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FEATURED ARTICLE

Protein disulfide isomerases as CSF biomarkers for the neuronal response to tau pathology

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Abstract
Introduction: Cerebrospinal fluid (CSF) biomarkers for specific cellular disease processes are lacking for tauopathies. In this translational study we aimed to identify CSF biomarkers reflecting early tau pathology-associated unfolded protein response (UPR) activation.

Methods: We employed mass spectrometry proteomics and targeted immunoanalysis in a combination of biomarker discovery in primary mouse neurons in vitro and validation in patient CSF from two independent large multicentre cohorts (EMIF-AD MBD, $n = 310$; PRIDE, $n = 771$).

Results: First, we identify members of the protein disulfide isomerase (PDI) family in the neuronal UPR-activated secretome and validate secretion upon tau aggregation in vitro. Next, we demonstrate that PDIA1 and PDIA3 levels correlate with total- and phosphorylated-tau levels in CSF. PDIA1 levels are increased in CSF from AD patients compared to controls and patients with tau-unrelated frontotemporal and Lewy body dementia (LBD).

KEYWORDS
Alzheimer's disease, CSF biomarker, PDI, tau pathology, UPR

Highlights

- Neuronal unfolded protein response (UPR) activation induces the secretion of protein disulfide isomerases (PDIs) in vitro.
- PDIA1 is secreted upon tau aggregation in neurons in vitro.
- PDIA1 and PDIA3 levels correlate with total and phosphorylated tau levels in CSF.
- PDIA1 levels are increased in CSF from Alzheimer's disease (AD) patients compared to controls.
- PDIA1 levels are not increased in CSF from tau-unrelated frontotemporal dementia (FTD) and Lewy body dementia (LBD) patients.

1 | BACKGROUND

Tau pathology is one of the core pathological hallmarks of Alzheimer's disease (AD) and is closely related to clinical symptoms.[1–3] Targeting of tau or the cellular response to tau pathology is therefore a promising therapeutic approach.[4] Total (t)-tau and phosphorylated (p)-tau levels in cerebrospinal fluid (CSF) are commonly measured in the diagnostic setting of AD and correlate with the presence of tau pathology in the brain.[5–8] It is thought that CSF t-tau levels reflect tau that passively releases from neurons as they degenerate and therefore marks a relatively late stage in the cellular pathogenesis. Tau has also been detected in different non-conventional secretory routes that may actively transport cytosolic tau out of the cell,[9–16] but it is unknown whether and how these processes contribute to CSF p-tau and t-tau levels in AD. Therefore, although CSF p- and t-tau are good markers that reflect, respectively, tau pathology and neurodegeneration in AD, the direct relationship between CSF t- and p-tau levels and specific cellular disease processes remains largely unknown. This limits their power as early biomarker for the monitoring of responses to pathogenesis-modifying treatments. Here we aim to identify fluid biomarkers reflecting a process early in AD pathogenesis, the activation of the unfolded protein response (UPR).

The UPR is a cellular stress response aimed to maintain and restore protein homeostasis (proteostasis), which is severely disturbed in AD as illustrated by the accumulation of misfolded and aggregated amyloid beta (Aβ) and tau proteins. The UPR is activated by endoplasmic reticulum (ER) stress and triggers an intricate transcriptionally and translationally regulated signaling network.[17] Immunohistochemical and biochemical analyses have shown that activation markers of the UPR are increased in brains of patients with AD and primary tauopathies.[18] Of note, the UPR activation markers follow the spatiotemporal distribution pattern of tau pathology in the brains of patients with AD.[19] At the cellular level, UPR activation markers are predominantly observed in neurons with early stages of tau pathology, characterized by hyperphosphorylated tau that is diffusely distributed, whereas it is absent from neurons with tangles.[19] Interestingly, there appears to be a specific association with tau pathology, because UPR activation markers are observed in FTD-tau, but not in FTD-FUS or FTD-TDP43.[20] These findings indicate that UPR activation is associated with an early stage of intraneuronal pathology.

The strong correlation between UPR activation and the early stages of intraneuronal tau pathology suggests that the UPR is activated as part of the disease process. This is supported by genetic studies that show that mutations in the gene encoding the UPR transducer protein kinase R–like ER kinase (PERK) is associated with increased risk for AD and other tauopathies: progressive supranuclear palsy (PSP) and primary age-related tauopathy (PART).[21–23] In addition, UPR activation initiates the phosphorylation and aggregation of tau in vitro and in vivo.[24–27] Although the mechanistic connection remains to be elucidated, inhibition of the UPR prevents tau phosphorylation

and aggregation as well as neurodegeneration in cell and animal tauopathy models.[25,27] Together the data suggest that the UPR is not only activated early in tau pathogenesis but that it is also an active contributor to the disease process. Hence, UPR-modifying treatments are explored as therapeutic intervention to slow down AD disease's progression.[28,29] Recently, it was demonstrated that several ER-resident UPR target proteins are secreted upon UPR activation (secreted [s]UPR proteins) in peripheral tissues and cell types.[30] sUPR proteins therefore hold a promise as body fluid biomarkers reflecting early tau pathology-related disturbance of cellular proteostasis.

In the present translational study, we aimed to identify a CSF biomarker for the early cellular response to tau pathology. To this end we first determined the neuronal UPR-induced secretome, using unbiased proteomics analysis of primary neuron cultures. We identify members of the protein disulfide isomerase (PDI) family as neuronal sUPR proteins and demonstrate that intraneuronal tau aggregation induces secretion of PDIA1 in vitro. Subsequently, we employed mass spectrometry CSF proteomics data and targeted protein immunoassay data of two independent large multicentre cohorts to show that t- and p-tau levels correlate with the levels of PDIA1 and PDIA3 in CSF. Interestingly, we show that PDIA1 levels are increased in CSF from patients diagnosed with AD, but not Lewy body dementia (LBD) or frontotemporal dementia (FTD). Subanalysis of the FTD group indicated that CSF PDIA1 levels in cases associated with TAR DNA-binding protein 43 (FTD-TDP43) but not those with tau pathology (FTD-tau) are significantly lower than in AD. Concluding, we identify PDIs as CSF biomarkers for the neuronal response to tau pathology.

2 | METHODS

2.1 | Animals and primary neuron cultures

Animal experiments were performed according to institutional and Dutch governmental guidelines and approved by the ethical committee of the Vrije Universiteit/Amsterdam UMC. Primary neuron cultures were prepared from new-born (P1) mice. Cerebral cortices were dissected in Hanks balanced salt solution supplemented with 10 mM HEPES and digested with 0.25% trypsin for 20 min at 37°C. Next, cortices were washed three times with Hanks–HEPES and triturated with fire-polished glass pipettes in DMEM containing 10% HI-FBS, 1% penicillin/streptomycin and 1% NEAA. Dissociated cells were centrifuged for 5 min at 800 revolutions per minute (rpm) and resuspended, counted and plated in Neurobasal medium (NB; Gibco), the most commonly used culture medium for long-term maintenance and maturation of neuronal cells (containing 25 mM D-glucose, 0.22 mM sodium pyruvate, amino acids, vitamins, inorganic salts, and other components), supplemented with 2% B-27, 1.8% HEPES, 0.25% GlutaMAX and 0.1% pen/strep at a density of 300,000 cells/well in six well-plates (for proteomics) or 99,000 cells/well in 24 well-plates (for dot blot analysis) coated with 5 μg/mL poly-L-ornithine and 2.5 mg/mL laminin. Cultures were maintained at 37°C/5% CO_2.

RESEARCH IN CONTEXT

1. **Systematic review**: Activation of the unfolded protein response (UPR) is increased in brain tissue of patients with Alzheimer's disease (AD) and primary tauopathies. Within the brain, UPR activation is associated with tau pathology at an early stage in cellular pathogenesis. It was shown that UPR target proteins could be secreted (sUPR proteins) during proteostatic stress. We hypothesized that neuronal sUPR proteins may provide an early tau-related CSF biomarker for tauopathies.

2. **Interpretation**: We identify the protein disulfide isomerase (PDI) family as neuronal sUPR proteins that are secreted by neurons with tau pathology in vitro. PDIA1 and PDIA3 levels closely correlate with total and phosphorylated tau levels in CSF. Our data are consistent with studies from different fields that link PDI proteins to tau pathology.

3. **Future directions**: We propose that PDI proteins could be used for the monitoring of responses to pathogenesis-modifying treatments. Future studies should characterize mechanisms of release and PDI dynamics in CSF.

2.2 | Proteomics neuronal secretome

At days in vitro (DIV) 14, neurons were washed with NB without supplements (NB-) for 1 h to remove bovine serum albumin (BSA). Next, 1 mL NB- was added and the UPR was activated by treatment with 5 μg/mL tunicamycin (TM; Sigma-Aldrich) dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich) for 6 h. Control cells were treated with equal volumes of DMSO (1:2000). After treatment, the medium of three wells was collected and pooled, filtered through a 0.2 μm filter to remove cellular debris and concentrated using ultrafiltration columns (10 kD MWCO). After concentrating the samples, the volumes were equalized (40 uL) and Laemmli buffer (4X; 10 uL; BioRad), a premixed sample buffer that ensures optimal band resolution during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; containing 62.5 mM Tris-HCl, pH 6.8 sample buffer 10% glycerol 1% LDS 0.005% bromophenol blue) was added. Next, the samples were boiled at 98°C for 5 min and equal volumes (40 uL) were run on a 10% SDS-PAGE gel. The gel was fixed and stained for 30 min with colloidal coomassie blue. The part of the gel containing proteins was cut into pieces of approximately 1 mm3, and destained by a sequential incubation in 50% acetonitrile/50 mM NH_3HCO_3; 100% acetonitrile; 50 mM NH_3HCO_3; 50% acetonitrile/50 mM NH_3HCO_3; 100% acetonitrile. The gel pieces were dried in SpeedVac vacuum concentrator, rehydrated in trypsin solution, and incubated at 37 °C overnight. Peptides were extracted twice with a solution containing 0.1% trifluoroacetic acid/50% acetonitrile for 20 min. The samples were dried in a Speed-Vac vacuum concentrator and stored at −20°C until further analysis.

Peptides were dissolved in 0.1% acetic acid, and analyzed by nano-LC MS/MS using an Ultimate 3000 LC system (Dionex, Thermo Scientific) coupled to the TripleTOF 5600 mass spectrometer (Sciex) as described previously.[31] In brief, peptides were fractionated on a 200 mm Alltima C18 column (100 μm i.e., 3 μm particle size) with increased acetonitrile concentration from 5 to 30% for 90 min, to 40% for 5 min, and to 90% for another 5 min, at a flow rate of 500 nL/min. The eluted peptides were electro-sprayed into a TripleTOF 5600 mass spectrometer. The nano-spray needle voltage was set to 2500 V. The mass spectrometer was operated in a data-dependent mode with a single MS full scan (m/z 350-1200) followed by a top 25 MS/MS. Ions were fragmented in the collision cell using rolling collision energy, and a spread energy of 10 eV. Match between Runs was applied and raw data from the mass spectrometer were searched by MaxQuant against the UniProt mouse proteome with a false discovery rate of 0.01. MaxLFQ normalization was enabled with a Label-free quantification (LFQ) minimal ratio count of 1. The minimal peptide length was set to 6; further MaxQuant settings were left at default. Inherent to the experimental paradigm there were several cases of missing data in the control condition. In these cases we did not use imputation to generate quantitative data for statistical analysis, but selected sUPR candidates from this group for downstream analysis based on higher abundance upon UPR activation in the media of all three independent biological experiments. Analyzed data are provided in Table S1. All obtained raw data has been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications (PRIDE; https://www.ebi.ac.uk/pride) partner repository with the dataset identifier PXD037592.

2.3 | Induction of tau aggregation

To induce tau aggregation, neurons were transduced with lentiviral 2N4R human tau constructs containing the FTD-associated P301L and S320F mutations (FTDtau[1+2]) at DIV3.[32–34] The construct was cloned in a 2nd-generation backbone vector under the cytomegalovirus (CMV) promoter. Untransduced neurons served as control.

2.4 | Immunofluorescence

At DIV 18, neurons were fixed in two steps of 10 min with 1.85% (added to culture medium) and 3.7% formaldehyde in phosphate buffered saline (PBS) at room temperature (RT). After washing with PBS, mouse cells were permeabilized with 0.5% Triton X-100 in PBS for 5 min and blocked with blocking solution consisting of 2% normal goat serum and 0.1% Triton X-100 in PBS for 30 min at RT. Next, incubations with MC1 (Kind gift from Dr. Peter Davies, 1:500) and MAP2 (Abcam Cat# ab5392, RRID:AB_2138153 1:250) primary antibodies were performed overnight at 4°C and incubations with fluorescent secondary antibodies (Thermo Fisher Scientific Cat# A-21449, RRID:AB_2535866 and Cat# A-11001, RRID:AB_2534069) for 1 h at RT. Between and after the antibody incubations, cells were washed three times for 5 min in PBS. Immunofluorescent imaging of single focal

planes was performed with a Nikon Eclipse Ti confocal microscope controlled by NisElements 4.30 software (Nikon) using a 40× oil immersion objective (NA = 198 1.3).

2.5 | Dot blot assay

At DIV12 medium was collected and centrifuged at 2000 rpm for 10 min to remove cellular debris. In five steps, 10 μL culture medium was spotted onto a 0.2 μM nitrocellulose membrane, drying the membrane for 5–10 min after every step. Next, the membrane was blocked with 5% milk in Tris buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 1 h at RT. Subsequently, PDIA1 (Abcam Cat# ab2792, RRID:AB_303304, 1:250) and α-tubulin (Synaptic Systems Cat# 302 211, RRID:AB_887862, 1:1000) primary and horseradish peroxidase (HRP)-conjugated secondary antibody (Agilent Cat# P0447, RRID:AB_2617137, 1:2000) incubations were performed overnight for 1 and 2 h at RT, respectively. Between and after the incubations, membranes were washed three times for 10 min with TBS-T. Membranes were developed with SuperSignal West Femto substrate (Thermo Scientific) for 5 min and chemiluminescence was visualized with the Odyssey Imaging System (LI-COR) and analyzed with Image Studio 5.2 software (LI-COR). PDIA1 levels were corrected for α-tubulin levels.

2.6 | PDI analysis in CSF of patients

CSF proteomics data were employed from two cohorts: the European Medical Information Framework for AD Multimodal Biomarker Discovery (EMIF-AD MBD) study[35,36] and the Protein Identification for Discrimination of Dementias (PRIDE),[37–39] multicenter studies that are well characterized with regard to both clinical diagnosis as well as AD biomarker profiles and APOE genotypes. The EMIF-AD MBD study included CSF proteomics data of 82 individuals with normal cognition and normal Aβ, p- and t-tau CSF markers and 228 individuals with abnormal CSF Aβ across the clinical spectrum of AD (57 normal cognition, 92 mild cognitive impairment [MCI], and 79 with AD-type dementia). CSF proteomics was measured with TMT MS as detailed in.[36] CSF Aβ 1-42 and 1-40 (Aβ42/40) were centrally measured with EUROIMMUN, and local measures of p- and t-tau, harmonized into Z scores according to the specific enzyme-linked-immunosorbent assays (ELISA) (as detailed in[35]), were used. From the PRIDE study[37–39] we included 220 individuals with normal cognition and normal Aβ, p- and t-tau CSF markers, 345 individuals with abnormal CSF Aβ across the clinical spectrum of AD (67 normal cognition, 48 MCI, and 230 with AD-type dementia), and 206 with other types of dementia (110 FTD and 96 dementia with DLB). The FTD group is mixed cohort tau-related ($n = 10$; MAPT mutation $n = 7$, pathologically confirmed $n = 4$) and TDP43-related pathology confirmed cases ($n = 53$; C9ORF72 mutation $n = 23$, GRN mutation $n = 33$, pathologically confirmed $n = 33$). All individuals underwent a full work-up at the Amsterdam Dementia Center[37,38] or University of Pennsylvania. PDIA1/P4HB levels were determined with targeted Proximity Extension Assay (PEA) technology (Olink) (Target 96 Development panel). All PDIA1 levels were above the

lower limit of detection. The measurements are in Normalized Protein eXpression (NPX), Olink's arbitrary unit in Log2 scale. Local assay specific cutoffs for measures of Aβ1-42, p-tau181 and t-tau were used and harmonized to Innotest for the correlation analyses.

2.7 | Statistical analysis

Statistical analysis of proteomics data from proteins that were detected in all samples (see the Results section for more details) was performed with Perseus software (MaxQuant): LFQ values were log2 transformed and analyzed by a two-tailed Student's t-test. GraphPad Prism 8.0.1 software was used to perform statistical analysis for all other in vitro experiments in this study. Shapiro-Wilk test was used to assess distribution normality and a two-tailed Student's t-test to compare two groups. R software (https://www.r-project.org/) was used to analyze CSF data. Associations between PDI protein levels and Aβ, p- and t-tau CSF markers were tested with linear regression models. Univariate models without covariates as well as models that corrected for age and sex were analyzed (Tables S2 and S3). Group differences between PDIA1 levels in different dementias were tested with ANOVA followed by Dunn's posthoc test. A p-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Activation of the UPR enhances the secretion of PDI proteins from neurons

In order to identify a potential sUPR biomarker, the neuronal secretome was determined upon UPR activation in vitro. Primary mouse neurons were treated with the UPR-activator TM for 6 h, after which the culture medium was analyzed by nano-liquid chromatography-mass spectrometry (nano-LC MS/MS) (Figure 1A). The number of proteins that accumulate above detection level in culture media is limited, yet in total 810 different proteins were identified of which 108 were detected in the secretome of both control neurons and UPR-activated neurons (Figure 1B). Quantitative analysis showed that the secretion of 15 of these proteins significantly increased upon UPR activation (sUPR proteins, Figure 1C). Because the secretion of sUPR proteins is induced by stress, the levels of a major part of the proteins in the neuronal secretome (686) were below detection limit in one or more replicates of the control condition (Figure 1B). Because this precludes statistical analysis, we selected additional sUPR candidates from this group for downstream analysis based on higher abundance upon UPR activation in the media of all three independent biological experiments (Figure 1D; 103 sUPR proteins). Hence, in total 118 sUPR proteins were identified in the neuronal secretome.

To determine which of the identified sUPR proteins are established UPR targets in primary neurons of which the expression increases upon UPR activation, the mRNA expression profiles determined by mRNA sequencing[40] (GSE200742, https://www.ncbi.nlm.nih.gov/geo/query/

acc.cgi?acc = GSE200742) were examined. This showed that mRNA expression of 10 sUPR proteins – *Pdia1, Pdia3, Pdia4, Calr, Hspa9, Ganab, Tpt1, Srm, Pabpc1* and *Eef1g* – significantly increased upon UPR activation in mouse neurons. Remarkably, three of these proteins— PDIA1 (also called P4HB), PDIA3 and PDIA4 - are ER-resident proteins of the disulfide isomerases (PDIs) family, a group of 21 related enzymes that play an important role in protein folding and the regulation of proteostasis in the ER.[41] Therefore, PDIs are an interesting target for further exploration as sUPR biomarker candidate.

Further analysis of the mRNA expression profiles[40] of the PDI family members showed that 15 PDI proteins are expressed by primary neurons in culture. Besides *Pdia1, Pdia3* and *Pdia4*, also the mRNA expression of *Pdia5, Pdia6, Erp29*/PDIA9, *Erp44*/PDIA10, *Tmx1*/PDIA11, *Txndc5*/PDIA15, *Txndc12*/PDIA16, and *Dnajc10*/PDIA19 significantly increased upon 24 h of UPR activation (Figure 2A). However, except for *Pdia1, Pdia3, Pdia4*, and *Pdia6*, the expression of these PDI family members is relatively low in neurons. In accordance, only the most highly expressed PDI family members are detected in the neuronal secretome (Figure 2B). Together, these data show that the majority of PDI family members are transcriptionally regulated UPR target genes in neurons. Importantly, only PDIA1, PDIA3, PDIA4, and PDIA6 are sUPR proteins that are actively secreted by neurons during UPR activation.

3.2 | Secretion of PDIA1 is induced by intraneuronal tau aggregation

To determine whether PDI secretion is induced by tau pathology, we employed tau containing the spontaneously aggregating FTD-associated P301L and S320F mutations (FTDtau[1+2])[32–34] by viral transduction (Figure 3A). This results in abundant insoluble tau aggregates in primary mouse neurons within 9 days that are positive for the MC1 antibody that detects a pathological conformation of tau (Figure 3B). In the timeframe of the experiment neurodegeneration is not observed. Culture media were collected 9 days later and the levels of PDIA1 - the best-studied PDI family member, for which a well-characterized and good-performing commercial antibody is available—were analyzed by dot blot analysis. This showed that PDIA1 was virtually absent in the culture medium of control neurons. However, PDIA1 was clearly detectable in the culture medium of FTDtau[1+2] expressing neurons that contain tau aggregation, demonstrating that tau pathology is sufficient to induce the secretion of PDIA1 (Figure 3C and 3D).

3.3 | CSF levels of PDI proteins correlate with CSF p-tau and t-tau levels in AD

Next, we assessed whether PDIs can be detected in CSF and their potential as biomarker for the cellular response to tau pathology. For this purpose, we employed the CSF proteomics data from control participants and patients across the clinical spectrum of AD from the EMIF-AD MBD study.[35,36] All four PDI biomarker candidates (PDIA1,
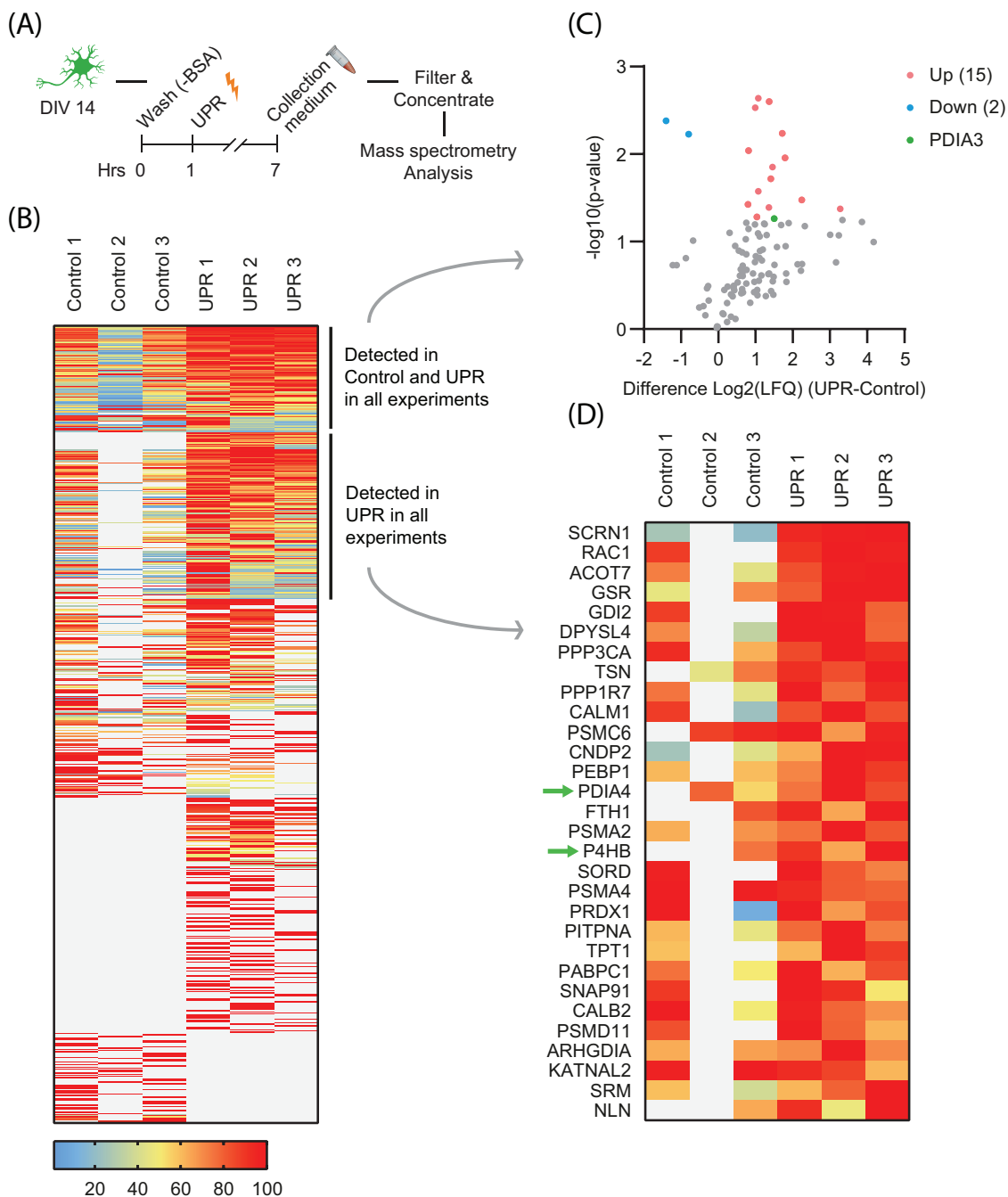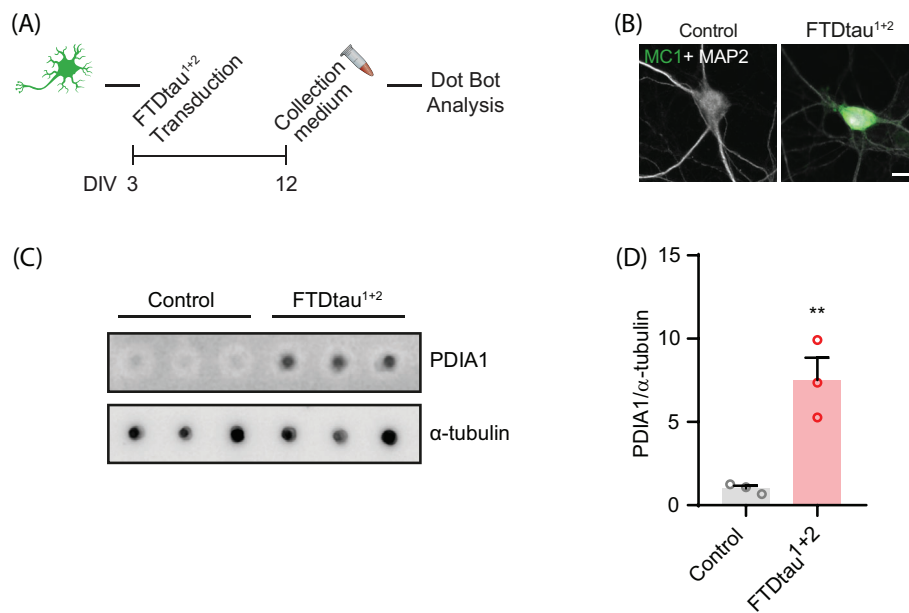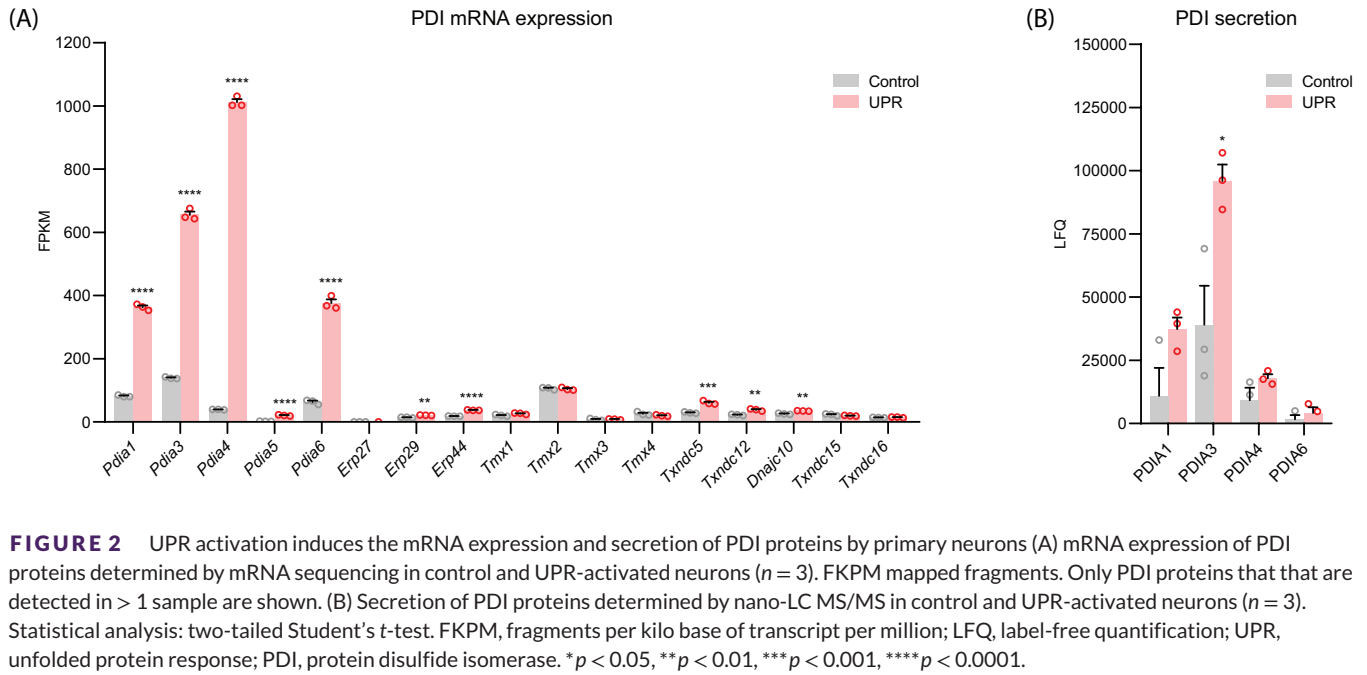
FIGURE 1 PDI proteins are present in the neuronal secretome upon UPR activation (A) Schematic overview of timeline and experimental setup. (B) Heatmap showing the relative LFQ intensity of all proteins detected in the secretome of control neurons ($n = 3$) and UPR-activated neurons ($n = 3$) determined by nano-LC MS/MS. Data were normalized to maximal LFQ intensity of each individual protein. Grey: not detected. (C) Volcano plot of differentially expressed proteins that are detected in the secretome of both control neurons and UPR-activated neurons in all experiments (biological replicates). (D) Heatmap showing the relative LFQ intensity of proteins that are detected and in all experiments (biological replicates) upon UPR activation and show increased secretion upon UPR activation in all experiments. Shown is a selection of 30 proteins with the highest relative LFQ intensity upon UPR activation. Green arrow: PDI family members PDIA4 and PDIA1/P4HB. The full list of proteins is provided in the Table S1. BSA, bovine serum albumin; DIV, days in vitro; LQF, label-free quantification; UPR, unfolded protein response.

PDIA3, PDIA4, and PDIA6) were detected in the CSF samples analyzed in this study, which allows correlation analysis of the sUPR biomarker candidates with the established diagnostic p-tau, t-tau and Aβ biomarkers (Figure 4). Across the group, higher CSF levels of PDIA1 and PDIA3 were correlated with both higher p- and t-tau levels, whereas they did not correlate with Aβ42/40 levels. In contrast, the levels of PDIA4 and PDIA6 in CSF did not significantly correlate with p- and t-tau and PDIA4 positively correlated with Aβ42/40, excluding them as potential biomarkers for the cellular response to tau pathology. Together these data demonstrate that the levels of PDIA1 and PDIA3 correlate with

FIGURE 2 UPR activation induces the mRNA expression and secretion of PDI proteins by primary neurons (A) mRNA expression of PDI proteins determined by mRNA sequencing in control and UPR-activated neurons ($n = 3$). FKPM mapped fragments. Only PDI proteins that that are detected in > 1 sample are shown. (B) Secretion of PDI proteins determined by nano-LC MS/MS in control and UPR-activated neurons ($n = 3$). Statistical analysis: two-tailed Student's t-test. FKPM, fragments per kilo base of transcript per million; LFQ, label-free quantification; UPR, unfolded protein response; PDI, protein disulfide isomerase. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

FIGURE 3 Tau pathology induces the secretion of PDIA1 (A) Schematic overview of timeline and experimental setup. (B) Representative confocal images of control neurons (untransduced) and neurons with tau pathology (FTDtau[1+2]-transduced). Dendrites (MAP2, white), tau pathology (MC1, green). Scale bar: 10 μm. (C) Dot blot of PDIA1 and α-tubulin protein levels in medium of neurons overexpressing FTDtau[1+2] ($n = 3$). (D) Quantitative analysis of dot blot. PDIA1 levels were corrected for α-tubulin levels. Statistical analysis: two-tailed Student's t-test. DIV, days in vitro; PDI, protein disulfide isomerase. **$p = 0.0086$.

t- and p-tau-levels in CSF, suggesting that the CSF levels of these proteins may reflect tau pathology-related UPR activation in AD.

To gain further support for the potential of PDI proteins as CSF biomarker for the cellular response to tau pathology, we analyzed PEA technology (Olink) data obtained in an independent well-characterized AD cohort, comprised of control individuals and patients across the clinical spectrum of AD from the PRIDE study.[37–39] This targeted immune-detection platform comprised PDIA1, but not other PDIs. This analysis confirmed that PDIA1 levels significantly correlated with p- and t-tau in CSF, whereas PDIA1 did not correlate with Aβ42 (Figure 5). These data are in agreement with our in vitro data that demonstrate that PDIA1 secretion is induced by tau pathology.
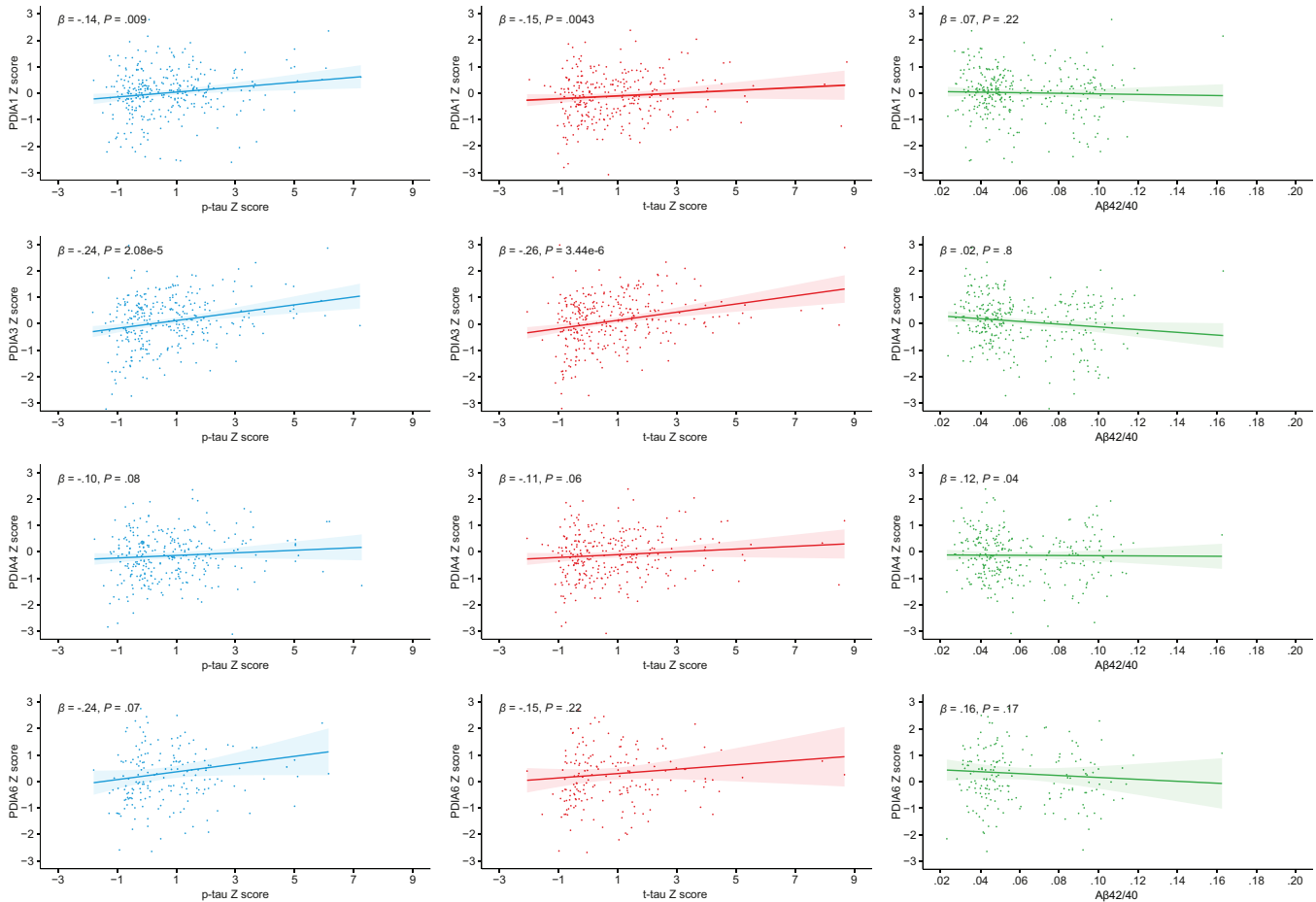
FIGURE 4 CSF levels of PDI proteins correlate with CSF p-tau and t-tau in the EMIF-AD MBD cohort analyzed were individuals with normal cognition and normal Aβ, p- and t-tau CSF markers ($n = 82$) and individuals with abnormal CSF Aβ across the clinical spectrum of AD ($n = 228$: 57 normal cognition, 92 MCI and 79 with AD-type dementia). Statistical analysis: Linear regression. Blue: p-tau, Red: t-tau, Green: Aβ42/40. β and p-values are shown in the top left corner of each graph and were corrected for sex and age. Analysis of univariate models without covariates are provided in Table S2. Aβ, amyloid beta; AD, Alzheimer's disease; CFS, cerebrospinal fluid; EMIF-AD MBD, European Medical Information Framework for AD Multimodal Biomarker Discovery; PDI, protein disulfide isomerase.

3.4 | PDIA1 levels are selectively increased in patients with tau-related dementia

To obtain further support for the hypothesis that CSF PDI levels reflect a response to tau pathology in the brain, we analyzed the PDIA1 CSF levels of patients with non-AD dementias as determined by the PEA technology. This demonstrates that PDIA1 levels were higher in CSF from AD patients compared to controls as well as FTD and DLB patients, suggesting a specific relation with AD (Figure 6A). A subset of the FTD group could aetiologically be classified as FTD-TDP43 or FTD-tau based on genetic and/or pathological diagnostic criteria. Although this cohort only contained a small number of FTD-tau patients, it is interesting to note that PDIA1 levels in CSF from FTD-tau patients are not different from those in AD CSF, in contrast to the significantly lower levels in FTD-TDP43 patients (Figure 6B). Together, these data indicate that CSF PDIA1 levels are specifically associated with cellular tau-pathology in the brain.

4 | DISCUSSION

In this study we first determined the neuronal UPR-activated secretome, using unbiased proteomics analysis of primary neuron cultures. We identify several members of the PDI family as neuronal sUPR proteins that are transcriptionally up-regulated and secreted upon UPR activation (Figure 1). PDIs are a family of 21 thiol–disulfide oxidoreductases and chaperones that are best known for their role in the (oxidative) folding of newly synthesized proteins in the ER.[41] PDIs are transcriptional target genes of the UPR (Figure 2) but also act as upstream regulators of the UPR transducers (reviewed in[41]). In line with their important role in ER homeostasis and the UPR, PDIs are luminal ER resident proteins. In addition, they can be found in the nucleus, cytoplasm, on the cell surface, and extracellularly.[42] Almost all PDIs, including PDIA1, PDIA3, PDIA4, and PDIA6, possess an ER-retention signal sequence.[41] Therefore, the trafficking out of the ER and subsequent secretion of PDIs is an active process. In agreement,
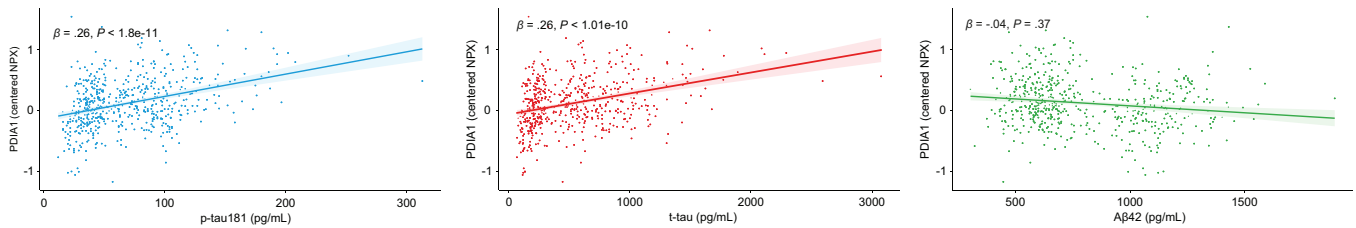
FIGURE 5 CSF levels of PDIA1 protein correlate with CSF p-tau181 and t-tau in the PRIDE cohort analyzed were individuals with normal cognition and normal $A\beta$, p- and t-tau CSF markers ($n = 220$) and individuals with abnormal CSF $A\beta$ across the clinical spectrum of AD ($n = 354$: 67 normal cognition, 48 MCI and 230 with AD-type dementia). Statistical analysis: Linear regression. Blue: p-tau, Red: t-tau, Green: $A\beta42$. β and p-values are shown in the top left corner of each graph and were corrected for sex and age. Analysis of univariate models without covariates are provided in Table S3. $A\beta$, amyloid beta; AD, Alzheimer's disease; CFS, cerebrospinal fluid; NPX, normalized protein eXpression; PDI, protein disulfide isomerase.
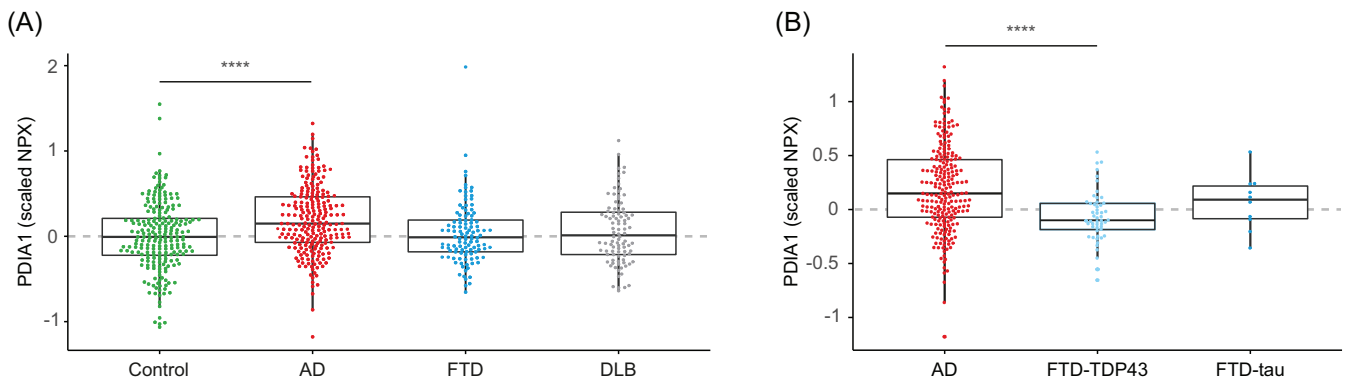
FIGURE 6 PDIA1 protein levels in CSF are selectively increased in tau-related dementia (A) Control: normal cognition and normal $A\beta$, p- and t-tau CSF markers ($n = 220$). AD ($n = 230$); FTD ($n = 110$); DLB ($n = 96$). (B) AD ($n = 230$); FTD-TDP43 ($n = 53$; C9ORF72 mutation $n = 23$, GRN mutation $n = 33$, pathologically confirmed $n = 33$); FTD-tau ($n = 10$; MAPT mutation $n = 7$, pathologically confirmed $n = 4$). Statistical analysis: ANOVA followed by Dunn's posthoc test. AD, Alzheimer's dementia; ANOVA, analysis of variance; $A\beta$, amyloid beta; DLB, dementia with Lewy bodies; FTD, frontotemporal dementia; FTD-tau, tau-related frontotemporal dementia; FTD-TDP43, TDP43-related frontotemporal dementia; NPX, Normalized Protein eXpression; PDI, protein disulfide isomerase. ****$p = < 0.0001$.

we show that the expression levels of different PDIs upon UPR activation do not always directly correspond with the extracellular protein levels (Figure 2), that is, *Pdia4*, shows the highest UPR-activated mRNA expression response in neurons (Figure 2A), but this is not reflected in the UPR-activated secretion of PDIA4 protein (Figure 2B). Hence, this excludes that the increased levels of secreted PDI proteins in the neuronal culture medium are merely the effect of higher transcription. Secretion of PDIs has previously been reported in a variety of cell types.[43] Recently, it has been shown that PDI is also secreted from dorsal root ganglion neurons in inflammatory and neuropathic pain models.[44]

Using dot blot analysis, we demonstrate that intracellular tau aggregation induces the secretion of PDIA1 in vitro. The EMIF-AD CSF mass spectrometry proteomics data and the PRIDE CSF PEA protein analysis provide a great resource to identify biomarker profiles in a hypothesis-free manner (e.g.,[36,39,45]). Here, we employed these data in a hypothesis-driven approach to study our candidate proteins. The CSF mass spectrometry proteomics data shows that CSF levels of PDIA1 and PDIA3 correlate with CSF t- and p-tau levels. Targeted protein

immunoassay analysis of PDIA1 in a separate cohort replicated the correlation with CSF t- and p-tau and showed no correlation with $A\beta$. Together with the close connection between tau but not $A\beta$ pathology and UPR activation in the brain observed previously[18,19] these data are supportive of a connection of PDI with tau-related disease, yet the correlation with tau biomarkers is modest. This suggests an association of PDI levels with a disease process that is tau-related, yet distinct from the tau pathology or neuro-degeneration that are reflected by p-tau and t-tau respectively. In accordance, UPR activation is observed in neurons with early stages of tau aggregation but is absent from neurons with end-stage tau pathology and degenerating neurons.[19,20]

We demonstrate that chronic UPR activation induces PDI secretion in neurons, but it is likely that other cell types including astrocytes and microglia also secrete PDIs during chronic UPR activation. Importantly, although the inflammatory response can be modulated by UPR signaling, activation of microglia and astrocytes per se is not accompanied by UPR activation.[46] In line with this, in the brain of tauopathy patients, UPR activation markers are very occasionally observed in glia cells and only if they have tau pathology.[20] It is therefore most likely that the

increased levels of PDI proteins in CSF derives from neurons with tau pathology, but we cannot exclude a contribution of other cell types.

Previous immunoblot and immunohistochemical data on the levels of PDIA1 in post mortem AD brain tissue showed conflicting results. Lee et al. (2010) reported a 9.49-fold increase in PDIA1 levels in the brain of AD patients compared to controls.[47] In this study, UPR activation was confirmed in the same brain tissue samples by increased XBP-1 mRNA splicing and expression of the UPR targets caspase-3, -4, and -12 and CHOP. Kim et al. (2000) did not observe differences in PDIA1 levels in brains of AD patients compared to age-matched controls. However, they did observe that the levels were highest in neurons that are vulnerable in AD and tend to have increased oxidative damage.[48] In the AD brain, PDIA1 is primarily expressed in neurons and the levels of PDI are increased in 78% to 100% of neurons bearing tau pathology.[47,49] Interestingly, a recent study of the p-tau interactome, identified PDIA1 as one of the most significant interactors of p-tau in neurons from patients with advanced AD.[50] In accordance, confocal microscopy and Co-IP data revealed that overexpressed human PDIA1 co-localizes and interacts with endogenous tau in the neuronal cell line SH-SY5Y.[51] Biochemical analysis suggests that PDIA1 inhibits fibrilization of tau by inhibiting both nucleation and elongation under physiological conditions,[51] indicating that PDI up-regulation might be a protective response against tau pathology. Interestingly, proteomics analysis of laser dissected neurons showed increased PDIA3 levels in neurons with an activated UPR, characterized by the presence of granulovacuolar degeneration bodies (GVBs), neuron-selective lysosomal structures that are associated with UPR markers and are typically detected in cells with early tau pathology.[19,52,53] In contrast, in tangle-bearing neurons without UPR activation markers the levels of PDIA3 were not altered and levels of PDIA4 and PDIA6 were decreased,[53] suggesting that PDI up-regulation specifically occurs in the early stages of cellular tau pathology, in accordance with UPR activation that also appears as early response in the pathogenesis.[19]

Although most prominently associated with tau pathology, UPR activation can be observed in other neurodegenerative proteinopathies as well.[18] Our targeted protein immunoassay data show that PDIA1 is selectively increased in CSF from AD patients, but not in FTD or DLB patients. Importantly, further analysis of the latter group, which is a mixed cohort of patients with tau-related and TDP43-related pathology, showed that PDIA1 levels were lower in CSF from FTD-TDP43 but not FTD-tau patients than AD patients, which is in agreement with the link between chronic UPR activation and cellular tau pathology and not TDP43 pathology in the human brain.[19,20,54]

It is tempting to speculate that extracellular PDI proteins function as chaperones that bind to extracellular proteins – for example, Aβ or tau seeds- and prevent their aggregation. Indeed, immunoblotting studies have shown that PDIA3 physically interacts with Aβ in the CSF of healthy individuals.[55] However, is has been shown that PDIA1 was not increased in the cortex of the Tg2576 Aβ pathology mouse, despite the widespread presence of senile plaques.[47] This suggests that Aβ is unlikely to be the trigger for the secretion of PDI proteins, in line with our results that show no correlation between PDI and Aβ CSF levels.

Importantly, both the oxidoreductase and chaperone function of PDI have been reported extracellularly.[43] Therefore, it is conceivable that the function of (secreted) PDIs in the brain stretches beyond the maintenance of extracellular proteostasis as chaperone. Alternatively, PDIs could be involved in the intercellular communication (danger signaling) of neurons with each other or other cell types in the brain.[30] In this respect, it is interesting to note that extracellular PDI has been reported to modulate the immune response in peripheral leukocytes and macrophages (reviewed in[43]). It is tempting to speculate on a potential functional role of PDI secretion from neurons with tau pathology as a signal that modulates the neuroinflammatory response in microglia or astrocytes.

In conclusion, we have identified PDIs as sUPR proteins that are secreted by neurons in response to tau pathology. PDI levels correlate with t- and p-tau levels in CSF of AD patients. In addition, PDIA1 levels are selectively increased in CSF of AD, but not other dementias that are not associated with intraneuronal tau pathology. CSF p-tau and t-tau biomarkers are robust identifiers to identify patients for disease modifying treatments, but because CSF t-tau and p-tau derive (at least in part) from degenerating neurons their potential as biomarker for the monitoring of responses to treatments that target early pathogenic processes is limited. We showed that it is feasible to detect differences in the CSF PDIA1 levels by antibody detection, suggesting that ELISA or more sensitive (immune) detection methods like single molecule array can be implemented to assess PDI levels in CSF. We propose that PDIA1 and PDIA3 may be utilized as biomarkers to monitor treatment responses to disease modifying interventions for tauopathies, including - but not limited to - interventions in the UPR itself.

CONFLICTS OF INTEREST

W.S. performs contract research for Alzinova and DiscovericBio and received grants from Janssen Pharmaceutica. She is associate editor of Acta Neuropathologica Communications and Alzheimer's Research & Therapy. L.V. received consultancy fees for Roche, paid to her institution. HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, ALZPath, Annexon, Apellis, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Passage Bio, Pinteon Therapeutics, Red Abbey Labs, reMYND, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave, has given lectures in symposia sponsored by Cellectricon, 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). C.E.T. has a collaboration contract with ADx Neurosciences, Quanterix and Eli Lilly, performed contract research or received grants from AC-Immune, Axon Neurosciences, Bioconnect, Bioorchestra, Brainstorm

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