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# ApoE and clusterin CSF levels influence associations between *APOE* genotype and changes in CSF tau, but not CSF Aβ42, levels in non-demented elderly

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ABSTRACT

Apolipoprotein E (*APOE*) ε4 genotype is associated with increased cerebral amyloid beta (Aβ) deposition in nondemented elderly and suggested to influence ApoE as well as ApoJ (clusterin [Clu]) and ApoA1 expression. We aimed to assess whether *APOE* affects early Alzheimer's disease pathophysiology via these apolipoproteins. Cerebrospinal fluid (CSF) ApoE, Clu, ApoA1, and CSF amyloid beta$_{1-42}$ (Aβ42) and tau levels were assessed in 403 individuals with subjective cognitive decline and mild cognitive impairment using enzyme-linked immunosorbent assay. Whether CSF apolipoprotein levels mediated *APOE*ε4 allele frequency effects on CSF Aβ42 and tau in nondemented elderly was investigated using mediation analysis, with age- and gender-adjusted linear regression analyses. CSF ApoE mediated 48% of the association between *APOE*ε4 and CSF tau, whereas Clu and ApoA1 did not. In addition, CSF Clu partially mediated the relation between CSF ApoE and tau (12%). CSF apolipoproteins did not mediate the inverse relation between *APOE*ε4 and CSF Aβ42, despite a strong association between the latter 2 biomarkers. In summary, our findings suggest that ApoE and Clu are involved in Aβ-independent pathways as part of the cascade leading to Alzheimer pathology.



## 1. Introduction

Apolipoprotein E (*APOE*) ε4 genotype is a major genetic risk factor for Alzheimer's disease (AD), associated with an earlier age of onset of dementia (Corder et al., 1993). The *APOE* gene encodes for the protein ApoE, which regulates lipid homeostasis in the brain and also supports injury repair (Corder et al., 1993). In the central nervous system, ApoE is primarily produced by astrocytes and microglia (Pfrieger and Ungerer, 2011). The 3 ApoE isoforms (ε2, ε3, and ε4) have different isoform-specific binding affinities for specific lipids and amyloid beta (Aβ) (Frieden and Garai, 2012). *APOE*ε4 carriers have more cerebral amyloid deposition than subjects with an ε3 or ε2 isoform, and ε4 carriership is associated with lower cerebrospinal fluid (CSF) amyloid beta$_{1-42}$ (Aβ42) concentrations (Castellano et al., 2011; Fagan et al., 2006; Prince et al., 2004). *APOE*ε4 carriership may increase the risk of AD via effects on Aβ clearance, through effects on either transport to the blood stream or glial cell uptake, or modulation of Aβ-induced glial cell activation (Bu, 2009). On the other hand, ApoE might influence AD pathophysiology also via Aβ-independent mechanisms, including its anti-inflammatory properties, its effects on alterations in neurovasculature, or the ApoE ε4 isoform-related deficits in cholesterol homeostasis, affecting synaptic integrity and plasticity (Liu et al., 2013; Wolf et al., 2013).

With the lack of therapeutic options once a diagnosis of dementia because of AD is made, predementia and preclinical stages of AD have become a focus of research (Sperling et al., 2011). In nondemented elderly *APOE*ε4 carriership is associated with an increased prevalence of amyloid positivity (Jansen et al., 2015). Patients with AD have altered levels of ApoE in CSF and plasma compared with individuals with normal cognition and mild cognitive impairment (MCI), although no consensus has been reached whether higher or lower ApoE levels are associated with AD (Gupta et al., 2011; Martínez-

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Morillo et al., 2014; Slooter et al., 1998; Taddei et al., 1997). In nondemented elderly, altered CSF and plasma ApoE levels are associated with an increased risk of AD, an effect most prominently observed in *APOE*ε4 carriers (Song et al., 2012; Thambisetty et al., 2010; van Harten et al., 2017). Also for other apolipoproteins, namely apolipoprotein A1 (ApoA1) and apolipoprotein J (also referred to as clusterin [Clu]), alterations in CSF and plasma levels have been associated with an increased risk of progression to dementia in nondemented elderly, and in some studies these effects were more prominent in *APOE*ε4 carriers too (Jongbloed et al., 2015; Saczynski et al., 2007; Schrijvers et al., 2011; Slot et al., 2017; Song et al., 2012). Besides ApoE, Clu is the main brain cholesterol transporter. In high density lipoproptein (HDL) particles Clu colocalizes with ApoA1, the third most abundant apolipoprotein in the central nervous system, and mainly involved in the reverse cholesterol transport in peripheral tissues (Pfrieger and Ungerer, 2011). Both Clu and ApoA1 have been suggested to affect Aβ-deposition and clearance (Castellano et al., 2011; Demattos et al., 2004; Merino-Zamorano et al., 2016; Verghese et al., 2013), and are considered to have neuroprotective properties, whereas Clu has been reported to be compensatory induced in response to low brain levels of ApoE in *APOE*ε4 carriers (Bertrand et al., 1995; Demattos et al., 2004; Koldamova et al., 2001; Narayan et al., 2012; Paula-Lima et al., 2009; Wyatt et al., 2011).

To investigate whether *APOE* affects the early pathophysiology of AD via apolipoproteins, we assessed whether the association between *APOE*ε4 allele frequency and CSF Aβ42 and tau levels could be explained by mediation of these associations by CSF ApoE, Clu, and ApoA1 levels in nondemented elderly with subjective cognitive decline (SCD) and MCI.

## 2. Materials and methods

### 2.1. Subjects

From the Amsterdam Dementia Cohort, we included 403 nondemented patients with available CSF and a baseline diagnosis of SCD (N = 191) or MCI (N = 212) (Albert et al., 2011; Jessen et al., 2014). All patients underwent a standardized dementia screening including neuropsychological, physical, and neurologic examination as well as laboratory tests, electroencephalography, and brain magnetic resonance imaging in a memory clinic setting (Van Der Flier et al., 2014). Diagnoses were made in a multidisciplinary consensus meeting. Patients were labeled as having SCD when they presented with memory complaints, but cognitive functioning was normal, and criteria for MCI, dementia, or any other neurologic or psychiatric disorder known to cause cognitive decline were not met (Jessen et al., 2014). MCI was diagnosed according to Petersen's criteria, and all MCI patients fulfilled National Institue on Aging (NIA) at National Institutes of Health (NIH) and the Alzheimer's Association core clinical criteria for MCI (Albert et al., 2011; Petersen et al., 1999).

### 2.2. Ethical procedures

The local medical ethics committee of the VU University Medical Center Amsterdam approved the collection of data and biomaterial from patients for research purposes. All patients gave written informed consent for the use of their data and biomaterial for research purposes. All research was conducted in accordance with the Helsinki Declaration of 1975.

### 2.3. Biomarker measurements

#### 2.3.1. APOE *genotyping*

*APOE* genotyping was performed after automated genomic DNA isolation from 7 to 10 mL blood collected in ethylenediamine tetraacetic acid blood. It was subjected to polymerase chain reaction, checked for size and quantity using a QIAxcel DNA Fast Analysis kit (Qiagen, Venlo, The Netherlands), and sequenced using Sanger sequencing on an ABI130XL.

#### 2.3.2. *CSF Aβ42 and tau analyses*

CSF analyses were performed at the Neurochemistry Laboratory at the Department of Clinical Chemistry of the VU University Medical Center Amsterdam. CSF was obtained by lumbar puncture between the L3/L4 or L4/L5 intervertebral space by a 25-gauge needle and collected in polypropylene tubes (Sarstedt, Numbrecht, Germany). CSF (2.5 mL) was used for routine chemical analyses such as erythrocyte count, and Aβ42, tau, and tau phosphorylated at threonine 181 (ptau) were measured using enzyme-linked immunosorbent assay (ELISA) (Innotest, Fujirebio, Ghent, Belgium) (Mulder et al., 2010). CSF for biobanking was centrifuged, aliquotted into 0.5 mL polypropylene vials (Sarstedt), and stored at −80 °C until further analysis (Del Campo et al., 2012).

#### 2.3.3. *CSF apolipoprotein analyses*

ApoA1 concentrations in CSF were measured using a commercial sandwich ELISA$^{PRO}$ kit for human ApoA1 (Mabtech AB, Nacka Strand, Sweden; Cat. No. 3710-1HP-10), according to the manufacturer's instructions. This assay uses ELISA strips precoated with capture monoclonal antibody (HDL110), to which samples were added. Captured ApoA1 was detected by adding another biotinylated ApoA1-specific monoclonal antibody (HDL44). Serial dilutions of purified human ApoA1 were used to prepare a standard curve (range 0.1–100 ng/mL) to calculate concentrations in the samples. CSF samples were tested at 1:1000 dilutions. Intra-assay coefficients of variance (CVs) for CSF ApoA1 results were on average 4.0%. Interassay CVs (26 plates) were 13.0% and 9.1% for the low and high ApoA1 plasma controls, respectively.

ApoE concentrations in CSF were measured using a commercial sandwich ELISA (Mabtech AB; Cat. No. 3712-1H-20). Monoclonal antibody (E276) was used to capture the ApoE present in the samples. Captured ApoE was subsequently detected by another biotinylated ApoE-specific monoclonal antibody (E887). Serial 3-fold dilutions of recombinant ApoE3 in Assay buffer (Mabtech AB) were used to prepare a standard curve ranging from 0.03 to 31.6 ng/mL. CSF samples were diluted 1:2000 in Assay buffer. Intra-assay CVs for CSF ApoE results were on average 3.0%. Interassay CVs (24 plates) were 10.3% and 10.4% for plasma high and low, respectively.

Clu concentrations in CSF were determined in a sandwich ELISA using a combination of Clu-specific mouse monoclonal antibodies (kindly provided by Dr Braesch-Andersen; Mabtech AB). Antibody J29 was used to capture Clu in samples, and another, biotinylated mouse monoclonal antibody, J84, for detection. Clu to prepare a standard curve (range 0.11–285 ng/mL) was isolated by affinity chromatography using Clu-specific monoclonal antibody G7 coupled to Sepharose 4B beads (Jongbloed et al., 2015). Dilutions of standard and patient samples were prepared in Assay buffer (Mabtech). The intra-assay CV was less than 5%. Interassay CV was 9.8%.

Detection of ApoA1, ApoE, and Clu in the ELISAs was visualized on subsequent incubations with streptavidin-horseradish peroxidase and 3,5,3′,5′-tetramethylbenzidine (Sigma, Germany).

### 2.4. Statistical analyses

In this cross-sectional study data were analyzed using statistical package for the social sciences for Macintosh, version 20 (IBM, Armonk, NY, USA). Demographic features were compared based on *APOE*ε4 carriership, and diagnosis (SCD vs. MCI), using *t* tests or $\chi^2$ tests as appropriate. Before analyses all biomarkers were log-

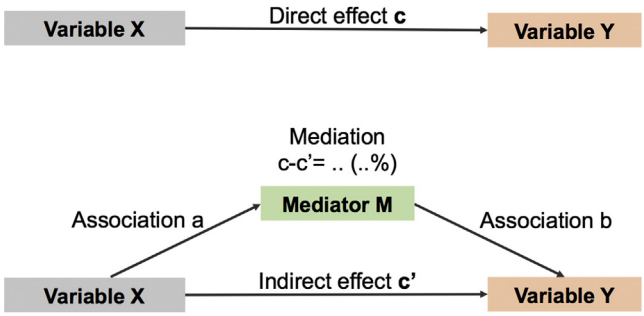


**Fig. 1.** Visual interpretation of the concept of mediation. $X$ = independent variable, $Y$ = outcome variable, $M$ = (possible) mediating variable. To evaluate possible mediation by mediator $M$ on the relation between $X$ and $Y$, variables $X$ and $Y$ and variable $X$ and mediator $M$ need to be significantly associated, otherwise one cannot speak of mediation. $a$ = association between variable $X$ and $M$; $b$ = association between *mediator M* and $Y$, adjusted for variable $X$; $c$ = association between $X$ and $Y$; $c'$ = association between $X$ and $Y$, adjusted for mediator $M$. Mediation = $c$-$c'$, percentage of mediation = $(c\text{-}c')/c \times 100$.

transformed, because they did not have a normal distribution. Subsequently, we transformed all biomarker values to $z$ scores.

Associations between *APOE*ε4 allele frequency, CSF Aβ42 and tau levels, and CSF ApoE, Clu, and ApoA1 concentrations were investigated using linear regression analyses, adjusted for age and gender. Subsequently, we used mediation analyses to assess whether CSF apolipoproteins influenced (mediated) the relation between *APOE*ε4 allele frequency and CSF Aβ42 and tau. Mediation analysis is a method to evaluate the possible influence of mediator $M$ on the relation between independent variable ($X$) and outcome variable ($Y$) (Baron and Kenny, 1986; Hayes, 2009; Mackinnon et al., 2007). We used linear regression analysis, adjusted for age and gender, to evaluate associations between independent variable $X$ (*APOE*ε4 allele frequency) and outcome variable $Y$ (CSF Aβ42 or tau), this direct association was referred to as relation $c$. Then we evaluated associations between $X$ and possible mediator $M$ (i.e., CSF ApoE, Clu, or ApoA1) (association $X$-$M$ was referred to as association $a$), and the association between $M$ and $Y$ adjusted for $X$ (referred to as association $b$). To evaluate possible mediation by mediator $M$ on the relation between $X$ and $Y$, associations $a$, $b$, and $c$ need to be significant, otherwise one cannot evaluate mediation. Finally, we evaluated the amount of change in regression coefficient (direct association $X$-$Y$ ($c$)) adjusted for mediator $M$, resulting in the indirect association $X$-$Y$ (referred to as association $c'$). The mediation effect was then calculated ($c$-$c'$) as well as the percentage of mediation ($(c\text{-}c')/c \times 100$), which represents the amount by which mediator $M$ explains the relation between $X$ and $Y$. See also Fig. 1 for a visual representation of the mediation concept. With regards to terminology we used the word mediate to indicate a statistical mediation by mediator $M$ on the relation between $X$ and $Y$. This does not automatically imply a causal relation, but does indicate a statistical influence of $M$ on the association between $X$ and $Y$.

Besides *APOE*ε4 allele frequency, we took CSF ApoE as a starting point and evaluated mediation of CSF Clu and ApoA on the association between CSF ApoE and CSF tau.

All analyses were adjusted for age and gender, $p < 0.05$ was considered significant.

## 3. Results

### 3.1. Demographic features and CSF AD biomarker and apolipoprotein levels

Table 1 shows the demographic features of the study participants. Distribution of *APOE*ε4 allele frequency (0/1/2 alleles) was 203/142/58. On average patients were aged 64 ± 9 years, with a mean Mini-Mental State Examination of 27 ± 2, and 166 (41%) were female. *APOE*ε4 carriers had lower CSF Aβ42, higher CSF tau, and higher CSF ApoE levels (3.1 ± 1.4 vs. 3.9 ± 3.1, $p = 0.000$). CSF Clu and ApoA1 concentrations did not differ between *APOE*ε4 carriers and noncarriers. CSF ApoE, Clu, and ApoA1 were all associated with age (respectively $\beta = 0.18$, $p = 0.000$; $\beta = 0.26$, $p = 0.000$; and $\beta = 0.14$, $p = 0.006$). There were no gender differences in CSF ApoE and Clu levels, whereas ApoA1 levels were lower in female participants (3.2 ± 1.5 vs. 4.0 ± 4.1, $p = 0.000$). Therefore, all analyses were adjusted for age and gender.

### 3.2. Associations between APOE*ε4, CSF Aβ42, tau, and CSF apolipoproteins*

Table 2 shows associations between *APOE*ε4 allele frequency, CSF Aβ42, tau and CSF apolipoproteins. We assessed these relations between *APOE*ε4 allele frequency and CSF analytes using linear regression analysis, adjusted for age and gender. Increased *APOE*ε4 allele frequency was associated with lower CSF Aβ42 ($\beta = -0.62$, $p = 0.000$), higher CSF tau (0.31, $p = 0.000$), and higher CSF ApoE levels (0.41, $p = 0.000$), but not CSF Clu or ApoA1. Higher CSF tau was associated with higher CSF ApoE ($\beta = 0.44$, $p = 0.000$) and higher CSF Clu levels ($\beta = 0.49$, $p = 0.000$), but not ApoA1. Meanwhile, CSF Aβ42 was not associated with any of the CSF apolipoproteins we measured. See Fig. 2 for scatterplots of associations between CSF Aβ42 and tau, and CSF apolipoproteins.

**Table 1**
Demographic features of the study population of nondemented elderly ($n = 403$)

| Variable | All | *APOE*ε4 negative | *APOE*ε4 positive | *p* | SCD | MCI | *p* |
|---|---|---|---|---|---|---|---|
| N | 403 | 203 | 200 | | 191 | 212 | |
| Age (y) | 64.0 ± 9.1 | 63.3 ± 9.5 | 64.8 ± 8.6 | 0.101 | 60.5 ± 8.8 | 67.2 ± 8.2 | 0.000 |
| Gender, female | 166 (41%) | 73 (36%) | 93 (47%) | 0.034 | 75 (39%) | 91 (43%) | 0.479 |
| MMSE | 27.3 ± 2.2 | 27.5 ± 1.1 | 27.2 ± 2.3 | 0.087 | 28.2 ± 1.6 | 26.5 ± 2.4 | 0.000 |
| *APOE*ε4 positive | 200 (50%) | | | | 74 (39%) | 126 (63%) | 0.000 |
| *APOE*ε4 allele frequency (0/1/2) | 203/142/58 | | | | 117/58/16 | 86/84/42 | 0.000 |
| CSF Aβ42 (ng/L) | 728 ± 281 | 843 ± 268 | 611 ± 243 | 0.000 | 841 ± 248 | 626 ± 270 | 0.000 |
| CSF tau (ng/L) | 407 ± 318 | 340 ± 245 | 475 ± 365 | 0.000 | 289 ± 203 | 513 ± 362 | 0.000 |
| CSF ApoE (mg/L) | 3.5 ± 1.5 | 3.1 ± 1.4 | 3.9 ± 3.1 | 0.000 | 3.3 ± 1.4 | 3.7 ± 1.5 | 0.000 |
| CSF clusterin (mg/L) | 9.1 ± 3.4 | 9.2 ± 3.6 | 9.1 ± 3.1 | 0.901 | 2.1 ± 0.4 | 2.2 ± 0.4 | 0.181 |
| CSF ApoA1 (mg/L) | 3.7 ± 3.3 | 3.7 ± 4.3 | 3.6 ± 2.0 | 0.585 | 3.7 ± 4.4 | 3.6 ± 1.9 | 0.448 |

Demographic features of 403 nondemented participants with SCD ($n = 191$) or MCI ($n = 212$). Data are presented as the mean ± standard deviation or $n$ (%); $t$ tests and $\chi^2$ tests were used to assess differences between *APOE*ε4 carriers versus noncarriers, and between diagnoses (SCD vs. MCI).
Key: Aβ42, amyloid beta$_{1-42}$; ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; SCD, subjective cognitive decline.

**Table 2**
Linear regression analyses between CSF analytes and *APOE*ε4 allele frequency in nondemented elderly ($n = 403$)

| Variable | Group | *APOE*ε4 allele frequency (1/2/3/) | CSF Aβ42 | CSF tau | CSF ApoE | CSF ApoJ (Clu) | Age |
|---|---|---|---|---|---|---|---|
| CSF Aβ42 | All | −0.62, $p = 0.000$ | | | | | −0.32, $p = 0.000$ |
| | SCD | −0.42, $p = 0.000$ | | | | | |
| | MCI | −0.65, $p = 0.000$ | | | | | |
| CSF tau | All | 0.31, $p = 0.000$ | −0.30, $p = 0.000$ | | | | 0.39, $p = 0.000$ |
| | SCD | 0.11, $p = 0.161$ | −0.07, $p = 0.255$ | | | | |
| | MCI | 0.32, $p = 0.000$ | −0.32, $p = 0.000$ | | | | |
| CSF ApoE | All | 0.41, $p = 0.000$ | −0.05, $p = 0.363$ | 0.49, $p = 0.000$ | | | 0.18, $p = 0.000$ |
| | SCD | 0.37, $p = 0.001$ | −0.02, $p = 0.805$ | 0.65, $p = 0.000$ | | | |
| | MCI | 0.41, $p = 0.000$ | −0.03, $p = 0.955$ | 0.44, $p = 0.000$ | | | |
| CSF ApoJ (Clu) | All | −0.03, $p = 0.670$ | 0.04, $p = 0.434$ | 0.32, $p = 0.000$ | 0.42, $p = 0.000$ | | 0.26, $p = 0.000$ |
| | SCD | −0.15, $p = 0.152$ | −0.06, $p = 0.511$ | 0.44, $p = 0.000$ | 0.38, $p = 0.000$ | | |
| | MCI | 0.06, $p = 0.494$ | 0.10, $p = 0.168$ | 0.33, $p = 0.000$ | 0.48, $p = 0.000$ | | |
| CSF ApoA1 | All | 0.06, $p = 0.406$ | 0.04, $p = 0.428$ | 0.07, $p = 0.219$ | 0.08, $p = 0.124$ | 0.37, $p = 0.000$ | 0.14, $p = 0.006$ |
| | SCD | 0.13, $p = 0.285$ | 0.09, $p = 0.323$ | −0.00, $p = 0.974$ | 0.05, $p = 0.492$ | 0.40, $p = 0.000$ | |
| | MCI | 0.02, $p = 0.831$ | 0.02, $p = 0.804$ | 0.12, $p = 0.073$ | 0.10, $p = 0.104$ | 0.35, $p = 0.000$ | |

Overview of associations between CSF analytes and *APOE*ε4 allele frequency in nondemented elderly ($n = 403$), subsequently stratified for diagnosis (SCD and MCI). All linear regression analyses were adjusted for age and gender, results are displayed as β with $p$ value. We also show unadjusted associations between age and CSF analytes.
Key: Aβ42, amyloid beta$_{1-42}$; Apo, apolipoprotein; Clu, clusterin; CSF, cerebrospinal fluid; MCI, mild cognitive impairment; SCD, subjective cognitive decline.

Higher CSF Clu was associated with both higher CSF ApoE (β = 0.42, $p = 0.000$) and higher CSF ApoA1 (β = 0.37, $p = 0.000$), but there was no relation between CSF ApoE and ApoA1. After stratification for diagnosis (SCD or MCI) results remained essentially the same, only the association between *APOE*ε4 allele frequency and CSF tau, and Aβ42 and CSF tau was not significant anymore in individuals with SCD (respectively β = 0.11, $p = 0.161$ and β = −0.07, $p = 0.255$, see Table 2).

### 3.3. Mediation analyses of CSF apolipoproteins on the relation between APOEε4 allele frequency and CSF Aβ42 and tau

Then, we investigated mediation of CSF apolipoproteins using linear regression analyses, adjusted for age and gender. The direct association between *APOE*ε4 allele frequency and CSF Aβ42 was −0.62 ($c$; $p = 0.000$). CSF ApoE, Clu, or ApoA1 did not mediate this relation (see Fig. 3). For more detailed results of linear

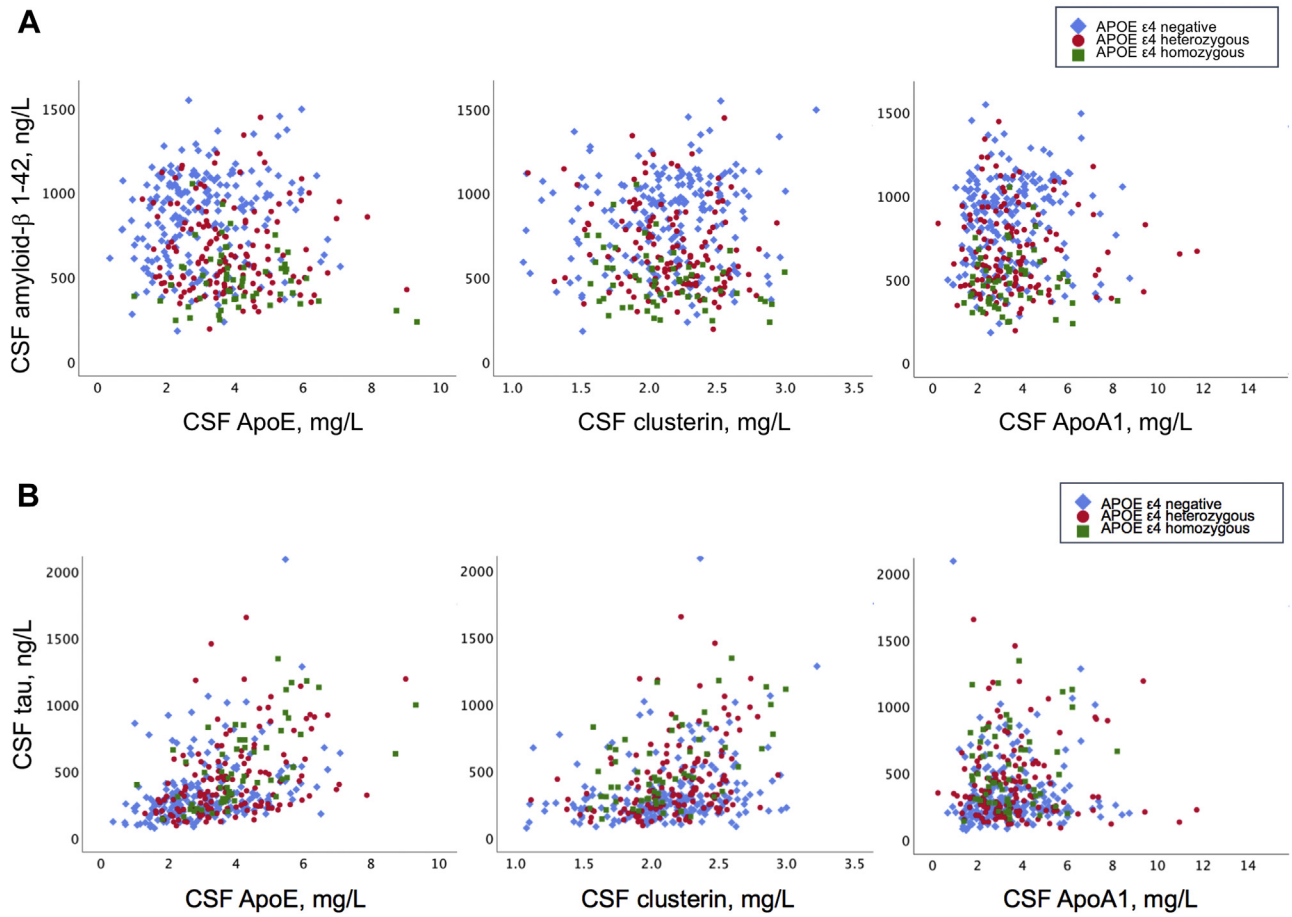


**Fig. 2.** Associations between CSF Aβ42, tau, and apolipoproteins. (A) Scatterplots of associations between CSF Aβ42 and CSF ApoE, clusterin, and ApoA1, marked by *APOE*ε4 status (*APOE*ε4 negative, ε4 heterozygous or homozygous). (B) Scatterplots of associations between CSF tau and CSF ApoE, clusterin, and ApoA1, marked by *APOE*ε4 status. Abbreviations: Aβ42, amyloid beta$_{1-42}$; ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; CSF, cerebrospinal fluid.

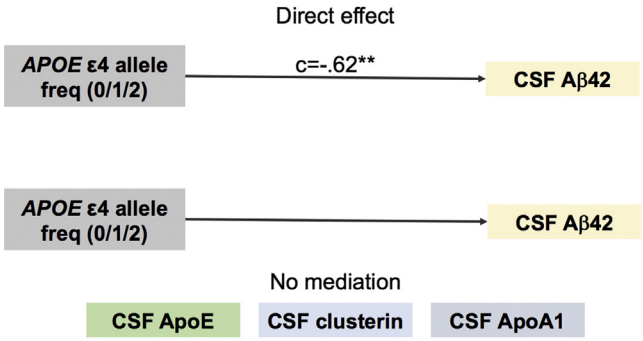


**Fig. 3.** No mediation by apolipoproteins of the association between *APOE*ε4 allele frequency and CSF Aβ42. Results displayed as standardized β, adjusted for age and gender, $^{**}p < 0.001$. Abbreviations: Aβ42, amyloid-beta$_{1-42}$; ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; *c*, association adjusted for age and gender; CSF, cerebrospinal fluid; freq, frequency.

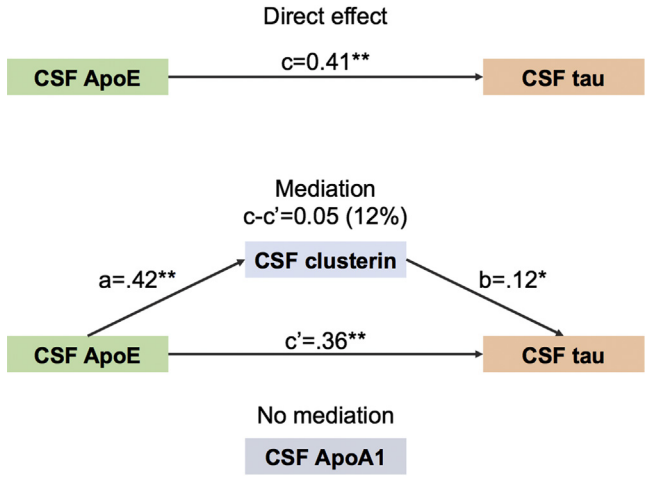


**Fig. 5.** Mediation (12%) by CSF clusterin of the association between CSF ApoE and CSF tau. Results displayed as standardized β, adjusted for age and gender, $^{*}p < 0.05$, $^{**}p < 0.001$. Abbreviations: ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; *c*, association adjusted for age and gender; *c'*, association adjusted for age, gender, and mediator; CSF, cerebrospinal fluid.

regression analyses used to evaluate mediation of CSF apolipoproteins see Supplementary Table A.

The direct association between *APOE*ε4 allele frequency and CSF tau was 0.31 (*c*; $p = 0.000$). This relation was partially mediated by CSF ApoE (*c'*; β = 0.16, $p = 0.000$; *c*-*c'* = 0.15, β ratio 48%, see Fig. 4). CSF Clu or ApoA1 did not mediate the association between *APOE*ε4 allele frequency and CSF tau.

Taking CSF ApoE as a starting point, the association between CSF ApoE and CSF tau was 0.41 (*c*; standardized beta, $p < 0.001$). This relation was partially mediated by CSF Clu (*c'*; standardized beta = 0.36, $p < 0.001$; *c*-*c'* = 0.05, β ratio 12%), but not by ApoA1, see Fig. 5.

After stratification for diagnosis (SCD vs. MCI) all results remained essentially the same. Only the association used to evaluate mediation of CSF ApoE on the relation *APOE*ε4 allele frequency and CSF tau was not significant anymore, but results were still in the same direction. For more detailed results after stratification for diagnosis see Supplementary Table A.

## 4. Discussion

*APOE*ε4 carriership is a major genetic risk factor for AD, but the exact pathophysiological mechanisms eventually leading to AD remain to be elucidated. To assess whether *APOE*ε4 genotype exerts its effect on AD pathology directly via ApoE levels or indirectly via effects on levels of other apolipoproteins (Clu or ApoA1) that share some of the lipid- and Aβ-carrier properties of ApoE, we examined the mediating effects of these apolipoproteins in CSF of non-demented elderly. We found that CSF ApoE levels partially explained the relation between *APOE*ε4 allele frequency and CSF tau, and that CSF levels of apolipoprotein Clu mediated the association between CSF ApoE and tau. Contrary to our expectation, CSF ApoE protein levels did not mediate the relation between *APOE*ε4 allele frequency and CSF Aβ42, despite a strong association between these latter 2 biomarkers.

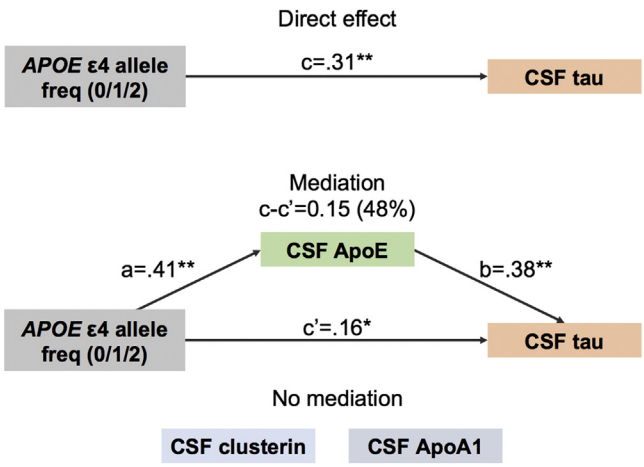


**Fig. 4.** Mediation (48%) by CSF ApoE of the association between *APOE*ε4 allele frequency and CSF tau. CSF clusterin and ApoA1 do not mediate the association. Results displayed as standardized β, adjusted for age and gender, $^{*}p < 0.05$, $^{**}p < 0.001$. Abbreviations: ApoA1, apolipoprotein A1; ApoE, apolipoprotein E; *c*, association adjusted for age and gender; *c'*, association adjusted for age, gender, and mediator; CSF, cerebrospinal fluid; freq, frequency.

Previous studies observed *APOE*ε4 allele dose-dependent effects on the risk of developing AD and amyloid plaque load in the brain, with an inverse effect of *APOE*ε4 allele frequency on CSF Aβ levels (Fagan et al., 2006; Prince et al., 2004). Effects of *APOE*ε4 may be because of effects on clearance and degradation of Aβ, as well as effects on Aβ production (Jiang et al., 2008; Schmechel et al., 1993). In line with previous research, we found a strong negative association between *APOE*ε4 allele frequency and CSF Aβ42 levels. However, the strong relation between *APOE*ε4 and CSF Aβ42 levels could not be explained by changes in CSF ApoE levels, despite the association of *APOE*ε4 allele frequency with both CSF Aβ42 and CSF ApoE levels in our cohort. The lack of association between CSF ApoE and CSF Aβ42 levels we observed was in contrast to some studies (Cruchaga et al., 2012; Fagan et al., 2000; LaDu et al., 2012) but not to other studies (Martínez-Morillo et al., 2014; Toledo et al., 2014; Verghese et al., 2013) studies.

We did find a dose-dependent effect of *APOE*ε4 allele frequency on CSF tau levels, for which CSF ApoE levels were a substantial mediator. Clinical studies have reported associations between CSF tau and CSF ApoE levels in patients with AD (Martínez-Morillo et al., 2014; Molina et al., 1999), but direct effects of *APOE*ε4 carriership on tau in AD are less frequently studied than associations with Aβ. In vitro the ApoE ε4 isoform was found to induce tau hyperphosphorylation, whereas ApoE ε3 increased the functional activity of protein phosphatase 2, which dephosphorylates phosphorylated tau in neurofibrillary tangles, suggesting opposite effects of ε3 and ε4 isoforms on AD-related neuronal damage (Brecht et al., 2004). Furthermore, ApoE ε4 has been shown to interact with cytoskeletal

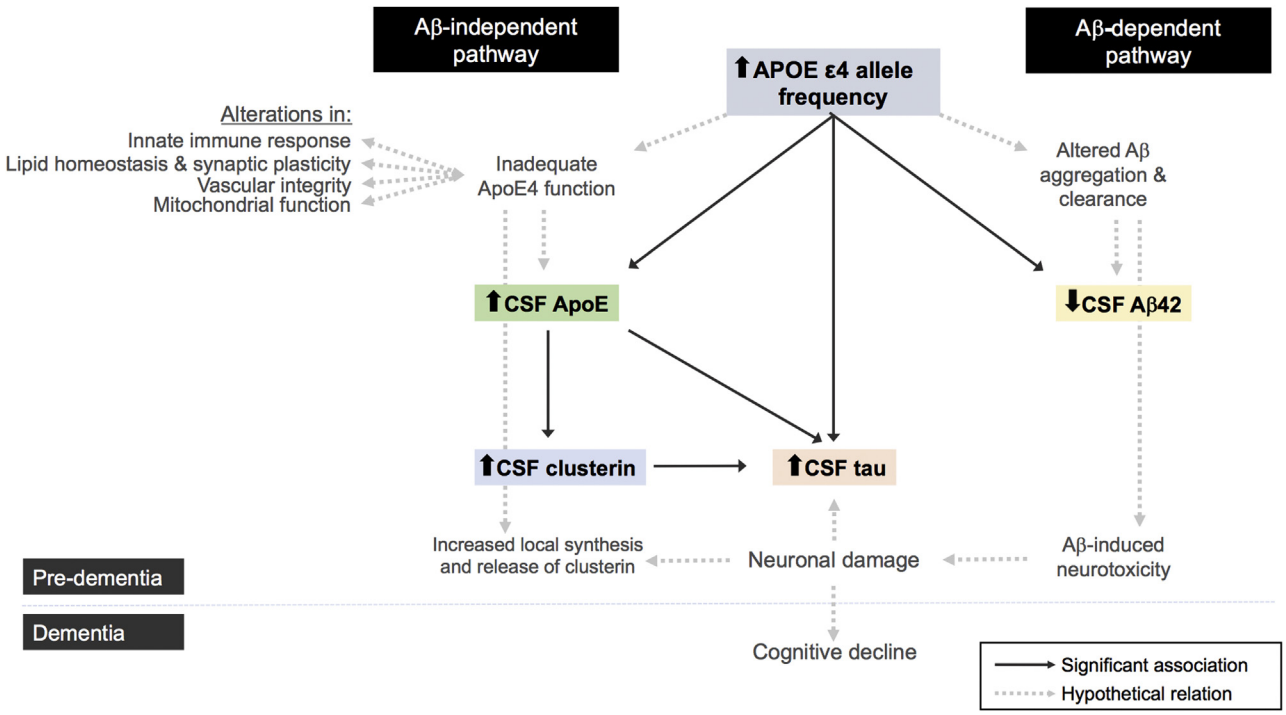


**Fig. 6.** Overview of associations between *APOE*, CSF Aβ42, tau, and apolipoproteins, and hypothetical underlying pathologic alterations. (Solid lines) significant associations, (dashed lines) hypothetical relations. Abbreviations: Aβ42, amyloid-beta$_{1-42}$; APOE, apolipoprotein E; CSF, cerebrospinal fluid.

proteins to form tangle-like structures containing phosphorylated tau, and truncated ApoE ε4 fragments might increase cytoskeletal disruption and mitochondrial dysfunction and neurotoxicity (Chang et al., 2005; Huang et al., 2001).

The lack of association between CSF ApoE and CSF Aβ42 levels in our study, together with the mediation of CSF ApoE in the relation between *APOE*ε4 and tau, may suggest that the ApoE ε4 isoform influences neuronal damage, reflected by increased CSF tau levels, indirectly via other mechanisms independent of Aβ. It has been proposed that tau-related pathology, independent of Aβ, might initiate and contribute to the pathogenesis of AD (Jack et al., 2013a, b). Possible Aβ-independent pathways affecting the pathophysiology of AD include the influence of *APOE* on inflammatory processes, cerebrovascular changes, and lipid homeostasis and thereby synaptic plasticity among others (Wolf et al., 2013). We theoretically evaluated these possible underlying mechanisms provoking AD pathophysiology independent of Aβ. First, innate immunity not only maintains homeostasis in cerebro through clearance of aged and obsolete proteins and cells but can also initiate neuronal damage when not properly controlled (Keene et al., 2011). The ApoE ε4 isoform has reduced anti-inflammatory properties compared with the other isoforms, which facilitates a proinflammatory environment with increased levels of proinflammatory cytokines tumor necrosis factor-α and interleukin-6 (Lynch et al., 2003; Zhu et al., 2012), and increased microglial activation, which in turn may facilitate neuronal damage (Keene et al., 2011; Zhu et al., 2012).

Second, cerebrovascular effects of ApoE include effects on integrity of the blood-brain barrier (BBB), which is more impaired in patients with AD carrying 1 or 2 *APOE*ε4 alleles, and may contribute to the disease via impaired Aβ clearance (Zipser et al., 2007). Independent of Aβ, BBB dysfunction may affect the pathophysiology of AD via impaired brain microcirculation, inducing neuronal dysfunction and injury (Zlokovic, 2011). How ApoE is involved in impaired vascular integrity and BBB dysfunction still remains largely unknown. A likely scenario suggested that expression of ApoE ε4 can lead to activation of a proinflammatory cyclophilin A–nuclear factor-κB–matrix-metalloproteinase-9 pathway in pericytes, that results in BBB breakdown (Bell et al., 2012), and ultimately to neuronal dysfunction. Taken together, these studies suggest that independent of Aβ, ApoE might influence vascular integrity early in AD pathogenesis.

A third Aβ-independent scenario beholds the influence of *APOE* on lipid homeostasis. Cholesterol is a vital component of cell membranes and maintaining lipid homeostasis is of great importance in the prevention of neurodegenerative diseases (Pfrieger and Ungerer, 2011). ApoE-mediated lipid redistribution is indispensable to the maintenance of synapse integrity and plasticity, both known to be affected in AD (Poirier et al., 2014). ApoE is mainly produced by astrocytes and microglia. Neuronal ApoE expression is upregulated after neuronal damage, possibly to induce neuronal repair (Aoki et al., 2003). The ApoE ε4 isoform is thought to confer the risk of AD via less effective lipidation and neuronal cholesterol delivery (Bu, 2009), which may lead to impaired neuronal plasticity and reduced neurogenesis, both involved in the pathogenesis of AD, independent of Aβ (Wolf et al., 2013). Although the ApoE ε4 isoform is less efficient in lipid transport, other apolipoproteins, such as Clu, may in turn be able to facilitate lipid transport and compensate for the ApoE ε4 isoform-associated loss in function. A graphical overview of associations and hypothetical underlying changes is provided in Fig. 6.

When we took the relationship between CSF ApoE and CSF tau as a starting point, we found that this relation was partially mediated (12%) by CSF Clu levels. A possible explanation is that Clu expression is upregulated in response to neuronal damage, in our study reflected by higher Clu levels associated with higher tau. In a mouse model overexpressing tau, Clu expression was found to be upregulated in the brain, whereas intracellular Clu interacted with tau in neurons (Zhou et al., 2013). However, in previous clinical studies we

have observed a correlation between CSF tau and Clu levels not only in nondemented patients frequenting our Alzheimer center (Jongbloed et al., 2015) but also in nondemented Parkinson patients and neurologically healthy control subjects (Van Dijk et al., 2013), which suggests that Clu may be involved in physiological processes as well. In the present study, we observed a relation between CSF Clu and CSF tau, but no significant association between CSF Clu and CSF Aβ42, whereas we did see a relation between CSF Clu and CSF Aβ42 in MCI cases, but not in AD or SCD, before (Jongbloed et al., 2015). Clu probably plays a physiological role in the clearance of Aβ42 toward the CSF through complex formation. The difference in associations between CSF Clu and CSF Aβ42 between our previous and present studies may be because of the extent of Clu-Aβ complex formation that may differ between health and disease, and may interfere with CSF Aβ measurements (Ghiso et al., 1993), thus influencing associations between CSF Clu and CSF Aβ42 levels determined in ELISAs. The composition of the nondemented elderly group in the present study comprised MCI and also SCD cases, and thus more cases without AD pathology. Moreover, the group size in the present study was 8-fold larger, and adjusted for age and gender, which may all to some extent have contributed to the observed differences in association between current and previous studies. Clu promotes neuroprotective or regenerative processes, including neurite outgrowth and network complexity in reaction to neuronal damage, a capacity thought to be impaired in the ApoE ε4 isoform (Lidström et al., 1998; Wicher et al., 2008). Because in brain homogenates of patients with AD with *APOE*ε4 carriership ApoE levels were lower and Clu higher, it has been suggested that Clu substitutes ApoE when levels or functional activity of ApoE is reduced (Bertrand et al., 1995). Such a substitution of ApoE by Clu may explain the mediating effect of Clu on the CSF ApoE and tau association.

Previous research indicated that ApoA1 was of influence in predementia stages of AD (Saczynski et al., 2007; Slot et al., 2017; Song et al., 2012), but we found no mediating effect of CSF ApoA1 on relations between *APOE*ε4 carriership and CSF Aβ42 or tau. In our study CSF ApoA1 was associated with CSF Clu, possibly because both colocalize in HDL particles, but ApoA1 did not relate to either CSF ApoE, Aβ, or tau levels. ApoA1 and Clu both have neuroprotective properties and may be upregulated in response to Aβ-related AD pathology (Dermattos et al., 2004; Paula-Lima et al., 2009; Wyatt et al., 2011). However, although CSF Clu levels probably increase because of local production in cerebro, those of ApoA1, which is mainly produced in the liver, probably increase because of enhanced transport over the BBB (Stukas et al., 2014). CSF or plasma ApoA1 have not been previously reported to be correlated with CSF or plasma Aβ42, but plasma ApoA1 levels were associated with plasma $A\beta_{1-40}$ in cerebral amyloid angiopathy patients, and apoA1 was suggested to be a physiological transporter of soluble Aβ at the peripheral level (Montañola et al., 2016). This may differ from the situation in the brain parenchyma as Clu facilitated $A\beta_{1-40}$ efflux over the BBB in an in vitro model using mouse cerebral capillary endothelial cells, whereas ApoA1 did not (Merino-Zamorano et al., 2016). In summary, further research is needed to investigate the differential roles of these 2 apolipoproteins, both with neuroprotective properties but with distinct roles in cholesterol and presumably also Aβ transport.

Our findings suggest a differential role for ApoE and Clu in individuals with SCD and MCI with AD pathology, which are both stages in which patients are not demented. Strengths of the study include the large sample of nondemented elderly with CSF biomarkers measured after stringent procedures and extensive standardized clinical investigation in a memory clinic setting. It is of note that not all individuals with MCI, and even less with SCD, eventually progress to dementia because of AD. Memory complaints and cognitive deficits may have various other underlying causes, such as vascular cognitive impairment and depression among others (Mewton et al., 2014; Serra et al., 2013). Therefore, evaluation of the observed mediation effects in relation to clinical subtypes and follow up of clinical progression of participants may be of much interest. Unfortunately, the limited sample size of subsets and the lack of postmortem neuropathologic data reviewing postmortem diagnoses did not allow such an evaluation. The lack of individuals with dementia because of AD in our project can be considered a limitation of this study, as we could not comprise the apolipoproteins within the whole AD continuum. Either way, evaluating nondemented patients may provide more information of the role of apolipoproteins in preclinical and prodromal stages of AD. Another limitation of the study includes the lack of experimental evaluation of the proposed associations, generated by a statistical approach using mediation analysis. Mediation analysis is a useful tool to evaluate the influence of a certain "mediator" on a proposed relationship. However, it does not imply a direction of the relationship, nor does it imply a causal relation. Evaluation of variables by mediation analysis is purely statistical and theoretical. Therefore further experimental evaluation of the type and extent of interaction between analytes, and also *APOE* genotype, is essential to further understand their impact on AD pathophysiology.

In conclusion, in nondemented elderly, CSF ApoE and Clu influenced the relation between *APOE*ε4 allele frequency and CSF tau, but not CSF Aβ42. These findings suggest that in predementia stages of AD, *APOE*ε4 genotype may contribute to the pathophysiology of AD via Aβ-independent pathways as part of the cascade leading to Alzheimer pathology.

## Disclosure

R.S., M.K., A.H., W.J., F.B., and R.V., report no disclosures. C.T. serves on the advisory board of Fujirebio and Roche, performed contract research for IBL, Shire, Boehringer, Roche, and Probiodrug, and received lecture fees from Roche and Axon Neurosciences. P.S. has received grant support for the institution Alzheimer Center, VU University Medical Center from GE Healthcare and MERCK; he has received speaker's fees paid to the institution Alzheimer Center, VU University Medical Center, from Lilly, GE Healthcare, and Roche. W.F. has received research funding and speaker honorarium from Boehringer Ingelheim, all funds were paid to her institution the Alzheimer Center, VU University Medical Center.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.neurobiolaging.2019.02.017.

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