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PAPER IN FOREFRONT

Nano-HPLC–MS analysis of phospholipids in cerebrospinal fluid of Alzheimer's disease patients—a pilot study

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Abstract There is emerging evidence that lipids play an important role in many neurodegenerative processes, for example in Alzheimer's disease (AD). Although different lipid alterations in the AD brain have been reported, there have only been very few investigations of lipid changes in the cerebrospinal fluid (CSF). Recent developments in mass spectrometry (MS) have enabled fast and sensitive detection of lipid species in different biological matrixes. In this study we developed an on-line HPLC–MS method for phospholipid profiling in the CSF based on nano-HPLC separation using an Amide column and detection with electrospray (ESI) quadrupole–time of flight (QTOF) MS. We achieved good separation, reproducibility, and sensitivity in monitoring of the major phospholipid classes, phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), and sphingomyelin (SM) in CSF. To emphasize the applicability of the method, a pilot study was performed on a group of CSF samples ($N=16$) from individuals with probable AD and non-demented controls. We observed a statistically significant increase of SM levels (24.3±2.4%) in CSF from probable AD individuals vs. controls. Our findings indicate that SM levels in the CSF could potentially provide a new lead in AD biomarker research, and show the potential of the method for disease-associated CSF phospholipid screening.

Keywords Liquid chromatography · Mass spectrometry · Phospholipids · Sphingomyelin · Cerebrospinal fluid · Alzheimer's disease

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Introduction

Lipids play various roles in living organisms. They form lipid bilayers that provide structural integrity to cells and organelles, create an optimum physiological and chemical environment for protein interactions and functions, and also serve as an energy reservoir and precursors for many messenger molecules. Lipid levels are tightly regulated in specific parts of the human body. Because the nervous system has the second highest concentration of lipids in human body, lipid metabolism is highly important for its proper

functioning. Many neurological disorders, including bipolar disorders and schizophrenia, and neurodegenerative diseases, for example Alzheimer's, Parkinson's, and Niemann–Pick diseases, are associated with alteration of lipid metabolism [1–3].

Alzheimer's disease (AD) is the most common form of dementia among the elderly population. It is estimated that by the year 2040, about 81 million people will be suffering from AD [4]. The major hallmark of AD is the formation of amyloid plaques (aggregates of amyloid-β (Aβ) peptides) in the brain [5–7]. Aβ peptides are generated by sequential cleavage of β-amyloid precursor protein (APP) by β-secretase and γ-secretase. Alternatively, APP can be processed successively by α and γ-secretase, generating non-amyloidogenic peptide p3. Given the fact that APP and all three secretases are integral membrane proteins and that the γ-secretase cleavage of APP occurs within its transmembrane domain, it is pertinent to conclude that lipid surroundings may play an important regulatory role in APP processing, formation of Aβ peptides, and the pathogenesis of Alzheimer's disease. In support to this concept are numerous studies which have confirmed a connection between AD and lipids, especially cholesterol and sphingolipids [8]. Possible mechanisms have recently been suggested [9].

Changes in the lipid profile occurring in the brain of AD individuals have been reported, but only a few studies have attempted to characterize the lipid profile of cerebrospinal fluid and to identify the potential differences between AD individuals and controls [3, 10]. Cerebrospinal fluid (CSF) is the most informative fluid source for neurodegenerative disease prognosis and diagnosis, because of its constant physical contact with brain, hence reflecting the neurological disease state in the molecular pattern of CSF. Besides, compared with brain tissue it is readily obtainable by lumbar puncture and in that sense is a less invasive diagnostic method. Therefore, analysis of phospholipids and other lipids in CSF offers a potential for lipid biomarker discovery in neurological diseases.

Han and co-workers observed a significant decrease of sulfatides in the brain and CSF of probable AD individuals with very mild dementia, and proposed that sulfatides in CSF could be a useful biomarker for early diagnosis of AD [11, 12]. Significantly increased levels of ceramides were observed in the CSF from probable AD individuals compared with age-matched neurological controls [13]. Changes in membrane phospholipids occur very early in AD pathogenesis and may provide the molecular basis for synaptic loss in AD [14]. Altered total phospholipid levels [15] and phosphatidylcholine metabolites [16, 17] in the CSF from AD individuals have been documented in comparison with controls. However, to our knowledge, a comprehensive CSF phospholipid profiling of AD individuals versus controls has not yet been pursued.

Lipidomics is a rapidly expanding research field focused on identifying alterations in lipid metabolism and changes during pathological processes. Traditional high-performance liquid chromatography (HPLC) for phospholipid analysis has been brought at the forefront in combination with electrospray ionization mass spectrometry (ESI–MS), enhancing the discovery of lipids in complex mixtures by exploration of different separation criteria and highly sensitive detection. Currently, the challenge for phospholipidomics is the confident characterization of compositional variability in the context of healthy cell function and disease pathology.

The objective of this work was to develop a high-throughput-amenable and sensitive nano-HPLC–ESI-QTOF-MS method for phospholipid screening in the CSF. A pilot analysis of phospholipid pattern changes in the CSF of probable AD individuals and non-demented controls was carried out using the developed method.

Materials and methods

Reagents

HPLC-analytical grade water and ammonium acetate were obtained from Merck (Darmstadt, Germany) and used without further purification. HPLC-grade acetonitrile, methanol, and methyl-tert-butyl ether were purchased from Sigma–Aldrich (Seelze, Germany). For sample preparation, distilled methanol from Merck was used. Internal standards (phosphatidylinositol PI (37:4), phosphatidylethanolamine PE (37:4), phosphatidylcholine PC (37:4) and sphingomyelin SM (d18:1, C12:0)) were obtained from Avanti Polar Lipids (Alabaster, USA).

Study participants

Sixteen patients of the Departments of Neurology at the University Hospitals in Zagreb and Split participated in this study. The individuals, clinically referred to as non-demented controls (N=7, M/F 3/4, age 55±4), were patients who had neurological problems that do not affect cognition, for example headache, vertigo, and back syndrome. Clinical diagnosis of probable Alzheimer's disease (N=9, M/F 4/5, age 71±3) was performed as part of a routine clinical work-up compliant with diagnostic criteria from the National Institute of Neurological and Communicative Disorders and Stroke, and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) and the Diagnostic and Statistical Manual of Mental Disorders, 4th edition (DSM-IV) [18] AD diagnosis was confirmed by measuring Aβ42, t-tau, and p181-tau levels in cerebrospinal fluid using ELISA assay (Innogenetics, Ghent, Belgium). As reported previously [19, 20], AD

individuals showed low Aβ42, high total tau, and/or high p181-tau levels in their CSF. Participants with other causes of cognitive impairment, including brain tumour, vitamin B_{12} deficiency, folate deficiency, thyroid dysfunction, depression, head trauma, CNS infection, and current alcohol abuse were not considered for study participation.

All patients, or their closest relative for patients judged unable to provide informed consent, gave written informed consent to participate and the study was approved by the local medical ethical committees. All subjects in the study underwent lumbar puncture (LP) for collection of CSF.

Sample preparation

Cerebrospinal fluid (2 mL) was collected in polypropylene tubes. Routine laboratory analysis confirmed that CSF samples were free from any blood contaminations, i.e. the white blood cell (WBC) and red blood cell (RBC) counts were within the normal range (0–5 for WBC count and 0 for RBC count). Samples were aliquoted to 0.5 mL into polypropylene tubes and stored at −80 °C. Before lipid extraction, CSF samples were centrifuged at 20,000*g* for 15 min (Eppendorf 5415 C micro centrifuge). Supernatant from each sample (200 μL) was used for lipid isolation. After adding internal standards (final concentrations of PI, SM, PE, and PC standards in CSF samples were 0.079, 0.579, 0.275, and 0.844 pmolμL^{-1}, respectively), lipids from CSF samples were extracted with methyl tert-butyl ether (MTBE) [21]. Briefly, after mixing the CSF samples with 1.5 mL methanol and 5 mL MTBE, the mixture was incubated for 1 h at room temperature with constant shaking. After adding 1.25 mL water followed by 10 min incubation at room temperature, the sample was centrifuged at 2,000*g* for 10 min (Hettich Universal Tabletop Centrifuge). The upper organic phase was collected and the lower phase was re-extracted with 2 mL theoretical upper phase (MTBE–methanol–water 10:3:2.5, *v*/*v*). Both upper phases were combined and dried under a nitrogen stream (Waldner–Captair, Type 5000 C), resolved in 200 μL of chloroform–methanol–water 30:60:8 (*v*/*v*), and stored at −20 °C.

Nano-HPLC–ESI-QTOF-MS

CSF phospholipids were separated on an Ultimate 3000 nano-HPLC system (Dionex/LC Packings, Sunnyvale, CA, USA) using a nano-scale TSK-Gel Amide 80 column (5 μm×75 μm×100 mm) purchased from Alltech Grom (Rottenburg, Germany). Throughout the experiments the column was maintained at 40 °C and the flow rate at 0.2 $\mu L min^{-1}$; 1 μL of sample was injected per analysis. CSF lipid extracts were prepared by dilution to a concentration of 5 pmolμL^{-1} (where total phospholipid concentration was 2.5 pmolμL^{-1}), considering the values for average lipid concentrations in CSF available from the literature [22–25]. For sample injection, solutions were evaporated to dryness and reconstituted in an adequate volume of acetonitrile–methanol 8:2 (*v*/*v*) containing 5 mmolL^{-1} ammonium acetate). The gradient program started with 100% solvent A (acetonitrile–methanol 99:1 (*v*/*v*) containing 5 mmolL^{-1} ammonium acetate) for 10 min. The mobile phase was linearly changed to 30% solvent B (methanol–water 80/20 (*v*/*v*) containing 5 mmolL^{-1} ammonium acetate) over 10 min, reached 40% solvent B in the following 15 min, and was maintained so for another 10 min, followed by a fast change to 100% solvent A in 5 min. At the end of the gradient, the column was reconditioned with 100% solvent A for 10 min. The overall run time was 60 min. All solvent mixtures were degassed before use (Branson Ultrasonic Bath 5510).

The nano-HPLC instrument was connected to a QTOF mass spectrometer (Micromass, Manchester, UK) equipped with an ESI ion source. For data acquisition the MassLynx software (Micromass) was used. Coupling of nano-HPLC and ESI–MS was performed via an in house-made sheathless interface involving distal coated silica emitters (New Objective, Woburn, MA, USA) with an inner diameter of 15 μm. MS analysis was carried out in negative-ion mode at an electrospray potential of 1500 V and sampling cone potential of 50 or 80 V; a desolvation nitrogen stream of 100 $L h^{-1}$ was applied. All mass spectra were externally calibrated using a reference sodium iodide solution.

Data processing and analysis

HPLC–MS data were processed by MassLynx software (Micromass). Representative spectra from each phospholipid fraction were further processed and quantified by LIMSA software [26]. Statistical validation of data was achieved by use of the non-parametric Mann–Whitney *U* test.

Results

Development of nano-HPLC–MS method for phospholipid analysis in cerebrospinal fluid

CSF is known to contain very small amounts of lipids [22–25]. The phospholipid concentration is approximately 5–6 ngμL^{-1} with average proportions of phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) in total phospholipid content of approximately 51%, 22%, 9%, and 4%, respectively. Because of low phospholipid levels (phospholipid levels in the CSF are approximately 300 times lower than in human serum) and relatively high concentrations of salts and proteins in the CSF we opted for methyl tert-butyl

ether (MTBE)-based extraction [21], which is suitable for profiling the complex lipidome from samples with excessive amounts of biological matrices. Recovery of lipid species is the same or higher than by the traditionally used Folch method [27], and lipid-containing phase collection is simpler because of the low density of MTBE [21].

A preliminary test of the MTBE extraction procedure using standard phospholipids (cf. Materials and methods) indicated MTBE extraction was the optimum choice for CSF. Chromatographic separation of phospholipid classes is typically carried out by normal-phase HPLC using a silica column [28]. In this study, HPLC separation on an Amide column was applied, because of its superior compliance with higher organic content in mobile phases compared with standard normal-phase chromatography. This is beneficial for solvent evaporation during electrospray ionization, hence spray stability, and for separation of glycolipids and other lipids [29], among other biomolecules.

Different gradients, solvent composition, and column temperatures, and sampling cone and electrospray potentials were tested using standard mixtures, and optimum conditions (cf. Materials and methods) for separation and detection of all phospholipid classes with minimum interfering salt adducts were devised (Fig. 1 and Electronic Supplementary Material). After optimization, the phospholipids from crude lipid extracts of cerebrospinal fluid samples were analyzed. The HPLC of order elution of each phospholipid class in CSF was: PE (~33.5–35.0 min), PC (~34.5–35.5 min), PI (~36.0–37.0 min), and SM (~37.5–38.5) as deduced from extracted ion chromatograms (Fig. 1).

The most intense signal corresponded to PC species, followed by SM, PI, and PE with ratios to PC intensity of approximately 0.3, 0.15, and 0.1, respectively. An efficient separation between PC, PI, and SM species was obtained under these conditions, yet partial overlap between PE and PC species (dashed line in Fig. 1) originated mainly from the different concentrations of the species in the biological sample. This is supported by the fact that when a defined standard mixture was applied, this overlap was not observed (Electronic Supplementary Material, Fig. S1). Spectra combined from each chromatogram were analyzed using LIMSA software [26] and the concentration corresponding to individual signal intensities of phospholipids was calculated as a relative value to the signal intensity of the internal standards. The method reproducibility, i.e. average relative standard deviation (RSD), was assessed by four replicate measurements of two different samples (Table 1). Serial dilution of a set of samples was monitored to investigate at which volume of CSF we could detect all four phospholipid classes. At 1:100 dilution (0.01 μL CSF) PC, SM, and PI internal standard signals corresponding to 8.44 fmol, 5.79 fmol, and 0.79 fmol, respectively, were detected as weak signals but with an appropriate isotopomer distribution for peak assignment. PE which would correspond to 2.75 fmol was still visible but without proper isotopomer distribution for peak assignment. The sensitivity for individual phospholipid classes varies because of ionization efficiency, but can reach a low fmol range, i.e. <10 fmol. However, for complete profiling of all four phospholipid species in CSF, including PE, it is estimated that >0.01 μL CSF is necessary.

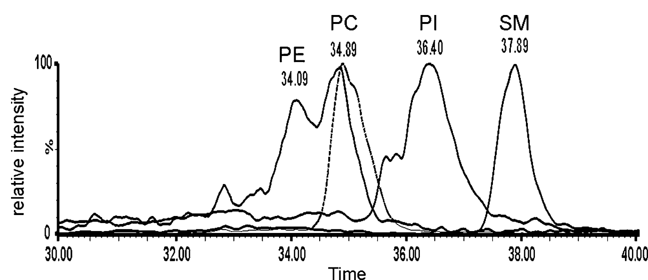


Fig. 1 Overlaid nano-HPLC–ESI–QTOF-MS extracted ion chromatograms of singly charged ions corresponding to different phospholipid classes in a crude extract of CSF. CSF crude extract (1 μL, equivalent to 0.4 μL CSF, lipid concentration approx. 5 pmolL^{-1}) was injected in the LC column (see Materials and methods). Extracted ion chromatograms are from singly charged ions from internal standards within CSF crude extract: PE, PI, and SM (*solid lines*) and PC (*dashed line*)

Phospholipid profile of CSF samples

In the brain, PE species are present in two forms—diacyl (PtdE) and alkenyl-acyl plasmalogen (PlsE), which is the dominant form in both neuronal and non-neuronal brain membranes [30]. Using the established method we detected three PlsE species in the CSF, PlsE(38:4), PlsE(38:6), and PlsE(40:6), and two PtdE species, PtdE(38:4) and PtdE (40:6) (Fig. 2), with average RSD of 12 and 21%, respectively (Table 1). All species were detected as deprotonated molecular ions.

PC species were detected mainly as acetate and chloride adducts. Partial loss of the choline head group from the PC acid adducts was also observed as a $[M-CH_3]^-$ signal (Fig. 3). In total, six distinct PC species were detected in

Table 1 Reproducibility and number of detected species from each phospholipid class. Two different CSF samples were measured four times and average relative standard deviation (RSD) of total phospholipid species in each phospholipid class was calculated

Phospholipid class	Detected species	RSD (%)
PE (PlsE)	3	12
PE (PtdE)	2	21
PC	6	3
PI	6	6
SM	8	8

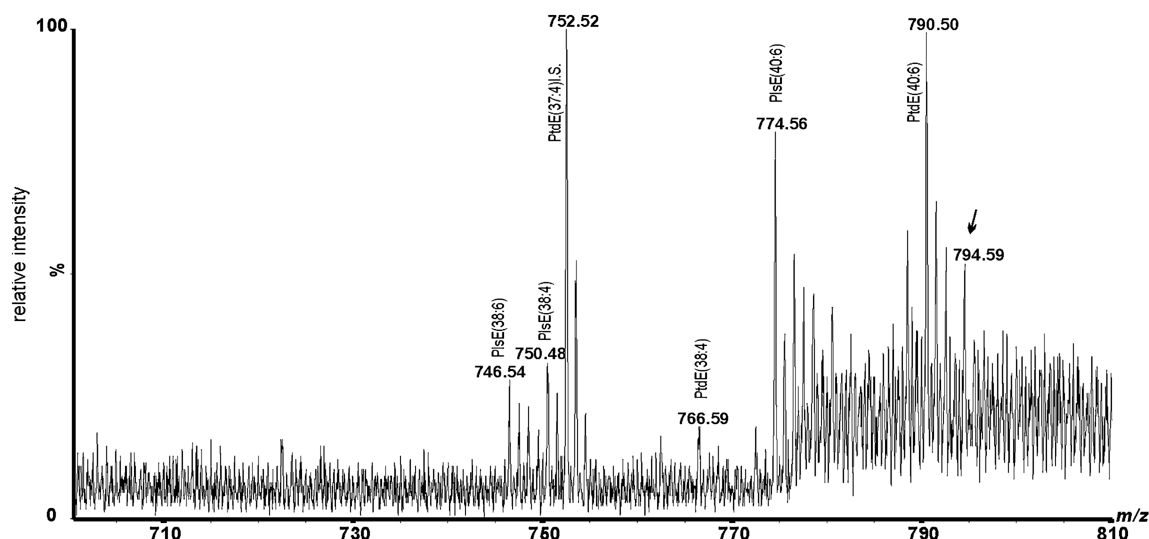
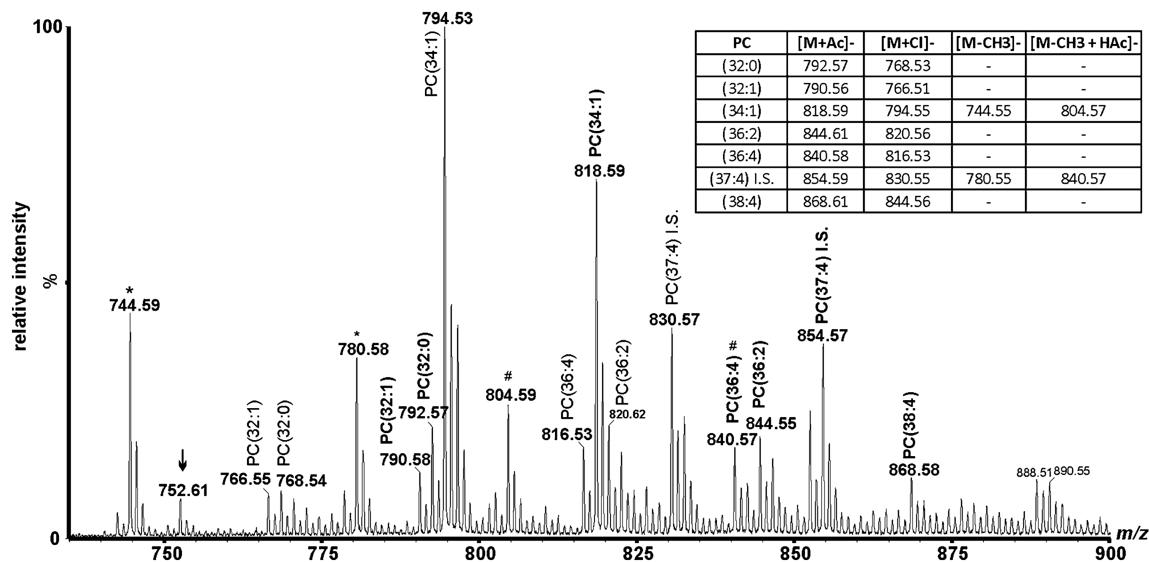


Fig. 2 Nano-HPLC–ESI–QTOF mass spectra of PE species combined from elution time 33.5–34.5 min. The diacyl PE (PtdE) and alkenyl–acyl plasmalogen (PlsE) were both detected as deprotonated singly charged ions. Interfering signal from PC at *m/z* 794.59 is indicated with an *arrow*

the crude extract of CSF sample: PC(32:0), PC(32:1), PC (34:1), PC(36:2), PC(36:4), and PC(38:4) (Fig. 3). For data assignment and further quantification, the partial overlap of PC with PE species was not found to be very detrimental, because the PC species yield a ten times more intense signal than PE (interfering signal from PE internal standard is indicated with an arrow in Fig. 3). For quantification of PC, both chloride and acetate adducts were considered, given their excellent reproducibility (RSD 3%; Table 1).

SM species were mainly detected as acetate adducts at a cone potential of 50 V. Because chloride adducts in SM spectra are of low abundance, only acetate adducts with RSD of 8% (Table 1) were considered for quantification. The distinct SM species detected are shown in Fig. 4a (only acetate adducts are assigned in Fig. 4a). Partial loss of choline head group was also visible as the $[M-CH_3]^-$ signal (marked with *). Interestingly, SM species with longer fatty acid chain had a shift in elution time compared with SM internal standard. More precisely, SM(d18:1, 20:0), SM (d18:1, 22:0), and SM(d18:1, 22:1) were partially separated from the SM internal standard, approximately 20 s before the maximum of the SM internal standard, and SM(d18:1, 24:1) and SM(d18:1, 24:2) eluted at 36.0–37.0 min along with the PI species. The acetate adduct of SM(d18:1, 24:1) is isobaric with the PI internal standard at nominal *m/z* 871. The different *m/z* values for the acetate adduct of SM(d18:1, 24:1) and the



PC	[M+Ac]-	[M+Cl]-	[M-CH3]-	[M-CH3 + HAc]-
(32:0)	792.57	768.53	-	-
(32:1)	790.56	766.51	-	-
(34:1)	818.59	794.55	744.55	804.57
(36:2)	844.61	820.56	-	-
(36:4)	840.58	816.53	-	-
(37:4) I.S.	854.59	830.55	780.55	840.57
(38:4)	868.61	844.56	-	-

Fig. 3 Nano-HPLC–ESI–QTOF mass spectra of PC species combined from elution time 34.5–35.5 min. Species were detected as singly charged acetate adducts (*bold letters*), chloride adducts (*regular letters*), and fragments with partial loss of head choline group with (*hash symbols*) or without (*asterisks*) acetic acid adduct. Signal at *m/z* 752.61 indicated with *an arrow* is interfering signal from PE spectra

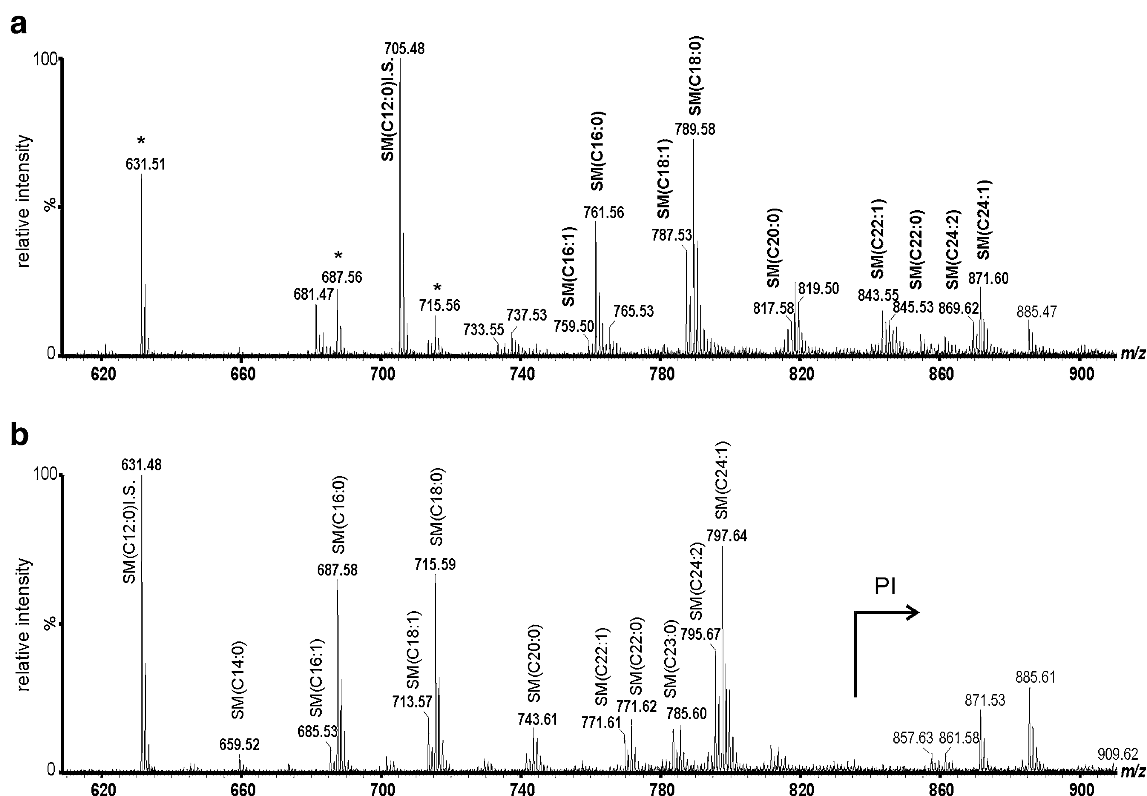


Fig. 4 **a** Nano-HPLC–ESI–QTOF mass spectra of SM species combined from elution time 37.5–38.5 min at cone potential 50 V. Species were detected as singly charged acetate adducts (*bold letters*) and fragments with partial loss of head choline group (*asterisks*). Chloride adducts signals were very weak (*unassigned numbers on spectra*). **b** Nano-HPLC–ESI–QTOF mass spectra of SM species combined from elution time 36–38.5 min (both PI and SM region) at cone potential 80 V. SM species were detected only as $[M-CH_3]^-$ ions. PI species were separated by *arrow*

PI internal standard at *m/z* 871.6 is not resolvable by the QTOF MS instrument. However, to achieve accurate quantification of these species, an alternative to changing the PI internal standard was increasing the sampling cone potential to 80 V. At these values, only $[M-CH_3]^-$ SM signals at *m/z* 795.6 and 797.6, respectively, were visible (Fig. 4b) and the overlap of isobaric peaks was thus avoided. This was probed by DDA LC–MS–MS analysis (data not shown). At 80 V for sampling cone potential, the PC species were still visible in all three forms, the only difference was that the intensity of the $[M-CH_3]^-$ signal was higher. PE species were detected in the deprotonated form, irrespective of sampling cone potential. PI species were detected as deprotonated molecular ions at both 50 and 80 V cone potential. PI pattern included six species: PI(36:2), PI(36:3), PI(36:4), PI(38:4), PI(38:5), and PI(40:6) (Fig. 5) with an average RSD of 6% (Table 1).

Sphingomyelin levels are elevated in the CSF of probable Alzheimer's disease patients

Using the on-line nano-HPLC–ESI-QTOF-MS method adapted for cerebrospinal fluid phospholipids, phospholipid profiling of sixteen CSF samples of clinically referred probable AD (N=9, M/F 4/5, age 71±3) and non-demented individuals (N=7, M/F 3/4, age 55±4) was performed as a pilot study. Duplicate measurements were carried out for each sample. The total concentration of PtdE, PlsE, PC, PI, and SM was calculated for each sample and statistically validated using the non-parametric Mann–Whitney U test. No statistically significant difference was observed in the total PtdE, PlsE, PC, and PI CSF levels between control and probable AD samples. Interestingly, total SM levels, at 24.3±2.4%, were increased in the CSF from the probable AD group ($p<0.05$) compared with the control group (Fig. 6a). Relative proportions of single SM species in total SM levels are shown in Fig. 6b. All eight SM species tend to be increased from 20 to 40% in CSF samples from probable AD group, but only three of them were statistically significant ($p<0.05$), probably because of the small sample size of our pilot study. SM(d18:1, 24:1) and SM (d18:1, 24:2) were not included in total calculations because of their different elution compared with SM internal standard, which would affect the accuracy of quantification. However, we calculated relative concentrations of those two SM species and their levels were twofold

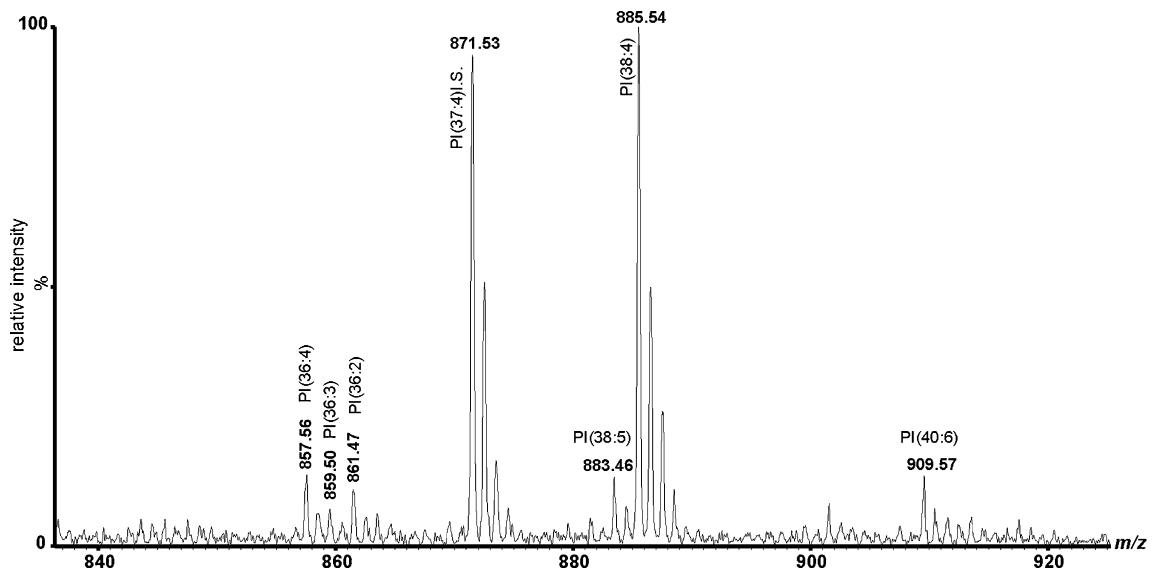


Fig. 5 Nano-HPLC–ESI–QTOF mass spectra of PI species combined from elution time 36–37 min. All species were detected as deprotonated singly charged ions

increased in CSF samples from the probable AD group compared with the control group, but with a much larger standard deviation than for the other SM species (data not shown). For more precise quantification one additional internal SM standard with longer fatty acid is required.

Discussion

Modern developments in mass spectrometry contributed substantially to advances in lipid screening and enabled the detection of a few hundred different lipid species in human CSF [31]. ESI–MS has evolved as the most sensitive method for cellular lipidome screening, with or without front-end HPLC separation [10]. However, the lipid content of CSF is much lower than at the cellular (for example brain) level, and in such cases separation of different phospholipid classes before ESI–MS is imperative to achieve high sensitivity of detection. In this work we developed a nano-HPLC–ESI–QTOF-MS method which was shown to be a superior approach to direct sample submission to ESI–MS for phospholipid qualitative and quantitative profiling in the CSF. Using this method we achieved an efficient separation of PE, PC, SM, and PI species, hence facilitating the different display of CSF phospholipids in AD versus controls.

Based on much evidence on the links between lipids and AD [8], this study was focused on comprehensive PE, PI, PC, and SM profiling in the CSF, in order to clarify their expression change with AD and their possibility as potential biomarker(s) for AD. Additionally, assessment of the phospholipid expression pattern in CSF of healthy versus AD individuals is of importance for understanding molecular processes implicated in AD and reflected in the CSF. Indeed, previous studies have reported that levels of sulfatides [11, 12] and ceramides [13] are reduced and increased, respectively, in both brain and CSF from AD vs. control samples

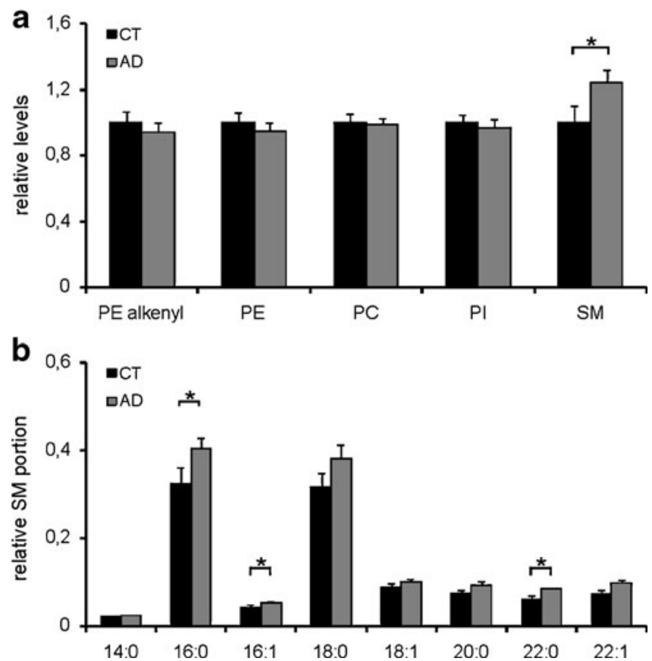


Fig. 6 Phospholipid level changes in cerebrospinal fluid of Alzheimer's disease individuals in comparison with control group. **a** Relative total levels of each phospholipid class in cerebrospinal fluid samples from individuals with probable Alzheimer's disease (*AD*) compared with control individuals (*CT*). We observed statistically significant elevation in total SM levels, i.e. 24.3±2.4%, in probable AD group compared with control ($p<0.05$). **b** Relative levels of each SM species in AD CSFs compared with control. *Error bar* represents the standard error of the mean

(age-matched neurological controls), suggesting that these lipid species could be potential lipid biomarkers for early AD diagnosis. In contrast, while there are several reports of phospholipid changes in the brain of AD individuals, there have only been a few analyses of phospholipid changes in the CSF. While in early AD brain samples no changes of PC, PI, and SM levels were detected [11], in advanced stages of the disease reduced phospholipid levels were reported [14, 32, 33]. In the CSF, a seventeenfold reduction of total phospholipid content was detected in an AD group compared with healthy controls, without a significant correlation with sex and age of individuals or the pH of the CSF sample [15]. Furthermore, reduced lysophosphatidylcholine/phosphatidylcholine ratio was found in CSF samples from post mortem AD individuals compared with post mortem CSFs of a control group (individuals with disorders other than neurological and psychiatric), with no changes in PC levels [16]. In addition, changes in water-soluble PC metabolites were found in CSF samples from probable AD individuals that were not detected in samples from individuals with vascular dementia [17], supporting the hypothesis that changes in CSF phospholipid levels could be potentially used for diagnosis of AD.

In contrast with previous phospholipid studies in the CSF, which were focused on a particular phospholipid species, we performed mass spectrometric analysis of the main phospholipid classes, PE, PC, PI, and SM, in the CSF. In this pilot study we showed a statistically significant increase in total SM levels, i.e. 24.3±2.4% in the probable AD group compared with a control group (individuals with disorders other than dementia). This finding is in agreement with a previous larger ^{31}P NMR study which, in addition to different lipid alterations in AD, reported a 21% increase of SM levels in five different AD brain regions compared with both healthy and other neurodegenerative controls [14]. Pettegrew et al. suggested that the SM change observed is more likely to be related to a unique process in AD than to general neurodegeneration processes [14]. In contrast with the increase of SM levels in AD, the SM levels were also reported to be reduced in the middle frontal gyrus from AD patients and unchanged in the midfrontal cortex grey matter membrane extract from AD patients at different stages [34]. Interestingly, recent measurement of SM and ceramides in serum suggested that serum levels of SM and ceramides could be used for monitoring and predicting AD progression [35], postulating that comparison of SM/ceramide species in CSF and serum from individuals with AD and other neurological controls could provide a new insight in SM metabolism changes during AD. Our findings of increased SM levels in the CSF of AD individuals need to be further validated on a larger sample size. The change of SM/ceramide ratio in the CSF during AD progression would be also an interesting aspect for further investigations.

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