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ORIGINAL PAPER

Total apolipoprotein E levels and specific isoform composition in cerebrospinal fluid and plasma from Alzheimer's disease patients and controls

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Abstract The apolipoprotein E (ApoE) ε4 allele is the strongest risk factor of sporadic Alzheimer's disease (AD), however, the fluid concentrations of ApoE and its different isoforms (ApoE2, ApoE3 and ApoE4) in AD patients and among APOE genotypes (*APOE* ε2, ε3, ε4) remain controversial. Using a novel mass spectrometry-based method, we quantified total ApoE and specific ApoE isoform concentrations and potential associations with age, cognitive status, cholesterol levels and established AD biomarkers in cerebrospinal fluid (CSF) from AD patients versus non-AD individuals with different *APOE* genotypes. We also investigated plasma total ApoE and ApoE isoform composition in a subset of these individuals. In total $n = 43$ AD and $n = 43$ non-AD subjects were included. We found that CSF and plasma total ApoE levels did not correlate with age or cognitive status and did not differ between AD and non-AD subjects deeming ApoE as an unfit diagnostic marker for AD. Also, whereas CSF ApoE levels did not vary between *APOE* genotypes *APOE* ε4 carriers exhibited significantly decreased plasma ApoE levels attributed to a specific decrease in the ApoE4 isoform concentrations. CSF total ApoE concentrations were positively associated with CSF, total tau, tau phosphorylated at Thr181 and Aβ1-42 of which the latter association was weaker and only present in *APOE* ε4 carriers indicating a differential involvement of ApoE in tau versus Aβ-linked neuropathological processes. Future studies need to elucidate whether the observed plasma ApoE4 deficiency is a life-long condition

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in *APOE* ε4 carriers and whether this decrease in plasma ApoE predisposes A*POE* ε4 carriers to AD.

Keywords Apolipoprotein E · Cerebrospinal fluid · Isoform · Mass spectrometry · Plasma

Abbreviations

Aβ	Amyloid-β
AD	Alzheimer's disease
ApoE	Apolipoprotein E
BBB	Blood–brain barrier
CNS	Central nervous system
CSF	Cerebrospinal fluid
HDL	High-density lipoprotein
LDL	Low-density lipoprotein
MMSE	Mini-mental state examination
QAlb	CSF/plasma albumin ratio
SNP	Single nucleotide polymorphism
T-tau	Total tau
P-tau	Tau phosphorylated at Thr181

Introduction

Human Apolipoprotein E (ApoE) is a 299-amino acid protein with a molecular mass of ~34 kDa encoded by the *APOE* gene [23]. The *APOE* gene has three major polymorphic alleles: ε2, ε3 and ε4, with a worldwide frequency of about 8, 78 and 14 %, respectively [9]. The three ApoE isoforms differ in only one or two amino acid residues at positions 112 and 158, where either cysteine or arginine is present: ApoE2 (Cys112, Cys158), ApoE3 (Cys112, Arg158) and ApoE4 (Arg112, Arg158). Thus, six genotypes are possible: ε2/2, ε2/3, ε2/4, ε3/3, ε3/4 and ε4/4 [46].

ApoE plays a key role in lipid and cholesterol transport and lipoprotein metabolism in the central nervous system (CNS) and periphery. This protein is produced differentially in the central and peripheral compartments and it does not cross the blood–brain barrier (BBB) [21]. In peripheral tissues, ApoE is primarily produced by the liver and macrophages while in the brain it is produced by astrocytes and microglia [20].

The ApoE2 and ApoE4 isoforms differ in only one amino acid as compared to ApoE3, but this substitution affects their structure and function [12]. In fact, these two isoforms seem to increase the number of atherogenic lipoproteins, accelerate atherogenesis [24] and are associated with greater cardiovascular disease risk [18].

APOE ε4 is the major risk factor of late-onset Alzheimer's disease (AD) with an average frequency of 49 % [48] versus 14 % in general population [9], whereas *APOE* ε2 has a protective effect [4]. Further, the *APOE* ε4 allele frequency is significantly higher across the Lewy body disease spectrum, including dementia with Lewy bodies and Parkinson's disease dementia [41].

Several mechanisms relating ApoE isoforms to AD pathology have been reported, including both amyloid-β (Aβ)-dependent and non-dependent mechanisms [20]. It has been suggested that ApoE negatively may influence clearance of soluble Aβ and deposition of insoluble Aβ at different stages of plaque formation [30]. For instance, in vitro studies suggest that ApoE negatively affects the cellular uptake of Aβ by primary human astrocytes and microglia isolated from post-mortem brains, in an Aβ aggregation-dependent manner [29, 30]. Also, the clearance of Aβ may be ApoE isoform-dependent [3], but the ability of ApoE to influence Aβ-clearance or aggregation appears not be mediated through direct interaction with soluble Aβ, but through its actions with other interacting receptors and transporters [47]. Recently, it was also demonstrated that introduction of human ApoE4 by use of gene therapy into the brains of two different AD mouse models at ages where these already had developed amyloid plaque pathology, aggravated amyloid pathology. In contrast, introduction of ApoE2 in the same mouse models led to enhanced Aβ clearance and reduced plaque pathology [15]. On the other hand, the alleged efficiency of ApoE-directed pharmaceutical therapeutics in the clearance of Aβ from brain [5] remains controversial and it has not been replicated yet [17]. Based on the strong evidence linking ApoE to neurodegenerative disease, numerous studies with varying outcomes have aimed to assess whether the APOE genotype affects cerebrospinal fluid (CSF), plasma and brain ApoE protein levels in individuals carrying different APOE genotypes. In various mouse models, ApoE levels have been shown to vary genotype dependently with ApoE4 expressing animals exhibiting the lowest and ApoE2 expressing animals the highest ApoE levels in CSF and plasma [31]. Thus, not only the different isoforms per se may alter risk of AD but the different ApoE concentrations may also be an AD risk-modulating factor.

Previous studies reporting total ApoE concentrations in patients with AD or mild cognitive impairment and non-demented individuals showed different and inconclusive results in CSF and plasma samples [6, 7, 31, 36, 39]. Importantly, studies aiming to elucidate the isoform composition of total ApoE in CSF and plasma as well as the association between these and established AD CSF biomarkers are incomplete. Therefore, the aims of this study were: (a) to determine the total ApoE as well as the specific ApoE isoforms levels in a cohort of non-demented elders and AD patients of various APOE genotypes using a mass spectrometry-based assay; (b) to evaluate the association between CSF and plasma levels of total ApoE; (c) to study the total ApoE isoform composition in CSF and plasma; and (d) to evaluate associations between total ApoE/ApoE isoform levels and age, gender, cholesterol levels and CSF AD biomarkers.

Methods

Sample collection

Cerebrospinal fluid samples were obtained at the Memory Clinic at Skåne University Hospital in Malmö (Sweden) from subjects with AD ($n = 38$) and non-AD individuals ($n = 37$). From these individuals also plasma samples were available from $n = 14$ non-AD and $n = 31$ AD subjects. We further included another $n = 11$ individuals, $n = 5$ were AD and $n = 6$ non-AD individuals and from whom only plasma samples were available. In total $n = 43$ non-AD and $n = 43$ AD, individuals were included in this study. The non-AD individuals experienced subjective cognitive symptoms at baseline, but detailed clinical investigation as well as clinical follow-up revealed that these subjects were not affected by any dementia disorder or neurological disease. Subjects diagnosed with AD met the DSM-IIIR criteria for dementia [27] and the criteria for probable AD, as defined by NINCDS–ADRDA [26]. Lumbar puncture was performed and the samples were handled as described before [49]. Briefly, 10–12 mL CSF was collected, centrifuged, aliquoted and frozen at −80 °C in polypropylene tubes within an hour of sample collection. Plasma samples were collected in tubes containing EDTA, centrifuged at 2,000*g* at 4 °C for 10 min, frozen and stored at −80 °C pending biochemical analysis. All individuals underwent brain imaging, neurological, psychiatric and cognitive examinations. Diagnostic laboratory testing including ApoE genotyping by use of restriction isotyping [14], quantitative determination of the CSF/plasma albumin quotient (QAlb) using nephelometry (as an indicator of the BBB function [1]) as well as CSF AD biomarker analysis were performed in clinical routine. All individuals gave informed consent either by use of a passive consent procedure where consent for retrospective use of banked clinical samples and data was assumed if individuals did not actively retract permission, as instructed in repeated local press advertisements, or by active written informed consent. Informed consent was documented in two separate registries including the patient medical chart and in the local clinical research database. The study protocol was approved by the local ethics committee at Lund University Sweden and conducted according to the Helsinki Declaration.

Analysis of CSF and plasma total cholesterol

CSF and plasma total cholesterol levels were analyzed using the fluorometric Amplex® Red Cholesterol Assay Kit (Invitrogen) according to instructions by the manufacturer. CSF and plasma samples were diluted to 1:2 and 1:500, respectively, prior to analysis.

Analysis of Aβ1-42, total tau and tau phosphorylated at Thr181

The CSF samples were analyzed in routine as previously described with commercially available enzyme-linked immunosorbent assays (ELISAs) (Innogenetics, Ghent, Belgium) to determine the levels of Aβ1-42, total tau (T-tau) and tau phosphorylated at Thr181 (P-tau) (INNOTEST® β-AMYLOID$_{(1-42)}$, hTAU Ag and PHOSPHO-TAU$_{(181P)}$), respectively [2, 43, 44].

Analysis of ApoE concentration

Total ApoE and ApoE isoform concentrations were determined, in a blinded manner, using a mass spectrometry-based assay as previously described [25]. In brief, three tryptic peptides derived from the two major ApoE single nucleotide polymorphisms (SNP112 and SNP158) were used for quantification of the three ApoE isoforms: CLAVYQAGAR (ApoE2), LAVYQAGAR (ApoE3) and LGADMEDVR (ApoE4). For ApoE phenotype identification, the presence of different combinations of four peptides (the three previously mentioned plus peptide LGADMEDVCGR (ApoE2 and 3)) was used.

Twenty-five microliters of CSF and 15 μL of plasma (previously diluted 1:100) were prepared as previously described [25]. Then, samples were analyzed by liquid chromatography-tandem mass spectrometry with an EASY-nLC 1000 (Thermo Fisher, Odense, Denmark) coupled online to a triple-quadrupole mass spectrometer (TSQ Vantage, Thermo Fisher, San Jose, USA).

The method showed excellent linearity (coefficient of determination, $r^2 = 0.99$) and reproducibility (within-laboratory imprecision <13 %) for the three ApoE isoforms studied. The ApoE phenotype was successfully assigned to all samples analyzed when compared with genetic testing results (100 % success rate). For a complete description of this mass spectrometry-based assay, see Ref. [25].

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistic software, version 20. A p value <0.05 was considered statistically significant. Normal distribution was evaluated using Shapiro–Wilk test and by inspection of Q–Q plots.

Student t- and Mann–Whitney U tests (two-tailed) were performed for comparisons between two groups of independent samples while one-way ANOVA and Kruskal–Wallis tests were performed for comparisons between more than two groups, when the variables were normally and not normally distributed, respectively. Associations between variables were reported by Spearman's rank correlation coefficient.

Results

Patient demographics

In total eighty-six individuals were included in this study whereof half ($n = 43$) were non-demented individuals and half ($n = 43$) were clinically diagnosed with AD. Study subject characteristics are described in Table 1. Statistically significant differences ($p < 0.001$) in age, total mini-mental state examination (MMSE) scores, CSF levels of T-tau, P-tau and Aβ1-42 concentrations were found between AD patients and non-AD individuals. No significant differences were observed for QAlb used as an indicator of BBB integrity between the two groups ($p = 0.75$) (Table 1). As previous studies have suggested an association between *APOE* ε4 and impairment of the BBB integrity [51] we also compared the QAlb between *APOE* ε4 carriers ($n = 40$) and non-carriers ($n = 44$) revealing no difference in BBB integrity between these two groups ($p = 0.17$).

APOE genotype and sample collection

APOE genotype frequencies are described in Table 2. As expected, the percentage of *APOE* ε4 carriers was significantly higher ($p < 0.001$) in the AD group (72 %) compared to the non-AD group (23 %).

ApoE concentration, age and gender

As previous results have suggested significant links between ApoE levels, gender and age of which the latter two were reported to explain 3.9 % of ApoE concentration variability in CSF [6], we also analyzed potential correlations between these factors in the current cohort. We found no significant associations between total ApoE and age in CSF (Spearman's rho = 0.16, $p = 0.17$) or plasma (Spearman's rho = 0.008, $p = 0.95$) samples, however, we acknowledge that our clinical sample size may be too small to detect significant age correlations. As the age and gender distribution indeed differed between the two investigated groups we modeled the effect of age and gender using multivariate analysis. We found no effect of gender (data not shown), however, we detected a significant effect of age on total CSF, but not plasma ApoE levels ($p = 0.017$), which was taken into consideration in the subsequent statistical analysis. In line with no detectable effect of gender on the quantified ApoE levels in plasma and CSF there was no significant difference in total ApoE concentration between men and women in CSF ($p = 0.68$) or plasma ($p = 0.29$).

Table 1 Subject characteristics

Diagnosis	Age (years)	Gender % (M/F)	MMSE score	Plasma cholesterol (μg/mL)	CSF cholesterol (μg/mL)	CSF T-tau (ng/L)	CSF P-tau[a] (ng/L)	CSF Aβ1-42 (ng/L)	QAlb[b]
Non-AD $n = 43$	61 (43–80)	47/53	29 (24–30)	1,012 ± 180	1.64 ± 0.35	323 (89–690)	41 (20–87)	539 ± 149	6.7 (2.4–19.1)
AD $n = 43$	78 (60–94)[d]	36/64	18 (4–23)[d]	1,029 ± 312	1.66 ± 0.35	700 (360–1,990)[d]	88 (44–226)[d]	335 ± 99[c]	7.1 (3.7–21.0)

Data are presented as mean ± standard deviation and median (minimum–maximum)

AD Alzheimer's disease, *MMSE* mini-mental state examination, *M* male, *F* female

[a] CSF P-tau levels were obtained from $n = 40$ non-AD and $n = 43$ AD patients

[b] CSF/plasma albumin ratio (QAlb) was obtained from $n = 41$ controls and $n = 43$ AD patients

[c] Indicates a significant difference at the $p < 0.001$ level compared to controls (independent *t* test)

[d] Indicates a significant difference at the $p < 0.001$ level compared to controls (Mann–Whitney *U* test)

Table 2 *APOE* genotype frequencies

Diagnosis	APOE ε4 carriers (%)	APOE ε2/3 (*n*)	APOE ε2/4 (*n*)	APOE ε3/3 (*n*)	APOE ε3/4 (*n*)	APOE ε4/4 (*n*)
Non-AD $n = 43$	23	7	1	26	7	2
AD $n = 43$	72[a]	2	1	10	23	7
Total $n = 86$	48	9	2	36	30	9

[a] Indicates a significant difference at the $p < 0.001$ level compared to controls

Total ApoE concentrations in AD versus non-AD cases and individuals with varying APOE ε4 status

Our results reveal no significant differences in CSF ($p = 0.52$) and plasma ($p = 0.77$) from AD and non-AD cases, (Fig. 1a, b). Also, neither CSF nor plasma levels of total ApoE were significantly linked to cognitive status as determined by MMSE test scores (data not shown). Similarly, the analysis of total ApoE concentrations across genotypes in the two diagnostic groups showed no significant differences in CSF samples ($p = 0.45$) (Fig. 1c). Clear differences were, however, found in the plasma samples ($p = 0.00008$) in which ApoE concentrations were genotype dependent, with the lowest levels found in *APOE* ε4 homozygotes ε2/3 > ε3/3 > ε3/4 > ε4/4 (Fig. 1d). When pooling the data from AD and non-AD individuals, to increase statistical power ($n = 86$), we documented pronounced differences in plasma total ApoE concentrations between *APOE* ε4 non-carriers and carriers. More specifically, significant differences in plasma but not CSF total ApoE concentrations were observed across *APOE* ε4 status groups (−/−, +/− and +/+) (Fig. 1e, f) with *APOE* ε4 non-carriers exhibiting the highest and *APOE* ε4 homozygotes the lowest ApoE levels (ApoE ε4: −/− > +/− > +/+) ($p = 0.0009$). Post hoc testing further confirmed significant differences between *APOE* ε4

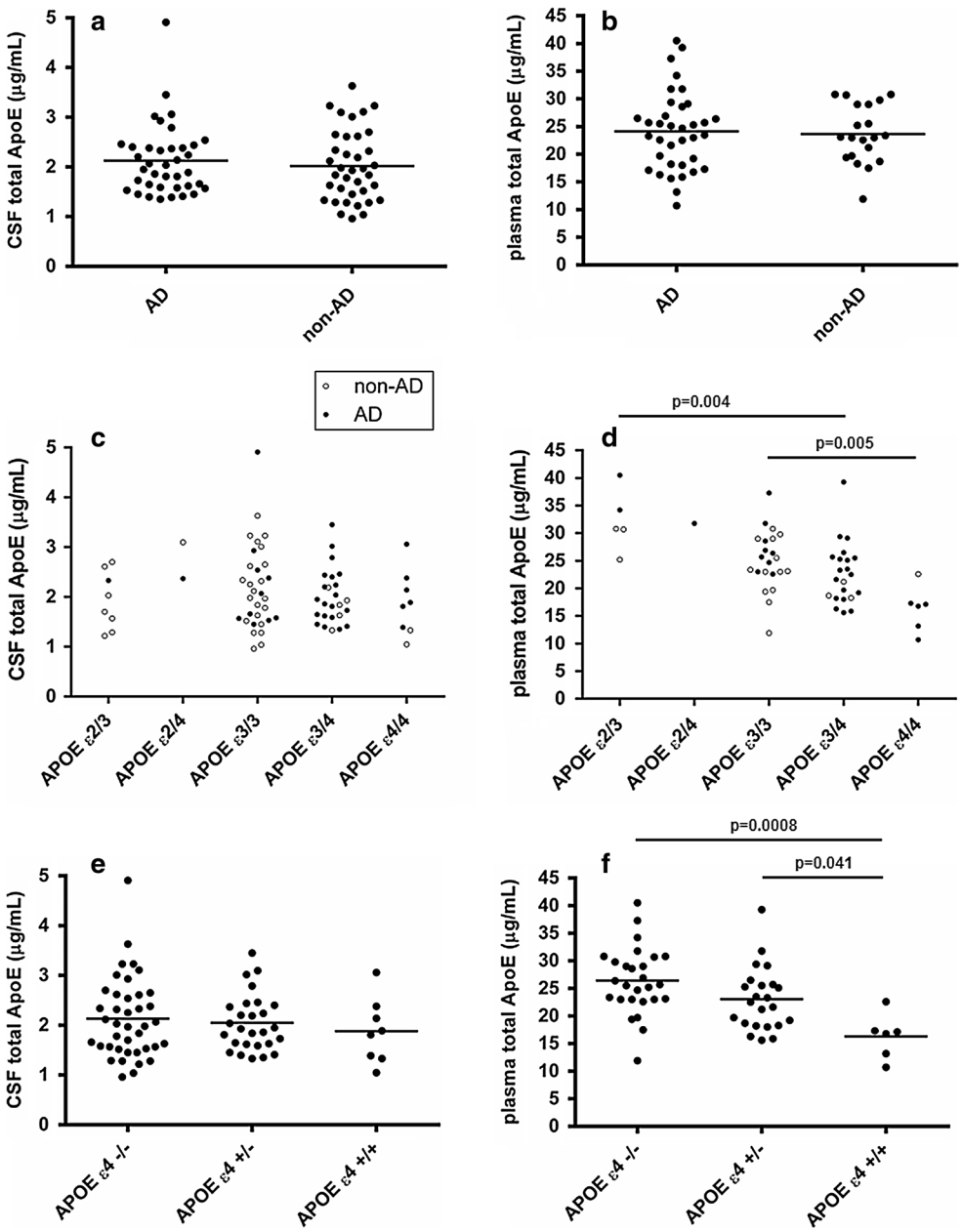

Fig. 1 Total ApoE concentration in CSF and plasma samples in AD and non-AD individuals (**a**, **b**), across different genotypes (**c**, **d**) and according to the *APOE* ε4 status (−/−, +/− and +/+) (**e**, **f**). Student *t* test and one-way ANOVA with bonferroni post doc testing were performed for comparisons between two and more than two groups of samples, respectively

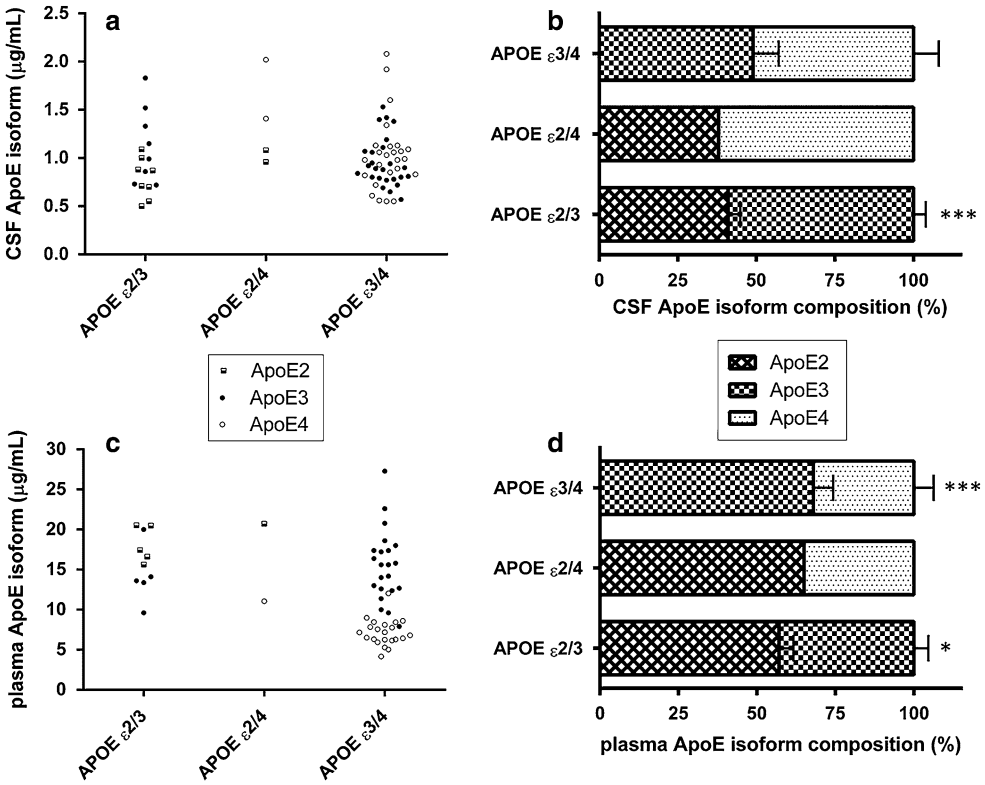

Fig. 2 ApoE isoform concentration and composition across heterozygous individuals (ε2/3, ε2/4 and ε3/4), in CSF (**a**, **b**) and plasma samples (**c**, **d**). Statistical analysis for ApoE isoform composition was performed with paired *t* test

−/− and *APOE* ε4 +/+ carriers ($p = 0.0008$) and between *APOE* ε4 +/− and *APOE* ε4 +/+ ($p = 0.041$) (Fig. 1f) suggesting an allele dose-dependent relationship between *APOE* ε4 and ApoE plasma concentrations. We found no significant association between CSF and plasma total ApoE concentrations (Spearman's rho = 0.07, $p = 0.67$) in the forty-five individuals with matching CSF and plasma samples.

CSF and plasma total ApoE isoform composition

To our knowledge, the specific ApoE isoform concentrations and total ApoE composition of different ApoE isoforms in heterozygous individuals have not yet been reported. Here we describe similar concentrations of the ApoE3 and ApoE4 isoforms in CSF from *APOE* ε3/4 individuals ($p = 0.42$) (Fig. 2a) yielding an approximate 50–50 % contribution of each isoform to the total CSF ApoE concentrations (Fig. 2b). In *APOE* ε2/3 individuals the total CSF ApoE concentrations, however, contained significantly ($p = 0.0004$) more ApoE3 than ApoE2 (Fig. 2a) of which the former constituted roughly 60 % of the total CSF ApoE levels (Fig. 2b). Comparing the total ApoE composition in CSF from *APOE* ε4 heterozygotes, ApoE4 concentrations were similar or equal to the concentrations of the other non-ApoE4 isoform (51.8 ± 8.4 vs. 48.2 ± 8.4 %, $p = 0.27$).

Contrasting to the CSF findings, subjects with the *APOE* ε3/4 genotype had significantly lower plasma levels of the ApoE4 isoform versus the ApoE3 ($p < 0.00001$) (Fig. 2c). The ApoE4 isoform concentrations only constituted about 30 % of the total plasma ApoE concentration in *APOE* ε3/4 individuals (Fig. 2d). The same results were observed in plasma of the ApoE ε2/4 phenotype. Further contradicting the corresponding CSF findings, the total plasma ApoE contained significantly ($p = 0.03$) more ApoE2 than ApoE3 in individuals with the *APOE* ε2/3 genotype (Fig. 2c) in which almost 60 % of the total plasma ApoE was composed of the ApoE2 isoform (Fig. 2d). There was no difference in the concentrations of ApoE3 and ApoE4 isoforms in plasma and CSF from AD versus non-AD individuals of which all were of the *APOE* ε3/4 genotype (data not shown). However, the distribution differences of ApoE4 in total plasma versus CSF ApoE concentrations differed significantly between the two compartments ($p < 0.001$). These results further support the notion of differential synthesis of ApoE between the central and peripheral compartments [19, 21].

ApoE concentration and total cholesterol

Since ApoE is known to be a major transporter of cholesterol, especially in the CNS, we investigated the levels of total cholesterol and potential associations with the ApoE levels both in CSF and plasma. Our analyses showed no difference in CSF or plasma total cholesterol concentrations between AD and non-AD individuals (Table 1), or between

APOE ε4 carriers and non-carriers (data not shown). Further, there was a highly significant association between total ApoE and total cholesterol in CSF samples (Spearman's rho = 0.46, $p = 0.00004$), but not in plasma (Spearman's rho = 0.25, $p = 0.07$) (Figure S1a, b). When looking into the association between CSF ApoE and cholesterol in *APOE* ε3/4 carriers only ($n = 25$), we found that the specific ApoE4, but not ApoE3, isoform concentrations were positively linked to CSF total cholesterol levels (Spearman's rho = 0.46, $p = 0.02$ vs. rho = 0.25, $p = 0.22$).

CSF ApoE concentration and AD biomarkers

To explore possible links between total ApoE and specific ApoE isoform concentrations, and well-established biomarkers of AD pathophysiological processes we analyzed the relationship between the CSF ApoE and CSF levels of T-tau, P-tau and Aβ1-42. We found that the total CSF ApoE concentrations were positively associated with CSF Aβ1-42, T-tau and P-tau (Figure S2a–c). Further, both the individual levels of the ApoE isoforms 3 and 4 were significantly and positively associated with CSF T-tau and CSF P-tau concentrations (Table 3). These associations were present in both diagnostic groups and hence appear to be linked irrespective of AD diagnosis. Further, whereas the strong correlations between CSF ApoE, T-tau and P-tau were present in both *APOE* ε4 carriers and non-carriers, the weaker link between total CSF ApoE and CSF Aβ1-42 was only present in *APOE* ε4 carriers (Table 4). No associations were found between total plasma ApoE or individual ApoE isoform concentrations, and the AD biomarkers (data not shown).

Our results demonstrating a significant, but rather weak association between CSF Aβ1-42 and ApoE are in line with those previously presented by Cruchaga et al. [6]. These authors also presented differences in CSF ApoE concentrations between individuals with high versus low CSF Aβ1-42 levels. Employing the same CSF Aβ1-42 cut-off level (500 pg/mL) which in an earlier study was shown to be indicative of cortical Aβ deposition as determined by Pittsburgh Compound-B (PiB) imaging [8] we found no difference in CSF total ApoE levels between subjects with high versus low Aβ burden (<500 pg/mL ($n = 54$) versus >500 pg/mL ($n = 21$) CSF Aβ1-42, Mann–Whitney *U* test, $p = 0.064$). Comparing the same groups, we also did not find any difference in plasma total ApoE levels (Mann–Whitney *U* test, $p = 0.312$).

Table 3 CSF ApoE isoform associations with tau

ApoE isoform	CSF T-tau	CSF P-tau
CSF ApoE3[a]	(A) 0.516	(A) 0.465
	$p = 0.002$	$p = 0.008$
	($n = 32$)	($n = 31$)
	(B) 0.514	(B) 0.531
	$p = 0.009$	$p = 0.006$
	($n = 25$)	($n = 25$)
CSF ApoE4[b]	(A) 0.162	(A) 0.237
	$p = 0.440$	$p = 0.253$
	($n = 25$)	($n = 25$)
	(B) 0.881	(B) 0.881
	$p = 0.004$	$p = 0.004$
	($n = 8$)	($n = 8$)

[a] CSF ApoE3 isoform concentrations from (A) ε3/ε3 ($n = 32$) and (B) ε3/ε4 ($n = 25$) individuals

[b] CSF ApoE4 isoform concentrations from (A) ε3/ε4 ($n = 25$) and (B) ε4/ε4 ($n = 8$) individuals

Table 4 Total CSF ApoE and AD biomarker associations in *APOE* ε4 carriers and non-carriers

CSF Total ApoE	CSF T-tau	CSF P-tau	CSF Aβ1-42
APOE ε4 Carriers	0.426	0.440	0.385
	$p = 0.011$	$p = 0.008$	$p = 0.023$
	($n = 35$)	($n = 35$)	($n = 35$)
APOE ε4 Non-carriers	0.497	0.552	0.263
	$p = 0.001$	$p < 0.001$	$p = 0.101$
	($n = 40$)	($n = 39$)	($n = 40$)

Discussion

To the best of our knowledge, this is the first study reporting both CSF and plasma human total ApoE levels as well as the specific ApoE isoform composition thereof using a mass spectrometry-based quantification assay. *APOE* ε4 is the strongest and most well-documented risk factor for late-onset AD, however, the mechanism by which this ApoE isoform is involved in the development of the disease remains unclear. In the present study, we found that CSF levels of ApoE did not correlate to cognitive performance as determined using the MMSE test and further did not differ between AD and non-AD cases or among individuals of different *APOE* genotypes. In contrast, *APOE* ε4 carriers exhibited a significant decrease in plasma total ApoE levels attributed to a specific decrease in the ApoE4 isoform. Whereas the total ApoE isoform distribution was roughly 50/50 % between ApoE3 and ApoE4 in CSF from *APOE* ε3/4 individuals, total plasma ApoE contained a 70/30 % ApoE3/ApoE4 isoform distribution. Last, we describe a strong association between CSF total ApoE as well as individual ApoE3 and ApoE4 isoform concentrations, and CSF

tau levels. Only a weak, but significant link between CSF Aβ1-42 and total CSF ApoE was detected specifically in *APOE* ε4 carriers suggesting that ApoE may be differentially associated with Aβ and tau pathology.

Several in vivo studies on rodents expressing human ApoE isoforms have reported genotype-dependent variability in ApoE concentrations with the lowest levels found for ApoE4 [15, 31] suggesting that concentration-dependent differences between the three isoforms may be one of the factors contributing to disease. Concentrations may be of relevance as ApoE was shown to interact with Aβ in a dose- and isoform-specific manner affecting Aβ oligomerization [22, 33]. Indeed ApoE can also be immunohistochemically identified in diffuse as well as neuritic plaques in the human brain [45] and appears to differently affect cellular uptake of Aβ oligomers and fibrils in primary human astrocytes and microglia [29, 30]. It is also well-known that especially *APOE* ε4 carriers begin to exhibit AD-related pathology already during middle age while still asymptomatic [16].

Previous attempts to quantify the total ApoE concentrations in human CSF and/or plasma samples [6, 7, 31, 36, 39] have yielded different results, probably due to the limitations of analytical method utilized for ApoE isoforms detection. Poor correlation between ApoE concentrations using different immunoassays has been reported [6]. Besides, the high similarity in the protein sequence of ApoE isoforms (only one or two different amino acids) may cause a lack of reliability of these methods. Mass spectrometry has emerged as a technology that offers novel applications and can solve some of the limitations of immunoassays, including the required sequence specificity to discriminate and measure ApoE isoforms. We previously developed an accurate and precise mass spectrometry-based assay for the analysis of ApoE isoforms in CSF and plasma samples [25], which in addition to allowing us to quantify the specific ApoE isoforms also enables us to identify the ApoE phenotype with 100 % accuracy.

In the present study, we analyzed the total ApoE concentrations as well as the individual ApoE isoform concentrations in CSF and plasma samples from patients with AD and non-AD subjects. We did not find any significant influence of age on CSF and plasma ApoE levels and total ApoE concentrations were similar between men and women. As previous studies have reported a positive association between ApoE concentrations, age and gender [6, 7] we do not rule out that such links do exist and acknowledge that the statistical power of the herein investigated sample may be too low to detect such associations. Further, no significant differences in CSF or plasma total ApoE levels were observed between AD patients and non-AD subjects and in line with these results we did not detect any association between ApoE levels and cognitive performance. Results from a meta-analysis including thirty-eight studies previously suggested a relatively small, but specific effect of the *APOE* ε4 genotype on certain domains of cognitive performance [37], however, attempts to investigate in detail the potential associations between ApoE fluid levels and different cognitive domains are still lacking. Acknowledging that the MMSE test is a rather bold method for the measurement of performance in different cognitive domains, our results are in line with other studies reporting missing links between ApoE levels and cognitive test scores confirming that total ApoE concentrations are not informative for the diagnosis of AD [34, 36]. In support, a study by Toledo et al. recently reported similar CSF ApoE levels between controls, MCI and AD patients included in the Alzheimer's disease neuroimaging initiative (ADNI). The same study, however, also demonstrated an association between lower CSF ApoE levels, longitudinal cognitive decline, structural MRI changes and conversion from MCI to AD. Interestingly, whereas the authors showed a stronger association between plasma ApoE levels and a baseline clinical diagnosis of MCI rather than AD they found no correlations between plasma ApoE levels, conversion from MCI to AD or longitudinal cognitive performance as indicated by ADAS-Cog scores. Although Toledo et al. [40] claimed it to be less probable, they discussed the possibility of altered plasma ApoE levels contributing to AD pathological events which plateau prior to the onset of cognitive symptoms, we would not rule out such a scenario. Future prospective studies including repeated determination of ApoE fluid concentrations to elucidate early effects of altered ApoE levels on cognitive performance and disease biomarkers are highly warranted.

In regard to previously proposed effects of *APOE* genotype on ApoE fluid levels, we did not find any significant differences in CSF total ApoE concentration between individuals of different *APOE* genotypes. Other authors reported contradictory results in CSF samples. Thus, while some studies showed significantly lower CSF ApoE4 levels in mice [31] and humans [6], Darreh-Shori et al. [7] reported higher CSF ApoE levels in *APOE* ε4 carriers. We believe that by use of a method that is not biased by antibody-affinity differences to different ApoE isoforms, the herein reported ApoE concentrations are highly reliable.

By specific quantification of the different ApoE isoforms, we were also able to determine the distribution between the two isoforms in total ApoE in both CSF and plasma from heterozygous individuals (*CSF* ApoE 2/3: 43/57 %, ApoE 2/4: 38/62 % and ApoE 3/4: 49/51 %. *Plasma* ApoE 2/3: 57/43 %, ApoE 2/4: 65/35 % and ApoE 3/4: 68/32 %). The distribution of the different ApoE isoforms was not reflected in total ApoE concentrations except for the ApoE levels in plasma from heterozygous *APOE* ε4 carriers. Thus, in line with previous studies [11, 28, 38] we

observed significantly lower plasma total ApoE concentrations in *APOE* ε4 carriers with the lowest concentrations observed in *APOE* ε4 homozygotes. The plasma ApoE concentration difference was *APOE* genotype dependent, with the following order: ε2/3 > ε3/3 > ε3/4 > ε4/4. As mentioned above, the percentage of ApoE4 in *APOE* ε3/4 carriers was, on average, 32 % which is similar to the findings of an earlier report showing that the contribution of the ApoE4 isoform to total plasma ApoE concentrations was on average 38 % [11]. Thus, the observed decrease in ApoE levels in *APOE* ε4 carriers is caused by a specific decrease in ApoE4 isoform concentrations. Very similar results were reported by other authors using a mass spectrometry assay. Wildsmith et al. [50] observed different turnover rates of ApoE in the periphery and CNS. While no significant differences between genotypes were observed in the CSF, statistically significant differential turnover rates for plasma ApoE isoforms (ε4 > ε3 > ε2) were reported. Simon et al. [36] reported the same influence of *APOE* genotype on total ApoE concentration in plasma samples, with concentrations: ε2/3 > ε2/4 > ε3/3 > ε3/4 > ε4/4. These results are in line with earlier findings presented by Gregg et al. [10] who showed that the catabolic rate of ApoE4 was significantly faster than that of ApoE3 when radiolabeled ApoE3 and ApoE4 were injected into normolipidemic subjects of the *APOE* ε3/ε3 genotype. The same authors showed that ApoE4 predominantly associates with the very low-density lipoprotein (VLDL) fraction whereas ApoE3 was found at a greater extent in high-density lipoproteins (HDL). Interestingly, the catabolism of ApoE4 was faster than that of ApoE3 irrespective of lipoprotein sub-fraction. Regardless of catabolic compartment, which appear to be several, the present determination of specific ApoE isoform ratios in heterozygous individuals will allow future ApoE-directed experimental studies to combine ApoE isoforms in the ratios physiologically occurring in humans.

As expected, we found no significant association between CSF and plasma total ApoE concentrations which is in line with previous studies [6, 50] confirming that ApoE is synthesized independently in the CNS and periphery and does not cross the BBB. Further we found a highly significant association between total ApoE and total cholesterol in CSF samples, but not in plasma. Plasma lipoproteins in humans are commonly divided into subclasses depending on size, density and electrophoretic mobility. Chylomicrons and VLDL carry mainly triglycerides whereas low-density lipoprotein (LDL) and HDL mainly transport cholesterol. ApoE in the periphery is associated with chylomicrons, VLDL and LDL, however, lipoproteins containing ApoE in the CSF have been described as resembling plasma HDL in size [13]. Thus the observed difference in association between plasma versus CSF concentrations of ApoE and total cholesterol is not entirely surprising. Interestingly, in *APOE* ε3/4 carriers in which the CSF isoform ratio was nearly identical (49 vs. 51 %), only ApoE4 levels were positively associated with CSF total cholesterol. These results do, therefore, not mirror the proposed differences in ApoE isoform-dependent cholesterol efflux from astrocytes and neurons in the CNS for which ApoE4 was reported to be less efficient than ApoE2 and ApoE3 (ApoE2 > ApoE3 > ApoE4) [42].

Last, we examined potential links between the levels of total ApoE and specific ApoE isoform concentrations, and levels of the three established AD biomarkers considered to reflect Aβ plaque load, neurofibrillary tangle formation and neurodegeneration [32]. Cruchaga et al. [6] previously reported a positive correlation between ApoE and Aβ1-42, however, Darreh-Shori et al. [7] reported a negative correlation between ApoE and Aβ1-42 but a positive correlation between ApoE and T-tau. We show that total CSF but not plasma ApoE concentration and CSF ApoE isoforms 3 and 4 were positively associated with higher CSF T-tau and P-tau levels. These results were irrespective of diagnostic group and further support the notion of CSF ApoE levels as an unfit diagnostic tool. Our findings are in line with the results reported by Toledo et al. who demonstrated positive correlations between CSF levels of ApoE and tau irrespective of *APOE* genotype and clinical diagnosis. They speculated that increased levels of tau (reflecting neuronal injury) would lead to increased levels of ApoE possibly to facilitate neuronal repair. Toledo et al. further hypothesized that truncated CSF ApoE4 fragments, which have been associated with tau phosphorylation, could lead to increased levels of P-Tau. As the authors of this particular study only measured total ApoE levels and not the individual ApoE levels they could not differentiate between associations between different ApoE isoforms and tau [40]. In the present study, we showed that both CSF ApoE3 and ApoE4 isoforms were linked to T-tau and P-tau thus a specific effect of truncated ApoE4 fragments only on the levels of P-tau is not supported by our results.

We found a significant but rather weak association between CSF total ApoE and Aβ1-42 concentrations. Further when dividing our sample into groups with low versus high CSF Aβ1-42 using a previously published cut-off value of 500 pg/mL [6, 8] we did not observe any differences in total CSF ApoE levels. Interestingly, the documented correlation between Aβ1-42 and total CSF ApoE in the present study was only present in *APOE* ε4 and not detected when screening for associations between Aβ1-42 and the individual ApoE isoforms. A previous study performed on post-mortem brain homogenates reported an inverse correlation between ApoE and Aβ tissue concentrations [35]. In the light of those previously reported results and the herein presented data it appears that increased levels of ApoE in the CNS may lead to increased Aβ clearance

which may be specifically important in *APOE* ε4 carriers, the latter as proposed by our results. However, the lack of individual correlations between Aβ1-42 and ApoE specific isoforms may in our study be due to insufficient power and thus potential individual associations should not be ruled out. Also, based on our cross-sectional study design the sequence of events cannot be determined. Thus, defining whether altered levels of Aβ and tau promote an increase in ApoE, or the other way around, remains to be determined in longitudinal follow-up studies in which the use of PiB imaging would be very informative.

Taken together, in this work employing a novel mass spectrometry-based method for ApoE quantification we aimed to clarify the reported inconsistencies in regard to CSF and plasma ApoE levels in AD patients versus non-AD individuals with different *APOE* genotypes. We show that there is no difference in total ApoE levels in neither plasma nor CSF between AD patients and non-AD individuals thus plasma and CSF levels of ApoE are unfit to serve as diagnostic biomarkers for AD. We further show that plasma ApoE levels but not CSF levels differ among *APOE* genotypes and that the observed decrease in plasma ApoE in *APOE* ε4 carriers is attributed to a specific ApoE4 isoform deficiency. Last we show that CSF ApoE levels correlate positively to both tau and Aβ of which the latter finding was evident in *APOE* ε4 carriers only. Future studies need to elucidate whether the observed ApoE4 plasma deficiency is a life-long condition in *APOE* ε4 carriers and whether this decrease in plasma ApoE concentrations predisposes these individuals to AD.

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