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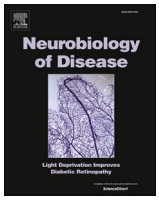

Apolipoprotein L1 is increased in frontotemporal lobar degeneration post-mortem brain but not in ante-mortem cerebrospinal fluid

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ABSTRACT

Aims: Frontotemporal Dementia (FTD) is caused by frontal-temporal lobar degeneration (FTLD), characterized mainly by brain protein aggregates of tau (FTLD-Tau) or TDP-43 (FTLD-TDP). The clinicopathological heterogeneity makes ante-mortem diagnosis of these pathological subtypes challenging. Our proteomics study showed increased Apolipoprotein L1 (APOL1) levels in CSF from FTD patients, which was prominently expressed in FTLD-Tau. We aimed to understand APOL1 expression in FTLD post-mortem brain tissue and to validate its potential as a CSF biomarker for FTD and its pathological subtypes.

Methods: APOL1 levels were analyzed in the frontal cortex of FTLD (including FTLD-Tau and FTLD-TDP) and non-demented controls by immunohistochemistry (FTLD total = 18 (12 FTLD-Tau and 6 FTLD-TDP); controls = 9), western blot (WB), and a novel prototype ELISA (FTLD total = 44 (21 FTLD-Tau and 23 FTLD-TDP); controls = 9). The association of APOL1 immunoreactivity with phosphorylated Tau (pTau) and TDP-43 (pTDP-43) immunoreactivity was assessed. CSF APOL1 was analyzed in confirmed FTD patients ($n = 27$, including 12 FTLD-Tau and 15 FTLD-TDP) and controls ($n = 15$) using the same ELISA.

Results: APOL1 levels were significantly increased in FTLD post-mortem tissue compared to controls as measured by immunohistochemistry, WB, and ELISA. However, no differences between the pathological subtypes were observed. APOL1 immunoreactivity was present in neuronal and glial cells but did not co-localize with pTau or pTDP-43. CSF APOL1 levels were comparable between FTD patients and controls and between pathological subtypes.

Conclusion: APOL1 is upregulated in FTLD pathology irrespective of the subtypes, indicating a role of this novel protein in FTD pathophysiology. The APOL1 levels detected in brain tissue were not mirrored in the CSF, limiting its potential as a specific FTD biofluid-based biomarker using our current immunoassay.

List of abbreviations

Analysis of covariance	ANCOVA
Amyotrophic lateral sclerosis	ALS
Alzheimer's disease	AD
Apolipoprotein 1	APOL1
Chromosome 9 open reading frame 72	*C9orf72*
Corticobasal degeneration	CBD
Cerebrospinal fluid	CSF
Coefficient variations	CV
Diaminobenzidine tetrahydrochloride dihydrate	DAB
Frontotemporal Dementia	FTD
Frontal-temporal lobar degeneration	FTLD
TAR DNA-binding protein 43	TDP-43

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Progranulin	GRN
High-density lipoprotein	HDL
Liquid permanent red	LPR
Microtubule-associated protein tau	MAPT
Pick's Disease	PiD
Progressive supranuclear palsy	PSP
Phosphorylated Tau	pTau
Phosphorylated TDP-43	pTDP43
Tissue protein Extraction reagent	T-PER

1. Introduction

Frontotemporal dementia (FTD) is the second most common form of dementia among people under the age of 65 (Onyike and Diehl-Schmid, 2013). FTD is a heterogeneous disorder presenting with different clinical phenotypes. It is caused by frontotemporal lobar degeneration (FTLD) in which two main and exclusive protein hallmarks have been identified: aggregates of the microtubulin-associated protein Tau (FTLD-Tau) and inclusions of the TAR DNA-binding protein 43 (TDP-43, FTLD-TDP) (Irwin et al., 2015; Mann and Snowden, 2017). Approximately 10–20% of the FTD cases is caused by genetic mutations that are linked to distinct neuropathologies (Lashley et al., 2015). Mutations in the microtubule-associated protein tau (*MAPT*) gene are related to FTLD-Tau, while mutations in progranulin (*GRN*) and chromosome 9 open reading frame 72 (*C9orf72*) genes are related to FTLD-TDP pathology (Irwin et al., 2015; Lashley et al., 2015). These two proteinopathies likely require distinct pharmacological treatments, and therefore, discrimination between these pathological subtypes (i.e. FTLD-Tau and FTLD-TDP) is needed. Currently, no fluid biomarkers exist that can identify the underlying pathology in sporadic FTD cases, and thus biomarkers that reflect different aspects of FTD pathophysiology are needed (Josephs et al., 2011; Swift et al., 2021; van der Ende and van Swieten, 2021).

Our previous unbiased proteomics study using cerebrospinal fluid (CSF) from FTD cases with known underlying pathology revealed that Apolipoprotein 1 (APOL1) was increased in FTLD-Tau compared to controls and FTLD-TDP patients (5- and 3-fold, respectively) (Teunissen et al., 2016). Single nucleotide polymorphisms in the ApoL gene family have been associated with schizophrenia and an upregulation of APOL1 expression has also been detected in the prefrontal cortex of schizophrenia patients (Mimmack et al., 2002; Takahashi et al., 2008). This is of interest considering that schizophrenia and FTD have overlapping clinical features (Cooper and Ovsiew, 2013; Momeni et al., 2010; Velakoulis et al., 2009). APOL1 is part of the high-density lipoprotein (HDL) complex, which plays a role in cholesterol transport and abnormalities in cholesterol turnover have been associated with FTD and other neurodegenerative disorders such as Alzheimer's disease (AD) or Niemann-Pick disease (Duchateau et al., 1997; Kim et al., 2018; Hottman et al., 2014; Brauer et al., 2019). Furthermore, overexpression of APOL1 induces lysosomal-dependent autophagic cell death (Wan et al., 2008; Zhaorigetu et al., 2008; Hu et al., 2012). Autophagy is an important mechanism for maintaining cellular homeostasis and is involved in the clearance of misfolded or aggregated proteins. Moreover, mutations in genes associated with lysosomal functioning or trafficking are related to the etiology of FTD (*CHMP2B*) or FTLD-TDP specifically (*C9orf72, GRN* and *TMEM106b*) (Baker et al., 2006; Hu et al., 2010; Clayton et al., 2015; Urwin et al., 2010; Brady et al., 2013; Farg et al., 2014), indicating that impaired lysosomal functioning is an important pathological pathway that might be dysregulated in FTD (Budini et al., 2017; Root et al., 2021). Whether APOL1 could play a role in FTD or the specific FTLD subtypes remains unknown. We hypothesize that APOL1 is increased in FTD, potentially reflecting lysosomal-related pathological brain changes and could be a novel CSF biomarker aiding in the discrimination of the FTLD pathological subtypes. In this study, we aimed to characterize APOL1 in post-mortem brain tissue of FTLD patients with definite neuropathology (FTLD-Tau and FTLD-TDP) and validate its potential as a discriminatory CSF biomarker for FTLD pathological subtypes. We additionally assessed the association of APOL1 with YKL-40, a promising FTD marker also detected in our previous unbiased proteomic study reflecting ongoing neuroinflammation.

2. Material and methods

2.1. Human samples

2.1.1. Post-mortem human brain tissue

Frozen frontal cortex tissue blocks of non-demented controls ($n = 9$) and individuals with FTLD pathology (FTLD = 44; FTLD-Tau = 21 and FTLD-TDP = 23) were obtained from the Netherlands Brain Bank ($n = 26$) and the CIEN Foundation Brain bank ($n = 27$; BT-CIEN, Madrid, Spain). The FTLD-Tau group included cases with different underlying tauopathies such as progressive supranuclear palsy (PSP, $n = 8$), Pick's disease (PiD, $n = 4$), and corticobasal degeneration (CBD, $n = 3$). Cases with underlying *MAPT* mutation ($n = 6$, related to Tau pathology) or *C9orf72* ($n = 7$) and *GRN* ($n = 1$) mutations (related to TDP pathology) were also included (Baker et al., 2006; Renton et al., 2011; Spillantini et al., 2000). Frontal cortex tissue blocks were homogenized with Tissue Protein Extraction Reagent (T-PER, 0.1 g/mL, Thermo Fisher Scientific, Waltham, USA) containing EDTA-free protease inhibitor cocktail (1:25, Roche, Basel, Switzerland) and phosphatase inhibitor (1:10, Roche, Basel, Switzerland). Tissue homogenates were centrifuged at 10.000 *g* for 5 min at 4 °C. Thereafter, total protein content was measured with the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Brain lysates were aliquoted and stored at −80 °C until further analysis.

Paraffin-embedded frontal cortex tissue of non-demented controls ($n = 9$) and individuals with FTLD pathology (FTLD = 18; FTLD-Tau = 12 and FTLD-TDP = 6) were similarly obtained from the Netherlands Brain Bank (n = 9) and BT-CIEN ($n = 18$). In the FTLD-Tau group, PSP ($n = 5$), PiD ($n = 3$), CBD (n = 1) patients and *MAPT* mutation carriers (n = 3) were included. The FTLD-TDP group included individuals with mutation in the *C9orf72* gene (n = 3). To explore whether APOL1 immunoreactivity was expressed in other brain areas, additional sections covering different brain areas (i.e. temporal cortex, cingulate cortex and parietal cortex) were included from one non-demented individual. Sections of 5-μm thick were mounted on tissue slides (Superfrost® plus, Menzel Glaser, Braunschweig, Germany) and dried overnight at 37 °C.

Pathological diagnosis was performed following established guidelines (Lund, 1994; Cairns et al., 2007). All donors or their next of kin provided written informed consent for brain autopsy and the use of medical records for research purposes. An overview of patient demographics is presented in Table 1.

2.2. Ante-mortem human CSF

CSF samples were obtained from the Amsterdam dementia cohort ($n = 27$) (van der Flier et al., 2014; van der Flier and Scheltens, 2018) and the Erasmus Medical Center ($n = 13$). The total CSF cohort included controls (subjective cognitive decline, $n = 14$) and FTD cases with known underlying neuropathology ($n = 26$, FTLD-Tau = 12, FTLD-TDP = 14). FTLD-Tau was confirmed based on autopsy ($n = 6$) or *MAPT* mutation ($n = 3$). The FTLD-Tau group was enriched with patients clinically diagnosed with PSP (n = 3), which is primarily associated with Tau neuropathology (Ingelsson et al., 2007). The FTLD-TDP group included autopsy-confirmed cases ($n = 9$) and patients with *GRN* ($n = 1$) or *C9orf72* ($n = 1$) mutations. The FTLD-TDP group was further enriched with FTD cases that presented with amyotrophic lateral sclerosis (FTD-ALS, $n = 3$), which associates with TDP pathology (Neumann et al., 2006).

Patients underwent cognitive and neurological assessments and FTD diagnosis was determined according to consensus criteria (Lund, 1994;

Table 1
Demographic data post-mortem tissue.

Frozen tissue blocks[a]	Control	FTLD (total)	FTLD-Tau	FTLD-TDP
n (M/F)	9 (5/4)	44 (20/24)	21 (8/13)	23 (12/11)
Age, years (mean ± SD)	69 (11)	68 (9)	69 (11)	67 (8)
PMD, hours (mean ± SD)	6,9 (2,8)	6,3 (3,5)	5,5 (2,1)	6,9 (4,1)
FTLD Subclassifications			8 PSP	7 *C9orf72*
			4 PiD	1 *GRN*
			3 CBD	15 sporadic[c]
			6 *MAPT*	
Paraffin-embedded tissue[b]	Control	FTLD (total)	FTLD-Tau	FTLD-TDP
n (M/F)	9 (3/6)	18 (9/9)	12 (5/7)	6 (4/2)
Age, years (mean ± SD)	69 (9)	69 (10)	67 (10)	72 (9)
PMD, hours (mean ± SD)	5 (3,4)	6 (1,9)	6,2 (2,3)	5,7 (1,6)
FTLD Subclassifications			5 PSP	3 *C9orf72*
			3 PiD	3 sporadic[c]
			1 CBD	
			3 *MAPT*	

FTLD, frontotemporal lobar degeneration; TDP, TAR DNA-binding protein; PSP, progressive supranuclear palsy; CBD, corticobasal degeneration; PiD, Pick's Disease; n, number of cases; M, male; F, female; PMD, post-mortem delay; SD, standard deviation.

[a] Frozen tissue blocks were used for western blot and ELISA analysis.

[b] Paraffin-embedded tissue cases were used for immunohistochemistry analysis.

[c] Sporadic cases were confirmed for TDP pathology by autopsy.

Neary et al., 1998). The control group consisted of cases with subjective cognitive decline, while other neurological or biochemical assessments were normal (CSF total-Tau/Aβ42 ratio < 0.52 (Duits et al., 2014)) and did not meet the criteria for mild cognitive impairment, dementia, or another neurological disorder (Sperling et al., 2011; Jessen et al., 2014). Informed consent from all participants in this study was obtained. Patient characteristics are presented in Table 2.

2.3. Western blot

CSF (30 μL), frontal cortex lysates (10 μg) or human APOL1 recombinant protein (5 ng, 1–398 aa, SinoBiological, Wayne, USA) were prepared in sample buffer (0.03 M Tris, 2% SDS, 10% glycerol, 50 mM DTT, 0.1 mM bromophenol blue) and heated for 5 min at 95 °C. Electrophoresis was performed with 1.5 mm NuPAGE Novex 4–12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and immunoblotting was performed as previously described (Hok-A-Hin et al., in press). The following primary antibodies were used: monoclonal mouse anti-human APOL1 antibody (1:20.000, ProteinTech, Manchester, UK), polyclonal rabbit anti-human APOL1 antibody (1:1000, Novus Biologicals, Centennial, USA), or monoclonal mouse anti-human Actin antibody (1:5000, clone AC-40, Sigma-Aldrich, Saint Louis, USA). Frontal cortex lysates were quantified for APOL1 (ProteinTech) and Actin (Sigma-Aldrich) protein signals with the ImageLab™ software version 3.0 (Bio-Rad, Hercules, USA). The specificity of the mouse anti-APOL1 antibody (ProteinTech) was supported by antibody pre-absorption. The anti-APOL1 antibody was incubated 24 h at 4 °C with 200 M excess of its specific antigenic peptide (1–238 aa, ProteinTech), before incubation with the immunoblot.

Table 2
Demographic data of CSF samples.

	Control	FTD (total)	FTLD-Tau	FTLD-TDP
n (M/F)	14 (6/8)	26 (12/14)	12 (4/8)	14 (8/6)
Age, years (mean ± SD)	63 (9)	60 (9)	59 (12)	60 (5)
FTLD Subclassifications			3 PSP	3 FTD-ALS
			3 *MAPT*	1 *GRN*
			6 sporadic[a]	1 *C9orf72*
				9 sporadic[a]

FTD, Frontotemporal dementia; FTLD, frontotemporal lobar degeneration; TDP, TAR DNA-binding protein; n, number of cases; M, male; F, female; y, years; PSP, progressive supranuclear palsy; PiD, Pick's Disease; ALS, Amyotrophic lateral sclerosis; SD, standard deviation.

[a] Sporadic cases were confirmed for FTLD pathology by autopsy.

2.4. Immunohistochemistry

Immunohistochemistry (IHC) was performed as previously described (Hok-A-Hin et al., in press). Post-mortem brain sections were deparaffinized and boiled for 15 min in a microwave with sodium citrate buffer (10 mmol/L pH 6.0) to perform antigen retrieval. Sections were incubated overnight at 4 °C in a humid environment with mouse anti-human APOL1 antibody (1:1000, ProteinTech) in antibody diluent (Immunologic, Duiven, The Nederlands). After washing with PBS, sections were incubated with EnVision (anti-mouse/rabbit HRP, undiluted; DAKO, Glostrup, Denmark) for 1 h at room temperature (RT). The colour was developed using 3,3′ diaminobenzidine tetrahydrochloride dihydrate (DAB; 0.1 mg/mL, 0.02% H_2O_2, DAKO) for 10 min as the chromogen. Nuclei were stained with hematoxylin and section were mounted with Quick-D mounting medium (Klinipath, Duiven, The Nederlands). The specificity of the mouse anti-human APOL1 antibody (ProteinTech) for IHC was also tested by pre-absorption. First, the anti-APOL1 antibody was pre-absorbed for 24 h at 4 °C with 200 M excess of its specific antigenic peptide (1–238 aa, ProteinTech), thereafter, sections were incubated with the pre-absorbed antibody overnight at 4 °C.

Double immunohistochemistry experiments were performed to determine the association of APOL1 with phosphorylated Tau (pTau). Sections were incubated with mouse anti-APOL1 (1:1000, ProteinTech) and rabbit anti-pTau antibodies (AT8, 1:1000, Abcam) diluted in antibody diluent (Immunologic) overnight at 4 °C. Thereafter, sections were incubated with polyclonal goat anti-mouse HRP (1:150, DAKO) and anti-rabbit biotin (1:300, DAKO) antibodies for 1 h at RT. Next, a one-hour incubation with streptavidin-alkaline phosphatase (1:100, 1000 U, Roche) diluted in antibody diluent (Immunologic) was performed. APOL1 immunoreactivity was first visualized with DAB (0.1 mg/mL, 0.02% H_2O_2, DAKO). Thereafter, sections were emerged in Tris-HCL buffer (0.2 M, pH 8.5) and pTau immunoreactivity was visualized with Liquid permanent red solution (LPR, 1:100, DAKO). Nuclei were stained with hematoxylin and section were mounted with Aquatex® (Merck, Darmstadt, Germany). Since some non-specific signal was detected using the phosphorylated TDP-43 (pTDP-43) antibody and LPR, we compared APOL1 and pTDP-43 staining patterns with single immunohistochemistry experiments using sequential sections. Sections were incubated overnight at 4 °C with mouse anti-APOL1 (ProteinTech) or 1 h with rabbit anti-pTDP-43 antibody (Ser409 + 410, 1:1000, ProteinTech) diluted in antibody diluent (Immunologic) as described above.

Semi-quantitative analysis of APOL1 immunoreactivity was performed with the QuPath software as described previously (Bankhead et al., 2017) (version 0.1.2, Queen's University of Belfast, Ireland) including five microscopic areas randomly selected per section, acquired at 200× magnification. APOL1 immunoreactivity was determined by using the DAB positive pixel count (thresholds: down-sample factor = 4, Gaussian sigma = 2, hematoxylin threshold (negative) = 0.1, DAB threshold (positive) = 0.3). This threshold was applied for all images (supplementary fig. 1). The analysis was performed by a researcher who was blinded from the diagnosis.

2.5. In-house APOL1 immunoassay

An immunoassay specific for APOL1 was developed following recommended procedures (Del Campo et al., 2015; Andreasson et al., 2015). High-binding 96-well microplates (Costar, New York, USA) were coated with capture antibody (1 μg/mL of mouse anti-human APOL1, ProteinTech) in coating buffer (0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH 9.6)

and incubated overnight at RT. Next, plates were rinsed with PBS and blocked with 0.5% Casein, 0.1% Gelatin in Tris buffer (20 mM Tris-HCl + 50 mM NaCl, pH 7.5) for 1.5 h at RT. Thereafter, plates were washed with washing buffer (Tris buffer containing 0.05% Tween-20) and incubated with samples (brain lysates (1:50) or CSF (1:4)) for 2 h at RT. The standard curve was prepared with human APOL1 Recombinant Protein (SinoBiological) using the following concentrations: 160, 80, 40, 20, 10, 5, and 2.5 ng/mL diluted in Tris buffer. After a washing step, samples were incubated with a detection antibody (5 μg/mL of rabbit anti-human APOL1, Novus Biologicals) for 1 h at RT. Plates were then washed and incubated with polyclonal swine anti-rabbit IgG/HRP (1:2000, DAKO). After washing, plates were incubated with substrate tetramethylbenzidine/dimethylsulfoxide (TMB/DMSO, 10 mg/mL) in substrate buffer (0.1 M $C_6H_8O_7$, 0.1 M NaOAc, pH 4.0) containing 0.03% H_2O_2 for 10 min and the reaction was stopped using 1 M H_2SO_4. The absorbance was read at 450 nm. APOL1 concentrations measured in brain lysates were corrected for the total protein content.

Our in-house APOL1 immunoassay was validated for CSF following the international guidelines for immunoassay validation (Andreasson et al., 2015). In brief, parallelism was performed by using a 2-times serial dilution of four CSF samples. Recovery was evaluated by spiking five CSF samples with a low (5 ng/mL), medium (50 ng/mL), or a high (100 ng/mL) spike of the APOL1 recombinant protein (SinoBiological). Dilution linearity was performed by spiking three CSF samples with APOL1 recombinant protein (1600 ng/mL, SinoBiological) following a 4-times serial dilution. The effect of freeze/thawing on APOL1 levels was also assessed. Pooled CSF samples were aliquoted and exposed for 1, 2, 3, 5 and 7 freeze/thaw cycles, samples were thawed for 2 h at RT and freezing at −80 °C for minimal 12 h. Reference aliquots were stored directly at −80 °C. The intra- and inter-assay coefficient variations (CV) were established as <3% and < 8% for brain lysates and < 4% and < 11% for CSF.

2.6. YKL-40 immunoassay

CSF protein levels for YKL-40, another FTD-relevant biomarker, were previously measured in a subset of cases (11 controls and 19 FTD patients) using the MicroVue YKL-40 enzyme immunoassay (Quidel Corporation, San Diego, USA) (Teunissen et al., 2016; Del Campo et al., 2018). This assay was validated for analysis in CSF with intra- and inter-assay of CV < 4% and 11% (Teunissen et al., 2016; Del Campo et al., 2018).

2.7. Statistical analysis

Statistical analysis was carried out using IBM SPSS statistics (version 26, IBM, Armonk, NY) and graphs were plotted with GraphPad Prism (version 9.1.0, San Diego, USA). The normality of the data was assessed with the Shapiro-Wilk test. The effect of potential covariates (i.e age, sex and center) were analyzed by Spearman correlation analysis and Mann-Whitney *U* tests. Differences in APOL1 levels between groups were evaluated either by analysis of covariance (ANCOVA) with log-transformed data including center as a covariate or Kruskal-Wallis test, when applicable. Bonferonni post-hoc analysis was applied. Pearson correlation analysis was performed to analyze the association of CSF APOL1 levels with YKL-40. Values of $p < 0.05$ were considered significant.

3. Results

3.1. APOL1 antibody characterization and immunoassay validation

Mouse and rabbit anti-APOL1 antibodies detected the recombinant APOL1 protein on western blot (WB) around 44 kDa, the expected molecular weight of the protein. A similar protein signal around 44 kDa was also observed in both CSF and post-mortem frontal cortex for the mouse anti-APOL1 antibody. The rabbit anti-APOL1 antibody showed an additional band between 37 and 50 kDa in post-mortem frontal cortex (Fig. 1A). Antibody preabsorption with its antigenic peptide abolished protein signals in both WB and IHC, supporting the specificity of the mouse APOL1 antibody (Fig. 1B-C). The mouse anti-APOL1 antibody was then used for WB, IHC, and as a capture antibody in our prototype ELISA. Rabbit anti-APOL1 antibody was used as a detection antibody for the prototype ELISA. Validation of the prototype APOL1 immunoassay for CSF analysis showed that parallelism, recovery and dilution linearity tests were within the acceptance criteria (i.e. 80% to 120%) (Supplementary fig. 2A-C). Furthermore, APOL1 levels in CSF remained stable up to seven freezing and thawing cycles (Supplementary fig. 2D).

3.2. APOL1 immunoreactivity and protein levels are increased in FTLD frontal cortex

APOL1 immunoreactivity was detected within the cytoplasm of neuronal and glial cells in all the brain areas examined (frontal temporal cortex, cingulate cortex and parietal cortex, Fig. 2 and Supplementary fig. 3) within a control case. Similar APOL1 immunoreactivity was detected in the frontal cortex of FTLD cases (Fig. 2A). We observed an increase of APOL1 immunoreactivity specially associated with glial cells in FTLD compared to controls ($p < 0.001$, Fig. 2B), which was present in both FTLD-Tau ($p < 0.001$) and FTLD-TDP ($p < 0.05$) groups, and no differences were detected between these pathological subtypes. Despite the limited sample size, a tendency towards a higher APOL1 immunoreactivity was observed in FTD mutation carriers (i.e. *MAPT* and *C9orf72*) (Fig. 2C). No specific association of APOL1 immunoreactivity to either pTau or pTDP-43 was observed (Fig. 2A and supplementary fig. 4–5). Similar to the immunohistochemistry data, we observed by WB that APOL1 was increased in FTLD cases compared to controls independently of the pathological subtype or any specific FTLD subclassification (e.g *MAPT*, *C9orf72*) (Fig. 3A-C, $p < 0.001$). Our novel prototype ELISA confirmed that APOL1 concentrations were increased in FTLD cases (Fig. 3D, $p < 0.05$), though this increase was especially driven by the FTLD-TDP group (Fig. 3E, $p < 0.01$). A tendency towards an increase of APOL1 in FTLD-Tau was also detected ($p = 0.03$), but differences did not survive correction for multiple testing (Fig. 3E). The significant correlation between APOL1 levels measured by either WB and ELISA (Fig. 3F, $r = 0.634$, $p < 0.01$) suggests that similar protein isoforms are measured by these technologies.

3.3. CSF APOL1 levels remain unchanged in FTD patients

To analyze whether APOL1 could be a useful biomarker for FTD or the FTLD subtypes we next analyzed APOL1 in ante-mortem CSF from confirmed FTD patients with our in-house APOL1 sandwich immunoassay. No difference in CSF APOL1 concentrations were detected between controls and FTD patients (Fig. 4A), nor between pathological subtypes or any FTLD subclassifications (Fig. 4B). We also assessed the correlation between CSF APOL1 and YKL-40 levels, a marker associated with neuroinflammatory processes, and found a moderate negative association between APOL1 and YKL-40 in CSF ($r = -0.398$, $p < 0.05$, Fig. 4C).

4. Discussion

In this study, we have characterized APOL1 protein expression in post-mortem human brain tissue using three different methods. We show that APOL1 is expressed in the cytoplasm of both neuronal and glial cells. APOL1 levels are increased in post-mortem frontal cortex of FTLD tissue independently of the pathological subtypes. However, such changes were not mirrored in the CSF, where APOL1 levels were comparable between pathological confirmed FTD patients and controls.

Characterization of the APOL1 antibodies in post-mortem frontal cortex by WB showed several protein bands around 44 kDa, which is the

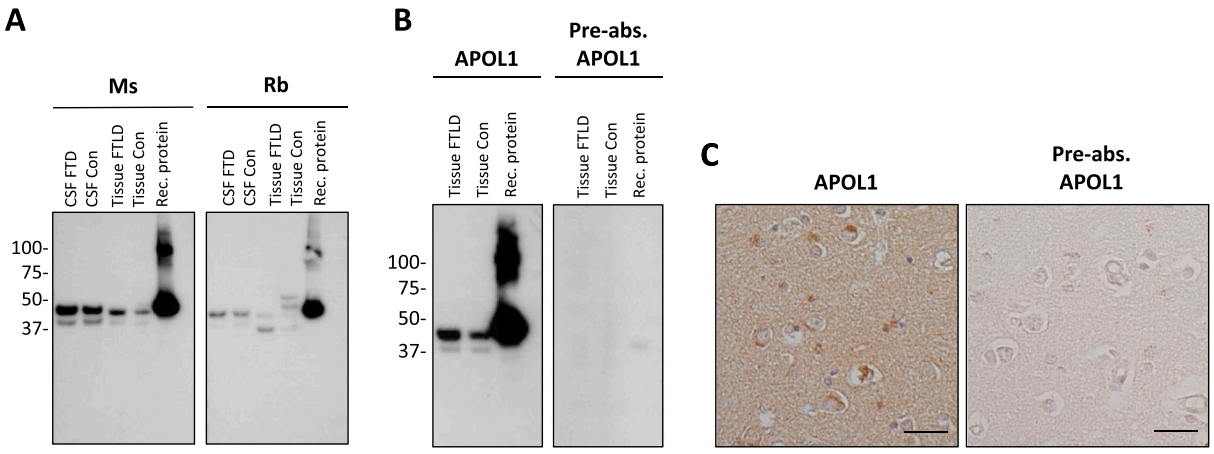

Fig. 1. APOL1 antibody characterization.
(A) Mouse (Ms) anti-APOL1 antibody detects similar protein bands around 44 kDa for CSF, frontal cortex tissue and the recombinant protein. Rabbit (Rb) anti-APOL1 antibody shows a protein band around 44 kDa in the CSF and the recombinant protein while in tissue lysates multiple protein bands between 37 and 50 kDa were detected (B). Mouse anti-APOL1 antibody was pre-absorbed (Pre-abs.) with a 200 M excess of the antigenic peptide. APOL1 immunoreactivity in FTLD and control frontal cortex and the APOL1 recombinant protein was abolished in western blot (A) and immunohistochemistry (C) after pre-absorption of the antibody with its antigenic peptide. Scale bars indicate 50 μM. Abbreviations: FTLD, Frontal temporal lobar degeneration; FTD, Frontotemporal dementia; Con, control; CSF, cerebrospinal fluid.

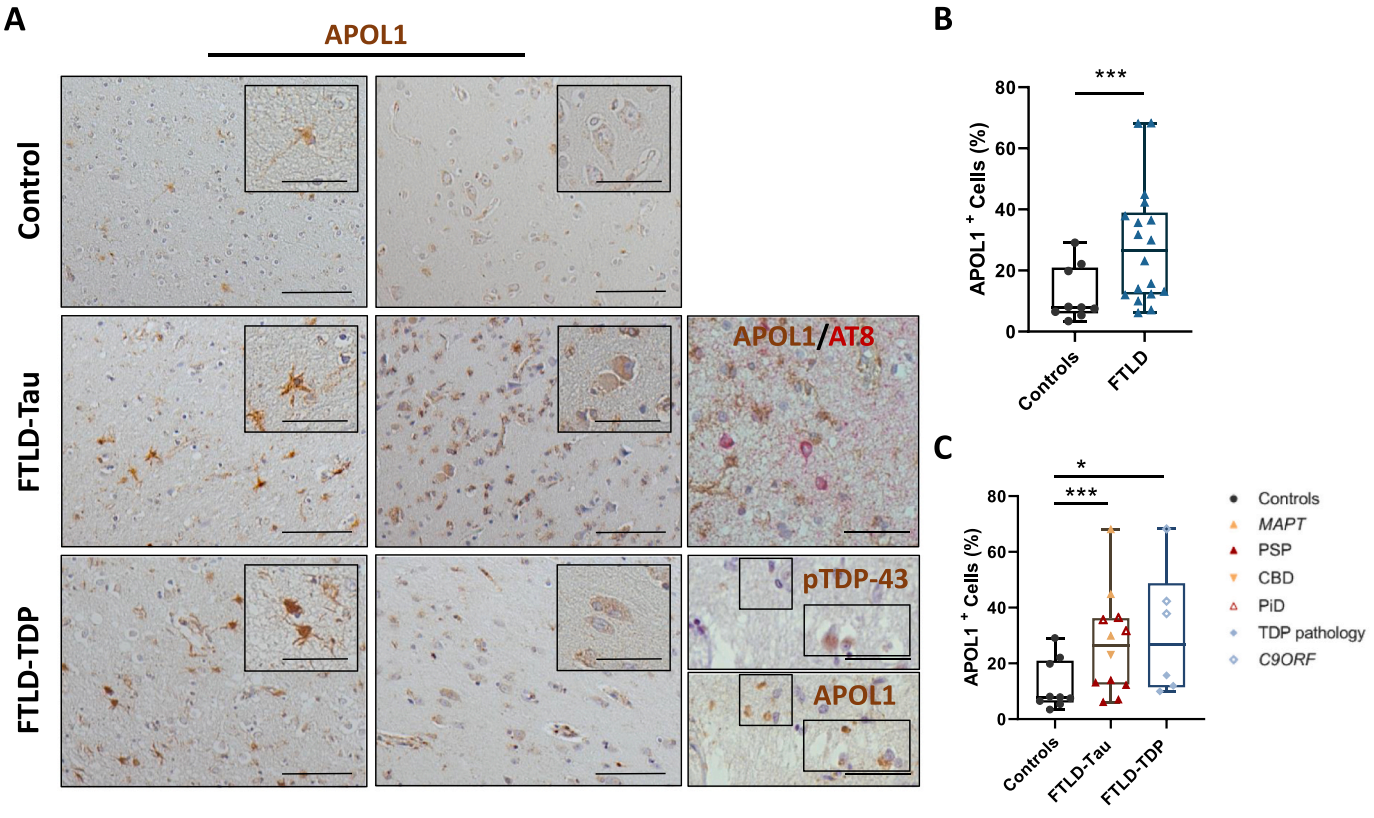

Fig. 2. APOL1 immunoreactivity increased in FTLD frontal cortex.
(A) Representative image of APOL1 immunoreactivity in frontal cortex sections of controls and FTLD (FTLD-Tau and FTLD-TDP) cases showed association of APOL1 to glia and neuronal-like cells but not with pTau (AT8) or pTDP-43 (black squares highlight areas where no similar staining was observed). (B) Single stained slides were quantified and APOL1 immunoreactivity was shown to be increased in FTLD cases. (C) Stratification of FTLD into the pathological subtypes showed higher APOL1 in both FTLD-TDP and FTLD-Tau compared to controls, but not between FTLD pathological subtypes. Boxplots represent median with interquartile range. *** $p < 0.001$, * $p < 0.05$. Scale bars indicate 100 μM, scale bars in the inserts and double staining represent 50 μM.

expected molecular weight of the APOL1 protein. These signals were abolished after pre-absorption with its specific APOL1 antigenic peptide, supporting the specificity of the monoclonal antibody used across the different methods. The different bands around 44 kDa may represent different protein isoforms or splice variants (Duchateau et al., 1997; Duchateau et al., 2001), as also observed in previous studies (Kumar et al., 2019). Previous studies showed that APOL1 is present in neuronal cells from controls, and gene expression analysis showed an up-

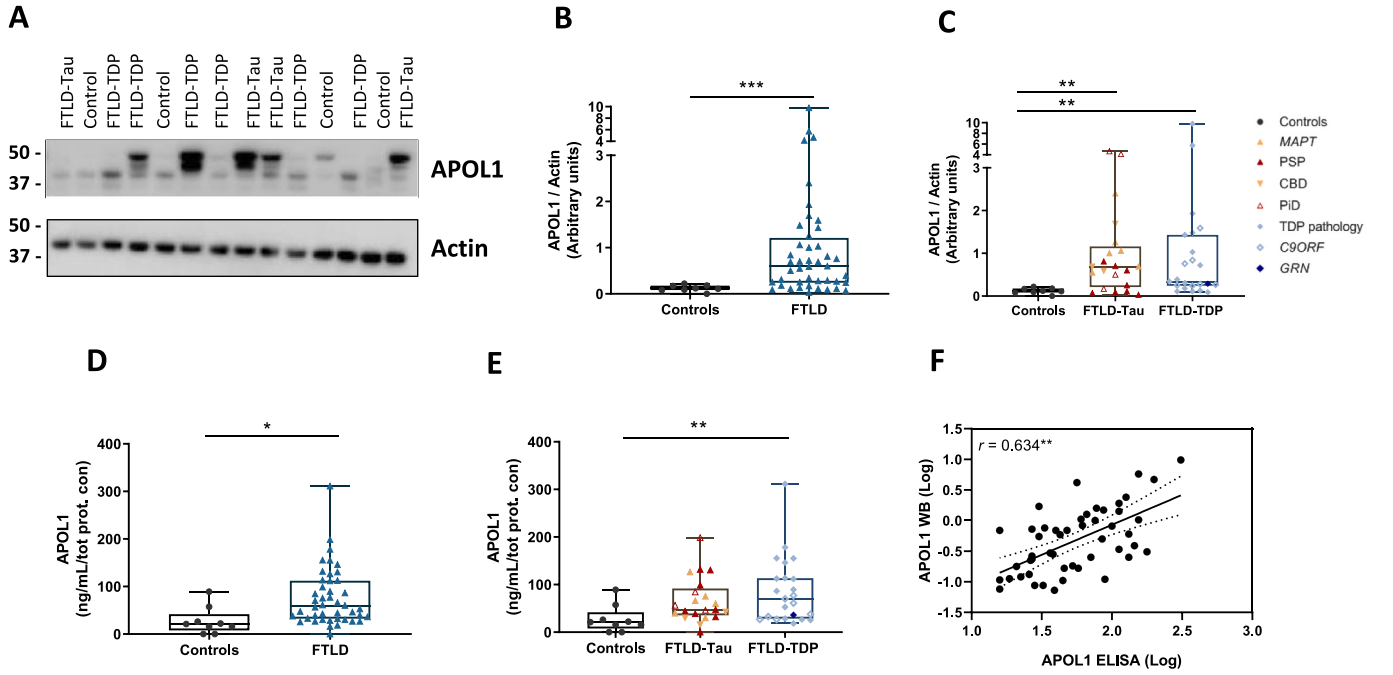

Fig. 3. APOL1 protein levels are increased in FTLD frontal cortex tissue.
(A) Representative western blot of APOL1 (44 kDa) and Actin (42 kDa) protein, tested in tissue lysates from controls and FTLD (including FTLD-Tau and FTLD-TDP subtypes) cases. (B) APOL1 reactivity was quantified and corrected for levels in actin and show that APOL1 reactivity was increased in FTLD compared to controls. (C) APOL1 was higher in both FTLD-Tau and FTLD-TDP compared to controls and no difference was observed between FTLD pathological subtypes. (D) APOL1 levels were measured by our in-house immunoassay and corrected for the total protein content (tot prot. con). (E) We show increased APOL1 levels in FTLD cases compared to controls which was specific for FTLD-TDP. (F) A positive correlation between the APOL1 protein measured by western blot and our in-house immunoassay is detected. Dotted lines represent the 95% confidence bands. Boxplots represent the median ± interquartile range. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.

regulation in schizophrenia patients (Mimmack et al., 2002; Takahashi et al., 2008). In line with these findings, we observed APOL1 neuronal reactivity in non-demented and FTLD cases. However, we also observed APOL1 immunoreactivity in glial-like cells in FTLD pathology, which might be related to the increased microglial activation and astrogliosis observed in FTD patients (Woollacott et al., 2020; Hartnell et al., 2021). Using three different (semi)-quantitative methods, we showed that APOL1 levels are increased in FTLD post-mortem brain tissue compared to controls, which was independent of the pathological subtypes. Immunohistochemistry characterization suggests that APOL1 reactivity tends to be higher in the FTD mutation carriers but this did not reach statistical significance. These trends were not observed in semi-quantitative (WB) and quantitative methods (ELISA) using a larger sample size, thereby suggesting that APOL1 increase is independent of subclassifications. This is supported by our double and sequential IHC analysis that showed no specific association of APOL1 immunoreactivity to the main FTLD pathological proteins (i.e pTau or pTDP-43). This suggests that APOL1 is not related to these pathological hallmarks and might be involved in independent molecular processes or in a common process downstream of FTLD pathology. APOL1 regulates lipid metabolism and cholesterol transport, mechanisms that are involved in FTLD pathophysiology (Duchateau et al., 1997; Kim et al., 2018; Zhaorigetu et al., 2008; Bain et al., 2019). In addition, APOL1 can induce lysosomal-dependent autophagic cell death (Zhaorigetu et al., 2008). The strong elevated APOL1 levels in FTLD may thus reflect the dysregulation of lipid metabolism or lysosomal processes downstream of the primary effect of the specific pathological aggregations. Interestingly, dysregulation of other lysosomal-related proteins (i.e C9orf72, GRN and TMEM106) causes lysosomal dysfunction, which leads to a defect in the autophagic pathway contributing to FTLD pathology (Feng et al., 2020; Zhou et al., 2020; Deng et al., 2017). Future experimental studies should define the exact biological processes by which APOL1 changes may contribute to the pathophysiology of FTD. It should be noted that the dysfunction of the *endo*-lysosomal system is also present in other neurodegenerative disorders (e.g. AD and dementia with Lewy bodies) (Szabo et al., 2022; Bras et al., 2014), highlighting the importance of proper lysosomal functioning and the need to understand the commonalities and specificities of this complex system across different dementia types. Therefore, since we only included FTD patients, the extensive characterization of APOL1 across these dementias might still be of interest.

We observed that CSF APOL1 levels were comparable between FTD and controls using our validated prototype ELISA that was also used for APOL1 tissue level quantification. These results are in contrast with previous mass-spectrometry findings, in which increased APOL1 levels were detected in CSF and serum of FTD patients (Teunissen et al., 2016; Katzeff et al., 2020). Translation of proteomic findings into immunoassays for routine analysis poses great challenges for the development of optimal fluid-based biomarkers (Teunissen et al., 2018). While mass-spectrometry approaches identify peptides from trypsinized proteins, antibody-based technologies detect proteins in their native conformation, which may explain the discrepancies between studies, in terms of detected isoforms and post-translational modifications. In addition, our previous proteomic study used a smaller sample size, which could increase the change of false-positive results. Furthermore, here we included additional subclassifications (i.e PSP and FTD-ALS) within the main pathologies that were not included in our original proteomic study (Teunissen et al., 2016), potentially increasing heterogeneity. In contrast, our negative CSF findings might also partly be explained by the limited cases included in this study. Thus we can not exclude that a larger sample size is needed to confirm APOL1 changes in CSF (Teunissen et al., 2016). Furthermore, it is striking that the APOL1 changes we observed in frontal cortex tissue were not detected in CSF using the same immunoassay. One explanation could be the differences in the sample preparation. A lysis reagent (i.e T-PER) was used to prepare the tissue lysates probably extracting proteins from various cellular

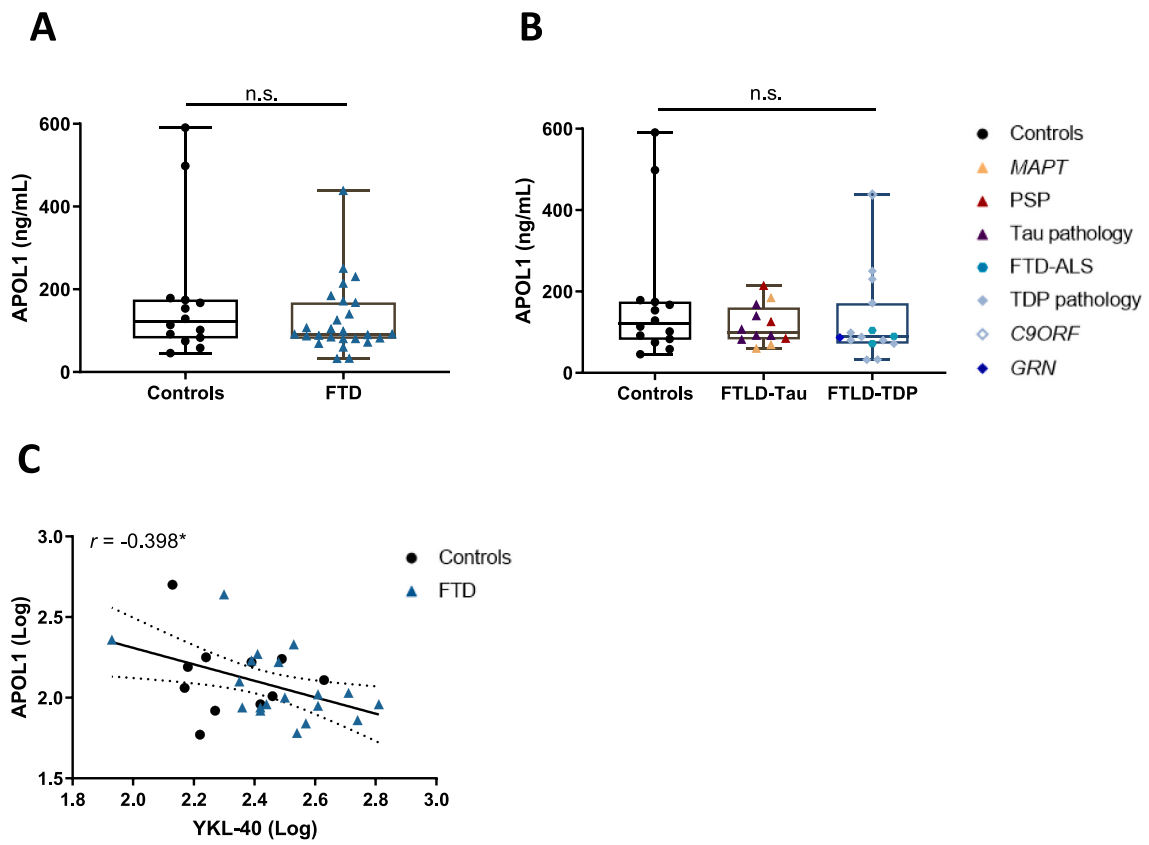

Fig. 4. APOL1 levels in CSF remained comparable between FTD patients and controls.
CSF APOL1 was analyzed in controls and FTD patients (with defined Tau or TDP pathology) with our in-house immunoassay. (A) APOL1 levels remained similar across clinical groups and no differences were observed between the pathological subtypes (B). (C) CSF APOL1 levels showed a negative correlation with CSF YKL-40 levels ($r = -0.398$). Boxplots represent the median ± interquartile range. n.s. indicates a non-significant difference between groups. Dotted lines represent the 95% confidence bands. * $p < 0.05$.

compartments while in CSF such additional steps were not performed and circulating APOL1 to some extent might still be associated with HDL particles (Madhavan and O'Toole, 2014) or exosomes (Chiasserini et al., 2014) and thus the relevant epitopes may be covered. Even though we did not detect differences in CSF APOL1 across clinical groups, we observed a negative correlation with YKL-40, a marker increased in CSF from FTD patients, reflecting ongoing neuroinflammation (Alcolea et al., 2017). Our data could indicate that the dysregulation of CSF APOL1 levels affects or is influenced by ongoing inflammatory processes.

This study is not without limitations. FTD is a heterogeneous disease with a range of clinical and pathological phenotypes. Here, to increase statistical power, different tauopathies and mutations associated with a specific FTLD pathological group (i.e. Tau and TDP) were grouped. This is not trivial considering that differences between genetic and sporadic FTD cases may exist (Bottero et al., 2021). Our data suggests that APOL1 changes are not associated with a specific FTD subclassification (e.g. specific mutation carriership, sporadic, etc) but a larger sample size is needed to confirm any subtype-specific changes. Additionally, we were not able to include post-mortem brain and ante-mortem CSF from the same patients, as these samples are rare. These limitations highlight the need to develop large well-characterized FTD cohorts and calls for strong collaborative work across different centers (Del Campo et al., 2022). The strengths of our study are the use of well-characterized cohorts including both post-mortem brain tissue and ante-mortem CSF and the use of complementary methods supporting the reliable measurement of APOL1 protein.

5. Conclusion

This study detected increased APOL1 levels in the post-mortem frontal cortex of FTLD cases independent of the underlying pathological hallmark, indicating that APOL1 is a novel unifying protein involved in FTD pathophysiology. Nevertheless, the APOL1 changes detected in brain were not mirrored in the CSF, limiting its potential as a specific FTD fluid-based biomarker using the developed prototype immunoassay. The lack of bodyfluid biomarkers for FTD or FTLD-related pathologies remains of importance to improve clinical diagnosis, calling for additional studies to identify and validate novel FTD-specific biomarkers.

Ethics approval

This study was approved by the ethical review boards from Amsterdam UMC and CIEN Foundation Brain bank. Informed consent was obtained from all subjects or their authorized representative.

Authors contributions

YSH contributed to the study design and was responsible for acquisition, statistical analysis, interpretation of the data and drafting the manuscript. AD, AR, HS, JH, JS, and YP provided material. All authors participated in data interpretation and critically revised the manuscript. CET and MC conducted the study concept and design, data acquisition and interpretation, critically revised the manuscript and supervised the study. All authors read and approved the final manuscript.

Availability of data and materials

The datasets used and or analyzed in the current study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2022.105813.

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