Novel CSF biomarkers in genetic frontotemporal dementia identified by proteomics

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Abstract

Objective: To identify novel CSF biomarkers in GRN-associated frontotemporal dementia (FTD) by proteomics using mass spectrometry (MS). Methods: Unbiased MS was applied to CSF samples from 19 presymptomatic and 9 symptomatic GRN mutation carriers and 24 noncarriers. Protein abundances were compared between these groups. Proteins were then selected for validation if identified by ≥4 peptides and if fold change was ≤0.5 or ≥2.0. Validation and absolute quantification by parallel reaction monitoring (PRM), a high-resolution targeted MS method, was performed on an international cohort (n = 210) of presymptomatic and symptomatic GRN, C9orf72 and MAPT mutation carriers. Results: Unbiased MS revealed 20 differentially abundant proteins between symptomatic mutation carriers and noncarriers and nine between symptomatic and presymptomatic carriers. Seven of these proteins fulfilled our criteria for validation. PRM analyses revealed that symptomatic GRN mutation carriers had significantly lower levels of neuronal pentraxin receptor (NPTXR), receptor-type tyrosine-protein phosphatase N2 (PTPRN2), neurosecretory protein VGF, chromogranin-A (CHGA), and V-set and transmembrane domain-containing protein 2B (VSTM2B) than presymptomatic carriers and noncarriers. Symptomatic C9orf72 mutation carriers had lower levels of NPTXR, PTPRN2, CHGA, and VSTM2B than noncarriers, while symptomatic MAPT mutation carriers had lower levels of NPTXR and CHGA than noncarriers. Interpretation: We identified and validated five novel CSF biomarkers in GRN-associated FTD. Our results show that synaptic, secretory vesicle, and inflammatory proteins are dysregulated in the symptomatic stage and may provide new insights.
into the pathophysiology of genetic FTD. Further validation is needed to investigate their clinical applicability as diagnostic or monitoring biomarkers.

Introduction

Frontotemporal dementia (FTD) is the second most common form of presenile dementia, with autosomal dominant inheritance in approximately 30% of the cases.\(^1,2\) Pathogenic mutations in granulin (GRN) are a major cause of hereditary FTD with underlying transactive response DNA-binding protein 43 (TDP-43) pathology.\(^2\) The vast majority of GRN mutations result in reduction of progranulin (PGRN) protein levels in blood and cerebrospinal fluid (CSF) by haploinsufficiency.\(^3\)–\(^6\) However, the exact mechanism by which PGRN reduction leads to neurodegeneration is poorly understood. Upcoming therapeutic interventions should ideally be applied in the presymptomatic or prodromal stage of the disease, when neuronal damage is minimal, highlighting the need for biomarkers that reflect early pathologic processes.\(^7\)

Most studies on fluid biomarkers in FTD have used targeted approaches, allowing measurement of known protein candidates only,\(^7,8\) while unbiased approaches have scarcely been performed.\(^9,10\) In autosomal dominant Alzheimer’s disease, unbiased approaches have uncovered early changes in the proteome.\(^11\)

In the present study, we investigated CSF proteomics by unbiased mass spectrometry (MS) in presymptomatic and symptomatic GRN mutation carriers. We aimed to identify novel proteins that reflect disease activity and/or give insight into the pathophysiology. We validated and quantified a selection of the identified proteins using parallel reaction monitoring (PRM), a high-resolution targeted MS-based approach, in an international cohort of GRN mutation carriers and other forms of genetic FTD, namely C9orf72 and MAPT mutation carriers.\(^1\)

Methods

Subjects

Discovery proteomics was applied on CSF of 9 symptomatic and 19 presymptomatic GRN mutation carriers and 24 healthy noncarriers ("discovery cohort"), who participate in the Dutch longitudinal FTD Risk Cohort (FTD-RisC).\(^12\) Briefly, patients with genetic FTD and asymptomatic 50% at-risk individuals (either presymptomatic mutation carriers or noncarriers) from families with genetic FTD are followed yearly or two-yearly by means of neurological examination, neuropsychological testing, MRI scanning, structured informant interviews, and collection of blood and, in a subset, CSF collection.

PRM was performed on a selection of the proteins identified by discovery proteomics in CSF of 61 GRN mutation carriers (31 presymptomatic, 30 symptomatic), 70 C9orf72 mutation carriers (16 presymptomatic, 54 symptomatic), 27 MAPT mutation carriers (12 presymptomatic, 15 symptomatic), and 52 noncarriers ("validation cohort"). CSF samples were collected from six research centers in Europe and the USA. Forty-six samples in the validation cohort overlapped with those in the discovery cohort.

The study was approved by the local ethics committee and all participants (or a legal representative) provided written informed consent.
Sample collection

CSF was collected in polypropylene tubes according to standardized local procedures and stored at −80°C after centrifugation within 2 h after withdrawal.

Discovery proteomics

Discovery proteomics was performed as described previously and details are reported in Data S2. In short, albumin and IgG were depleted from 50 μl of CSF sample to maximize peptide detection (Pierce, PN 85162). After overnight in-solution trypsin digestion, samples were analyzed by LC–MS/MS in a randomized order on a nano LC system coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). For peptide and protein identification, MS/MS spectra were extracted using ProteoWizard software (version 3.0.9248) and analyzed with the database search engine Mascot (Matrix Science, UK) against the Uniprot database (downloaded November 12, 2015; taxonomy: Homo sapiens; 20,194 entries). Next we combined the search results of the individual samples, applied scoring of hits (local false discovery rate ≤1%), and conducted protein grouping using the software Scaffold. For label-free quantitation MS raw data were processed with Progenesis QI (version 2.0) and analyzed with the database search engine Mascot (Matrix Science, UK) against the Uniprot database (downloaded November 12, 2015; taxonomy: Homo sapiens; 20,194 entries). Next we combined the search results of the individual samples, applied scoring of hits (local false discovery rate ≤1%), and conducted protein grouping using the software Scaffold.16,17 For label-free quantitation MS raw data were processed with Progenesis QI (version 2.0) and linked with identification results to finally determine peptide and protein abundances. Abundances were normalized to the total ion current to compensate for experimental variations using an algorithm available in the analysis software. Subsequently, the data were exported in Excel format.

Statistical analyses of discovery proteomics

For all peptides identified by discovery proteomics, we compared peptide abundances in: (1) symptomatric mutation carriers versus noncarriers; (2) symptomatic versus presymptomatic mutation carriers; (3) presymptomatic mutation carriers versus noncarriers. As the data were not normally distributed, a Wilcoxon rank-sum test was used. Corresponding proteins were regarded as significantly differentially abundant when they satisfied all of the following criteria, as described before with minor adjustments: (1) the protein was identified by two or more peptides; (2) 25% or more of the peptides of the protein were significant at P < 0.01; (3) 50% or more of the peptides of the protein were significant at P < 0.05; (4) 75% or more of the peptides were changed in the same direction (i.e., up- or downregulated). Statistical background levels were determined by permutation tests on all samples and all identified peptides/proteins. The number of differentially abundant proteins was regarded as significant when the observed number in the true analysis exceeded the threshold from the permutation analysis: mean ± three times the standard deviation. Fold changes based on median abundances were calculated for all group comparisons on peptide levels and peptides with a median of zero were excluded. Next, protein fold changes were calculated by the mean of corresponding peptide fold changes.

PRM validation

Differentially abundant proteins from discovery proteomics were selected for PRM validation based on the following criteria: (1) the protein was identified by four or more peptides and (2) protein fold change was ≤0.5 or ≥2.0.

PRM was essentially performed as described previously and details are reported in Data S2. In short, 20 μL of CSF was digested overnight by trypsin. LC-MS analysis was carried out on a nano LC system coupled to an Orbitrap Fusion mass spectrometer (Thermo Fisher Scientific). For PRM of the peptide panel of candidates a time scheduled targeted MS/MS method was used and the referring peptide-specific parameters are listed in Table S1. To allow absolute quantification of peptides, synthetic stable isotope labeled (SIL) peptides were added as listed in Table S1. As technical quality check (QC), a pool of 80 CSF samples was prepared and loaded as 8-fold replicate on each well-plate. During LC-MS measurements, every 12th run a QC sample was measured to determine the reproducibility of the assay. For assessment of sensitivity of the assay an eight-point dilutions series of the peptide panel in CSF digest matrix was prepared and measured in triplicate. MS data processing was conducted using the software package Skyline.20 Peak ratios were exported and used for calculation of CSF concentrations of the samples and determining analytical parameters limit of detection (LOD), lower limit of quantitation (LLOQ), and coefficients of variance (CV) (Tables S2a and S2b) using the software package R.21

Statistical analyses of demographic data and PRM validation

Statistical analyses were performed in IBM SPSS Statistics 24.0 applying a significance level of P < 0.05. Demographic and PRM data for each genotype (GRN, C9orf72, and MAPT) were compared between symptomatic mutation carriers, presymptomatic mutation carriers, and noncarriers. For PRM results, per candidate protein one corresponding targeted peptide was chosen based on the suitability for quantification and lowest LOD, LLOQ, and CV as indicated in Tables S2a and S2b. Peptides with CV >15% were excluded from further analyses. As the data...
were not normally distributed, a Kruskall-Wallis test with post hoc Dunn’s test was performed to compare peptide concentrations between groups. Analysis of covariance (ANCOVA) of log-transformed peptide concentrations was used to correct for age at CSF sampling. All post hoc analyses were adjusted for multiple testing by means of Bonferroni correction.

Mass spectrometry data has been made available via the PRIDE partner repository with the dataset identifiers PXD012178 (discovery study) and PXD012179 (validation study).²²

Gene set enrichment analyses
Gene set enrichment analyses to the Gene Ontology database²³ were performed on a selection of proteins identified by discovery proteomics in symptomatic mutation carriers versus noncarriers, and separately on proteins identified in symptomatic versus presymptomatic mutation carriers. We relaxed the protein selection criteria to allow for separation of multiple enriched pathways in our dataset, aiming to include 50–150 proteins per enrichment analysis. Proteins with a fold change ≥0.83 or ≥1.2 and with ≥25% of the peptides significantly up- or down-regulated (P < 0.05) were included. Enrichment was performed to the whole genome as statistical background, accepting false discovery rate (FDR)-corrected results of P < 0.05 as significantly enriched Gene Ontology (GO) terms. The most significant nonredundant terms for Biological Processes (GOBP), Cellular Components (GOCC), and Molecular Functions (GOMF) were extracted and a protein network was created based on these terms using Cytoscape (v3.4.0).

Results

Subjects
Subject characteristics are shown in Table 1. In the discovery cohort, no differences were found in age at CSF collection or gender among symptomatic and presymptomatic mutation carriers and noncarriers. In the validation cohort, symptomatic GRN (median 61 years) and C9orf72 mutation carriers (59 years) were significantly older than presymptomatic GRN (54 years) and C9orf72 mutation carriers (45 years, both P < 0.001) and noncarriers (54 years, P < 0.001) at the time of CSF collection.

Discovery proteomics
We identified a total of 4539 peptides corresponding to 572 proteins, of which 503 proteins were identified by ≥2 peptides. Twenty proteins were considered significantly differentially abundant in symptomatic GRN mutation carriers compared to noncarriers. In the comparison between symptomatic and presymptomatic GRN mutation carriers, nine differentially abundant proteins were found (Fig. 1, Table S3). No significant differences were found between presymptomatic GRN mutation carriers and noncarriers. All differentially abundant proteins were identified by peptides, which were matched exclusively to that protein.

Validation by PRM
Seven proteins fulfilled our criteria for validation by PRM (Table 2). The protein Ig alpha-1 chain C region (IGHA1)

## Table 1. Subject characteristics.

|                  | N     | Age at CSF collection, years | Gender, male (%) | Age at symptom onset, years | Disease duration, years |
|------------------|-------|-----------------------------|------------------|----------------------------|-------------------------|
| Discovery cohort |       |                             |                  |                            |                         |
| Noncarriers      | 24    | 51 (40–58)                  | 14 (58%)         | n/a                        | n/a                     |
| Presymptomatic GRN | 19   | 56 (47–60)                  | 9 (47%)          | n/a                        | n/a                     |
| Symptomatic GRN  | 9     | 58 (53–60)                  | 3 (33%)          | 57 (51–58)                 | 2.3 (1.5–3.6)           |
| Validation cohort |      |                             |                  |                            |                         |
| Noncarriers      | 52    | 54 (43–59)                  | 24 (46%)         | n/a                        | n/a                     |
| Presymptomatic GRN | 31  | 54 (42–59)                  | 12 (39%)         | n/a                        | n/a                     |
| Symptomatic GRN  | 30    | 61 (57–66) *                | 11 (37%)         | 58 (55–63)                 | 1.9 (1.2–3.0)           |
| Presymptomatic C9orf72 | 16 | 45(36–52)                      | 3 (19%)          | n/a                        | n/a                     |
| Symptomatic C9orf72 | 54 | 59 (54–65) *                      | 31 (57%)         | 56 (50–62)                 | 2.4 (1.2–5.2)           |
| Presymptomatic MAPT | 12 | 48 (44–53)                      | 5 (42%)          | n/a                        | n/a                     |
| Symptomatic MAPT | 15    | 53 (51–60)                  | 7 (47%)          | 51 (46–55)                 | 3.0 (1.4–5.0)           |

Continuous variables are presented as medians (interquartile range). FTD, frontotemporal dementia; CSF, cerebrospinal fluid.
*Symptomatic GRN mutation carriers significantly older than presymptomatic GRN mutation carriers and noncarriers (P < 0.001).
†Symptomatic C9orf72 mutation carriers significantly older than presymptomatic C9orf72 mutation carriers and noncarriers (P < 0.001).
‡Symptomatic C9orf72 mutation carriers and noncarriers significantly more males than presymptomatic C9orf72 mutation carriers (P = 0.024).
was excluded from validation analyses as just one peptide was targeted and this peptide had a CV > 15%.

Symptomatic GRN mutation carriers had significantly lower concentrations of Neuronal pentraxin receptor (NPTXR), Receptor-type tyrosine-protein phosphatase N2 (PTPRN2), Neurosecretory protein VGF (VGF), Chromogranin-A (CHGA), and V-set transmembrane domain-containing protein (VSTM2B) compared to both presymptomatic carriers and noncarriers by PRM (Table 3, Fig. 2, Fig. S1). Complement component C8 gamma chain (C8G) levels were higher in symptomatic mutation carriers, however, this difference was no longer statistically significant after correction for age at CSF sampling.

Figure 1. Flow chart of differentially abundant proteins. The number of identified peptides and proteins are displayed and are then split to the differentially abundant proteins per group comparison: (1) symptomatic versus presymptomatic carriers, and (2) symptomatic versus noncarriers. No differentially abundant proteins were found in the comparison presymptomatic versus noncarriers (not shown). In the lower row, proteins are displayed that were selected for validation by PRM. CaM, Calcium/calmodulin-dependent; NPTXR, neuronal pentraxin receptor; PTPRN, receptor-type tyrosine-protein phosphatase-like N; PTPRN2, receptor-type tyrosine-protein phosphatase N2; TNF, tumor necrosis factor; VSTM2B, V-set and transmembrane domain-containing protein 2B.
Significant differences were found between presymptomatic mutation carriers and noncarriers. Field change (SYM/PRE): fold change in discovery proteomics in the comparison between symptomatic and presymptomatic mutation carriers. NPTXR, neuronal pentraxin receptor; PTPRN2, receptor-type tyrosine-protein phosphatase N2; VGF, neurosecretory protein VGF; CHGA, chromogranin-A; VSTM2B, V-set and transmembrane domain-containing protein 2B; C8G, complement component C8 gamma chain; IGHA1, Ig alpha-1 chain C region.

Symptomatic C9orf72 mutation carriers had significantly lower concentrations of NPTXR, PTPRN2, CHGA, and VSTM2B compared to noncarriers (Fig. 2, Fig. S1). Lower concentrations of NPTXR, PTPRN2, CHGA, and VSTM2B were found in presymptomatic mutation carriers than in noncarriers, although not statistically significant.

Symptomatic MAPT mutation carriers had significantly lower concentrations of NPTXR and CHGA compared to noncarriers, while the other proteins did not show any significant differences between groups (Fig. 2, Fig. S1).

For all proteins included in validation analyses, no significant differences were found between presymptomatic carriers and noncarriers.

| Peptides, n | Fold change (SYM/NC) | Fold change (SYM/PRE) |
|------------|----------------------|-----------------------|
| NPTXR      | 6                    | 0.34                  | 0.39                  |
| PTPRN2     | 5                    | 0.35                  | –                     |
| VGF        | 21                   | 0.45                  | –                     |
| CHGA       | 18                   | 0.46                  | –                     |
| VSTM2B     | 4                    | 0.49                  | –                     |
| CBG        | 4                    | 2.00                  | –                     |
| IGHA1      | 6                    | 2.39                  | –                     |

Fold change (SYM/NC): fold change in discovery proteomics in the comparison between symptomatic GRN mutation carriers and noncarriers. Fold change (SYM/PRE): fold change in discovery proteomics in the comparison between symptomatic and presymptomatic GRN mutation carriers. NPTXR, neuronal pentraxin receptor; PTPRN2, receptor-type tyrosine-protein phosphatase N2; VGF, neurosecretory protein VGF; CHGA, chromogranin-A; VSTM2B, V-set and transmembrane domain-containing protein 2B; C8G, complement component C8 gamma chain; IGHA1, Ig alpha-1 chain C region.

Gene set enrichment analyses

For gene set enrichment analyses, 116 proteins were included in the comparison of symptomatic mutation carriers versus noncarriers, and 72 proteins were included in the comparison of symptomatic versus presymptomatic mutation carriers. In total, 44 GOBP and 7 GOCC terms were significantly enriched (Data S1). The most significantly enriched terms for both comparisons included acute inflammatory response, response to axonal injury and modulation of synaptic transmission. The generated protein interaction network is shown in Figure S2.

Discussion

In this proteomics study, we identified several differentially regulated proteins in CSF of GRN-associated FTD. Validation of our results by targeted mass spectrometry revealed significantly lower levels of NPTXR, CHGA, VSTM2B, PTPRN2, and VGF in symptomatic GRN mutation carriers compared to presymptomatic and noncarriers. Here, we provide some background information on these proteins.

NPTXR is a transmembrane protein expressed on neurons and glia and is a member of the neuronal pentraxin (NP) family. NPs are multifunctional proteins that have been implicated in synaptic plasticity. NPTXR has been identified as a progression biomarker in Alzheimer’s disease (AD), with elevated levels in mild cognitive impairment and low levels in AD patients. In autosomal dominant AD, NPTXR levels were elevated in presymptomatic carriers, an effect we did not observe in our presymptomatic GRN carriers. This discrepancy may result from differences in underlying pathophysiology, or...
The last candidate protein we identified is VSTM2B, this is a membrane protein but its exact function has scarcely been studied.

The observed decrease in synapse proteins could represent synaptic turnover or loss occurring during the course of the disease. Increasing evidence suggests that altered synaptic function may contribute to the early pathogenesis of FTD, especially in GRN mutations, a concept previously recognized primarily in AD. In rat hippocampal neurons, knocking down PGRN decreases synapse density, and in GRN-knockout mice, PGRN-deficiency causes synaptic dysfunction prior to the occurrence of other neuropathological changes. It has been hypothesized that PGRN deficiency could cause synaptic pruning through activation of microglia and complement factors. Strategies aimed at increasing or maintaining synaptic connectivity could prove beneficial in future therapeutic interventions.

Four of the five protein decreases (NPTXR, VSTM2B, CHGA, and PTPRN2) observed in symptomatic GRN carriers were also seen in symptomatic C9orf72 carriers, suggesting that these changes are not specific for GRN-associated FTD. The trend toward lower levels of these proteins in presymptomatic C9orf72 carriers compared to noncarriers, must be interpreted with caution due to the lack of statistical significance. However, if confirmed in a larger genetic FTD cohort, this could support the hypothesis that C9orf72-associated FTD has a more protracted onset than GRN-associated FTD. In MAPT mutation carriers, significant differences in protein concentrations were only found for NPTXR and CHGA. This may reflect differences in underlying pathophysiology or it may be due to the smaller sample size in MAPT mutation carriers.

Strengths of this study include the unique sample set with a large cohort of presymptomatic and symptomatic GRN mutation carriers. Restricting our discovery cohort to GRN mutation carriers allowed us to create a pathologically homogeneous group of FTD-patients. The unbiased proteomics approach enabled us to identify novel biomarkers without predefined hypotheses. Validation of our discovery proteomics results by PRM has provided convincing evidence for our findings.

The depletion step in the discovery proteomics, removing albumin, and IgG, has considerably improved the detection of low abundancy proteins. Very low abundancy proteins could, however, be below the detection limit despite the depletion step. This may explain why we did not find PGRN, known to be decreased in GRN mutation carriers, or neurofilament light chain (NfL), known to be increased in symptomatic carriers, both of which have average CSF concentrations below 10 ng/ml. Furthermore, relevant proteins may bind to the depleted proteins,
thereby impeding their detection. Finally, our stringent selection criteria for validation likely reduced the number of false-positive findings, however, may also have excluded certain relevant potential biomarkers.

In conclusion, we present five promising novel CSF biomarkers in genetic FTD. Further verification and correlation with clinical features is needed in larger cohorts of genetic FTD, such as GENFI (Genetic FTD Initiative) and LEFFTDS (Longitudinal Evaluation of Familial Frontotemporal Dementia Subjects). Validation by immunoassays is necessary to reveal whether clinical implementation of these biomarkers is feasible.

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Author Contributions

E.L.v.d.E. and L.H.M. contributed to study design, data acquisition, statistical analysis and interpretation, and drafting of the manuscript. C.S., M.P.S., and D.N. contributed to data acquisition and analysis (i.e., mass spectrometry experiments) and drafting of the manuscript. J.G.J.v.R. contributed to study design, data analysis and interpretation (i.e., gene set enrichment analysis) and drafting of the manuscript. J.C.v.S., H.S., and T.M.L. contributed to study design, data acquisition and interpretation, and provided critical revision of the manuscript. All other authors contributed to data acquisition and revised the manuscript.

Conflict of Interest

The authors report no conflict of interest relevant to this work.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Protein levels measured by PRM in presymptomatic and symptomatic GRN, C9orf72 and MAPT mutation carriers.

Figure S2. Network of enriched Gene Ontology (GO) terms coupled to related proteins.

Data S1. List of enriched Gene Ontology terms.

Data S2. Methods

Table S1. Peptide specific settings of the PRM method.

Table S2a. PRM settings: Peptide assay characteristics.

Table S2b. Peptide quantification information.

Table S3a. Differentially abundant proteins (n = 20) in symptomatic GRN mutation carriers versus noncarriers.

Table S3b. Differentially abundant proteins (n = 9) in symptomatic versus presymptomatic GRN mutation carriers.