Endo-lysosomal protein concentrations in CSF from patients with frontotemporal dementia caused by CHMP2B mutation

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Abstract
Introduction: Increasing evidence implicates proteostatic dysfunction as an early event in the development of frontotemporal dementia (FTD). This study aimed to explore potential cerebrospinal fluid (CSF) biomarkers associated with the proteolytic systems in genetic FTD caused by CHMP2B mutation.

Methods: Combining solid-phase extraction and parallel reaction monitoring mass spectrometry, a panel of 47 peptides derived from 20 proteins was analyzed in CSF from 31 members of the Danish CHMP2B-FTD family.

Results: Compared with family controls, mutation carriers had significantly higher levels of complement C9, lysozyme and transcobalamin II, and lower levels of ubiquitin, cathepsin B, and amyloid precursor protein.

Discussion: Lower CSF ubiquitin concentrations in CHMP2B mutation carriers indicate that ubiquitin levels relate to the specific disease pathology, rather than all-cause neurodegeneration. Increased lysozyme and complement proteins may indicate innate immune activation. Altered levels of amyloid precursor protein and cathepsins have previously been associated with impaired lysosomal proteolysis in FTD.
1 | INTRODUCTION

Frontotemporal dementia (FTD) comprises a group of early-onset dementia syndromes characterized by high heritability. Pathogenic variants in a dozen genes have been demonstrated, of which the C9orf72, GRN, and MAPT genes are implicated in more than half of familial cases. A point mutation in the CHMP2B gene (c.532-1G > C) has been identified as a cause of autosomal dominant dementia in a Danish family with behavioral variant FTD. The CHMP2B protein is a core component of the heteromeric ‘endosomal sorting complex required for transport III’ (ESCRT-III). This protein complex is involved in biogenesis of endocytic multivesicular bodies (MVBs), membrane repair, and regulation of autophagy. Most well-described is the role of ESCRT-III in the endo-lysosomal sorting of ubiquitinated cargo: The protein complex binds transiently to endosomal surfaces and is responsible for the scission of membranes budding into the MVB compartment during the formation of intraluminal vesicles. The mutation renders CHMP2B and thereby the ESCRT-III unable to dissociate from its binding partners and disassemble from endosomal membranes, where the protein accumulates. The result is defective trafficking of endosomes and impaired fusion of endosomes and autophagosomes with lysosomes. Correspondingly, enlarged endosomes have been observed in CHMP2B-FTD patient brain tissue and fibroblasts, as well as animal and cell models expressing the CHMP2B mutation. It has not been fully established whether the organelle malformation itself or mechanisms downstream of it are driving neurodegeneration. However, the mutations that cause FTD converge on defects in the autophagy-lysosomal and ubiquitin-proteasomal systems. Disturbed proteostasis particularly affects neurons, presumably owing to their specialized morphology and post-mitotic longevity. A distinct neuropathology has been described in post-mortem tissue from CHMP2B-FTD patients, namely the presence of cytoplasmic ubiquitin and p62 inclusions (FTLD-UPS), in the absence of tau, TDP-43 or FUS deposits. This hallmark has been recapitulated in three independent, transgenic mouse models expressing mutant CHMP2B. Interestingly, reactive gliosis was present in all CHMP2B-mice, and one study showed that the endo-lysosomal defects are present in both microglia and neurons, indicating involvement of the innate immune system – another common feature of FTD subtypes.

At present, there are no disease-modifying treatment options and no disease-specific biomarkers for FTD that can inform clinicians about expected disease onset, rate of progression, timing of potential treatment, or monitoring of the response to pharmacological intervention. Collection of cerebrospinal fluid (CSF) is essential for establishing diagnosis, or monitoring of the response to pharmacological intervention. Collection of cerebrospinal fluid (CSF) is essential for establishing disease-specific biomarkers for FTD subtypes. CHMP2B animal and cell models have shown that endo-lysosomal components such as certain cathepsins, LAMPS and Rab proteins are affected by CHMP2B mutation, but the mediators of proteostasis have not yet been assessed in CSF from CHMP2B-FTD patients. To this end, we quantified a panel of CSF peptides in patients and controls. The targeted mass spectrometry panel was identified in an explorative proteomics study as tryptic peptides of proteins associated with endocytosis, lysosomal function, and the ubiquitin-proteasome system, that could be reliably quantified using solid-phase extraction and parallel reaction monitoring mass spectrometry.
2 | METHODS

2.1 | Participants

This cross-sectional study included 31 members of the Danish CHMP2B-FTD family: 11 symptomatic and 7 presymptomatic CHMP2B mutation carriers and 13 non-carrier controls. Patients were diagnosed at the Danish Dementia Research Centre, Rigshospitalet, Denmark, using international consensus criteria for behavioral variant FTD. Members of the CHMP2B-FTD family are continuously offered pre-clinical genetic testing and genetic counseling. If consented to, serum and CSF are obtained from each family member for research purposes, without disclosing the genetic status, if so desired by the individual. Family members who do not carry the CHMP2B mutation and have no neurological or cognitive deficits on examination serve as the control group. Addenbrooke’s Cognitive Examination (ACE) was employed as a measure of cognitive disease burden. The study was approved by the Ethics Committee of the Capital Region of Denmark (H-1-2012-041).

2.2 | CSF acquisition

Lumbar punctures were performed according to standard protocol. CSF samples were analyzed in clinical routine at the Department of Clinical Biochemistry, Rigshospitalet. Samples intended for PRM-MS were centrifuged at 2000 g for 10 min, and aliquots of 250 μL were immediately stored at −80°C in the Danish Dementia Biobank, pending biochemical assay at the Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy, University of Gothenburg.

2.3 | Pre-analysis sample preparation

The methods have previously been described in detail, see Brinkmalm et al., Sjödin et al., and Fernström et al. In short, 100 μL CSF was mixed with 25 μL internal standard (IS) containing heavy isotope-labeled peptides (C-term 13C/15N-labeled K and L, and cysteine carbamidomethylation) corresponding to all peptides included in the analysis (JPT Peptide Technologies and Thermo Fisher Scientific) as well as uniformly 13C-labeled ubiquitin (Silantes). The samples were reduced and alkylated by the addition of 25 μL 250 μg/mL iodoacetamide (in 50 mM NH4HCO3; shaking at room temperature for 30 min and then 30 min at room temperature) and 25 μL 70 mM iodoacetamide (in 50 mM NH4HCO3; shaking at room temperature in the dark for 30 min), respectively. Digestion was performed by adding 25 μL 0.08 μg/μL sequencing grade modified trypsin (Promega Co.) diluted in 50 mM NH4HCO3 followed by shaking incubation for 18 h at +37°C which was ended by the addition of 25 μL 10% trifluoroacetic acid. SPE was performed using standard procedure and Oasis HLB 96-well μElution Plates (2 mg sorbent and 30 μm particle size; Waters Co.). The plate was conditioned (2 × 300 μL methanol) and equilibrated (2 × 300 μL H2O). The samples were loaded followed by a wash (2 × 300 μL H2O) and were finally eluted (2 × 100 μL methanol). The samples were dried by vacuum centrifugation and frozen at −80°C pending analysis.

2.4 | PRM-MS

The target panel of interest included 47 peptides derived from 20 proteins. Table S1 shows all peptide measurements and protein estimates. Frozen and dried samples were dissolved, and injected and separated using a Dionex UltiMate 3000 standard-LC system (Thermo Fisher Scientific) in reversed phase on a Hypersil GOLD HPLC C18 column (length 200 mm; inner diameter 2.1 mm; particle size 1.9 μm; Thermo Fisher Scientific). Analysis was performed on a hybrid Q Exactive mass spectrometer (Thermo Fisher Scientific) using electrospray ionization and a HESI-II ionization probe (Thermo Fisher Scientific). The instrument operated in positive ionization mode and data acquisition was performed using a scheduled PRM method with a retention time isolation window of 2 min, an isolation window of m/z 3, a resolution setting of 70k, an automatic gain control target of 1 × 106 and a maximum injection time of 300 ms.

Peak detection and area integration were performed using Skyline v3.6,0 targeting [M+ H]1+ y-ions from a data-independent acquisition method with a fixed isolation window of m/z 3 and an orbitrap analyzer resolution setting of 70k at m/z 200. The ratio of the sum of the product ion areas of tryptic to isotope-labeled peptide was used for quantification.

RESEARCH IN CONTEXT

1. Systematic review: PubMed searches were conducted to compare data from biomarker, animal, and cell model studies on impaired proteostasis in neurodegenerative diseases, with focus on frontotemporal dementia (FTD). Knowledge is lacking regarding which components of the endo-lysosomal and ubiquitin-proteasomal systems are affected, and whether such proteins might be utilized as biomarkers.

2. Interpretation: This is the first study to assess CSF levels of proteostatic markers in a genetically homogenous FTD cohort. From mass spectrometry data, we identified six significantly different protein levels when comparing CHMP2B mutation carriers with family controls. Particularly interesting was the finding of lower CSF ubiquitin in CHMP2B-FTD patients, challenging the view that ubiquitin levels solely reflect neurodegeneration.

3. Future directions: The proposed markers should be included in upcoming studies on fluid biomarkers in familial FTD to confirm the alterations. For certain peptides, for example, ubiquitin, quantification of full-length protein in CSF from CHMP2B-FTD patients is relevant.
### TABLE 1  Patient characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Presymptomatic CHMP2B mutation carriers</th>
<th>Symptomatic CHMP2B mutation carriers</th>
<th>Healthy family members</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of participants</td>
<td>7</td>
<td>11</td>
<td>13</td>
<td>0.405</td>
</tr>
<tr>
<td>Sex, females/males</td>
<td>4/3</td>
<td>3/8</td>
<td>5/8</td>
<td>0.511</td>
</tr>
<tr>
<td>Mean age, years (range)</td>
<td>52.0 (32.7–68.1)</td>
<td>62.9 (53.6–73.3)</td>
<td>60.5 (38.1–71.1)</td>
<td>0.060</td>
</tr>
<tr>
<td>Mean ACE score (range)</td>
<td>92 (88–95)</td>
<td>74.9 (60–89)</td>
<td>93.3 (87–100)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

### 2.5 Immunoprecipitation and PRM-MS

SNAP-25/synaptotagmin-1 were quantified with an in-house assay consisting of enrichment with immunoprecipitation (mouse monoclonal antibodies clone 41.1 (Synaptic Systems) and SM181 (Biosite)) followed by quantitation with liquid chromatography/tandem mass spectrometry (LC-MS/MS, see section above). The instrument was set to acquire scheduled pairs or triplets of fragmentation scans (PRM scans) in profile mode, allowing simultaneous detection of the CSF peptide and the corresponding IS. LC-MS/MS raw files acquired with Xcalibur software version 2.2 SP1.48 (Thermo Fisher Scientific) were imported into Pinpoint software version 1.3.0 (Thermo Fischer Scientific), and peak areas of the CSF and IS peptides were generated. CSF levels of SNAP-25tot, and synaptotagmin-1 were calculated by multiplying the ratio of the LC-MS peak areas with the concentration of the corresponding IS.

### 2.6 Statistics

Statistical analyses were carried out using R statistical software, version 1.2.1335. Normal distribution of peptide values was checked with QQ-plots, histograms, and Shapiro-Wilk normality tests. Non-normally distributed data were logarithmically transformed. Comparisons of mean protein or peptide levels in the clinical groups were calculated with ANOVA and post-hoc comparisons were conducted using Tukey test with single-step adjustment as implemented in the multcomp toolbox. Since there was a tendency towards significantly different ages in the clinical groups, age was included as a covariate in these comparisons. Correlations between continuous variables were assessed using the Spearman’s Rank correlation coefficient. Descriptive statistics were carried out with Fisher’s exact test or one-way ANOVA with post-hoc Tukey test. p-values below 0.05 were considered significant.

### 3 RESULTS

#### 3.1 Patient characteristics

Participants’ demographics and ACE scores are presented in Table 1. Female-to-male ratios did not significantly differ between groups. Mean age differed between symptomatic and presymptomatic patients by 10.8 years (p = 0.055, Tukey test). As expected, ACE scores were significantly lower in symptomatic participants compared with both presymptomatic and healthy participants.

### 3.2 CSF protein concentrations

Comparing CHMP2B mutation carriers with controls revealed significantly increased protein levels of complement C9, lysozyme C (lysozyme), and transcobalamin II (TCN2) in mutations carriers, and significantly lower levels of ubiquitin, amyloid precursor protein (APP) and cathepsin B (CTSB). No significant differences were detected for AP2B1, CTSD, CTSF, CTSL, CTSZ, DPP7, GM2A, HEXB, LAMP1, LAMP2, FUCAT1, TPP1, SYT1, or SNAP25 (Table S2). The six protein estimates significantly associated with CHMP2B mutation were based on the levels of 14 peptides in total. Peptide pairs and triplets originating from the same protein were highly correlated, except the combination of the pro-peptide CTSB58-71 and the light chain peptide CTSB210-220 (Rs = 0.38, p = 0.062), which were both highly correlated with the heavy chain peptide, CTSB80-87 (Rs = 0.73–0.77, p < 0.0001). When dividing the CHMP2B mutation carriers into symptomatic and presymptomatic participants, the levels of 10 of the 14 peptides were significantly different among the three clinical groups (Figure 1, Table S2).

We have recently found an increased level of NfL in CSF from both symptomatic and presymptomatic CHMP2B mutation carriers, as well as an increased CSF/serum albumin ratio in symptomatic CHMP2B-FTD patients – the former being a well-established marker of axonal degeneration, the latter reflecting potential compromise of BBB integrity. In the current study, none of the 10 peptides were significantly correlated with CSF NfL levels in CHMP2B mutation carriers (Table S2). The complement C9, lysozyme, and TCN2 peptides correlated significantly with the CSF/serum albumin ratios, as shown in Figure 2. The complement C9 peptides and one of two lysozyme peptides were significantly correlated with age (Table S2). Notably, CSF/serum albumin ratios did not correlate with age in the mutation carrier group (Rs = 0.11, p = 0.670). No correlations between peptide levels and ACE-scores were observed in mutation carriers (Table S2).

### 4 DISCUSSION

Increasing evidence implicates proteostatic dysfunction as an early event in the development of neurodegenerative diseases, including...
FTD and CHMP2B-FTD specifically. To explore potential mediators of impaired proteostasis in CHMP2B-FTD, this study investigated CSF levels of a panel of peptides functionally involved in endo-lysosomal or ubiquitin-proteasome function. A total of 47 peptides originating from 20 proteins were analyzed and their concentrations compared among 31 members of the Danish CHMP2B-FTD family. PRM-MS revealed significantly higher levels of complement C9, lysozyme, and TCN2 in CHMP2B mutation carriers compared with controls, and significantly lower levels of ubiquitin, APP, and CTSB (Figure 1).

Neuronal inclusions containing ubiquitin and the ubiquitin-binding autophagy receptor p62 remain the only immunohistochemical hallmarks of CHMP2B-FTD postmortem brain tissue. How the cerebral deposition of ubiquitin is reflected in CSF, has not been investigated. Here, both presymptomatic carriers and symptomatic CHMP2B-FTD patients had significantly lower ubiquitin levels than controls.

Two studies have previously included measurements of CSF ubiquitin in FTD patients. Both found the concentration of ubiquitin to be at the same level as healthy controls. Patients with AD and Creutzfeldt-Jakob disease (CJD) showed significantly increased levels of CSF ubiquitin, demonstrated across several studies. Elevated CSF ubiquitin in AD and particularly in CJD has been linked to the extent of neuroaxonal damage. This is supported by correlations between CSF ubiquitin and established markers of neurodegeneration, that is, NfL and total-tau, as well as observed rise in CSF ubiquitin following traumatic brain injury. However, seemingly unaltered CSF levels in other neurodegenerative disorders such as PD indicate that ubiquitin is not exclusively a biomarker of nonspecific neurodegeneration, but instead suggests some degree
of association with disease mechanisms or affected brain region. Of note, the studies that have analyzed CSF ubiquitin levels in behavioral type FTD did not distinguish between genetic subtypes, which may differ in terms of ubiquitin dynamics, as it might be the case with NFL.\textsuperscript{20}

A possible explanation for the lower CSF ubiquitin levels in CHMP2B mutation carriers lies in the impairment of the ESCRT machinery, responsible for internalizing ubiquitinated proteins into MVBs, where they are sorted for either lysosomal degradation with accompanying release of ubiquitin or trafficking back to the plasma membrane. We speculate that the accumulated MVBs observed in CHMP2B models are maintaining an intracellular reservoir of ubiquitin, not reaching the CSF. One might expect that such neuronal ubiquitin deposits would increase CSF levels as neurodegeneration progresses, similar to levels of NFL in CHMP2B-FTD.\textsuperscript{19} In this regard, an apparent spatial mismatch between the frontotemporal cortical neurodegeneration and the neuronal ubiquitin inclusions – which are predominantly found in the hippocampus\textsuperscript{11} – might partly explain the seemingly non-parallel courses of CSF ubiquitin and NFL in CHMP2B-FTD. Another contributing possibility is compromised ESCRT-dependent formation of exosomes, potentially leading to fewer ubiquitinated proteins being transported to the extracellular space. However, silencing one of the direct binding partners of CHMP2B, VPS4B, in HeLa cells has previously been shown to increase overall exosome secretion, not reduce it.\textsuperscript{38} If validated, decreased CSF ubiquitin levels may distinguish CHMP2B-FTD or FTD collectively from other neurodegenerative disease entities, as it has only been found significantly reduced in one other neurodegenerative disease cohort.\textsuperscript{23}

CSF levels of complement C9 and lysozyme were increased in CHMP2B-FTD patients compared with controls. The complement proteins constitute part of the innate immune system and are produced, in the CNS, by neurons and glial cells.\textsuperscript{39} They mediate proinflammatory stimulation and chemotraction between these cell types, as well as clearance of pathogens and damaged cells via the membrane attack complex (MAC), and tagging of synapses for phagocytosis by microglia.\textsuperscript{39} Lui et al. have demonstrated that lysosomal defects and complement activation in microglia can lead to neurodegeneration through excessive synaptic pruning in a mouse model of FTD caused by GRN mutation.\textsuperscript{40} Astrocytes generated from induced pluripotent stem cells from CHMP2B-FTD patients have been shown to be more reactive and display increased production of complement C3, which was confirmed in the brains of mice expressing mutant CHMP2B.\textsuperscript{41} Complement C9, the terminal constituent of the complement cascade and major part of the cytoplasmic MAC, has not previously been investigated in CSF or in vitro models in FTD, but its elevation in CSF indicates involvement of innate immune function. This is supported by increased levels of CSF lysozyme – a glycan cleaving polypeptide produced by microglia.\textsuperscript{42} Findings of higher CSF lysozyme in AD patients than controls have been inconsistent,\textsuperscript{22,23,43,44} although a protective effect of the protein has been proposed based on rescue experiments in Drosophila models.\textsuperscript{43,44} To our knowledge, the role or quantities of lysozyme in FTD have not previously been studied. Importantly, both complement C9 and lysozyme were associated with the CSF/serum albumin ratio (Figure 2).\textsuperscript{20} Correlations between soluble MAC components and BBB dysfunction is known from patients with traumatic brain injury,\textsuperscript{45} and the positive correlation between complement C9 and age has previously been observed in AD.\textsuperscript{46} Earlier studies have reported increased levels of CSF lysozyme as part of an inflammatory reaction to a variety of CNS pathologies.\textsuperscript{47} Passage of lysozyme from blood through an impaired BBB as well as local production by microglia within the CNS, have been suggested as the underlying mechanisms. Thus, it is plausible that complement C9 and lysozyme emanate from serum and contribute to the measured CSF amounts in CHMP2B-FTD patients. In relation to this, we have previously observed a systemic immune response in CHMP2B-FTD patients.\textsuperscript{48} Innate immune activation is also supported by results from transgenic CHMP2B Drosophila models\textsuperscript{49–51} and CHMP2B mouse models, which have all exhibited reactive gliosis.\textsuperscript{13–16} One such mouse model revealed that accumulation of endo-lysosomal byproducts was present in microglia as well as neurons.\textsuperscript{14} A follow-up study showed an early microglial activation in the transgenic mice, progressing into overt microgliosis with a proinflammatory phenotype, neuronal loss, and behavioral changes.\textsuperscript{17} In addition, the authors detected increased levels of lysosomal cathepsin D and LAMP-1,\textsuperscript{14} which has also been reported in Grn knockout mice and patients with GRN-FTD.\textsuperscript{52}

In the present study, CSF concentrations of these lysosomal proteins were unchanged, whereas CTSB was significantly reduced in CHMP2B-FTD patients. Several studies have demonstrated increased CSF levels of CTSB in AD, traumatic brain injury, inflammatory neurological diseases, and aging.\textsuperscript{23,53,54} Lysosomal leakage of CTSB with ensuing unchecked proteolytic activity in the cytosol has been suggested as the deleterious mechanism.\textsuperscript{53,54} Since ESCRT-III is involved in endo-lysosomal membrane repair, one might expect CHMP2B mutation to increase the concentration of CTSB in CSF, rather than reduce it in CHMP2B-FTD patients, which we observe. The potential reduction in CTSB requires further exploration.

In this study, the quantity of APP was significantly lower in CSF from CHMP2B-FTD patients compared with controls. The transmembrane APP protein functions as a cell surface receptor on neurons, involved in neurite growth and synaptogenesis.\textsuperscript{55} It is cleaved by secretases into smaller fragments relating to the amyloidogenic or non-amyloidogenic pathways.\textsuperscript{55} In AD, the CSF concentrations of soluble APP did not differ between patients and controls in a recent meta-analysis,\textsuperscript{56} neither did the levels of the major isoforms of APP in studies using targeted proteomics.\textsuperscript{22,23} In FTD, albeit less well studied, findings point to lower levels of soluble APP in patients with different types of FTLD.\textsuperscript{57–60} Inside neurons, APP is ubiquitinated and targeted to the intraluminal vesicles of MVBs through interaction with the ESCRT machinery.\textsuperscript{61} The internalization of APP and delivery to lysosomes has been shown to parallel that of the epidermal growth factor receptor (EGFR),\textsuperscript{61} the degradation of which is delayed in CHMP2B mutation models\textsuperscript{62} – supposedly due to entrapment of EGFR in mutant CHMP2B positive compartments.\textsuperscript{5} Thus, as described for ubiquitin, APP may be retained in the MVB compartment, reducing the proportion of APP available for cleavage into soluble forms at the plasma membrane, lowering the CSF concentration in CHMP2B-FTD patients.
Lastly, TCN2 was significantly elevated in the patient group. It is responsible for transporting vitamin B12 into tissues via its receptor (CD320), where it is degraded in lysosomes, while dissociated B12 is exported into the cytosol. It is unknown whether the ESCRT system is involved in this process, but ubiquitination of CD320 does not appear to occur. A strong correlation between TCN2 levels in plasma and CSF has been demonstrated elsewhere and it is likely that the bulk of TCN2 in CSF originates from serum. In line with this, TCN2 was significantly associated with the CSF/albumin ratio in our study.

Research into mediators of disrupted proteostasis has been limited in FTD, particularly for genetic subtypes. This study has screened a panel of potential biomarkers associated with endo-lysosomal function in CHMP2B-FTD. Of note, the proteins investigated are multifunctional and hence significantly different CSF levels do not necessarily equate to significant differences in cellular proteostasis. Although the number of statistical comparisons has been attempted to be kept at a minimum, the rarity of the disorder means that the size of our cohort is modest in relation to the number of analytes tested, increasing the risk of false positives. However, the cohort is pathogenetically homogenous, which is necessary for disease entities with such diverse genetic backgrounds as FTD. The findings require validation in an independent cohort and for certain peptides, for example, ubiquitin, further quantification of the full-length protein in CSF from CHMP2B-FTD patients will be relevant. The ubiquitin peptides generated by our enzymatic digestion approach can be derived from both free and conjugated forms of the protein – thus, we cannot assess if intact, free ubiquitin is decreased in CHMP2B-FTD. In AD, however, previous studies have shown that both free and conjugated ubiquitin levels are increased. Furthermore, a recent study employing the same technique as herein could confirm that the levels of these particular ubiquitin peptides are increased in CSF from AD patients. Taken together, the data support different concentrations of CSF ubiquitin in AD, FTD, and healthy controls. The lack of correlation between the studied peptides and CSF NfL suggests that the altered protein levels in mutation carriers are not secondary to axonal neurodegeneration, as expressed by CSF NfL levels, but perhaps represent another part of the disease mechanism.

ACKNOWLEDGMENTS

K.B. has served as a consultant, at advisory boards, or at data monitoring committees for AbbVie, Alector, ALZPath, Annexon, Apellis, Araya Therapeutics, AZTherapeutics, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pintech Therapeutics, Red Abbey Labs, rEMYN, Roche, Samumed, Siemens Healthineers, Triple Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectriocz, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. H.Z. has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, ALZPath, Annexon, Apellis, Araya Therapeutics, AZTherapeutics, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pintech Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triple Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectriocz, Fujirebio, Alzecure, Biogen, and Roche, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). The remaining authors (A.T.S., A.H.S., P.E., P.R., C.S.M., E.E.H., T.T.N., A.B., and J.E.N.) have no conflicts of interest to declare. Author disclosures are available in the supporting information.

REFERENCES


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