2017/928) human research ethics committees. All methods were carried out in accordance with the relevant guidelines and regulations. Blood samples were obtained following written informed consent from the participant and/or primary carer. All patients underwent a neurological examination, a comprehensive cognitive assessment and structural brain MRI, and met current consensus diagnostic criteria for bvFTD\textsuperscript{19}, ALS\textsuperscript{20} or no neurological disease. Blood samples (9 mL) were collected in tubes (BD Vacutainer SST II Advance Tube #367958), and serum prepared by centrifugation at 3,500 rpm for 10 min at 4 °C, which was then aliquoted and stored at −80 °C until use.

**Protein depletion.** The ‘top 14’ high abundant proteins were depleted from the samples using a 4.6 mm × 100 mm Multiple Affinity Removal System column (MARS, Agilent, Santa Clara, CA, USA) based on the depletion method\textsuperscript{21} and following the manufacturer’s instructions. Briefly, 40 µl of serum was diluted with 120 µl buffer A, passed through a 0.22 µm filter and centrifuged at 16,000g. The supernatant was injected into a MARS column and the flow through collected. The column was washed with buffer B, which elutes the bound proteins, before re-equilibrating with buffer A prior to the next sample. The collected fractions were buffer exchanged into 100 mM TEAB.

**Mass spectrometry.** The buffer exchanged flow through from the depletion step was mixed with an equal volume of 8 M urea, and the proteins were reduced and alkylated then digested with trypsin. The peptides were purified using Oasis hydrophilic–hydrophobic-balanced (HLB) plus short cartridges (Waters Corp., Milford, MA, USA) and resuspended in 30 µl water, the peptide concentration assessed using a QUBIT (Invitrogen, Carlsbad, CA, USA), and the pH adjusted to pH 8.0. The concentration was adjusted to 10 µg/30 µl with 100 mM TEAB and labelled with 10plex Tandem Mass Tags (TMT, Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer’s instructions. The TMT-labelled peptides were purified using a HLB column and fractionated by hydrophilic interaction liquid chromatography (HILIC) in off-line mode using an in-house packed TSK-Amide 80 HILIC column with PEEK filter and an Agilent 1200 chromatography system (Agilent Technologies, Santa Clara, CA, USA). Each of the ten HILIC fractions were dried down and then resuspended in MS loading buffer (3% (v/v) acetonitrile/0.1% (v/v) formic acid) and analyzed by nano-capillary liquid chromatography–tandem mass spectrometry (LC–MS–MS) using a Dionex Ultimate 3000 HPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to an in-house nitro fiber nano 75 µm × 30 cm column packed with ReproSil Pur 120 C18 stationary phase (1.9 µm, Dr. Maisch GmbH, Germany). Separated compounds were analyzed with an Orbitrap Fusion Trifrid Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). A synchronous precursor selection MS3 method\textsuperscript{22} was used for data collection. Proteome Discoverer 2.2 (Thermo Fisher Scientific, Waltham, MA, USA) was used to analyze the MS data as the runs progressed and any TMT plex with less than 500 protein groups was re-run. The raw mass spectrometry data was first processed using MaxQuant\textsuperscript{23}. The variable modification included oxidation for methionine and protein N-terminal acetylation. A single fix modification of carbamidomethyl cysteine was included. Global parameters included a 1% false discovery rate.

**Gene ontology analysis.** Two gene ontology software programs, Bioprofiling\textsuperscript{24} (https://www.bioprofiling.de, 16 Dec 2019) and STRING\textsuperscript{25} v11 (16 Dec 2019), were used to interpret and predict function or pathway on a set of proteins identified by the proteomics analysis. The 23 proteins that were significantly altered in FTD (APOL1, C1S, C3, C7, CILP2, COMP, CRTAC1, CTSH, EFEMP1, EIF5A, FBLN1, GSN, HSPG2, IGHV1, ITIH2,
samples per individual (i.e. Year-1 and Year-2; 12-month apart) for all analysis. We used the protein depletion spectrometry (LC–MS–MS) technology. In total 144 bvFTD, 56 ALS and 44 control samples were analyzed; two bvFTD and ALS serum proteins using proteomics based on the advanced liquid chromatography-tandem mass software (v1.45s).

Western blotting. Equal volumes of serum from randomly chosen samples were heated with sample buffer (3.2% SDS, 32% glycerol, 0.16% bromophenol blue, 100 mM Tris–HCl, pH 6.8, 8% 2-mercaptoethanol). They were then electrophoresed on Criterion Stain-free 4–20% SDS-PAGE gels (Bio-Rad) and transferred onto nitrocellulose membranes at 100 V for 30 min. The membranes were blocked with PBS containing 5% nonfat dry milk and probed separately with each of the antibodies: anti-CKM (mouse monoclonal, 1:1,000, Santa Cruz, sc-365046), anti-COMP (mouse monoclonal, 1:1,000, Santa Cruz, sc374660), anti-EFEMP1 (mouse monoclonal, 1:1,000, Santa Cruz, sc-33722, 1:1,000), anti-FBLN1 (mouse monoclonal, 1:1,000, Santa Cruz, sc25281), anti-GSN (mouse monoclonal, 1:1,000, Abcam, ab11081), anti-PROS1 (rabbit monoclonal, 1:1,000, Santa Cruz, sc-52720). The membranes were washed three times in PBS containing 0.1% Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. Signals were detected using enhanced chemiluminescence and Gel Doc System (Bio-Rad). The blots were stripped and probed for the housekeeper protein transferrin. The signal intensity was quantified using Image Lab (Bio-Rad) and NIH ImageJ software (v1.45s).

Statistical analysis. In total, 855 proteins were detected across the entire experiment, with the percentage of missing proteins ranging from 27.1 to 51.9% across the different MS runs. Protein peak intensities were first log2 transformed, then any missing values were imputed using the k nearest neighbor algorithm (impute.knn function from the impute package in R). Following imputation, protein intensities were normalized. All proteins altered in ALS were identified by using the RUV-III (Removing Unwanted Variation-III) algorithm36. In the experimental design, samples were replicated across different runs to enable us to utilize RUV-III, which uses negative controls and replicates to remove systematic errors of unknown origin. All 855 proteins were used as negative controls. Default parameters from the RUVIII function were used. After normalization, any proteins that were originally missing were removed, and samples with replicates averaged. Then we fitted linear models using the R/Bioconductor software package limma27. A design matrix which included age and sex as covariates was entered separately into each of the programs following their instructions.

Results

Proteomics analysis of bvFTD and ALS serum. Here we undertook a comprehensive analysis of bvFTD and ALS serum proteins using proteomics based on the advanced liquid chromatography-tandem mass spectrometry (LC–MS–MS) technology. In total 144 bvFTD, 56 ALS and 44 control samples were analyzed; two samples per individual (i.e. Year-1 and Year-2; 12-month apart) for all analysis. We used the protein depletion method in which 96% of 14 high-abundant proteins (e.g. albumin, IgG) were removed37. This strategy allows for the identification of less abundant proteins with a greater accuracy. Our aim was to identify peripheral proteins that are altered in bvFTD and ALS compared to controls, to interpret and understand the pathophysiology associated with the protein changes observed.

Firstly, each disease was compared with controls, independent of year and covarying for age and sex. In bvFTD, 23 proteins were significantly altered (Fig. 1). Eight were increased—APOL1, C3, CTSH, EIF5A, MYH2, S100A8, SUSD5, WDR1—and 15 were decreased—C1S, C7, CILP2, COMP, CRTAC1, EFEMP1, FBLN1, GSN, HSPG2, IGHV1, ITIH2, PROS1, SHBG, UMOD, VASN (Table 1). In ALS, 14 proteins were significantly altered (Fig. 2) with 6 proteins increased—APOL1, CKM, CTSH, IGHG1, IGKC, MYH2—and 8 proteins decreased—C7, COMP, CRTAC1, EFEMP1, FBLN1, GSN, HSPG2, SHBG (Table 2). Interestingly, there was a substantial overlap in the proteins that were altered in the two diseases with 11 of the proteins altered in both diseases, i.e. APOL1, CTSH and MYH2 were upregulated, and C7, COMP, CRTAC1, EFEMP, FBLN1, GSN, HSPG2 and SHBG were decreased (Fig. 3).

Secondly, Year-1 sample with Year-2 sample were compared within each group. There were no proteins that were significantly altered between the two time points for all three groups. The protein that was closest to significance was ENO1. ENO1 levels were non-significantly increased in bvFTD (P = 0.08), and unchanged in ALS and controls (Fig. 4).

Validation of protein changes in bvFTD and ALS. The LC–MS–MS proteomics data was validated using western blotting. Proteins were selected for validation based on the availability of antibodies and suitability/sensitivity of protein detection in human serum by this method. Gene ontology results (see below) were also taken into account when selecting proteins for validation. Based on the gene ontology results, calcium ion binding was the prominent predicted function. The proteins selected for validation were therefore—COMP, EFEMP1, FBLN1, GSN and PROS (changed in bvFTD) and CKM, COMP, and GSN (changed in ALS). The same serum samples used for the proteomics analysis were electrophoresed on SDS-PAGE gels and probed with specific antibodies. All proteins tested were significantly altered in bvFTD and ALS compared to controls, supporting the proteomics data (Fig. 5).
Figure 1. Volcano plot representation of bvFTD versus controls proteomics data. The significantly altered proteins in bvFTD (N = 72) compared to controls (N = 22) are shown as red circles. Age and gender were included as covariates, Benjamini–Hochberg method was used to correct for multiple testing, and proteins with an adjusted $P < 0.05$ to be statistically significant; FC fold change.

| Protein                                      | Symbol | Uniprot code | LogFC | P-value |
|----------------------------------------------|--------|--------------|-------|---------|
| Increased                                    |        |              |       |         |
| Complement C3                                | C3     | P01024       | 0.59  | 0.0016  |
| Cathepsin H                                  | CTSH   | P09668       | 17.19 | 0.0016  |
| Myosin-2                                     | MYH2   | Q9UKX2       | 4.50  | 0.0033  |
| Sushi domain-containing protein 5            | SUSD5  | O60279       | 11.93 | 0.0117  |
| Eukaryotic translation initiation factor 5A  | EIF5A  | P63241       | 3.01  | 0.0230  |
| Protein S100A-8                               | S100A8 | P05109       | 0.87  | 0.0230  |
| Apolipoprotein L1                            | APOL1  | O14791       | 0.59  | 0.0401  |
| WD repeat-containing protein 1               | WDR1   | Q75083       | 5.94  | 0.0422  |
| Decreased                                    |        |              |       |         |
| Cartilage acidic protein 1                   | CRTAC1 | Q9NQ79       | 0.64  | 0.0024  |
| Fibulin-1                                    | FBLN1  | P23142       | 0.59  | 0.0024  |
| Complement C1S                               | C1S    | P09871       | 0.38  | 0.0033  |
| EGF-containing fibulin-like extracellular matrix protein 1 | EFEMP1 | Q12805 | 0.62 | 0.0067 |
| Gelsolin                                     | GSN    | P06396       | 0.40  | 0.0069  |
| Sex hormone-binding globulin                 | SHBG   | P04278       | 0.69  | 0.0074  |
| Inter-alpha-trypsin inhibitor heavy chain H2 | ITH2   | P19823       | 0.45  | 0.0074  |
| Cartilage intermediate layer protein 2       | CILP2  | Q8IU8L       | 6.47  | 0.0099  |
| Cartilage oligomeric matrix protein          | COMP   | P49747       | 0.42  | 0.0118  |
| Perlecans                                    | HSPG2  | P98160       | 0.37  | 0.0242  |
| Uromodulin                                   | UMOD   | P07911       | 0.66  | 0.0259  |
| Complement C7                                | C7     | P10643       | 0.41  | 0.0259  |
| Vasoerin                                     | VASN   | Q6EMK4       | 0.48  | 0.0259  |
| Vitamin K-dependent Protein S                 | PROS1  | P07225       | 0.29  | 0.0345  |
| Immunoglobulin heavy variable 1–2            | IGHV1  | A0A0G2JM13   | 2.63  | 0.0422  |

Table 1. Proteins that were significantly altered in bvFTD compared to controls.
Assessing pathophysiological changes in bvFTD and ALS. To understand the pathophysiology associated with the peripheral protein changes in bvFTD and ALS serum, two gene ontology software programs, bioprofiling and STRING, were used; gene ontology is a technique for interpreting and predicting functions or pathways based on changes in a set of genes or proteins. Firstly, we assessed the 23 proteins (APOL1, C1S, C3, C7, CILP2, COMP, CRTAC1, CTSH, EFEMP1, EIF5A, FBLN1, GSN, HSPG2, IGHV1, ITIH2, MYH2, PROS1, S100A8, SHBG, SUSD5, UMOD, VASN and WDR1) that were significantly altered in bvFTD by Bioprofiling. The prominent function/pathways generated were “calcium ion binding” pathway with 9 protein hits and “innate immunity” pathway with 5 protein hits (Fig. 6A), with the innate immunity pathway having the most clustering of upregulated proteins. The same two pathways (calcium ion binding and innate immunity) were identified using STRING with 10 and 8 protein hits respectively (Fig. 6B). Interestingly, all of the proteins (except S100A8) allocated to the two pathways using STRING were downregulated. In addition, four of the proteins (CIS, GSN, MYH2, CTSH, and EFEMP1) were upregulated in bvFTD by Bioprofiling. These results suggest that the peripheral protein changes in bvFTD and ALS may be associated with a calcium ion binding and innate immunity response, which may play a role in the pathophysiology of these disorders.

Table 2. Proteins that were significantly altered in ALS compared to controls.

| Protein                                            | Symbol | Uniprot Code | LogFC | P-value |
|----------------------------------------------------|--------|--------------|-------|---------|
| Increased                                          |        |              |       |         |
| Myosin-2                                           | MYH2   | Q9UKX2       | 4.72  | 0.0047  |
| Cathepsin-H                                        | CTSH   | P09668       | 18.99 | 0.0047  |
| Apolipoprotein L1                                  | APOL1  | O14791       | 0.84  | 0.0133  |
| Immunoglobulin heavy constant gamma 1              | IGHG1  | P01857       | 2.07  | 0.0240  |
| Immunoglobulin kappa constant                      | IKG1   | P01834       | 1.02  | 0.0322  |
| Creatine kinase M-type                             | CKM    | P06732       | 1.26  | 0.0322  |
| Decreased                                          |        |              |       |         |
| EGF-containing fibulin-like extracellular matrix protein 1 | EFEMP1 | Q12805       | 0.82  | 0.0047  |
| Complement C7                                      | C7     | P10643       | 0.56  | 0.0133  |
| Cartilage acidic protein 1                         | CRTAC1 | Q9NG79       | 0.68  | 0.0133  |
| Cartilage oligomeric matrix protein                | COMP   | P49747       | 0.49  | 0.0240  |
| Sex hormone-binding globulin                       | SHBG   | P04278       | 0.76  | 0.0240  |
| Gelsolin                                           | GSN    | P06396       | 0.41  | 0.0389  |
| Fibulin-1                                          | FBLN1  | P23142       | 0.54  | 0.0447  |
| Perlecan                                           | HSPG2  | P98160       | 0.42  | 0.0447  |
Figure 3. Overlap of proteins that were significantly altered in bvFTD and ALS serum. The blue circle represents those proteins that were significantly altered in bvFTD and red circle represents those proteins that were significantly altered in ALS. Three proteins were increased in both bvFTD and ALS. Eight proteins were decreased in both bvFTD and ALS.

Figure 4. Changes in ENO1 abundance in a 12-month period in control, bvFTD and ALS serum. Blue dots represent Year-1 samples and red dots represent Year-2 samples; each individual is connected by a grey line. No significant change was observed for all three groups. Thick lines represent mean and boundary lines represent SE.
Figure 5. Validation of proteomics data by western blotting. (A) Proteomics data of individual proteins in bvFTD, ALS and controls. (B) Western blotting of individual proteins in bvFTD compared to controls normalized to the housekeeper protein transferrin (Transf), and optical density (OD) measurements of the bands. (C) Western blotting of individual proteins in ALS compared to controls normalized to the housekeeper protein transferrin (Transf), and optical density (OD) measurements of the bands. Data represent mean and SE as error bars, *P < 0.05, **P < 0.01, ***P < 0.001. The blots have been cropped from full-size blots as shown in the Supplementary Fig. 1.
PROS1, S100A8) (Fig. 6B) overlapped in both pathways, suggesting a potential link between calcium ion binding and innate immunity in bvFTD.

Secondly, we assessed the 14 proteins (APOL1, C7, COMP, CKM, CRTAC1, CTSH, EFEMP1, FBLN1, GSN, HSPG2, IGHG1, IGKC, SHBG, MYH2) that were significantly altered in ALS by Bioprofiling and STRING. Similar to the protein changes in bvFTD serum, the prominent pathway generated by both programs was calcium ion binding with 5 and 6 protein hits respectively (Fig. 6B). There were also 4 protein hits in the innate immunity pathway identified using STRING (Fig. 6B).

**Discussion**

Proteomics analysis of FTD and ALS has been conducted primarily in CSF with limited reproducibility across studies. To date, no studies have been reported for FTD using serum or plasma. To address these shortfalls in knowledge, a comprehensive analysis of protein changes in bvFTD and ALS serum was performed using quantitative discovery proteomics. Twenty-three proteins were significantly altered in bvFTD and 14 proteins were significantly altered in ALS compared to controls, with 11 of these proteins altered in both diseases, supporting overlap in pathophysiological changes between FTD and ALS. Assessment of the altered proteins using two gene ontology programs established that the dysfunction primarily related to calcium ion binding pathways.

Calcium is recognized as critical to neurons given its multifunctional involvement in membrane excitability, signal transduction, neurotransmitter release, synaptic plasticity, cell cycle regulation and axon growth. Existing evidence supports a link between calcium dysregulation and neurodegenerative processes in both FTD and ALS. The drug Riluzole currently used to treat ALS works via inhibiting calcium signaling. In a functional network study of an independent cohort of sporadic FTD patients, calcium/cAMP homeostasis and energetic metabolism impairments were identified as primary causes of the loss of neuroprotection and neural cell damage in FTD. The authors suggested that calcium homeostasis in addition to DNA damage and oxidative stress could be among major molecular underpinnings for a significant proportion of unexplained FTD etiology. In separate studies, calcium dysregulation was reported to contribute to neurodegeneration in iPSC-derived neurons from FTD patients and ALS patients. In C9ORF72 iPSC-derived motor neurons from ALS and FTD patients, decreased cell survival correlated with calcium homeostasis, suggesting a novel pathogenic link between C9ORF72, dysregulated calcium signaling and altered proteostasis. Furthermore, in mutant models of TDP-43 pathology, increased calcium was shown to drive TDP-43 mediated neuronal toxicity.

Collectively, these studies indicate that calcium dysregulation may be a critical component of neurodegenerative pathogenesis in both FTD and ALS. Given the current study shows dysregulation of multiple serum proteins associated with calcium binding pathways in both bvFTD and ALS, it may be necessary to monitor calcium homeostasis in living FTD, ALS and cognitively normal individuals to distinguish normal calcium...
homeostasis from pathological conditions. Of the calcium related proteins identified in our study, GSN has the strongest established relationship with neurodegeneration. GSN is primarily an actin binding protein, however it has other roles, including in neurotransmission, synaptic plasticity, cytoskeleton remodeling, apoptosis and inflammation\(^{23,24}\). Elevated calcium concentration causes GSN conformational changes that facilitate its binding to actin filaments\(^{25}\). Plasma GSN levels are decreased in ALS patients\(^{15}\), corroborating our results.

PROS1 is another protein involved in calcium ion binding. It has a well-established role in blood anticoagulation and is dependent on calcium binding for structural stability\(^{45}\). PROS1 is also an agonist for the TAM family of receptor tyrosine kinases, which are regulators of immunity. Given PROS1 is primarily produced by microglia in the brain, this suggests that it may play a role in neuroinflammation\(^{46}\). COMP is an extracellular matrix glycoprotein that contains calcium ion binding domains. Currently, little is known whether COMP plays a role in the human brain. FBLN1 and EFEMP1 are part of the fibulin family of extracellular matrix glycoproteins with numerous calcium binding domains\(^{37,48}\). FBLN1 is abundantly expressed in the human brain. It has been shown to bind to the amyloid precursor protein and modulate its activity\(^{47}\). EFEMP1 is thought to have anti-angiogenic properties\(^{49}\). CKM was upregulated only in ALS serum in the current study. CKM is a creatine kinase involved in the brain, this suggests that it may play a role in neuroinflammation\(^{46}\). COMP is an extracellular matrix glycoprotein that contains calcium ion binding domains. Currently, little is known whether COMP plays a role in the human brain. FBLN1 and EFEMP1 are part of the fibulin family of extracellular matrix glycoproteins with numerous calcium binding domains\(^{37,48}\). FBLN1 is abundantly expressed in the human brain. It has been shown to bind to the amyloid precursor protein and modulate its activity\(^{47}\). EFEMP1 is thought to have anti-angiogenic properties\(^{49}\). CKM was upregulated only in ALS serum in the current study. CKM is a creatine kinase involved in energy consumption and, interestingly, elevated serum creatine is linked to muscle damage\(^{50}\). CKM has not been previously studied in the context of ALS. This protein is potentially a good candidate for follow-up studies, given ALS causes muscle weakness. Although the upregulation of ENO1 (enolase enzyme) in bvFTD serum did not reach significance over a one-year period, it will be useful to test this marker across longer disease progression. Enolase levels were shown to increase in AD CSF compared to controls, and these increases correlated with amyloid-β, total-tau and phosphorylated-tau levels\(^{51,52}\).

Whilst further investigation is required to understand the precise mechanisms and involvement of calcium dysregulation in neurodegeneration, serum calcium binding proteins, such as those validated in the current study, could be explored as potential indicators for neuronal vulnerability to disease\(^{53,54}\). The utility for such serum biomarkers to measure and detect calcium dysregulation in vivo in human subjects may facilitate tracking of specific at-risk neuronal populations in key neural networks within the CNS, that could allow for the development of neuroprotective approaches in FTD and ALS\(^{55,56}\).

Another prominent function/pathway generated by the gene ontology studies was innate immunity for both bvFTD and ALS. Innate immune-mediated mechanisms and chronic neuroinflammation are recognized as pathological hallmarks of neurodegenerative diseases, involved in both their development and progression\(^{56,57}\). In both FTD and ALS several key lines of evidence implicate innate immunity and neuroinflammation in the pathogenesis of disease\(^{58,59}\). Specifically, increased microglial activation and astrogliosis in disease affected brain regions\(^{60,61}\), altered expression of pro- and anti-inflammatory factors in blood and CSF\(^{29,62}\) and the most substantial evidence to date; the association between immune-related gene variants and susceptibility to FTD and ALS as recently reviewed in detail elsewhere\(^{63,64}\). Particularly in FTD, genome wide association studies consistently identify an association between the HLA locus (immune system) and FTD, and suggest a critical role for microglial and inflammation-associated genes in the mechanisms that drive FTD progression\(^{65,66}\) and particularly TDP-43 pathogenesis\(^{67}\).

In this study, multiple innate immune and complement cascade serum proteins are dysregulated predominantly in bvFTD, some of which overlapped with ALS, indicating similar and generalized immune mechanisms are involved. Three complement component proteins representing multiple pathways of the complement cascade were shown to be dysregulated in bvFTD serum (C1S, C3, C7). Corroborating with this data, C3 was shown to be increased in bvFTD serum by western blotting in a recent study\(^{67}\). In ALS, only one complement component (C7) representing a single complement pathway was dysregulated. The complement cascade is a critical arm of innate immunity and its chronic activation is a key mediator of neuroinflammation, shown to be upregulated in the aging brain and implicated in a number of neurodegenerative diseases\(^{68-70}\), including ALS\(^{71,72}\), but largely unexplored in FTD. In one of very few studies that have addressed complement in FTD, CSF samples from FTD patients carrying the GRN mutation showed a progressive increase in C1q and C3 that correlated with cognitive decline\(^{74}\). Given this, and the dysregulation of a number of innate immune proteins primarily within the serum in bvFTD shown in this study, the immune/inflammatory profile in bvFTD may differ from that of ALS. Indeed, FTD and ALS have been shown previously to be characterized by a different neuroinflammatory profile using ELISA analysis of targeted inflammatory factors in CSF and blood from asymptomatic mutation carriers\(^{75}\). Collectively, these findings suggest the potential to utilize a panel of innate immune and complement serum proteins as targets for future development of disease-specific neuroinflammation in FTD and ALS. Furthermore, in the present study the dysregulated serum proteins involved in calcium binding pathways (C1S, GSN, PROS1, S100A8) overlapped with innate immunity and complement cascade pathways, suggesting a potential interplay between these two pathways and their mechanisms in bvFTD pathogenesis. Whilst distinct mechanisms in their own right, calcium and neuroinflammatory signaling pathways exhibit extensive crosstalk and bi-directional interactions\(^{76}\). Alterations in neuroinflammation with aging and disease are proposed to have strong links to dysregulated calcium signaling in glial cells of the CNS, given both microglia and astrocytes express highly developed calcium signaling machinery\(^{77,78}\).

In summary, we have demonstrated that there are significant peripheral protein changes in bvFTD and ALS serum compared to controls. In particular, proteins relating to calcium ion binding and innate immunity are dysregulated in bvFTD and ALS serum. Future work will involve further validation of these proteins in different and larger independent cohorts, and investigating their role in calcium signaling in the pathogenesis of FTD and ALS, as well as furthering the study of innate immunity. This study represents the first proteomics analysis of sporadic FTD and sporadic ALS serum together, providing new insights into under-recognized perturbed peripheral pathways and the potential for biomarker development for both FTD and ALS.
References

1. Figuet, O., Hornberger, M., Mioshi, E. & Hodges, J. R. Behavioural-variant frontotemporal dementia: diagnosis, clinical staging, and management. Lancet Neurol. 10, 162–172 (2011).
2. Kiernan, M. C. et al. Amyotrophic lateral sclerosis. Lancet 377, 942–955 (2011).
3. Westeneng, H. J. et al. Prognosis for patients with amyotrophic lateral sclerosis: development and validation of a personalised prediction model. Lancet Neurol. 17, 423–433 (2018).
4. Burrell, J. R. et al. The frontotemporal dementia-motor neuron disease continuum. Lancet 388, 919–931 (2016).
5. Neumann, M. Frontotemporal lobar degeneration and amyotrophic lateral sclerosis: molecular similarities and differences. Rev. Neurol (Paris) 169, 793–798 (2013).
6. Blair, I. P. & et al. FUS mutations in amyotrophic lateral sclerosis: clinical, pathological, neurophysiological and genetic analysis. J. Neurol. Neurosurg. Psychiatry 81, 639–645 (2010).
7. Williams, K. L. et al. Pathophysiological insights into ALS with C9ORF72 expansions. J. Neurol. Neurosurg. Psychiatry 84, 931–935 (2013).
8. Feneberg, E., Gray, E., Ansorge, O., Talbot, K. & Turner, M. R. Towards a TDP-43-based biomarker for ALS and FTLD. Mol. Neurobiol. 55, 7789–7801 (2018).
9. Foiani, M. S. et al. Searching for novel cerebrospinal fluid biomarkers of tau pathology in frontotemporal dementia: an elusive quest. J. Neurol. Neurosurg. Psychiatry 90, 740–746 (2019).
10. Zetterström, P., Andersen, P. M., Brandström, T. & Marklund, S. L. Misfolded superoxide dismutase-1 in CSF from amyotrophic lateral sclerosis patients. J. Neurochem. 117, 91–99 (2011).
11. Rohrer, J. D. et al. Serum neurofilament light chain protein is a measure of disease intensity in frontotemporal dementia. Neurology 87, 1329–1336 (2016).
12. Verde, P. et al. Neurofilament light chain in serum for the diagnosis of amyotrophic lateral sclerosis. J. Neurol. Neurosurg. Psychiatry 90, 157–164 (2019).
13. Pandey, A. & Mann, M. Proteomics to study genes and genomes. Nature 405, 837–846 (2000).
14. Reichenberg, A. et al. Deep undepleted human serum proteome profiling toward biomarker discovery for Alzheimer’s disease. Clin. Proteomics 16, 16 (2019).
15. Xu, Z., Lee, A., Nouwens, A., Henderson, R. D. & McCombe, P. A. Mass spectrometry analysis of plasma from amyotrophic lateral sclerosis and control subjects. Amyotroph. Lateral Scler. Frontotemporal Degener. 17, 362–376 (2016).
16. Bereman, M. S., Beri, J., Enders, J. R. & Nash, T. Machine learning reveals protein signatures in CSF and plasma fluids of clinical value for ALS. Sci. Rep. 8, 16334 (2018).
17. Zubiri, I. et al. Tissue-enhanced plasma proteomic analysis for disease stratification in amyotrophic lateral sclerosis. Mol. Neurodegener. 13, 60 (2018).
18. Ahmed, R. M. et al. Systemic metabolism in frontotemporal dementia. Neurology 83, 1812–1818 (2014).
19. Rascovsky, K. et al. Sensitivity of revised diagnostic criteria for the behavioural variant of frontotemporal dementia. Brain 134, 2456–2477 (2011).
20. Al-Chalabi, A. et al. Amyotrophic lateral sclerosis: moving towards a new classification system. Lancet Neurol. 15, 1182–1194 (2016).
21. Zokotjova, N., Mrzinjiska, P., Chen, H. & Martosella, J. Combination of affinity depletion of abundant proteins and reversed-phase fractionation in proteomic analysis of human plasma/serum. J. Chromatogr. A. 1189, 332–338 (2008).
22. Ting, L., Rad, R., Gygi, S. P. & Haas, W. MS3 eliminates ratio distortion in isobaric multiplexed quantitative proteomics. Nat. Methods 8, 937–940 (2011).
23. Cox, J. & Mann, M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat. Biotechnol. 26, 1367–1372 (2008).
24. Antonov, A. V. BioProfiling.de: analytical web portal for high-throughput cell biology. Nucleic Acids Res. 39, W323–W327 (2011).
25. Sklarczyk, D. et al. STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 47, D607–D613 (2019).
26. Molania, R., Gagnon-Bartsch, J. A., Dobrovic, A. & Speed, T. P. A new normalization for Nanostring nCounter gene expression data. Nucleic Acids Res. 47, 6073–6083 (2019).
27. Ritchie, M. E. et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43, e47 (2015).
28. Davidson, P. et al. Studies of the pathophysiological mechanisms in frontotemporal dementia by proteome analysis of CSF proteins. Brain Res. Mol. Brain Res. 109, 128–133 (2002).
29. Teunissen, C. E. et al. Novel diagnostic cerebrospinal fluid biomarkers for pathologic subtypes of frontotemporal dementia identified by proteomics. Alzheimer’s Dement. (Amst) 2, 86–94 (2016).
30. van der Ende, E. L. et al. Novel CSF biomarkers in genetic frontotemporal dementia identified by proteomics. Ann. Clin. Transl. Neurol. 6, 698–707 (2019).
31. Barschke, P. Oechl, P., Steinacker, P., Ludolph, A. & Otto, M. Proteomic studies in the discovery of cerebrospinal fluid biomarkers for amyotrophic lateral sclerosis. Expert Rev. Proteomics 14, 769–777 (2017).
32. Sudhof, T. C. Calcium control of neurotransmitter release. Cold Spring Harbor Perspect. Biol. 4, a011353 (2012).
33. Emmgaye, N., Reid, C. A. & Fine, A. Calcium stores in hippocampal synaptic boutons mediate short-term plasticity, store-operated Ca2+ entry, and spontaneous transmitter release. Neuron 29, 197–208 (2001).
34. Lu, B. et al. Extracellular calcium controls background current and neuronal excitability via an UNC79–UNC80–NALCN cation channel complex. Neuron 68, 488–499 (2010).
35. Grosskreutz, J., Van Den Bosch, L. & Keller, R. U. Calcium dysregulation in amyotrophic lateral sclerosis. Cell Calcium 47, 165–174 (2010).
36. Palluzzi, F. et al. A novel network analysis approach reveals DNA damage, oxidative stress and calcium/cAMP homeostasis-associated biomarkers in frontotemporal dementia. PLoS ONE 12, e0185797 (2017).
37. Beltran-Parrazal, L. & Charles, A. Riluzole inhibits spontaneous Ca2+ signaling in neuroendocrine cells by activation of K+ channels and inhibition of Na+ channels. Br. J. Pharmacol. 140, 881–888 (2000).
38. Imamura, K. et al. Calcium dysregulation contributes to neurodegeneration in FTLD patient iPSC-derived neurons. Sci. Rep. 6, 34904 (2016).
39. Bursch, F. et al. Altered calcium dynamics and glutamate receptor properties in iPSC-derived motor neurons from ALS patients with C9orf72, FUS, SO1D1 or TDP43 mutations. Hum. Mol. Genet. 28, 2835–2850 (2019).
40. Dafina, R. et al. C9orf72 hexanucleotide expansions are associated with altered endoplasmic reticulum calcium homeostasis and stress granule formation in induced pluripotent stem cell-derived neurons from patients with amyotrophic lateral sclerosis and frontotemporal dementia. Stem Cells 34, 2063–2078 (2016).
Author contributions
W.S.K. designed the study, analyzed the data, and wrote the manuscript. G.M.H. designed the study, analyzed the data, and revised the manuscript. J.S.K. carried out the validation and gene ontology studies, analyzed the data, and wrote the manuscript. E.B. analyzed the data, and wrote the manuscript. K.L. performed the statistical analysis. A.C. and B.C. performed the proteomics analysis. M.C.K., O.P. and J.R.H. recruited the patients for technical assistance.

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**Competing interests**
The authors declare no competing interests.

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