ORIGINAL RESEARCH

Different CSF protein profiles in amyotrophic lateral sclerosis and frontotemporal dementia with *C9orf72* hexanucleotide repeat expansion

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ABSTRACT

Objectives The hexanucleotide repeat expansion in the *C9orf72* gene is the most common mutation associated with amyotrophic lateral sclerosis (C9-ALS) and frontotemporal dementia (C9-FTD). Until now, it is unknown which factors define whether *C9orf72* mutation carriers develop ALS or FTD. Our aim was to identify protein biomarker candidates in the cerebrospinal fluid (CSF) which differentiate between C9-ALS and C9-FTD and might be indicative for the outcome of the mutation.

Methods We compared the CSF proteome of 16 C9-ALS and 8 C9-FTD patients and 11 asymptomatic *C9orf72* mutation carriers (CAR) by isobaric tags for relative and absolute quantitation. Eleven biomarker candidates were selected from the pool of differentially regulated proteins for further validation by multiple reaction monitoring and single-molecule array in a larger cohort (n=156).

Results In total, 2095 CSF proteins were identified and 236 proteins were significantly different in C9-ALS versus C9-FTD including neurofilament medium polypeptide (NEFM) and chitotriosidase-1 (CHIT1). Eight candidates were successfully validated including significantly increased ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) levels in C9-ALS compared with C9-FTD and controls and decreased neuronal pentraxin receptor (NPTXR) levels in C9-FTD versus CAR.

Conclusions This study presents a deep proteomic CSF analysis of C9-ALS versus C9-FTD patients. As a proof of concept, we observed higher NEFM and CHIT1 CSF levels in C9-ALS. In addition, we also show clear upregulation of UCHL1 in C9-ALS and downregulation of NPTXR in C9-FTD. Significant differences in UCHL1 CSF levels may explain diverging ubiquitination and autophagy processes and NPTXR levels might reflect different synapses organisation processes.

INTRODUCTION

The GGGGCC hexanucleotide repeat expansion (HRE) in the non-coding region of the *C9orf72* gene is the most common mutation associated with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD).[1] The mutation accounts for ~20%–40% of familial ALS, 10%–25% of familial FTD, 3%–6% of sporadic ALS (sALS) and 6% of sporadic FTD (sFTD) cases.[1 2] The frequency of *C9orf72* HRE in ALS and FTD overlapping disorders was shown to be higher compared with pure ALS or FTD in pan-European population.[3] Little is known about the physiological function of the C9orf72 protein, but it acts as a guanosine diphosphate/guanosine triphosphate exchange factor and there are several lines of evidences indicating that C9orf72 plays a role in autophagy and regulates axonal actin dynamics.[4 5]

Unaffected individuals have up to 20 hexanucleotide repeats, whereas tens to thousands of repeats are pathogenic.[6] Three main disease mechanisms caused by HRE are currently discussed: loss of function of the C9orf72 protein, toxic gain of function due to the accumulation of RNA foci and production of dipeptide repeat proteins by repeat-associated non-ATG translation.[1 7–9] All of these mechanisms have been observed in both ALS and FTD patients with *C9orf72* HRE (C9-ALS, C9-FTD). However, until now it is unclear how the *C9orf72* HRE is associated with different clinical presentations.

The aim of our study was to identify cerebrospinal fluid (CSF) protein biomarkers that can differentiate between C9-ALS and C9-FTD cases and thus provide insight into the segregating clinical presentations. Until now, proteomic studies of patients with C9-ALS and C9-FTD have focused mainly on frontal cortex brain autopsy material.[10 11] We decided to focus on CSF because sampling is usually done at the time of diagnosis and thus early proteomic changes can be detected. Furthermore, due to its proximity to affected neuroanatomical regions CSF better reflects pathomechanisms in the brain compared with other biofluids. We analysed asymptomatic *C9orf72* HRE carriers (CAR) to determine whether proteomic changes between C9-ALS and C9-FTD are caused by an increase or

decrease in one of the study groups compared with the CAR group. In the validation step, we increased the number of *C9orf72* HRE carriers and also investigated sporadic patients to determine whether the proteomic changes are specific for the *C9orf72* HRE. Additionally, we included non-neurodegenertaive controls to identify any regulations in the CAR group before symptom onset, since anatomical changes were already described in presymptomatic CAR.[12 13]

To our knowledge, this is the first unbiased CSF proteome analysis comparing *C9orf72* HRE carriers with different clinical presentations. The analysis was performed using isobaric tags for relative and absolute quantitation (iTRAQ) and liquid chromatography/tandem mass spectrometry (LC-MS/MS). Validation of 11 promising biomarker candidates was performed by a multiplex targeted MS approach (multiple reaction monitoring, MRM).

MATERIALS AND METHODS

Patients and CSF collection

Collection and analysis of CSF samples were approved by the local Ethics Committees of Ulm University (proposal number 20/10). CSF samples were stored at −80°C until analysis. All persons or their relatives gave written informed consent to participate in the study. Diagnoses of ALS and FTD were made according to the El Escorial criteria[14] and consensus criteria for FTLD,[15] respectively. Disease severity was evaluated by the Amyotrophic Lateral Sclerosis Functional Rating Scale—Revised (ALSFRS-R)[16] and FTLD-Clinical Dementia Rating scale (FTLD-CDR).[17]

In total, 157 participants were investigated: The initial discovery CSF proteome analysis was performed with 16 C9-ALS, 8 C9-FTD and 11 CAR CSF samples. Due to a low availability of CSF samples from *C9orf72* mutation carriers, all iTRAQ samples were also used for MRM validation except for one sample (C9-ALS, female, 45 years) and filled up with additional CSF samples. In total, the MRM validation cohorts consisted of 28 C9-ALS, 18 C9-FTD, 5 C9-ALS patients with behavioral variant FTD signs (C9-ALS/bvFTD), 28 CAR and 29 control (CON) patients. In addition, we analysed the CSF levels of two protein biomarker candidates in 27 sALS and 21 sFTD cases during validation. CON patients had no known neurodegenerative diseases. Additionally, the CON group was divided into an old (CON-o, n=17) and a young (CON-y, n=12) control group in the validation step. Specific diagnoses of the CON groups are shown in online supplementary table S1. CSF was collected by lumbar puncture at the Department of Neurology, Ulm University Hospital, Germany (C9-ALS, CAR and CON), at the Department of Neurology, Erasmus University Medical Center Rotterdam, the Netherlands (C9-ALS, C9-FTD and CAR) and at different clinical centres of the German Consortium for Frontotemporal Lobar Degeneration: Ulm, Munich, Erlangen, Leipzig, Rostock, Hamburg and Homburg (C9-FTD, C9-ALS/bvFTD, sFTD). Patients with C9-ALS, C9-FTD, C9-ALS/bvFTD and CAR were tested positive for the *C9orf72* HRE by Southern blot analysis and PCR[18] and tested negative for other common ALS and FTD mutations: *SOD1*, *FUS*, *MAPT* and *GRN*.

Materials

All materials are listed in the Methods section of the online supplementary material.

Sample preparation for discovery CSF proteome analysis

Two hundred microlitres of CSF sample were spiked with a solution of internal standard (IS) proteins to a final concentration of 200 ng flagellin, 2.5 pmol β-lactoglobulin, 100 ng ovalbumin and 100 mM triethylammonium bicarbonate buffer (TEAB). Samples were reduced and alkylated with 5 mM tris(2-carboxyethyl)phosphine and 10 mM chloroacetamide for 20 min at 60°C and 400 rpm. With the help of Amicon Ultra centrifugal filters (3 kDa molecular weight cut-off; Merck Millipore, Darmstadt, Germany), buffer was exchanged with 500 mM TEAB and samples were concentrated to 25 µL. Proteins were digested with trypsin/Lys-C in a ratio of 1:50 enzyme to protein ratio for 16 hours at 27°C and 400 rpm. Digests were diluted in ethanol to a final concentration of 75% and labelled with the iTRAQ reagents for 1 hour at 22°C and 400 rpm. C9-ALS, C9-FTD and CAR samples were labelled with iTRAQ reagents 115–117. Samples were systematically randomised into 4-plex sets. We made sure that the samples of study group were equally often labelled with all three reagents, and samples of at least two different study groups were analysed in a 4-plex set. A CSF pool was labelled with the iTRAQ reagent 114 in all 4-plex approaches. To stop the reaction, formic acid (FA) was added to a final concentration of 10%. The four iTRAQ samples were combined and dried by vacuum centrifugation. Samples were re-dissolved in 1% trifluoroacetic acid (TFA)/water by mixing and sonication. Peptides were captured with in-house prepared STAGE tips containing solid phase cation extraction disks and eluted into low-binding tubes with increasing concentration of ammonium acetate in 20% acetonitrile (ACN)/0.5% FA (fraction 1–5: 125 mM, 160 mM, 220 mM, 300 mM, 450 mM) and followed by 5% ammonium hydroxide/80% ACN (fraction 6). Eluates were dried by vacuum centrifugation and dissolved in 12 µL 0.5% TFA for MS analysis. Detailed LC-MS/MS methods and data analysis are described elsewhere (online supplementary methods, table S3).

Sample preparation for MRM analysis

Two hundred microlitres of CSF sample were mixed with 28 µL IS solution containing heavy labelled peptides (see online supplementary table S2) and 20 µL 1M TEAB. Samples were digested with 12 µL trypsin/Lys-C solution (0.1 µg/µL) for 18 hours at 37°C and 400 rpm. The reaction was stopped by adding 700 µL water and 100 µL 10% TFA. Digested MRM samples were fractionated using STAGE tips with increasing ammonium acetate concentrations in 20% ACN/0.5% FA (fraction 1–5: 75 mM, 125 mM, 200 mM, 300 mM, 450 mM) followed by 5% ammonium hydroxide/80% ACN (fraction 6). Eluates were dried by vacuum centrifugation and dissolved in 27.5 µL 0.1% TFA/6% ACN for MS analysis. Detailed LC-MS/MS methods and data analysis are described elsewhere (online supplementary methods, table S4).

Single-molecule array (SIMOA) assay

For SIMOA validation, the MRM sample cohort was used without samples from Rotterdam. Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) CSF concentrations were quantified using the SIMOA UCHL1 discovery kit (Quanterix, Lexington, Massachusetts, USA) on a SIMOA HD-1 analyser according to the manufacturer's instructions. The lower limit of detection and quantification are 1.05 pg/mL and 3.43 pg/mL, respectively. Coefficient of variation (CV) was tested by the measurement of five technical replicates with a CV of 5.36% and signals were stable for up to four freeze-thaw cycles (data

Table 1 Demographic characteristics of the study cohorts

	C9-ALS	C9-FTD	CAR	P-value					
iTRAQ discovery cohort									
n	16	8	11	–					
Gender (male/female)	10/6	5/3	2/9	0.0515					
Age	60 (51–67)	56 (46–60)	46 (42–49)	0.0061					
ALSFRS-R	39.5 (34.75–42.75)	–	–	–					
FTLD-CDR	–	6.5 (4.13–11.38)	–	–					
	C9-ALS	**sALS**	**C9-FTD**	**sFTD**	**C9-ALS/bvFTD**	**CAR**	**CON-o**	**CON-y**	**P-value***
MRM validation cohort									
n	28	27	18	21	5	28	17	12	–
Gender (male/female)	15/13	17/10	12/6	14/7	2/3	8/20	10/7	3/9	0.0627 (0.6979)
Age	59 (55–67)	60 (51–66)	57 (47–60)	63 (59–68)	64 (64–66)	45 (38–49)	58 (52–64)	46 (43–51)	<0.0001 (0.2419)
Study site: Ulm/ Rotterdam	27/1	27/0	13/5†	21/0†	5/0†	14/14	17/0	12/0	–
ALSFRS-R	39.5 (35.25–41.75)‡	44 (40–46)	–	–	–	–	–	–	–
FTLD-CDR	–	–	5.5 (3.63–10)	5.5 (4.5–6.75)	–	–	–	–	–
	C9-ALS	**C9-FTD**	**C9-ALS/ bvFTD**	**CAR**	**CON-o**	**CON-y**	**P-value***		
SIMOA validation cohort									
n	26	13	5	13	17	10	–		
Gender (male/female)	15/11	8/5	2/3	3/10	10/7	2/8	0.1000 (0.8673)		
Age	61 (55–67)	56 (45–60)	64 (64–66)	47 (43–51)	58 (52–64)	48 (43–51)	<0.0001 (0.0928)		
ALSFRS-R	39.5 (35.25–41.75)‡	–	–	–	–	–	–		
FTLD-CDR	–	6 (4–8.5)	–	–	–	–	–		

Values are given in median and IQR in parentheses. Kruskal-Wallis test and Dunn's post hoc test were used to test for age and χ^2 test was used to test for gender differences.
*P-value in parentheses after testing between the groups C9-ALS, C9-FTD, C9-ALS/bvFTD and CON-o.
†Study site: FTLD Consortium/Rotterdam.
‡ALSFRS-R scores were available from 14 patients.
ALSFRS-R, Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised; C9-ALS, amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion; CAR, *C9orf72* hexanucleotide repeat expansion carriers; C9-FTD, frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; CON-o, control-old group; CON-y, control-young group; FTLD-CDR, FTLD-Clinical Dementia Rating scale; iTRAQ, isobaric tags for relative and absolute quantitation; sALS, sporadic amyotrophic lateral sclerosis; sFTD, sporadic frontotemporal dementia; SIMOA, single-molecule array.

not shown). Kruskal-Wallis test and Dunn's post hoc test were conducted for multiple group comparison. For correlation analyses, the Spearman correlation coefficient was used.

RESULTS

Clinical data

Demographic characteristics of all study groups are presented in table 1. To reduce the influence of age and gender during validation, we differentiated two non-neurodegenerative CON groups. The CON-o group is matched for age and gender to the patient groups and CON-y is matched to the CAR group.

Discovery CSF proteome analysis

The CSF proteomes of patients with C9-ALS and C9-FTD as well as CAR were analysed by iTRAQ LC-MS/MS. In total, 2095 proteins with at least one unique peptide were identified (online supplementary table S5) and on average 1367 proteins per sample without any significant differences between the three groups (Kruskal-Wallis test, Dunn's post hoc test p=0.2498). Eight hundred forty-one proteins were detected in all 35 samples.

To identify differentially expressed proteins across the three groups, we compared the CSF proteomes pairwise by generating volcano plots using the Perseus software (figure 1). Only proteins with at least three valid values per group were considered (C9-ALS vs C9-FTD: 1319 proteins; C9-ALS vs CAR: 1357 proteins; C9-FTD vs CAR: 1315 proteins). Two hundred thirty-six proteins were differentially regulated in C9-ALS CSF samples compared with C9-FTD with a log2 difference of at least 0.5 (online supplementary table S6). By comparing C9-ALS and C9-FTD CSF samples to the CAR group, 513 and 38 proteins were significantly different with a log2 fold change higher than 0.5, respectively. As a proof of concept, higher CSF levels of neurofilament medium polypeptide (NEFM) and chitotriosidase-1 (CHIT1) were detected in C9-ALS CSF, consistent with

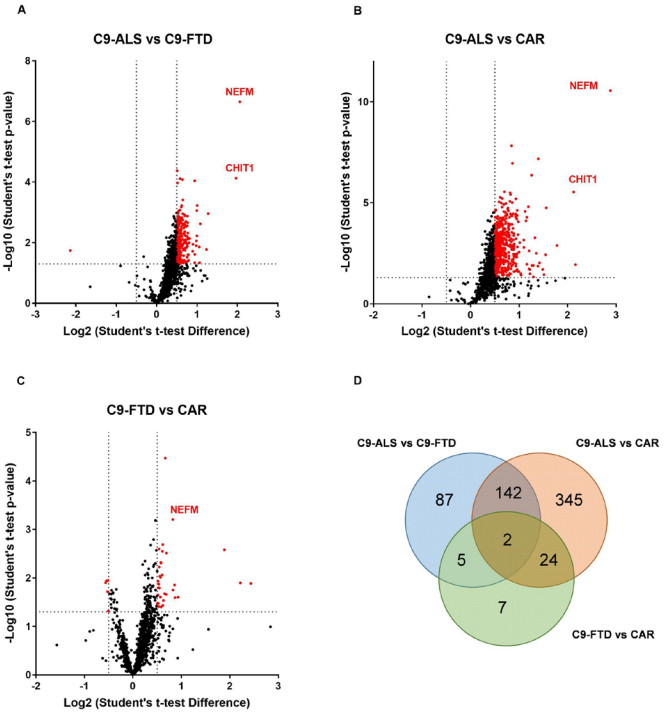

Figure 1 Pairwise CSF proteome comparison of C9-ALS versus C9-FTD, C9-ALS versus CAR and C9-FTD versus CAR (A–C). Coloured points represent proteins with significant different CSF concentrations (Student's t-test, $p<0.05$, |log2 difference|>0.5). (D) Venn diagram of all significantly regulated proteins in all three pairwise CSF proteome comparisons. C9-ALS, amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion; C9-FTD, frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; CAR, asymptomatic *C9orf72* hexanucleotide repeat expansion carriers; CHIT1, chitotriosidase-1; CSF, cerebrospinal fluid; NEFM, neurofilament medium polypeptide.

previous findings of elevated CSF levels of CHIT1 and neurofilament light and heavy polypeptide in ALS.[19 20]

We also compared the lists of differentially regulated proteins between the three pairwise proteome comparisons (figure 1D). The results indicate that the major fraction of identified protein regulations is driven by changes in C9-ALS CSF proteome. Additionally, we performed a Gene Ontology (GO) term enrichment analysis and results are presented in the online supplementary results, table S7.

Validation of selected promising biomarker candidates

For further validation we selected a set of 11 promising biomarker candidates from our pool of significantly regulated proteins. The following criteria favoured the selection of a candidate: significantly different CSF levels in C9-ALS versus C9-FTD with high fold change and/or high significance, almost exclusive expression in CNS according to reported protein expression levels from Human Protein Atlas (www.proteinatlas.org) and already described function in neurological diseases of the candidate or other protein family members.[21–29] All candidates and their respective iTRAQ regulations are listed in table 2.

Validation by MRM

For validation, we established an MRM assay for all 11 candidates (online supplementary table S8). We analysed the correlation of CSF protein levels with age and gender in the CON and CAR groups and results are listed in online supplementary tables S9 and S10.

For five protein biomarker candidates, we could successfully validate higher CSF levels in C9-ALS CSF compared with CAR: chitinase-3-like protein 2 (CHI3L2), alpha-crystallin B chain (CRYAB), profilin-1 (PFN1), neural proliferation differentiation and control protein 1 (NPDC1) and UCHL1 (figure 2). CSF levels of CHI3L2 (SEV…SLK) peptide were upregulated in C9-ALS and C9-ALS/bvFTD compared with all control groups (figure 2A). Additionally, CHI3L2 levels were elevated in C9-FTD CSF samples in comparison to CON-y group. No significant correlation with age nor gender was observed for this peptide. Higher CRYAB CSF levels in C9-ALS samples were also shown in comparison to C9-FTD, CAR and CON-y group based on the quantification of two peptides and mean values showed the same results (figure 2B–D). CRYAB CSF were significantly higher in CON-o versus CON-y subjects and a strong correlation with age was shown for both peptides (FSV…DVK) ($r_s=0.6522$, $p=0.0001$) and (HFS…ELK) ($r_s=0.6872$, $p<0.0001$). Additionally, CSF levels were significantly upregulated in male patients in the total CON group ($p<0.05$). Elevated PFN1 CSF levels in C9-ALS versus CAR and CON-y group were only significant for (TFV…VGK) peptide, and mean values of both peptides showed a weaker significance (figure 2E–G). For both peptides no correlation with age nor gender differences were observed in CON or CAR groups. CSF levels of NPDC1 (LED…LAR) peptide were elevated in C9-ALS compared with C9-FTD, CAR and CON-y group (figure 2H). A weak but significant correlation with age was found in the total CON group for this peptide ($r_s=0.3818$, $p=0.0410$). UCHL1 CSF levels were also upregulated in patients with C9-ALS compared with C9-FTD and all control groups (figure 2I–K). Levels were also elevated in C9-ALS/bvFTD CSF compared with the CAR and CON-y group and for (QFL…TEK) peptide compared with C9-FTD samples. A weak to moderate correlation with age was observed in the CAR group for both peptides (QFL…TEK) ($r_s=0.3847$, $p=0.0476$) and (MPF…LLK) ($r_s=0.4095$, $p=0.0305$). For UCHL1 we additionally analysed sALS cases. CSF UCHL1 levels were also

Table 2 Protein biomarker candidates for validation with their respective iTRAQ regulations

Candidate for validation	Gene	C9-ALS versus C9-FTD	C9-ALS versus CAR	C9-FTD versus CAR
Chitinase-3-like protein 2	CHI3L2	↑	↑↑	↑
Neural cell adhesion molecule L1-like protein	CHL1	↑	ns	↓
Alpha-crystallin B chain	CRYAB	↑↑	↑↑	ns
Neural proliferation differentiation and control protein 1	NPDC1	↑	ns	↓
Neuronal pentraxin receptor	NPTXR	↑	ns	↓
Profilin-1	PFN1	↑	↑	ns
Transferrin receptor protein 1	TFRC	↑	↑	ns
Triggering receptor expressed on myeloid cells 2	TREM2	↑	↑↑	ns
Thioredoxin domain-containing protein 17	TXNDC17	↑	↑	ns
Ubiquitin carboxyl-terminal hydrolase isozyme L1	UCHL1	↑	↑	ns
Neurosecretory protein VGF	VGF	↑	ns	↓

↑/↓significant upregulation or downregulation; ↑↑: at least twofold significant upregulation. C9-ALS, amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion; CAR, *asymptomatic C9orf72* hexanucleotide repeat expansion carriers; C9-FTD, frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; iTRAQ, isobaric tags for relative and absolute quantitation; ns, not significant.

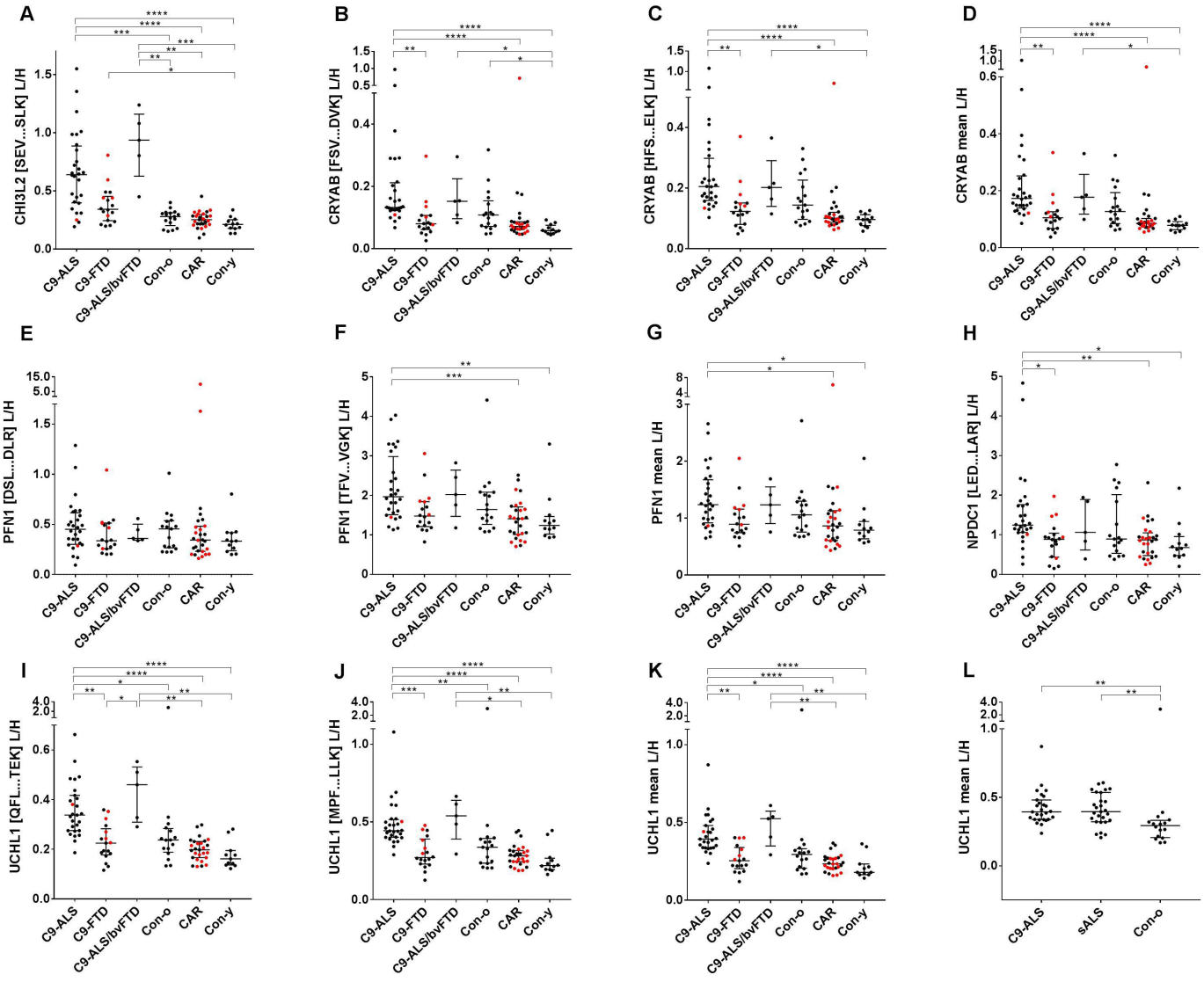

Figure 2 MRM results of upregulated biomarker candidates in C9-ALS CSF. Median and IQR are shown for the ratio of light peptides to spiked heavy labelled peptides (L/H). Samples were collected from two different study sites: Ulm/FTLD Consortium (black) and Rotterdam (red). Kruskal-Wallis test and Dunn's post hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. C9-ALS,amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion;C9-FTD, frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; CAR, asymptomatic *C9orf72* hexanucleotide repeat expansion carriers; CHI3L2, chitinase-3-like protein 2; CON-o, control-old group; CON-y, control-young group; CRYAB, alpha-crystallin B chain; CSF,cerebrospinal fluid; MRM, multiple reaction monitoring; NPDC1, neural proliferation differentiation and control protein 1; PFN1, profilin-1; sALS, sporadic amyotrophic lateral sclerosis; UCHL1, ubiquitin carboxyl-terminal hydrolase isozyme L1.

elevated in sALS CSF compared with non-neurodegenerative controls (figure 2L). No difference was observed in sALS versus C9-ALS.

One protein biomarker candidate was shown to be differentially regulated in C9-FTD CSF: neuronal pentraxin receptor (NPTXR). Both NPTXR peptides (MDQ…LEK) and (VAE…AFK) were decreased in C9-FTD compared with CAR CSF samples (figure 3A–C). We additionally analysed CSF of sFTD cases with similar median FTLD-CDR scores (table 1). In contrast to C9-FTD patients, the mean values of both peptides in sFTD cases are not significantly different from CON-o samples (figure 3D). No association with age nor gender was found for these two peptides in the CAR or CON group.

For two protein biomarker candidates, we observed lower CSF levels in the CAR group compared with patients with C9-ALS: triggering receptor expressed on myeloid cells 2 (TREM2) and transferrin receptor protein 1 (TFRC) (figure 3E,F). TFRC levels were also significantly decreased compared with C9-FTD and CON-o group. For TREM2, significant lower CSF levels were shown in female participants in the CAR group compared with male carriers (p=0.0406).

For three protein biomarker candidates we were not able to validate the iTRAQ results: neurosecretory protein VGF (VGF), neural cell adhesion molecule L1-like protein and thioredoxin domain-containing protein 17 (online supplementary figure S1), although all MRM peptides correlate moderately to very strongly with the iTRAQ data except for TFRC (VSA…IEK) (online supplementary table S11).

Validation by SIMOA technology

To further validate the proteomic results for UCHL1, we performed an SIMOA assay and analysed the UCHL1 CSF levels in all validation groups: C9-ALS (n=26), C9-FTD (n=13),

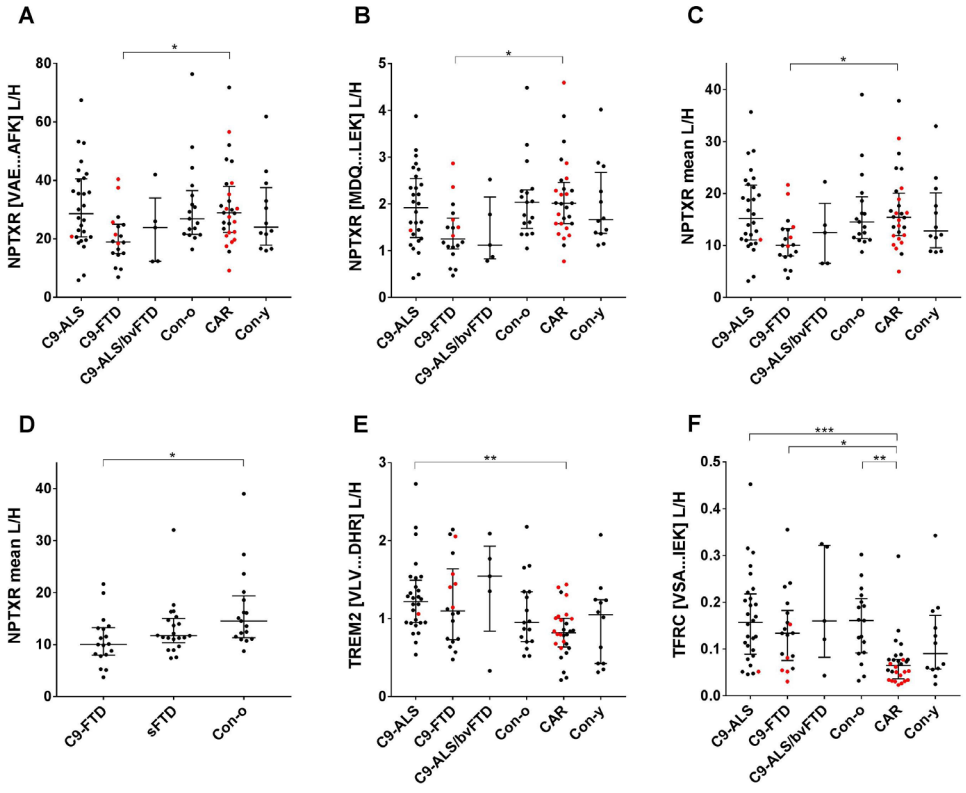

Figure 3 MRM results of downregulated NPTXR CSF levels in C9-FTD (A–D) and decreased protein biomarker candidates in CAR group (E, F). Median and IQR are shown for the ratio of light peptides to spiked heavy labelled peptides (L/H). Samples were collected from two different study sites: Ulm/FTLD Consortium (black) and Rotterdam (red). Kruskal-Wallis test and Dunn's post hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. C9-ALS,amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion;C9-FTD, frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; CAR, asymptomatic *C9orf72* hexanucleotide repeat expansion carriers; CON-o, control-old group; CON-y, control-young group; CSF,cerebrospinal fluid; MRM, multiple reaction monitoring; NPTXR, neuronal pentraxin receptor; TFRC, transferrin receptor protein 1; TREM2, triggering receptor expressed on myeloid cells 2.

C9-ALS/bvFTD (n=5), CAR (n=13), CON-o (n=17) and CON-y (n=10). We could successfully validate higher UCHL1 CSF levels in C9-ALS and C9-ALS/bvFTD compared with all other groups (Kruskal-Wallis test, Dunn's post hoc test $p<0.0001$) and there was a strong correlation of the SIMOA results with both peptides quantified by MRM: (QFL…TEK) ($r_s=0.7698$, $p<0.0001$) and (MPF…LLK) ($r_s=0.7726$, $p<0.0001$) (figure 4).

DISCUSSION

In this study, we performed a deep proteomic CSF analysis of C9-ALS, C9-FTD and asymptomatic CAR by using the iTRAQ technology. In total, we identified almost 2100 CSF proteins. We found 236 differentially regulated CSF proteins in C9-ALS compared with C9-FTD. We also identified elevated CHIT1 and NEFM CSF levels in C9-ALS reflecting the neuroinflammation and neurodegeneration processes, respectively. The upregulation of neurofilaments and CHIT1 in ALS CSF has been described previously[19 20] and serves as a proof of concept of our experimental approach.

The largest proportion of CSF proteome differences was observed in the ALS proteome. On the basis of our GO enrichment analysis, we suggest that this is due to the massive degree of neuroinflammation in combination with neurodegeneration in ALS and thus results in a large proportion of brain proteins entering the CSF.

We selected 11 promising protein biomarker candidates for further validation by targeted mass spectrometry (MRM) and SIMOA technology. We were able to validate our results for eight candidates. Five candidates were shown to be upregulated in C9-ALS CSF: CHI3L2, CRYAB, PFN1, NPDC1 and UCHL1. CHI3L2 is a macrophage-derived chitinase and elevated CSF levels in ALS in comparison to healthy controls, ALS mimics, patients with Parkinson's disease and patients with primary lateral sclerosis were already reported previously.[26] Here, we could also confirm elevated CSF levels in C9-ALS patients with or without additional bvFTD. The increase of CHI3L2 CSF levels in C9-ALS, but not C9-FTD, compared with age-matched non-neurodegenerative controls is an indication of different roles of microglia in the pathophysiology of these two disease phenotypes, although not specific for the *C9orf72* HRE. It also confirms our recent observation of a different inflammatory profile in ALS versus FTD.[30] Moreover, we also confirmed elevated CRYAB CSF levels in C9-ALS versus C9-FTD. CRYAB is a small heat shock protein playing a role in various diseases and has anti-inflammatory and neuroprotective functions.[31] We therefore conclude that CRYAB CSF levels are generally elevated in ALS independent from the presence of a *C9orf72* HRE. Additionally, we found increased PFN1 CSF levels in C9-ALS in comparison to the CAR group and CON-y. A limitation of our study is that we do not have an age- and gender-matched CAR group because asymptomatic CAR are usually young and the samples are rare. To overcome this, we included two non-neurodegenerative control groups in our validation cohort and analysed the correlation with age in all control groups. However, for both PFN1 peptides we did not observe any association with age nor gender in the control groups. PFN1 is an actin-binding

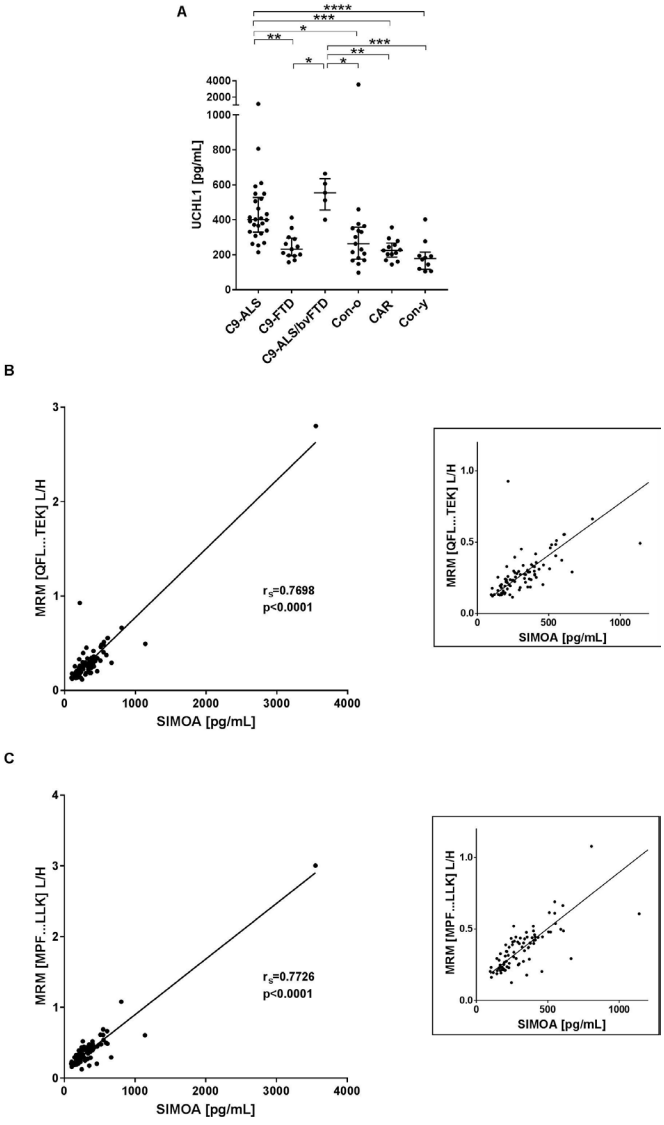

Figure 4 Validation of UCHL1 by SIMOA technology. (A) Median and IQR are shown for UCHL1 CSF levels. Kruskal-Wallis test and Dunn's post hoc test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. (B, C) Correlation of SIMOA results with two UCHL1 MRM peptides. Figures on the right side represent a section of the left graph. C9-ALS,amyotrophic lateral sclerosis patients with *C9orf72* hexanucleotide repeat expansion; C9-FTD,frontotemporal dementia patients with *C9orf72* hexanucleotide repeat expansion; CAR, asymptomatic *C9orf72* hexanucleotide repeat expansion carriers; CON-o, control-old group; CON-y, control-young group; CSF,cerebrospinal fluid; L/H, ratio of light peptides to spiked heavy labelled peptides; MRM, multiple reaction monitoring; r_s, Spearman correlation coefficient; SIMOA, single-molecule array; UCHL1, ubiquitin carboxyl-terminal hydrolase isozyme L1.

protein and *PFN1* mutations are known to cause ALS.[22] C9orf72 protein was reported to regulate axon actin dynamics by interacting with cofilin and other actin-binding proteins. We therefore conclude that elevated PFN1 CSF levels in C9-ALS could indicate a mechanistic link between *C9orf72* and *PFN1* mutations in ALS.

In addition to the cytoskeleton and inflammatory proteins, we could also show clearly increased UCHL1 CSF levels in C9-ALS and C9-ALS/bvFTD in comparison to C9-FTD and all control groups with two different validation methods (MRM and SIMOA). UCHL1 is an enzyme playing a role in ubiquitination processes with hydrolase and ubiquitin ligase activity.[32] In Parkinson's disease, multiple system atrophy and progressive supranuclear palsy decreased UCHL1 CSF levels were reported compared with controls and lower UCHL1 expression was identified in Parkinson's diseases and Alzheimer's disease frontal cortex tissues.[33 34] Here, we show a clear upregulation in C9-ALS CSF. Additionally, UCHL1 levels were also upregulated in patients with sALS indicating a general role of UCHL1 in ALS disease.[27] Since ubiquitination processes play a crucial role in autophagy, UCHL1 could be a promising candidate to explain the diverging outcomes of the *C9orf72* HRE.

We also observed significantly increased NPDC1 CSF levels in C9-ALS compared with the CAR group and CON-y subjects, but this can be explained by an age correlation in the CON cohort. NPDC1 is highly expressed in adult hippocampus and frontal and temporal lobes[28] and is colocalised with synaptic vesicles.[35] Therefore, we suppose that decreased NPDC1 CSF levels in C9-FTD compared with C9-ALS are due to synaptic dysfunction. Whether this decrease is specific for *C9orf72* HRE and could explain the different outcomes of the mutation should be investigated by further experiments.

For one protein biomarker candidate we could successfully validate decreased CSF levels in C9-FTD versus CAR group: NPTXR. NPTXR is a transmembrane synaptic protein belonging to the neuronal pentraxin family. NPTXR plays a role in organisation of excitatory and inhibitory synapses by interaction with neuronal pentraxin 1 and 2[36] and lower CSF levels were also reported for Alzheimer's disease and multiple sclerosis.[37 38] Interestingly, NPTXR levels were not significantly altered in sFTD cases compared with non-neurodegenerative controls in our study, although we observed a trend to lower CSF levels. Recently, van der Ende and colleagues also reported decreased NPTXR CSF levels in symptomatic *GRN* and *MAPT* mutation carriers,[39] suggesting that lower NPTXR CSF levels are specific for genetic FTD cases.

Two protein biomarkers showed decreased CSF levels in the CAR group compared with disease groups: TREM2 and TFRC. To discuss this, one should be aware that the CON-o and CON-y groups are not completely healthy and one can classify the CAR group rather as a phenotypically healthy control group. TFRC is a ubiquitously expressed transferrin receptor involved in various diseases including neurodegenerative diseases and cancer.[40] TREM2 is an immunomodulatory receptor playing a role in neurodegenerative and other inflammatory diseases.[29] We assume that TREM2 and TFRC are general disease markers reflecting inflammation and altered iron homeostasis and CSF levels are already altered in the CON groups. CSF levels in CAR group may more reflect the normal physiological state.

In summary, our study presents a deep comparative proteomic CSF analysis of ALS and FTD patients carrying a *C9orf72* HRE. We found several differentially regulated proteins, mostly cytoskeletal or extracellular-located and vesicle-associated proteins, playing a role in cell cycle and immune response processes. We are aware that this is a preliminary study and limitations are discussed in a supplementary extended discussion section. However, UCHL1 and NPTXR are two promising candidates that could possibly explain the different manifestations of the *C9orf72* HRE. UCHL1 may explain diverging mechanisms in ubiquitination and autophagy processes in C9-ALS versus C9-FTD and NPTXR may reflect different excitatory and inhibitory synapses' organisation processes. Further experiments to study the link between the *C9orf72* HRE and the cellular

pathways in which UCHL1 and NPTXR act will be of interest in the future.

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