NEUROPATHOLOGY

Apolipoprotein E Proteinopathy Is a Major Dementia-Associated Pathologic Biomarker in Individuals with or without the *APOE* Epsilon 4 Allele

Jozsef Gal,^{*,†} Yuriko Katsumata,^{‡§} Haining Zhu,^{¶||} Sukanya Srinivasan,[§] Jing Chen,[¶] Lance Allen Johnson,^{‡§} Wang-Xia Wang,^{§**} Lesley Renee Golden,[†] Donna M. Wilcock,^{‡§††} Gregory A. Jicha,^{§††} Matthew D. Cykowski,^{§§} and Peter Tobias Nelson^{§**}

From the Spinal Cord and Brain Injury Research Center (SCoBIRC),^{*} Department of Neuroscience,[†] Department of Biostatistics,[‡] Sanders-Brown Center on Aging,[§] Department of Molecular and Cellular Biochemistry,[¶] Department of Pathology,^{**} Department of Physiology,^{††} and Department of Neurology,^{‡‡} University of Kentucky, Lexington, Kentucky; Research & Development,^{||} Lexington VA Medical Center, Lexington, Kentucky; and Houston Methodist Hospital,^{§§} Houston, Texas

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Address correspondence to
Peter Tobias Nelson, M.D.,
Ph.D., Division of Neuropathology,
Department of Pathology,
Sanders-Brown Center on Aging,
University of Kentucky, Rm 311, 800 S.
Limestone St., Lexington, KY
40536-0230. E-mail: peter.nelson@uky.edu.

The amygdala is vulnerable to multiple or “mixed” mis-aggregated proteins associated with neurodegenerative conditions that can manifest clinically with amnesic dementia; the amygdala region is often affected even at earliest disease stages. With the original intent of identifying novel dementia-associated proteins, the detergent-insoluble proteome was characterized from the amygdalae of 40 participants from the University of Kentucky Alzheimer’s Disease Center autopsy cohort. These individuals encompassed a spectrum of clinical conditions (cognitively normal to severe amnesic dementia). Polypeptides from the detergent-insoluble fraction were interrogated using liquid chromatography-electrospray ionization-tandem mass spectrometry. As anticipated, portions of peptides previously associated with neurologic diseases were enriched from subjects with dementia. Among all detected peptides, Apolipoprotein E (ApoE) stood out: even more than the expected Tau, APP/A β , and α -Synuclein peptides, ApoE peptides were strongly enriched in dementia cases, including from individuals lacking the *APOE* ϵ 4 genotype. The amount of ApoE protein detected in detergent-insoluble fractions was robustly associated with levels of complement proteins C3 and C4. Immunohistochemical staining of *APOE* ϵ 3/ ϵ 3 subjects’ amygdalae confirmed ApoE co-localization with C4 in amyloid plaques. Thus, analyses of human amygdala proteomics indicate that rather than being only an “upstream” genetic risk factor, ApoE is an aberrantly aggregated protein in its own right, and show that the ApoE protein may play active disease-driving mechanistic roles in persons lacking the *APOE* ϵ 4 allele. (*Am J Pathol* 2022, 192: 564–578; <https://doi.org/10.1016/j.ajpath.2021.11.013>)

Typically, the brains of persons who died with amnesic dementia harbor multiple aberrantly aggregated proteins.^{1–3} In prior studies that discovered the now widely recognized dementia-associated misfolded proteins (eg, Tau and A β), those polypeptides were isolated from the detergent-insoluble protein fraction.^{4–6} The amount of detergent-insoluble pathogenic proteins (DIPPs) in the aged brain at autopsy has been consistently correlated with cognitive impairment before death.^{7–10}

Complementing clinical-pathologic correlation studies, genetic research has provided important insights into disease-driving mechanisms. The genes that encode each of

the common neurodegenerative disease (ND)-associated DIPPs (*APP*, *MAPT*, *SNCA*, and *TARDBP*) may, when mutated, cause NDs. However, only an extremely small minority of ND cases are caused by those genetic mutations.

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The ND-associated genetic risk factor with the largest known public health impact is the *APOE* $\epsilon 4$ allele. (In this publication, the apolipoprotein E gene is referred to as *APOE* and the cognate protein as ApoE.) Compared with the more common *APOE* $\epsilon 3$ allele, the *APOE* $\epsilon 4$ gene variant is defined by a nucleotide substitution inducing a cysteine→arginine amino acid change in the ApoE protein.¹¹

The *APOE* $\epsilon 4$ allele is associated with an increased risk for Alzheimer disease (AD) neuropathologic change, Lewy body diseases, and limbic-predominant age-related TDP-43 encephalopathy neuropathologic change.^{12–16} Differing mechanisms have been proposed by which the risk-associated *APOE* $\epsilon 4$ polymorphism may cause or exacerbate ND pathophysiology.¹⁷ For example, the *APOE* $\epsilon 4$ allele may promote altered processing of the other misfolded protein(s), may stimulate deleterious neuroinflammation, and/or may have an adverse impact on brain lipid processing.^{18,19} ApoE immunostaining in amyloid plaques and Tau tangles has been reported, and a gain-of-toxic ApoE protein function has been hypothesized in AD.^{11,17}

Although *APOE* pathobiology is a very active area of scientific investigation, whether the ApoE protein participates in ND pathogenesis among individuals who lack the *APOE* $\epsilon 4$ allele is presently unknown. This is a potentially important issue because most persons with NDs lack the *APOE* risk allele. More specifically, whereas the *APOE* $\epsilon 4$ risk allele is present in approximately 25% of individuals in most human populations,^{17,20} approximately 75% of persons of advanced age have at least some AD-type pathology.^{21,22} Thus, well over one-half of persons with AD-type pathology lack the *APOE* risk allele.

Driven at least partly by genetic factors, dementia-related diseases tend to affect specific vulnerable anatomic regions, expanding through the brain in a stereotypic manner. The biological phenomena underlying the region-specific anatomic vulnerability are still mostly unknown. However, a common paradigm has emerged: the amygdala and immediate peri-amygdala regions of the human brain harbor misfolded proteins early in the course of multiple NDs.^{23–29} For example, even in cognitively unimpaired individuals, the amygdala region often contains the following: pathologic Tau (neurofibrillary tangles) and A β (amyloid plaques) peptide, which signal incipient AD neuropathologic change; α -Synuclein aggregates that herald Lewy body diseases; and/or TDP-43 proteinopathy that indicates limbic-predominant age-related TDP-43 encephalopathy neuropathologic change.^{12,25,28,30,31} For these reasons, the amygdala may be a special source of fresh insights into biologic phenomena relevant to early ND pathogenesis. Candidate amygdala DIPP were previously studied from individuals who had been followed up to autopsy with tissue available at the University of Kentucky AD Research Center biobank.³²

This study reports the results of additional amygdala proteomics studies with a larger sample of cases and controls from the University of Kentucky AD Research Center

biobank. Consistent with prior work, expected dementia-associated DIPP are present in amygdala and enriched in the brains of individuals with documented antemortem cognitive impairment. In the present study, there was clear evidence that the ApoE protein is a conspicuous dementia-associated misfolded polypeptide in the amygdala, even among individuals lacking the *APOE* $\epsilon 4$ risk allele.

Materials and Methods

Fractionation of Amygdala Proteins from Autopsy Brains

Research protocols including consent forms were approved by the University of Kentucky Institutional Review Board, and all research volunteers consented to research autopsies. Details regarding the recruitment of University of Kentucky AD Research Center research volunteers, as well as clinical and pathologic assessments, have been described previously.^{33–37} Research participants with unusual dementia syndromes (eg, prions or trinucleotide repeat diseases) or brain tumors were excluded. Information was also obtained on agonal events for each subject, and additional criteria for exclusion from the study were an extended interval of pre-mortem hypoxia, any medical ventilator use, brain edema, or large infarct. A convenience sample of cases was selected from among autopsies with a postmortem interval <5 hours. Dissected amygdalae were snap-frozen in liquid nitrogen at autopsy and then stored at -80°C until these experiments were performed.

Protein sample fractionation followed the published methodology of Sampathu et al,³⁸ with some modifications. Each amygdala was weighed, then homogenized in 5 mL of low-salt (LS) buffer [10 mmol/L TRIS-HCl, pH7.5, 5 mmol/L EDTA, and 10% (w/v) sucrose] per gram tissue using tissue grinders (#47732-446; VWR, Radnor, PA). The homogenates were centrifuged at $25,000 \times g$, 4°C for 30 minutes, and the supernatants saved as the LS fractions. The pellets were re-extracted with LS buffer, centrifuged as above, and the supernatants were discarded. The pellets were extracted with 5 mL TX buffer [LS buffer supplemented with 1% (v/v) Triton-X-100 and 0.5 mol/L NaCl] per gram original tissue, centrifuged at $180,000 \times g$, 4°C for 30 minutes, and the supernatants saved as the TX fractions. The pellets were re-extracted with TX buffer, centrifuged as above, and the supernatants were discarded. The pellets were extracted with 5 mL myelin flotation buffer (TX buffer containing 30% [w/v] sucrose) per gram original tissue, centrifuged at $180,000 \times g$, 4°C for 30 minutes, and the supernatants were discarded. The pellets were extracted with 5 mL SARC buffer (LS buffer supplemented with 1% [w/v] N-lauroylsarcosine and 0.5 mol/L NaCl) per gram original tissue, incubated at 22°C for 2 hours on an end-over-end shaker, centrifuged at $180,000 \times g$, 22°C for 30 minutes, and the supernatants were saved as the SARC fractions. The pellets were extracted with 0.75 mL urea buffer [7 mol/L

urea, 2 mol/L thiourea, 4% (w/v) CHAPS, and 30 mmol/L TRIS-HCl, pH 8.5] per gram original tissue at 22°C, centrifuged at $25,000 \times g$, 22°C for 30 minutes, and the supernatants saved as the urea soluble fractions. The LS, TX, SARC, and myelin flotation buffers were supplemented with protease inhibitor cocktail (P8340, 1:300; Millipore Sigma, Burlington, MA), phenylmethylsulfonyl fluoride (0.2 mmol/L), *N*-ethylmaleimide (5 mmol/L), nicotinamide (20 mmol/L), trichostatin A (1.5 μ mol/L), sodium orthovanadate (1 mmol/L), and PhosSTOP phosphatase inhibitor tablets (1 tablet per 10 mL; 4906837001; Millipore Sigma). The LS, TX, SARC, and myelin flotation homogenates were supplemented with 1 mmol/L DL-dithiothreitol immediately before centrifugation, and the supernatants were saved with SDS-PAGE loading buffer and heated at 94°C for 5 minutes. The urea (ie, detergent-insoluble) fractions were saved similarly but without heating to avoid protein carbamylation.

Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry and Data Analysis

The processing of the amygdalae and the generation and analysis of the mass spectrometric data were performed in a blinded fashion (ie, without the experimenter knowing which samples represented cognitively normal control subjects and which represented patients with dementia). All mass spectra reported in this study were acquired at the University of Kentucky Proteomics Core Facility (<https://www.research.uky.edu/proteomics-core-facility>; last accessed October 27, 2021). Equal total protein amounts of the detergent-insoluble fractions were resolved by denaturing SDS-PAGE on 4% to 12% Bis-TRIS gradient protein gels (catalog #NP0335BOX; Thermo Fisher Scientific, Waltham, MA) using MES SDS Running Buffer (catalog #NP0002; Thermo Fisher Scientific), followed by staining with Sypro Ruby protein gel stain (catalog #S-12000; Molecular Probes, Eugene, OR). Gel pieces were cut above the 171 kDa protein molecular weight marker band followed by dithiothreitol reduction, iodoacetamide alkylation, and in-gel trypsin digestion. The resulting tryptic peptides were extracted, concentrated, and subjected to liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as previously described.^{39,40} Briefly, LC-MS/MS analysis was performed by using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with a cHiPLC-nanoflex system (Eksigent, Dublin, CA) through a nano-electrospray ionization source. The peptide samples were separated with a reversed-phase cHiPLC column (75 μ m $\times$ 150 mm) at a flow rate of 300 nL/min. Mobile phase A was water with 0.1% (v/v) formic acid, and mobile phase B was acetonitrile with 0.1% (v/v) formic acid. A 50-minute gradient condition was applied: initial 3% mobile phase B was increased linearly to 40% in 24 minutes and further to 85% and 95% for 5 minutes each before it was decreased to 3% and re-equilibrated.

Figure 1 Overview of study design and experiments performed. With the goal of identifying detergent-insoluble pathogenic proteins (DIPPs) enriched in dementia brains, experiments began with protein fractionation followed by comparison of high molecular weight (MW), urea-soluble (detergent-insoluble) proteins from cognitively impaired and control subjects. Optimally, a novel DIPP candidate would be more likely to be present in a preparation that was found to also contain established DIPPs: Tau, α -Synuclein, and/or amyloid precursor protein (APP). Apolipoprotein E (ApoE) emerged as being highly enriched in the detergent-insoluble fraction, and follow-up experiments were performed with immunoblots and immunohistochemistry. PMI, postmortem interval.

LC-MS/MS data were acquired in an information-dependent acquisition mode. Each data collection cycle consisted of 11 scan events: one Orbitrap mass spectrometer scan (300 to 1800 m/z) with 60,000 resolution for parent ions followed by MS/MS for fragmentation of the 10 most intense ions using collision-induced dissociation.

The LC-MS/MS data referent to gel slices were submitted for MS/MS protein identification using the MASCOT algorithm via Proteome Discoverer (version 1.3; Thermo Fisher Scientific), applying a database containing human protein sequences from Uniprot⁴¹ (last downloaded February 2, 2020). Parameters used in the MASCOT MS/MS ion search were: trypsin digestion with maximum of two missed cleavages, cysteine carbamidomethylation, and methionine oxidation. Post-translational modifications were also examined in MASCOT searches, including lysine acetylation,

Table 1 Summary Data for Included Case and Control Subjects

Case #	Age, years	Sex	Final MMSE score	Final clinical diagnosis	Braak NFT score	APOE genotype	ApoE MS results
1308	85	F	28	Normal	II	2/3	0
1298	96	F	24	Dementia	VI	2/3	36.4
1068	92	F	22.5	Dementia	VI	2/3	51.1
5388	87	F	28	MCI	I	3/3	0
1313	79	F	26	Normal	I	3/3	0
1293	86	F	29	Normal	I	3/3	0
1312	94	M	25	Normal	II	3/3	0
1106	79	M	29	Normal	II	3/3	44.1
1309	83	F	30	Normal	II	3/3	0
5415	86	F	28	Normal	III	3/3	0
1087	82	F	29	Normal	III	3/3	0
976	85	M	30	Normal	III	3/3	0
5368	96	F	1	Dementia	IV	3/3	0
1105	84	F	30	Normal	IV	3/3	0
1141	84	F	29	Normal	IV	3/3	0
5384	87	M	0	Dementia	V	3/3	151.9
5387	98	F	22	Dementia	V	3/3	206.4
1318	100	M	27	Dementia	V	3/3	0
1169	93	F	24	Dementia	V	3/3	0
5372	94	M	24	MCI	V	3/3	113.1
972	89	F	1	Dementia	VI	3/3	0
1113	89	F	14.5	Dementia	VI	3/3	0
1053	90	F	22.5	Dementia	VI	3/3	85.9
1033	71	M	18	Dementia	VI	3/3	0
5389	92	M	15	Dementia	VI	3/3	152.4
1096	84	M	23	Dementia	VI	3/3	151.4
5396	90	F	N/A	Dementia	III	2/4	0
965	82	F	22	Dementia	V	2/4	0
1306	83	F	30	Normal	I	3/4	0
1311	85	F	30	Normal	II	3/4	0
5380	100	F	21	Dementia	V	3/4	116.4
1035	84	F	19	Dementia	V	3/4	46.2
1052	80	F	19	Dementia	VI	3/4	348.9
1037	82	M	17.5	Dementia	VI	3/4	253.7
5394	64	F	6	Dementia	VI	3/4	248.5
1101	86	F	7	Dementia	VI	3/4	374.5
5344	84	M	2	Dementia	VI	3/4	156.2
5361	87	M	13	Dementia	V	4/4	59
1182	71	F	20	Dementia	VI	4/4	0
5335	62	F	21	Dementia	VI	4/4	0

F, female; M, male; ApoE, Apolipoprotein E; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MS, mass spectrometry; NFT, neurofibrillary tangle.

ubiquitylation, and serine, threonine, and tyrosine phosphorylation. Mass error tolerance was set to be <10 ppm for MS and 0.8 Da for MS/MS. A decoy database was built and searched to determine the false discovery rates. Peptides with false discovery rates lower than 0.01 were assigned as high confidence identification. The MASCOT software returns probability-based scores, calculated from the spectra detected of the individual peptides. These are described in the manufacturer's manual for the Proteome Discoverer software. The protein scores, based on the scores of the identified peptides, were calculated by using Proteome Discoverer.

Further details on how the protein score is calculated are provided in the Proteome Discoverer Version 1.3 User Guide XCALI-97358 Revision A (available from the manufacturer). Only proteins with a score ≥ 30 were included in the analysis in this study.

Immunohistochemistry

Immunohistochemical experiments were performed by using methods previously described,^{32,42} with slight modifications. Before immunohistochemistry, brain tissue was immersion-fixed in 10% neutral-buffered formalin

Table 2 Association between Detected Protein Levels (Detergent-Insoluble Fraction) and Last Clinical Diagnosis in All Participants (N = 40)

Protein	Last clinical diagnosis, n (%)		P value*
	Normal + MCI	Dementia	
ApoE			
Undetected†	15 (93.8)	9 (37.5)	6.71 × 10 ⁻⁴
Detected	1 (6.2)	15 (62.5)	
APP			
Undetected†	10 (62.5)	5 (20.8)	0.018
Detected	6 (37.5)	19 (79.2)	
MAPT			
Undetected†	3 (18.8)	2 (8.3)	0.37
Detected	13 (81.2)	22 (91.7)	
SNCA			
Undetected†	16 (100)	13 (54.2)	0.0012
Detected	0 (0)	11 (45.8)	

*P value was calculated by Fisher's exact test (undetected versus detected and normal + MCI versus dementia).
†MASCOT score <30.
ApoE, Apolipoprotein E; MCI, mild cognitive impairment.

(catalog #C4320-105; Cardinal Health, Dublin, OH) for 2 to 4 weeks before paraffin embedding. Immunohistochemical stains were performed as previously described⁴³ except that formic acid pretreatment was used for all slides. Briefly, sections cut at 8 μm thickness from formalin-fixed, paraffin-embedded tissue blocks were deparaffinized before microwave antigen retrieval for 6 minutes (power 8) using citrate buffer (Declere buffer, Cell Marque, Rocklin, CA). The sections were then placed in 100% formic acid (catalog #A-119P; Thermo Fisher Scientific) for 3 minutes. Sections were blocked in 5% normal goat or rabbit serum in Tris-buffered saline (5% S+TBS) for 1 hour at room temperature, then incubated in primary antibodies diluted in 5% S+TBS, for 22 hours at 4°C. Secondary antibodies were biotinylated IgG (catalog #BA-1000, #BA-2000, #BA-4000, and #BA-5000; Vector Labs, Burlingame, CA) diluted at 1:200 in 5% S+TBS for 1 hour at 19 to 22°C. After washing, the Vectastain ABC kit (Vector Labs) was used with Vector NovaRED (Vector Labs), followed by counterstain with hematoxylin, rinsing, dehydration, clearing, and mounting in coverslip media.

For triple-label immunofluorescence, sections cut at 8 μm thickness were deparaffinized before microwave antigen retrieval for 6 minutes (power 8) using citrate buffer (Declere buffer, Cell Marque). The sections were then placed in 100% formic acid for 3 minutes. Sections were next incubated for 45 seconds at room temperature in a 1× solution of TrueBlack (catalog #23007; Biotium, Fremont, CA) prepared in 70% ethanol to reduce auto-fluorescence. Sections were blocked in 5% normal donkey serum in TBS for 1 hour at room temperature, then incubated in primary antibodies anti-ApoE (rabbit monoclonal, 1:500 dilution; catalog #ab52607; Abcam, Waltham, MA), anti-C4a (mouse monoclonal, 1:250 dilution, catalog

#ab187278; Abcam), and anti-Tau (goat polyclonal, 1:2500 dilution; catalog #AF3496; R&D Systems, Minneapolis, MN) diluted in 5% normal donkey serum in TBS, for 48 hours at 4°C. Secondary antibodies conjugated to Alexa Fluor probes 488, 568 (diluted 1:500, catalog #A21206 and #A10037; Life Technologies, Carlsbad, CA), and 647 (catalog #A21447, Life Technologies), diluted 1:2500 in 5% normal donkey serum in TBS for 1 hour at room temperature. Slides were cover-slipped by using Invitrogen ProLong Gold mounting medium with DAPI (catalog #P36935; Thermo Fisher Scientific).

Immunoblotting

Proteins were resolved by denaturing SDS gel electrophoresis on 3% to 8% TRIS-acetate gradient protein gels (catalog #EA0378BOX; Thermo Fisher Scientific) using TRIS-acetate SDS running buffer (catalog # LA0041; Thermo Fisher Scientific). The resolved proteins were transferred to nitrocellulose membranes (catalog #1704158; Bio-Rad, Hercules, CA), followed by blocking with 5% nonfat dry milk in TBST (50 mmol/L Tris-HCl, 0.85% [w/v] NaCl, 0.1% [v/v] Tween-20, pH 7.5). The primary and secondary antibodies were applied in blocking buffer. The secondary antibody was IRDye 800CW goat anti-rabbit IgG (catalog #926-32211; Li-Cor, Lincoln, NE). The images were acquired on the Li-Cor Odyssey CLx Imaging System.

Statistical Analysis

Comparisons of detected (≥30 MASCOT score) and undetected (<30 MASCOT score) proteins between subjects with dementia and normal people or people with mild cognitive impairment were performed with Fisher's exact

Table 3 Association between Detected Protein Levels (Detergent-Insoluble Fraction) and Last Clinical Diagnosis in Participants without the APOE ε4 Allele (n = 26)

Protein	Last clinical diagnosis, n (%)		P value*
	Normal + MCI	Demented	
ApoE			
Undetected†	13 (92.9)	5 (41.7)	0.0093
Detected	1 (7.1)	7 (58.3)	
APP			
Undetected†	8 (57.1)	5 (41.7)	0.69
Detected	6 (42.9)	7 (58.3)	
MAPT			
Undetected†	2 (14.3)	2 (16.7)	1
Detected	12 (85.7)	10 (83.3)	
SNCA			
Undetected†	14 (100)	6 (50)	0.0040
Detected	0 (0)	6 (50)	

*P value was calculated by Fisher's exact test [undetected versus detected and normal + MCI versus dementia].
†MASCOT score <30.
ApoE, Apolipoprotein E; MCI, mild cognitive impairment.

Figure 2 Apolipoprotein E (ApoE) is enriched in the detergent-insoluble fraction of subjects with dementia, compared with other proteins, including in cases that lacked the *APOE* ε4 allele. Shown are the results of proteins detected via mass spectrometry, stratified according to last clinical diagnosis. For comparison's sake, results are shown for ApoE (A), APP (B), MAPT/Tau (C), and SNCA/α-Synuclein (D). MCI, mild cognitive impairment.

tests. The multivariable Tweedie compound Poisson model was applied to handle zero-inflated continuous outcome by specifying a “Tweedie” distribution (from the “statmod” R package) in the generalized linear model framework.⁴⁴ The “tweedie.profile” function from the “tweedie” R package was used to estimate the maximum likelihood estimation of the Tweedie power parameter with the range of 1 to 2 by 0.01 step.⁴⁵ The covariates included sex, age at death, and postmortem interval. All statistical analyses were conducted with R version 4.0.3 (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was set at 0.05.

Results

With the goal of identifying dementia-associated DIPPs in a relatively unbiased manner, the detergent-insoluble fractions of a set of amygdalae were analyzed, representing a spectrum of cognitive impairment before death. The study design and workflow are shown in Figure 1. Information on the biosamples used in LC-electrospray ionization-MS/MS experiments is presented in Table 1. After sequential processing with increasingly strong detergents, the proteinaceous pellets from amygdala samples were re-extracted with a solution that contained 7 mol/L urea and 2 mol/L thiourea, yielding the urea-soluble, “detergent-insoluble” fraction, the

high-molecular weight portion of which was analyzed with mass spectrometry.

In total, >800 proteins with a MASCOT protein score of at least 30 were identified in at least one amygdala sample. A spreadsheet with all of the detected peptides, for all of the cases, is provided as Supplemental Table S1. The presence of the known DIPPs Tau, Aβ, and α-Synuclein were confirmed in the amygdalae of patients with dementia. The co-incidence of the detection of these DIPPs with a MASCOT score of at least 30 was correlated with the last clinical diagnosis using Fisher's exact test. As expected, the detection of APP (Aβ) and SNCA (α-Synuclein) peptides in

Table 4 Associations between Proteins Levels and Dementia: Tweedie Generalized Linear Models (Outcome Is Protein Level, Predictor Is Last Clinical Diagnosis) in All Participants (*N* = 40) and among those without the *APOE* ε4 Allele (*n* = 26)

Protein	Including all subjects* <i>N</i> = 40		Subjects lacking <i>APOE</i> ε4* <i>n</i> = 26	
	β (SE)	<i>P</i> value	β (SE)	<i>P</i> value
ApoE	3.35 (1.20)	0.0085	3.30 (1.47)	0.035
APP	1.20 (0.46)	0.013	1.01 (0.51)	0.063
MAPT	1.21 (0.40)	0.0048	1.01 (0.30)	0.0032

*Adjusted for age at death, sex, and postmortem interval in hours.
ApoE, Apolipoprotein E.

Figure 3 Apolipoprotein E (ApoE) protein is strongly enriched in the detergent-insoluble fraction of subjects with dementia detected via mass spectrometry, compared with other proteins. Proteins detected via mass spectrometry were assessed, comparing those with dementia versus non-demented subjects. A “volcano plot” is shown to depict the *P* value (y axis, $\log_{10}$ -transformed) and β estimate (x axis) that conveys the size of the difference comparing the results of patients with dementia versus non-demented participants. Individual proteins that were up-regulated in the detergent-insoluble fraction of participants with dementia are shown in red, and those down-regulated are shown in blue. Because most of the proteins had undetected levels in most of the cases, the statistical analyses assessed continuous protein levels with a zero inflation (ie, it was assumed that each protein level follows a Tweedie distribution) among those proteins with ≥ 4 people having undetected levels. The dotted line depicts the $P < 0.05$ threshold for statistical significance.

the detergent-insoluble amygdala fractions correlated strongly with the clinical diagnosis of dementia (Table 2). For SNCA, the significant detection remained even in the subsample of participants who lacked the *APOE* $\epsilon 4$ allele (Table 3). Tau and A β proteins, but not α -Synuclein, were occasionally detectable in the detergent-insoluble fraction of amygdalae from cognitively normal subjects and also patients diagnosed with mild cognitive impairment, albeit usually with lower MASCOT scores (Table 2 and Figure 2). Therefore, differences in the detected protein levels of Tau and A β between normal/mild cognitive impairment and cases of dementia were analyzed using the multivariable Tweedie compound Poisson model. People with dementia had significantly higher Tau and APP/A β protein levels than the normal/mild cognitive impairment group (Table 4 and Figure 3). Overall, the findings for Tau, SNCA, and APP/A β were in line with expectations of preclinical disease among elderly individuals⁴⁶ (Table 1). However, it is also notable that a reliable signal for TDP-43–related peptides was not detected in the current study.

In addition to the widely recognized pathologic markers Tau, APP/A β , and α -Synuclein, statistical analysis of the mass spectrometric data revealed a close correlation between dementia diagnosis and the detection of ApoE

peptides in the detergent-insoluble fraction (Table 2, Figure 3, and Supplemental Figure S1). Overall, the correlation for ApoE peptides with dementia was even stronger than that seen for Tau, APP, or α -Synuclein peptides. The *APOE* gene variation is well known for playing important roles in the susceptibility to developing AD. Stratification according to the *APOE* $\epsilon 4$ status suggested that the presence of $\epsilon 4$ allele(s) led to high levels of insoluble ApoE in a subset of patients (Figure 2). Moreover, ApoE peptides were considerably enriched in the detergent-insoluble fraction of the amygdalae of patients with dementia even in the adjustment for (Table 4) and in the absence of (Tables 3 and 4) the $\epsilon 4$ allele. These results indicate that ApoE insolubilization correlates closely with dementia and may be pathogenetically impactful even in cases that lack the *APOE* $\epsilon 4$ allele.

Analysis of the tryptic peptides of ApoE identified with mass spectrometry in the detergent-insoluble amygdala fractions showed that they were clustered centrally, away from either the N- or C-terminal portions of the protein (Figure 4). Supplemental Table S2 presents all of the ApoE peptides detected across all cases. No phosphorylated serine, threonine or tyrosine, or acetylated or ubiquitinated lysine residue were identified in ApoE in the detergent-insoluble fractions.

To determine the distribution of ApoE solubility in the various protein extract fractions, immunoblotting was performed on the fractions from a cognitively normal control subject and a patient with dementia, both with *APOE* $\epsilon 3/\epsilon 3$ genotypes. In both cases, ApoE was mostly detected in the LS and the Triton-X100 fractions, close to the expected processed monomeric molecular weight of 34.2 kDa

Figure 4 Apolipoprotein E (ApoE) peptides identified via mass spectrometry from detergent-insoluble protein extracted from human amygdalae. The peptides identified with the highest confidence appeared to be clustered in the middle of the ApoE protein. Peptides from the N- and C-termini were not detected in the detergent-insoluble fraction, suggesting that proteolytic trimming may have occurred in the aggregated ApoE species. The peptides with green highlight were detected at a high confidence level in at least one subject. The peptide with the red highlight was repeatedly detected only with low confidence. Labeled in the figure are rs429358 (a.a. 130; blue arrow) and rs7412 (a.a. 176; red arrow), which confer ApoE ϵ -isoform status, as well as several notable protein domains. Also shown with underlining are a.a. 154 to 168, LDR receptor binding domain (blue underline), and a.a. 262 to 290, lipid binding domain (red underline).

Figure 5 Immunoblotting showing an enhanced Apolipoprotein E (ApoE) signal in the detergent-insoluble fraction of dementia patient amygdala. Amygdalae from a nondemented patient (Case 976) and a dementia patient (Case 5387), both with the *APOE* 3/3 genotype, were subjected to fractionation based on solubility, as described in the main text. The fractions were subjected to denaturing gel electrophoresis in the order of solubility with the low-salt (“LS”) fraction being the most soluble, and the “urea” fraction (the detergent-insoluble fraction) the least soluble. Immunoblotting with an anti-ApoE antibody detected ApoE at the expected monomeric size, and also likely oligomeric forms, in the LS and TX [LS buffer supplemented with 1% (v/v) Triton-X-100 and 0.5 mol/L NaCl] fractions. The detergent-insoluble fraction of patient 5387 with dementia contained enhanced ApoE signal in the form of a continuous smear.

(Figure 5). ApoE signal corresponding to likely multimers, primarily in the LS fractions, was also detected. The detergent-insoluble fraction of the patient with dementia had a continuous, smeared ApoE signal that was stronger, especially in the high molecular weight range, than in the control case. It is likely that this high molecular weight signal corresponded to the ApoE protein detected by using mass spectrometry.

Correlation analysis was performed to identify proteins in the detergent-insoluble high molecular weight fraction that were positively and negatively correlated with the ApoE peptides detected with mass spectrometry (Table 5 and Supplemental Figure S2). Strong correlations were found between levels of ApoE and a subset of proteins, including complement proteins C3, C4A and C4B, Tau (MAPT), ubiquitin (represented by UBB, UBC, RPS27A, and UBA52), and the Collagen alpha-1(XXV) chain (COL25A1).

The histopathologic relationship between ApoE, C4, and Tau proteins was investigated using immunohistochemistry, focusing on brains from participants with the *APOE* ε3/3 genotype. C4 was chosen as antigen out of convenience because of the availability of a robust antibody for immunohistochemistry (Table 6). Results of immunohistochemical staining of ApoE are depicted in Figures 6 to 8 and Table 7. In a cognitively normal participant with the highest

possible final Mini-Mental State Examination (MMSE) score (MMSE = 30) and no ApoE detected by mass spectrometry (Panel A, Case 1309), ApoE was primarily detected in reactive astrocytes and capillary profiles. In the only cognitively normal control subject in our cohort (MMSE = 29) with ApoE detected in the detergent-insoluble fraction (Case 1106), the ApoE staining of microglia and astrocytes was less pronounced. Instead, structures with the histomorphologic appearance of diffuse amyloid plaques were apparent (Figure 6B). In an individual who died with dementia (Case 1068; MMSE = 22.5), multiple stained structures, including astrocytes, blood vessels, and amyloid plaques, were apparent (Figure 6C). ApoE immunohistochemistry staining was performed on additional amygdalae from 11 patients with dementia (MMSE ≤ 24) with *APOE* ε2/3, 3/3, and 3/4 genotypes. Our findings showing extensive AD-type pathology in patients with dementia are summarized in Table 7. In a post hoc convenience sample of *APOE* ε3/3 cases (*n* = 5) that were not among the subjects sampled for proteomics studies, the same general findings were observed: in cases lacking AD pathology, ApoE immunohistochemistry predominantly labeled astrocytes, whereas in cases with AD pathology, ApoE immunohistochemistry labeled amyloid plaques and some cells that looked like neurofibrillary tangles (Supplemental Table S3 and Supplemental Figure S3).

As indicated by immunofluorescence microscopy, the ApoE and C4 proteins were co-localized extensively in senile plaque-like structures (Figure 7). Some areas showed more ApoE immunohistochemical signal, particularly small blood vessels, in areas lacking AD-type plaques and tangles. Particular microdomains of amyloid plaques were more strongly stained for C4 than for ApoE. The Tau protein was immunolocalized, as expected, in neurofibrillary tangles, neuritic threads, and neuritic plaques, and, although nearby,

Table 5 ApoE Peptides Detected in Detergent-Insoluble Fraction According to Mass Spectrometry Data: Correlations (r^2) with Other Proteins (Top 15) Across All Included Participants (*N* = 40)

Positive correlations		Negative correlations	
C3	0.889885	EPB41L3	−0.50981
MAPT	0.794382	CAMK2B	−0.45293
UBB	0.761242	CAMK2D	−0.42138
COL25A1	0.741231	CAMK2A	−0.40312
C4A	0.600414	NEFL	−0.3862
C4B	0.598196	IQSEC1	−0.37179
TRAF3IP1	0.565691	EPB41L1	−0.36325
PTGDS	0.560328	IQSEC2	−0.36027
CLU	0.53809	NEFM	−0.35971
PSAP	0.525282	INA	−0.35919
PAK3	0.498618	CAMKV	−0.35605
PAK1	0.498618	DNM3	−0.35087
CTSD	0.493292	TUBA4B	−0.34833
SNCA	0.492116	GNAI2	−0.33298
OLFM2	0.472014	GNAI2	−0.33213

ApoE, Apolipoprotein E.

Table 6 Antibodies and Antisera Used in the Current Study

Antigen	Vendor	Catalog #	Epitope/antigen	Uses in current study
ApoE	Abcam	Ab52607	“Near C-Terminal”	WB, IHC-P/IF
C4a	Abcam	Ab187278	Full length protein	IHC-P/IF
Tau	R&D Systems	AF3494	Residues Gln624-Gln756	IHC-P/IF

ApoE, Apolipoprotein E; IF, immunofluorescence; IHC-P, immunohistochemistry-paraffin embedded tissues; WB, Western blot.

the Tau protein did not co-localize specifically with ApoE or C4 proteins in amyloid plaques. A high-magnification image of a plaque-like structure in the brain of Case 1068 shows strong ApoE signal in the plaque core, whereas the C4 signal was also strong in the periphery of the plaque (Figure 8). The Tau-immunoreactive structures were often nearby but outside the ApoE or C4 signal. The ApoE antibody also highlighted small vessels and glial profiles that were not immunolabeled by the C4 antibody.

Discussion

The ApoE protein was robustly detected in detergent-insoluble amygdala extracts via mass spectrometry in dementia brains, from research participants with or without the *APOE* $\epsilon 4$ genotype. The study goals initially were not focused on ApoE; rather, the study was designed to identify and characterize novel DIPPp relevant to NDs. With this aim, proteins from samples of human amygdalae were extracted and analyzed with LC-electrospray ionization-MS/MS. Polypeptides present in detergent-insoluble extracts of patients with antemortem cognitive impairment were queried and compared with those in control brains. Relative to other proteins identifiable in the mass spectrometry data, including the signals referent to established DIPPp such as Tau, A β , and α -Synuclein, the ApoE results stood out. The strength of signal from the ApoE in the detergent-insoluble fraction was also positively correlated with the presence of complement proteins, particularly C3, C4A, and C4B.

There have been previous analyses of the human amygdala proteome.^{47–49} However, no studies have reported on the amygdala proteome in a sample of this size with dementia subjects and control subjects for the sake of comparison. Other prior studies examined DIPPp preparations from human brain samples outside of the amygdala, including mass spectrometric analyses of brain extracts from subjects with dementia-associated pathologies such as AD, limbic-predominant age-related TDP-43 encephalopathy neuropathologic change, and frontotemporal lobar degeneration.^{8,50–54}

The full-length ApoE protein includes an N-terminal signal peptide (Met1-Ala18) that is removed upon sorting.⁵⁵ No ApoE peptide was identified in the current study before the Ala80 residue. The ApoE region between Ala80 and Ala255 comprises eight 22-residue tandem repeats, in which the majority of the identified peptides were clustered. The C-terminal segment of the ApoE protein (residues Leu279

to His317) was not represented by identified peptides either. This C-terminal region is responsible for lipid binding, lipoprotein association, and oligomerization.^{56–58} Importantly, aggregation-prone regions were previously identified in the central region of ApoE.⁵⁹

The *APOE* $\epsilon 4$ allele has been associated clinically with increased dementia risk and pathologically with increased A β plaque load.^{16,60–65} Our results emphasized the relevance of the ApoE protein as a particularly important protein aggregate constituent in itself and not only as an “upstream” genetic risk factor. These results may indicate

ApoE IHC in amygdala: *APOE* $\epsilon 3/3$ cases

Figure 6 Brightfield immunohistochemical (IHC) staining of Apolipoprotein E (ApoE) in amygdala, from cases with the *APOE* $\epsilon 3/3$ genotype. The antibody used here is the same as the one used in the immunoblot in Figure 5. Cells with histologic features of neurons are shown with pale blue arrowheads in Panels A–C. Panel A shows ApoE structures in the amygdala of Case 1309, a nondemented participant. Note the reactive astrocytes (red arrows) and the capillary profiles (orange arrows). Panel B shows structures with the histomorphologic appearance of diffuse amyloid plaques (blue arrows) (Case 1106, a nondemented participant). In the photomicrograph in Panel C (dementia Case 1068) are multiple stained structures, including astrocytes (red arrow), blood vessels (orange arrow), and amyloid plaques (blue arrows). Scale bars: 60 μ m (A); 40 μ m (B); 50 μ m (C).

Figure 7 Immunofluorescence microscopy showing the localization of Apolipoprotein E (ApoE) (green; **A** and **E**), C4 (red; **B** and **F**), and Tau (white; **C** and **G**) proteins in two different brains with the *APOE* $\epsilon 3/3$ genotype. Panels **D** and **H** show the photomicrographs with the signals merged, with yellow indicating signal overlap. These photomicrographs are from amygdala sections from dementia Case 1068 (**A–D**) and dementia Case 1096 (**E–H**). Although the ApoE and C4 signals co-localized extensively, some areas show more ApoE, particularly small blood vessels (**white arrow** in panels **A** and **D**). Some regions of plaques were more strongly stained for C4 than for ApoE (**beige arrow** in panels **B** and **D**). Tau protein was seen, as expected in neurofibrillary tangles (**white arrows** in panel **G**) and neuritic threads and neuritic plaques (**beige arrow** in Panel **H**), and did not co-localize substantially with ApoE or C4. Scale bars: **C** (for **A–C**), 50 μm ; **D**, 70 μm ; **G** (for **E–G**), 50 μm ; **H**, 70 μm .

prevalent phenomena, particularly because this convenience sample was drawn from a community-based clinical cohort.

Although the current study design was unique, the key findings are compatible with (and confirmatory of) data reported in prior studies from other laboratories. The focus on human studies highlights three points that were previously described in various cohorts and contexts. First, ApoE was found to be aberrantly aggregated in dementia brains, and several potentially pathogenetic fragments of ApoE

have been implicated. Second, immunohistochemical stains showed ApoE protein to be co-localized with both amyloid plaques and Tau tangles in AD brains, including in *APOE* $\epsilon 3/3$ cases. Third, ApoE has been proposed to play roles in modulating brains' neuroinflammatory signaling.

Consistent with the findings in the current study, ApoE protein was previously shown to be aberrantly deposited in dementia brains, and proteolytic cleavage fragments of the protein were identified that may be neurotoxic. The ApoE

Figure 8 Immunofluorescence microscopy showing a single neuritic amyloid plaque with the localization of Apolipoprotein E (ApoE) (green; **A**), C4 (red; **B**), and Tau (white; **C**) proteins in amygdala of a person with the *APOE* ε3/ε3 genotype (Case 1068). In the depicted plaque-like structure, the ApoE signal is strongest in the plaque core (**arrow** in panel **A**), whereas the C4 signal is also strong in the periphery (**arrow** in panel **B**). The Tau-immunoreactive structures are often outside the ApoE or C4 signal (**arrow** in panel **C**). Panel **D** shows all three colors, and yellow depicts green-red regions of overlap. The ApoE antibody labels small vessels (**large arrowhead** in panel **A**) and glial profiles (**smaller arrowhead** in panel **A**) that are not labeled by the C4 antibody. Scale bar = 50 μm.

protein was enriched in subjects with dementia in prior analyses of proteomes (particularly the detergent-insoluble fractions) of human brains.^{51,53,66–70} An N-terminal ApoE cleavage product was characterized in AD and Down syndrome brains, and co-localized via immunohistochemistry

in Tau tangles.^{71–75} Extensive work has focused on how that N-terminal ApoE fragment may be neurotoxic and may participate in the AD pathogenesis mechanism(s).^{72,76,77} By contrast, when Aβ peptide was isolated from AD brains, it was bound to a C-terminal cleavage product of ApoE.^{78,79}

Table 7 Immunohistochemical Staining for ApoE in Amygdala Sections from a Convenience Sample of 14 Cases from the University of Kentucky AD Research Center Biobank

Case #	Age, years	Sex	Final MMSE score	Braak NFT stage	CERAD neuritic amyloid plaque severity	<i>APOE</i> allele status	ApoE MS result	ApoE stained astrocytes*	ApoE IHC-stained AD-type pathology†
1068	92	F	22.5	VI	Moderate	2/3	51.1	+	+
1298	96	F	24	VI	Severe	2/3	36.4	++	++
5384	87	M	0	V	Moderate	3/3	151.9	++	+
1096	84	M	23	VI	Severe	3/3	151.4	++	++
1309	83	F	30	II	None	3/3	0	+++	—
1106	79	M	29	II	Severe	3/3	44.1	+	++
1053	90	F	22.5	VI	Severe	3/3	85.9	++	++
5372	94	M	24	V	Severe	3/3	113.1	++	++
5394	64	F	6	VI	Severe	3/4	248.5	+	+++
1101	86	F	7	VI	Severe	3/4	374.5	+	++
1052	80	F	19	VI	Moderate	3/4	348.9	+	+++
1035	84	F	19	V	Severe	3/4	46.2	+	+++
1037	82	M	17.5	VI	Severe	3/4	253.7	+	+++
5361	87	M	13	V	Severe	4/4	59	+	+++

*ApoE IHC staining of cells resembling reactive astrocytes in amygdala: minimal (+), moderate (++), and widespread (+++).
†AD neuropathologic change features stained with ApoE IHC in amygdala: none (—); a minimal number of focal senile plaque-like structures (+), moderate densities of senile-plaque-like structures (++), or widespread senile plaques with some structures that resemble NFTs (+++).
F, female; M, male; AD, Alzheimer disease; ApoE, Apolipoprotein E; IHC, immunohistochemistry; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MS, mass spectrometry; NFT, neurofibrillary tangle.

Despite these observations, there is no widespread awareness of the potential for misfolded and/or cleaved ApoE in the brain to be strongly relevant to *APOE* $\epsilon 4$ noncarriers.

Previous studies have used immunohistochemical stains in human brain to show that ApoE protein is co-localized with both amyloid plaques and Tau neurofibrillary tangles, including in some Down syndrome and *APOE* $\epsilon 3/3$ cases.^{73,74,80–87} Although *APOE* is preferentially transcribed in astrocytes and microglia,^{88,89} the ApoE protein also exists in neurons according to immunohistochemical stains.⁸¹ The intraneuronal ApoE was present in control subjects and AD, not only co-localized with tangles.⁹⁰ ApoE deposition has also been linked to vascular disease, including cerebral amyloid angiopathy.^{80,83,91–93} It has been hypothesized that ApoE deposition is an early event often preceding the presence of N-terminal epitopes of A β in the evolving plaque morphology in amyloid plaques,⁹² providing a scaffolding for ensuing protein deposition.⁹⁴ The presence of ApoE in amyloid plaques was associated with dystrophic neurites.⁹⁵ However, not all amyloid plaques with dystrophic neurites are immunopositive for ApoE.⁹⁶ According to Cho et al,⁹⁷ “Double-labeling demonstrated that the plaque cores contained the entire ApoE protein, but that outer regions contained only a C-terminal fragment, suggesting a cleavage in the random coil region of ApoE.” Together, these findings point to intriguing connections between ApoE proteolytic cleavage and the development and toxicity of the microscopic markers of AD. However, there was no evidence of either extreme N- or C-terminal peptides in the detergent-insoluble proteome in the current study.

In addition to being aberrantly aggregated and/or cleaved in AD brains, and its presence in the disease-defining microscopic lesions of AD, ApoE has also been implicated in modifying neuroinflammatory signals.¹⁸ In the current study, C3 and C4 proteins were strongly associated with ApoE in the detergent-insoluble proteome detected with mass spectrometry. This correlation was robust in *APOE* $\epsilon 4$ noncarriers, and immunohistochemical studies confirmed that ApoE and C4a co-localized in both plaques and tangles, including in *APOE* $\epsilon 3/3$ participants’ brains. ApoE was previously shown to be associated with complement protein, particularly in cerebrospinal fluid.^{98–100} The exact implications of these correlations are unknown, but they indicate the existence of interactions between ApoE and complement activation that may be targetable for potential disease modification.

There are limitations and caveats that apply to the current study. Although established pathologic markers are commonly identifiable in the amygdala at autopsy of older persons, it only represents a specific subset of the findings related to dementia. Additional limitations of the current study include the relatively small sample sizes, intrinsic imperfections in the proteomic analyses that do not include all peptides due to various biochemical factors, and the potential pitfalls of false-negative and false-positive results

when undertaking follow-up studies with commercially available antibodies. Furthermore, all the samples used in the current study were from a single center. Although this reduces the likelihood of handling differences, there is a possibility that these results may not be generalizable. We note that there were a number of different proteins (ie, other than ApoE, Tau) that showed evidence of enrichment in the detergent-insoluble proteome, and we will be investigating these proteins in future studies.

Although the aforementioned caveats deserve consideration, the current study adds to an evolving appreciation of multiple misfolded proteins in the human brain. There is general agreement that there are four prevalently deposited/misfolded polypeptide species that are strongly associated with the dementia phenotype: A β , Tau, α -Synuclein, and TDP-43. ApoE not only deserves to be considered a fifth member of this group, but, even in persons lacking the *APOE* $\epsilon 4$ allele, ApoE may indeed be among the most impactful misfolded proteins in aging brains.

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Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2021.11.013>.

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