



Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Imaging mass spectrometry (IMS) of cortical lipids from preclinical to severe stages of Alzheimer's disease[☆]

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ARTICLE INFO

Article history:

Received 20 December 2016

Received in revised form 12 May 2017

Accepted 14 May 2017

Available online xxxxx

Keywords:

Alzheimer's disease

Brain

Lipids

Imaging mass spectrometry

Frontal cortex

Human

ABSTRACT

Alzheimer's disease (AD) is a progressive neurodegenerative disease affecting millions of patients worldwide. Previous studies have demonstrated alterations in the lipid composition of lipid extracts from plasma and brain samples of AD patients. However, there is no consensus regarding the qualitative and quantitative changes of lipids in brains from AD patients. In addition, the recent developments in imaging mass spectrometry methods are leading to a new stage in the *in situ* analysis of lipid species in brain tissue slices from human *postmortem* samples. The present study uses the matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), permitting the direct anatomical analysis of lipids in *postmortem* brain sections from AD patients, which are compared with the intensity of the lipid signal in samples from matched subjects with no neurological diseases. The frontal cortex samples from AD patients were classified in three groups based on Braak's histochemical criteria, ranging from non-cognitively impaired patients to those severely affected. The main results indicate a depletion of different sulfatide lipid species from the earliest stages of the disease in both white and gray matter areas of the frontal cortex. Therefore, the decrease in sulfatides in cortical areas could be considered as a marker of the disease, but may also indicate neurochemical modifications related to the pathogenesis of the disease. This article is part of a Special Issue entitled: Membrane Lipid Therapy: Drugs Targeting Biomembranes edited by Pablo Escriba-Ruiz.

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1. Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease affecting millions of patients worldwide. It is clinically characterized by progressive cognitive impairment, and neuropathologically, by the appearance of extracellular neuritic plaques and intraneuronal neurofibrillary tangles (NFT), together with a loss of synapses and neurons and the accumulation of lipid granules [1–3]. The neuritic plaques are largely composed of a dense amyloid core of β -amyloid (A β) peptide, which is generated by the amyloid precursor protein (APP) via the

sequential cleavage of APP by the β -secretase (β -site APP cleavage enzyme 1; BACE1) [4,5]. The NFT consist of a hyperphosphorylated form of the tau protein [6]. Moreover, the NFT are used as *postmortem* diagnostic criteria and also for the classification of AD patients in Braak's stages I to VI based on the severity of the disease [7].

Furthermore, there is now evidence that lipid molecules have a relevant role both in cell membrane composition and neurotransmitter signaling in different neurodegenerative diseases, such as AD [8]. Lipid molecules could also be involved in amyloidogenesis by the regulation of APP, BACE1 and the component of γ -secretase complex, since all of them are components of lipidic bilayers [9]. The composition and organization of lipids in the cell membranes determine the trafficking properties of proteins and metabolites with important consequences in cell signaling and physiology [10].

On the other hand, tau is a cytoplasmic protein that stabilizes microtubules that can be modulated by lipid molecules in different ways [11–14]. Some neurodegenerative diseases such as Niemann-Pick type C is characterized by the accumulation of cholesterol and glycolipids in lysosomes, together with the presence of NFT tangles, suggesting that lipid deregulation is involved in tau pathologies [15].

The lipid composition has also been analyzed in tissue homogenates (lipid fraction) from brain samples of AD patients. Total phospholipid

Abbreviations: MALDI-IMS, matrix assisted laser desorption/ionization imaging mass spectrometry; AD, Alzheimer's disease; ST, sulfatide; NFT, neurofibrillary tangles; A β , β -amyloid; CSF, cerebrospinal fluid; BACE1, β -site APP cleavage enzyme 1; PC, phosphatidylcholine; (MBT), 2-mercaptobenzothiazole; PI, phosphatidylinositol; CPE, ceramide-1-phosphoethanolamine; CPI, ceramide-1-phosphoinositol; TIC, total ion current; PA, phosphatidic acid; SM, sphingomyelin; GlcCer, glucosylceramide; Cer, ceramide; APP, amyloid precursor protein; APOE, apolipoprotein E; S/N, signal/noise.

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<http://dx.doi.org/10.1016/j.bbamem.2017.05.009>

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Please cite this article as: E. González de San Román, et al., Imaging mass spectrometry (IMS) of cortical lipids from preclinical to severe stages of Alzheimer's disease, *Biochim. Biophys. Acta* (2017), <http://dx.doi.org/10.1016/j.bbamem.2017.05.009>

Table 1
Values are means \pm SEM.

| Samples | n | | | Age (years) | Postmortem (hours) |
|---------|-------|---|---|----------------|--------------------|
| | Total | ♂ | ♀ | | |
| Control | 5 | 5 | 0 | 60.8 \pm 4.7 | 6.15 \pm 2.19 |
| AD | | | | | |
| I–II | 5 | 3 | 2 | 66.4 \pm 3.4 | 6.10 \pm 1.3 |
| III–IV | 5 | 4 | 1 | 75.8 \pm 4.3 | 9.10 \pm 2.0 |
| V–VI | 5 | 2 | 3 | 74.6 \pm 2.0 | 5.45 \pm 1.0 |

and sulfatide (ST) composition were found to be decreased in AD patients as compared to control subjects [16–19], in contrast to the increase in ceramide (Cer) and cholesterol content [18,20]. Cer levels in the cerebrospinal fluid (CSF) were also found to be increased in patients with AD [21], as was the level and activity of acid ceramidase in the fronto-temporal gray matter [22]. However, more recent studies described a decrease in ceramide synthase 2 activity, necessary for myelin biosynthesis, which precedes tau pathology [23]. Other changes have also been reported in the phospholipid composition of the brain, in CSF and also in plasma during different stages of AD [24–27]. A recent study analyzing more than 500 plasma samples from AD patients, reported eight phosphatidylcholine lipid species (PC) as possible biomarkers for AD and which predicted phenoconversion to either

amnesic mild cognitive impairment (MCI) or to Alzheimer's disease within a 2 to 3 year timeframe, with over 90% accuracy [28].

In this context, the development of mass spectrometry during the last decade has provided us with a very useful tool not only for lipidomics, but also for the imaging of lipid species directly *in situ* with unprecedented anatomical localization, enabling the simultaneous analysis of numerous phospholipid classes using a single slice of tissue. The great potential of the new technique of matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS) in anatomical analysis has accelerated its development and applications [29–32], providing information about lipid mapping in different tissues [33]. Nevertheless, there are very few studies applying IMS to human *postmortem* brain tissue samples [34–36].

There is extensive knowledge of the biochemistry of AD, but the biochemical mechanisms underlying the pathogenesis of the disease still remain unknown, and the approved therapeutic approaches are not effective. The aim of the present study was to find possible lipid regulations during the progression of AD in cortical areas that are affected by tau and amyloid histopathological markers by clearly discriminating the cortical layers from the adjacent white matter. Using MALDI-IMS we analyzed the anatomical distribution of lipids in *postmortem* human brain samples of frontal cortex (Brodmann area 8) from AD patients classified in three different groups (I–II, III–IV and V–VI) based on disease severity according to I–VI Braak's stages.

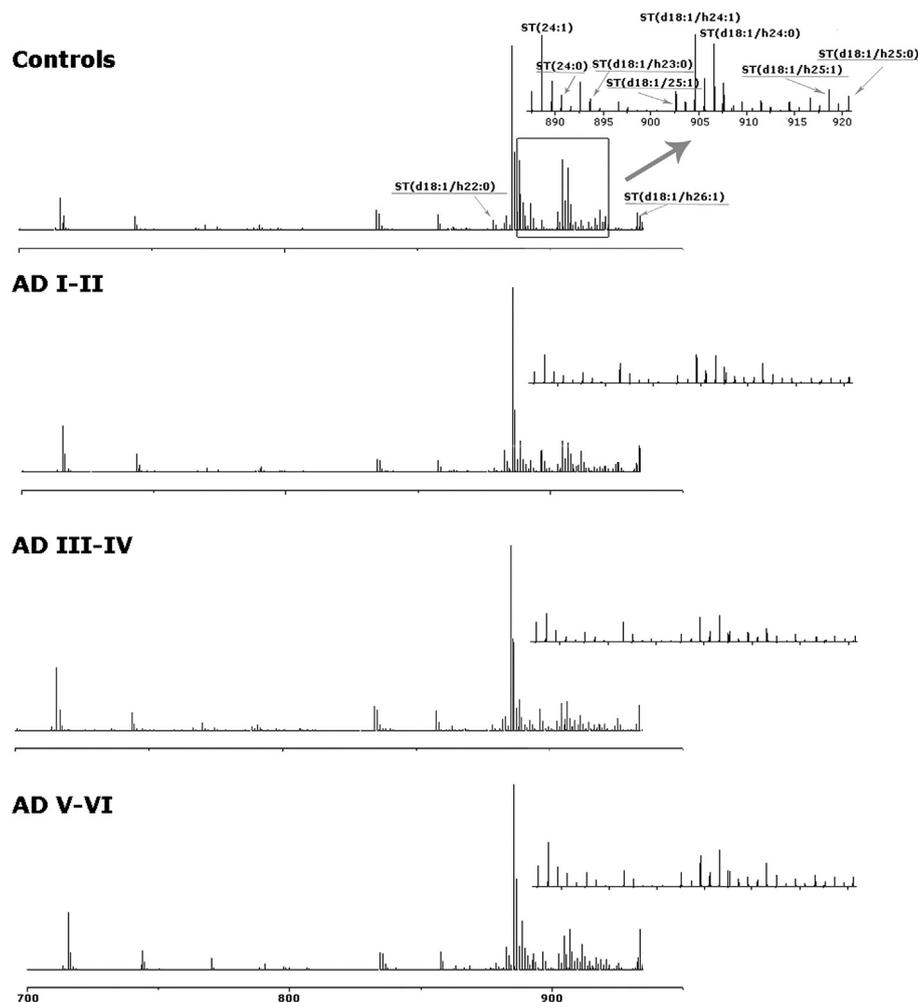


Fig. 1. Average spectra comparison between controls and AD patients. Mass spectra were obtained from the average spectra recorded directly from the tissue slice, selecting only the gray matter from the frontal cortex of the tissue slices. The spectra parameters were obtained using 2 micro scans/step with 10 laser shots and a raster step size of 150 μ m with the laser power at 15–20 μ J and using MBT matrix. Detection was performed in negative ion mode. The “y” axis represents the intensity of the measured MS signal as a percentage of the maximal peak. The maximal peak is the most intense peak of the spectrum, in this case 885.5494 PI (20:4/18:0) which is set at 100%.

Table 2

Percentage of the relative intensity of representative lipids in gray matter of *postmortem* human frontal cortex of controls and AD patients in negative ion detection mode. The maximal peak is the most intense peak of the spectrum, in this case 885.5494 PI (20:4/18:0) which is set at 100%.

| Assignment | Cal <i>m/z</i> | Exp <i>m/z</i> | % intensity | | | |
|--------------------------------------|----------------|----------------|-------------|-------------------------|-------------------------|------------------------|
| | | | Control | AD I–II | AD III–IV | AD V–VI |
| CPE (38:1) | 715.5760 | 715.5735 | 23.5 ± 6.3 | 37.4 ± 6.4 | 35.5 ± 10.3 | 29.5 ± 1.5 |
| SM (d37:1) | 743.6073 | 743.6052 | 12.9 ± 2.0 | 13.7 ± 2.9 | 9.6 ± 2.5 | 10.6 ± 0.5 |
| PS (18:0/18:1) | 788.5442 | 788.5429 | 1.8 ± 0.9 | 0.5 ± 1.5 | 2.0 ± 1.1 | 1.7 ± 0.9 |
| PE (18:0/22:6) | 790.5381 | 790.5373 | 4.3 ± 1.4 | 3.8 ± 1.3 | 3.5 ± 0.7 | 3.9 ± 0.7 |
| ST (d18:1/18:0) | 806.5451 | 806.5443 | 1.7 ± 0.2 | 0.5 ± 0.2 ^a | 0.5 ± 0.1 ^b | 0.8 ± 0.1 |
| PS (40:6) | 834.5291 | 834.5282 | 13.6 ± 3.2 | 12.3 ± 2.6 | 13.9 ± 4.1 | 10.2 ± 1.2 |
| PI (16:0/20:4) | 857.5186 | 857.5174 | 9.3 ± 0.8 | 10.7 ± 1.0 | 10.5 ± 1.0 | 10.1 ± 0.7 |
| ST (d18:1/h22:0) | 878.6033 | 878.6078 | 6.7 ± 1.5 | 2.8 ± 0.8 | 2.6 ± 0.3 ^b | 2.8 ± 0.7 ^c |
| PI (18:1/20:4) | 883.5337 | 883.5320 | 8.5 ± 1.3 | 8.8 ± 1.8 | 7.6 ± 1.3 | 7.1 ± 1.1 |
| ST (d18:1/h24:1) | 888.6240 | 888.6215 | 37.6 ± 4.7 | 18.6 ± 6.6 ^a | 16.0 ± 2.3 ^b | 24.0 ± 2.8 |
| ST (d18:1/h24:0) | 890.6395 | 890.6348 | 8.1 ± 1.5 | 2.9 ± 0.9 ^a | 3.8 ± 0.2 ^b | 7.3 ± 0.9 |
| ST (d18:1/h23:0) or ST (d18:0/h24:0) | 892.6189 | 892.6352 | 16.5 ± 3.9 | 5.0 ± 1.5 ^a | 6.4 ± 0.2 | 8.1 ± 1.0 |
| CPI (40:2) + MBT | 896.5779 | 896.5738 | 3.5 ± 0.9 | 20.5 ± 5.8 ^a | 11.3 ± 2.8 | 9.7 ± 1.3 |
| ST (d18:1/h25:1) or (d18:2/h25:0) | 902.6396 | 902.6380 | 13.7 ± 2.0 | 5.3 ± 2.2 | 5.0 ± 0.6 ^b | 8.3 ± 0.6 |
| ST (d18:1/h24:1) | 904.6188 | 904.6150 | 38.2 ± 7.5 | 16.2 ± 4.4 | 15.5 ± 1.3 ^b | 17.2 ± 0.8 |
| ST (d18:1/h24:0) | 906.6345 | 906.6295 | 43.6 ± 10.3 | 11.6 ± 3.7 ^a | 15.2 ± 0.5 | 18.4 ± 1.9 |
| PI (18:0/22:6) | 909.5488 | 909.5472 | 5.8 ± 0.8 | 5.1 ± 0.5 | 5.7 ± 0.8 | 5.8 ± 0.2 |
| ST (d18:1/h25:1) or (d18:2/h25:0) | 918.6346 | 918.6308 | 10.9 ± 2.1 | 3.8 ± 1.1 ^a | 3.6 ± 0.4 ^b | 5.0 ± 0.5 |
| ST (d18:1/h25:0) | 920.6502 | 920.6464 | 7.7 ± 2.2 | 4.9 ± 1.6 | 3.6 ± 1.2 | 5.0 ± 0.5 |
| ST (d18:1/h26:1) | 932.6502 | 932.6471 | 11.8 ± 2.7 | 4.7 ± 1.3 | 3.2 ± 0.7 ^b | 6.1 ± 0.6 |

Data are mean ± SEM. The p values were calculated by the Kruskal-Wallis non-parametric test followed by Dunn's test. Control (n = 5), AD I–II (n = 5), AD III–IV (n = 5), AD V–VI (n = 5). Cal: calculated, Exp: experimental. The notation (hX:Y) denotes hydroxylated sulfatide species.

^a AD I–II vs control.

^b AD III–IV vs control.

^c AD V–VI vs control.

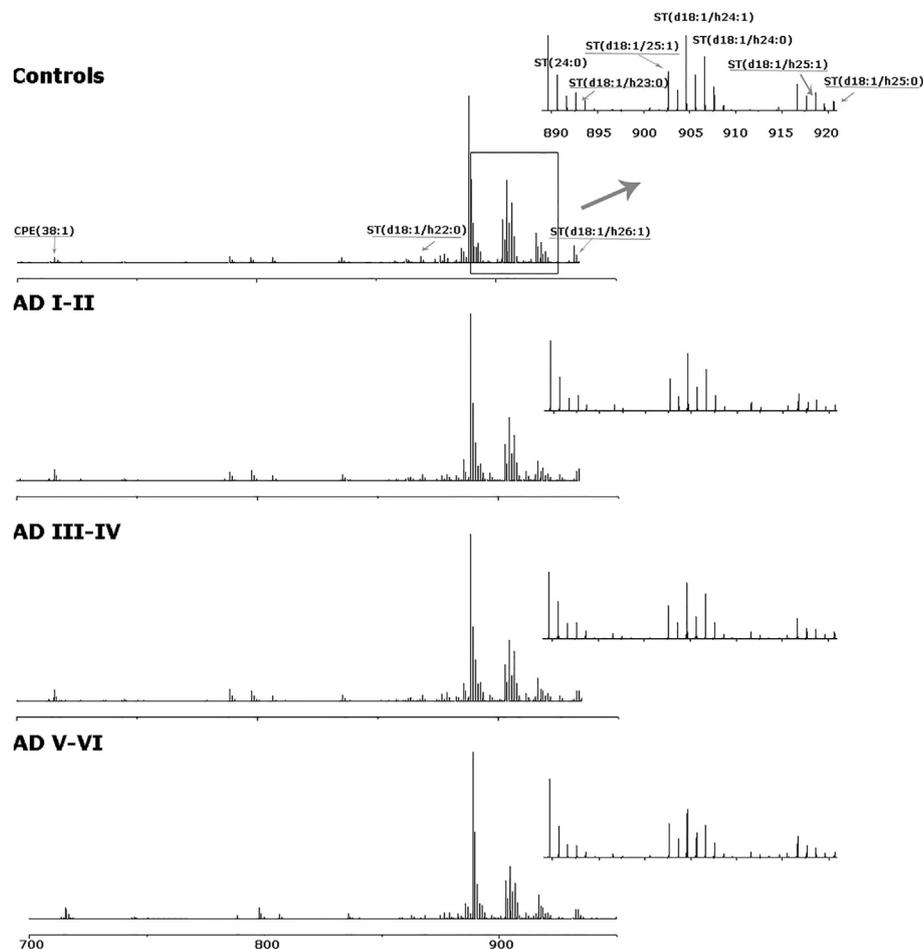


Fig. 2. Comparison of average mass spectra from controls (n = 5) and AD patients, obtained from the average spectra recorded directly from the tissue slice, selecting only white matter. The spectra parameters were 2 micro scans/step with 10 laser shots and a raster step size of 150 μm with the laser power at 15–20 μJ and using MBT matrix. Detection is performed in negative ion mode. The “y” axis represents the intensity of the measured MS signal as percentage of the maximal peak. The maximal peak is the most intense peak in this case 888.6254 ST (18:0/20:4) of the spectrum, which is set at 100%.

Table 3
Percentage of the relative intensity of representative lipids in white matter of *postmortem* human frontal cortex of controls and AD patients in negative ion detection mode. The maximal peak is the most intense peak of the spectrum, in this case 888.6240 ST (d18:1/24:1) which is set at 100%.

| Assignment | Cal <i>m/z</i> | Exp <i>m/z</i> | % intensity | | | |
|------------------------------------|----------------|----------------|-------------|-------------------------|------------------------|-------------------------|
| | | | Control | AD I–II | AD III–IV | AD V–VI |
| CPE (38:1) | 715.5760 | 715.5735 | 3.3 ± 0.8 | 7.0 ± 0.8 | 8.1 ± 0.6 ^b | 6.9 ± 1.6 |
| PS (18:0/18:1) | 788.5442 | 788.5428 | 6.1 ± 2.1 | 9.2 ± 3.1 | 9.2 ± 3.7 | 1.7 ± 0.2 |
| SM (d41:2) | 797.6542 | 797.6533 | 3.9 ± 0.9 | 6.8 ± 1.3 | 5.2 ± 1.3 | 6.1 ± 0.6 |
| ST (18:0) | 806.5451 | 806.5443 | 3.7 ± 0.8 | 3.1 ± 0.5 | 2.3 ± 0.2 | 2.1 ± 0.2 |
| PI (34:1) | 835.5342 | 835.5318 | 2.9 ± 0.5 | 3.8 ± 1.4 | 3.9 ± 1.2 | 2.6 ± 0.3 |
| PI (16:0/20:4) | 857.5186 | 857.5165 | 0.7 ± 0.2 | 1.8 ± 1.1 | 0.5 ± 0.2 | 0.5 ± 0.3 |
| ST (22:0) | 862.6077 | 862.6072 | 2.3 ± 0.3 | 1.2 ± 0.5 | 1.2 ± 0.5 | 1.6 ± 0.2 |
| ST (d18:1/h22:0) | 878.6033 | 878.6078 | 6.8 ± 0.8 | 5.4 ± 1.3 | 4.3 ± 1.2 | 3.2 ± 0.7 |
| PI (18:0/20:4) | 885.5494 | 885.5473 | 12.1 ± 1.1 | 9.8 ± 1.5 | 10.1 ± 1.1 | 9.2 ± 1.6 |
| ST (24:0) | 890.6395 | 890.6348 | 29.9 ± 4.1 | 22.3 ± 2.1 | 24.0 ± 0.8 | 19.9 ± 2.3 |
| ST (d18:1/23:0) or ST (d18:0/24:0) | 892.6189 | 892.6169 | 15.5 ± 2.0 | 9.3 ± 2.1 | 11.5 ± 2.8 | 8.0 ± 2.6 |
| CPI (40:2) + MBT | 896.5779 | 896.5779 | 0.2 ± 0.1 | 6.7 ± 2.4 ^a | 4.3 ± 1.6 | 1.8 ± 0.6 |
| ST (d18:1/25:1) or (d18:2/25:0) | 902.6396 | 902.6380 | 32.1 ± 3.0 | 21.3 ± 1.5 ^a | 26.1 ± 0.6 | 23.2 ± 0.3 |
| ST (d18:1/h24:1) | 904.6189 | 904.6150 | 49.7 ± 5.7 | 35.9 ± 2.2 | 33.7 ± 5.7 | 30.8 ± 3.2 ^c |
| ST (d18:1/h24:0) | 906.6345 | 906.6316 | 37.4 ± 5.0 | 22.8 ± 2.2 | 28.5 ± 4.5 | 20.0 ± 3.9 |
| ST (d18:1/h25:1) or (d18:2/h25:0) | 918.6346 | 918.6308 | 12.3 ± 2.0 | 7.2 ± 0.7 | 7.5 ± 0.9 | 6.1 ± 0.9 ^c |
| ST (d18:1/h25:0) | 920.6502 | 920.6455 | 7.4 ± 1.2 | 4.0 ± 0.2 | 6.1 ± 0.5 | 3.7 ± 0.9 ^c |
| ST (d18:1/h26:1) | 932.6502 | 932.6471 | 10.3 ± 0.8 | 5.3 ± 0.5 ^a | 7.8 ± 0.4 | 6.7 ± 1.4 |

Data are mean ± SEM. The p values were calculated by the Kruskal-Wallis non-parametric test followed by Dunn's test. Control (n = 5), AD I–II (n = 5), AD III–IV (n = 5), AD V–VI (n = 5). Cal: calculated, Exp: experimental. The notation (hX:Y) denotes hydroxylated sulfatide species.

^a AD I–II vs control.

^b AD III–IV vs control.

^c AD V–VI vs control.

2. Materials and methods

2.1. Brain sectioning

The human brain samples were supplied by the Institute of Neuropathology (University Hospital Bellvitge, Barcelona). Human brains were obtained at autopsy after prior informed consent and with the institutional approval of the ethics committees of the University of the Basque

Country (UPV/EHU), following the Code of Ethics of the World Medical Association (Declaration of Helsinki), and ensuring the privacy rights of the human subjects. The control samples were obtained from 5 males who had shown no evidence of neurological or metabolic disease. The neuropathological study disclosed no abnormalities in the brain. The AD samples were obtained from 15 males and females with AD at different Braak's stages. The intervals between death and autopsy were from 2.5 to 19 h (Table 1). Following autopsy, the brain samples were

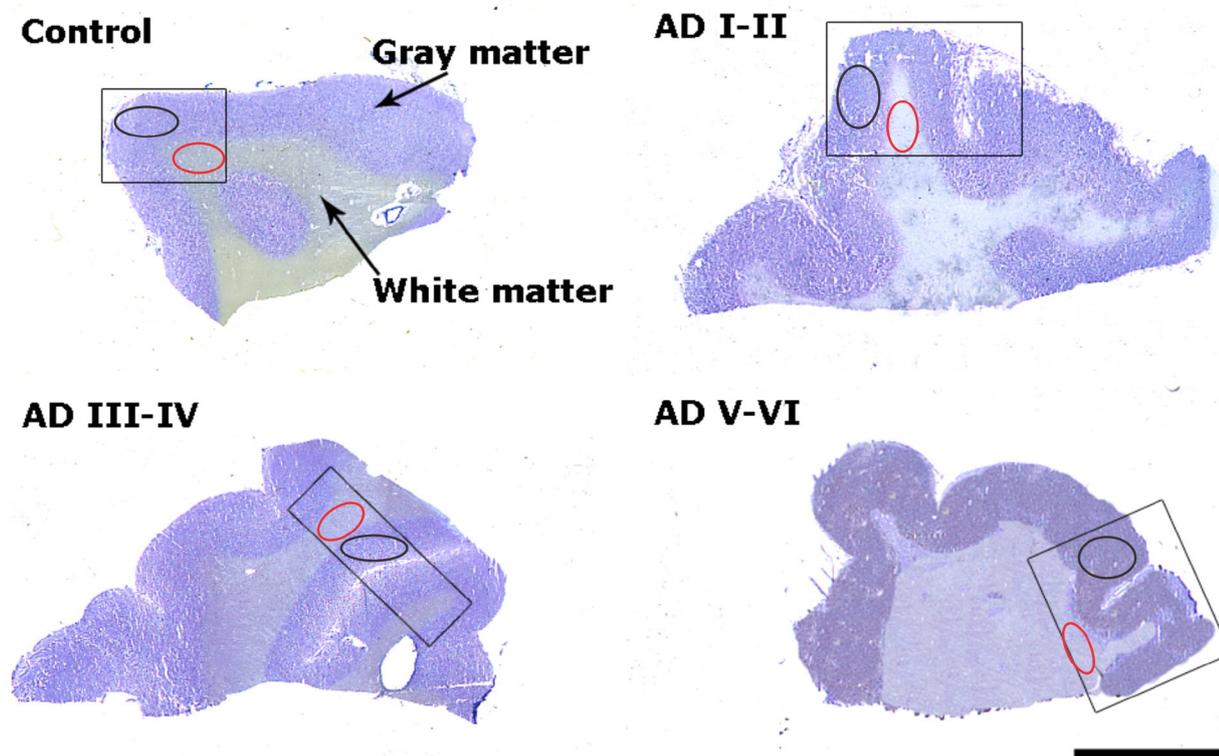


Fig. 3. Representative images of thionine stained brain sections of control (A), AD I–II (B), AD III–IV (C), AD V–VI (D). The black oval areas are the regions of interest (ROI) selected for measurement in gray matter and the red oval areas are the ROI in white matter. Bar: 1 cm.

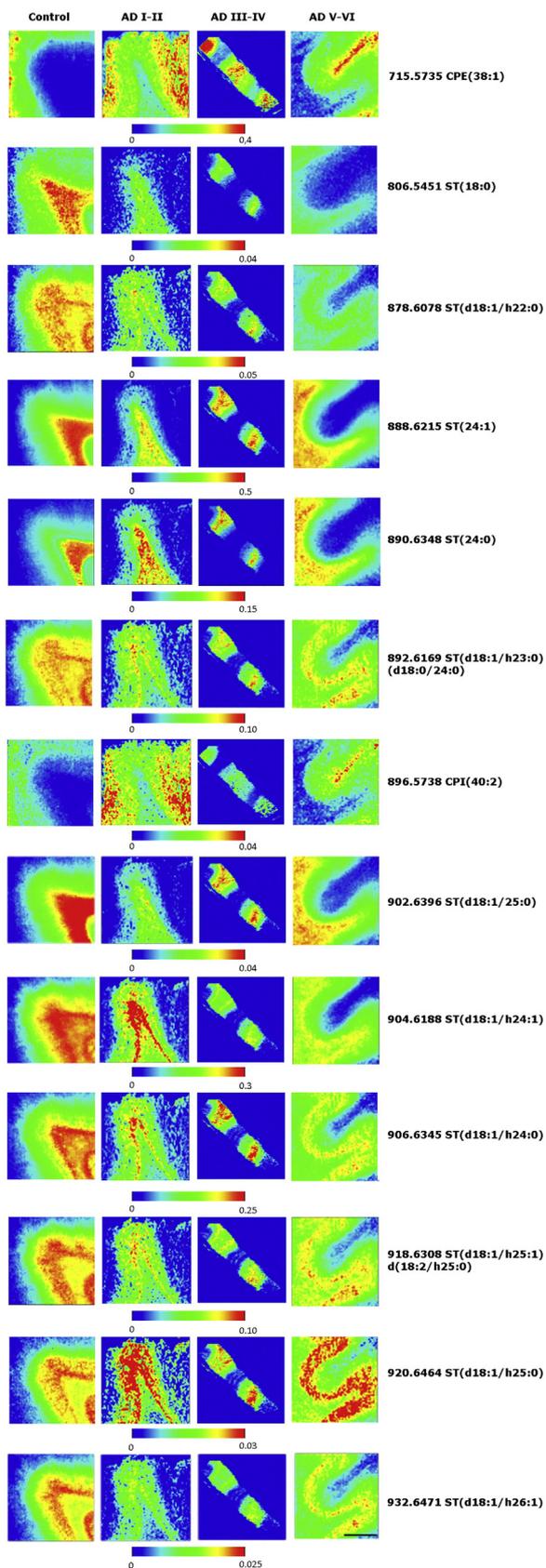


Fig. 4. The image shows the distribution of representative lipid species that were found modified when controls were compared with AD patients at different stages of the disease. The color-coded images show areas of gray and white matter in the frontal cortex of human brain tissue. The spectra were recorded with 150 μm spacing. Ten shots were accumulated at each point with the spectrometer working in negative ion mode. Laser power was set at 15–20 μJ . Bar : 3 mm.

Controls

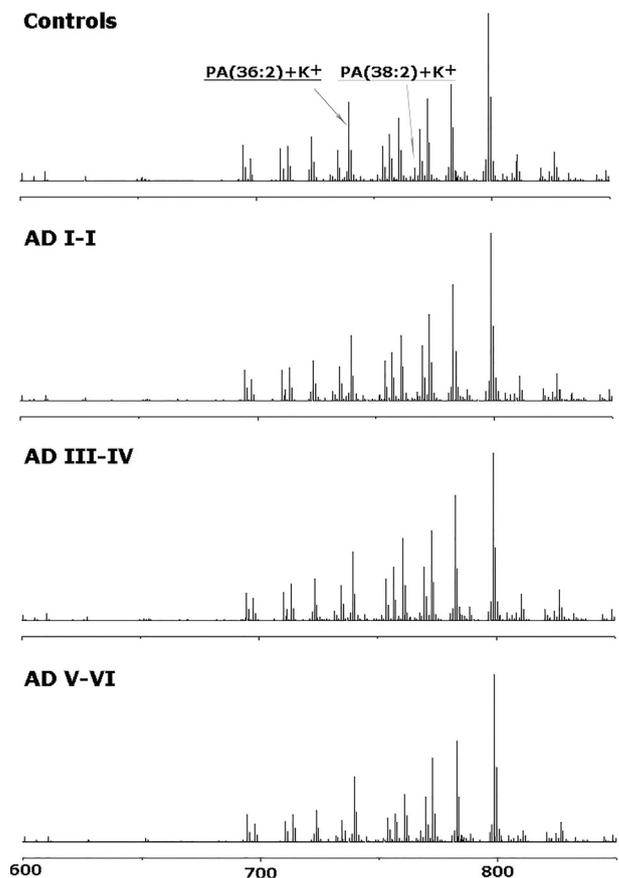


Fig. 5. Average mass spectra in positive ion mode from control subjects and AD patients selecting only gray matter. The spectra parameters were 2 micro scans/step with 10 laser shots and a raster step size of 150 μm with the laser power at 15–20 μJ and using MBT matrix. The “y” axis represents the intensity of the measured MS signal as percentage of the maximal peak. The maximal peak is the most intense peak of the spectrum, in this case 798.60 PC (34:1) + K⁺, which is set to 100%.

immediately frozen at -80°C . The frozen tissue was brought to -20°C , and 20 μm thick slices were obtained in a cryostat (Microm HM550, Walldorf, Germany). Two consecutive sections were mounted on slides and kept at -25°C until they were treated with the chemical matrix and introduced into a MALDI metal holder (LTQ orbitrap XL).

2.2. Thionine staining

Thionine staining was performed in consecutive sections to those used in MALDI-IMS experiments to facilitate the identification of neuro-anatomical structures. Tissue slices mounted onto gelatine-coated slides were hydrated after thawing. Hydration was performed by incubating tissue slices for 5 min in ethanol concentrations in a descending order (100%, 96%, 70% and 50%). Then, sections were submerged in thionine solution for 5 min. Finally, the sections were washed in deionized water and introduced in an ethanol ascendant order solution (50%, 70%, 96% and 100%) to dehydrate the tissue. The thionine stained images allowed us to delineate the area for MALDI scanning, including the six layers of the cortex and the adjacent white matter.

2.3. Sample preparation

The original lipid composition and anatomical characteristics of the tissue must be preserved throughout the sample-preparation process [37]. The frontal cortex brain samples were sectioned and stored until the moment of use, as described above.

Table 4
Percentage of the relative intensity of representative lipids in gray matter of *postmortem* human frontal cortex of controls and AD patients in positive ion detection mode. The maximal peak is the most intense peak of the spectrum, in this case 798.60 PC (34:1) + K⁺, which is set at 100%.

| Assignment | Cal <i>m/z</i> | Exp <i>m/z</i> | % intensity | | | |
|-----------------------------------|----------------|----------------|-------------|-------------------------|-------------|------------|
| | | | Control | AD I–II | AD III–IV | AD V–VI |
| PA (34:1) + Na ⁺ | 697.4779 | 697.4785 | 12.9 ± 3.4 | 12.6 ± 3.8 | 10.7 ± 2.1 | 10.5 ± 2.1 |
| GlcCer (d32:1) + K ⁺ | 710.4968 | 710.4912 | 20.0 ± 1.7 | 15.2 ± 3.2 | 14.9 ± 3.1 | 15.5 ± 0.7 |
| PA (34:1) + K ⁺ | 713.4518 | 713.4525 | 29.1 ± 1.8 | 20.4 ± 3.6 | 21.7 ± 4.0 | 24.3 ± 2.3 |
| PA (36:2) + Na ⁺ | 723.4935 | 723.4941 | 26.4 ± 4.9 | 23.8 ± 5.7 | 25.6 ± 3.5 | 22.3 ± 2.6 |
| SM (d18:1/18:0) | 731.6067 | 731.6083 | 2.7 ± 1.3 | 7.1 ± 0.9 | 7.6 ± 2.0 | 4.5 ± 0.6 |
| PC (32:0) | 734.5654 | 734.5688 | 18.1 ± 2.4 | 22.0 ± 1.6 | 22.7 ± 6.0 | 16.5 ± 3.4 |
| PA (36:2) + K ⁺ | 739.4680 | 739.4685 | 50.9 ± 1.3 | 33.4 ± 3.6 ^a | 38.5 ± 4.0 | 40.9 ± 1.6 |
| PA (38:2) + Na ⁺ | 751.5258 | 751.5248 | 4.3 ± 1.2 | 2.8 ± 0.5 | 2.9 ± 0.3 | 2.8 ± 0.5 |
| SM (d18:1/18:0) + Na ⁺ | 753.5886 | 753.5896 | 20.8 ± 3.7 | 23.8 ± 4.9 | 24.2 ± 2.9 | 16.6 ± 2.0 |
| PC (32:0) + Na ⁺ | 756.5519 | 756.5534 | 27.4 ± 5.1 | 31.7 ± 5.3 | 27.2 ± 5.1 | 28.8 ± 4.6 |
| PC (34:2) | 758.5654 | 758.5690 | 2.9 ± 0.5 | 3.0 ± 0.5 | 2.9 ± 0.4 | 2.3 ± 0.3 |
| PC (34:1) | 760.5856 | 760.5858 | 36.9 ± 5.7 | 46.3 ± 7.6 | 48.9 ± 10.8 | 38.1 ± 6.3 |
| PA (38:2) + K ⁺ | 767.4988 | 767.5006 | 8.3 ± 0.5 | 2.2 ± 0.3 ^a | 5.7 ± 0.4 | 6.1 ± 0.5 |
| SM (d18:1/18:0) | 769.5626 | 769.5641 | 15.3 ± 1.8 | 14.5 ± 1.4 | 16.4 ± 1.4 | 11.4 ± 1.3 |
| PC (32:0) + K ⁺ | 772.5259 | 772.5261 | 49.0 ± 4.3 | 54.6 ± 7.4 | 51.2 ± 5.1 | 53.3 ± 6.2 |
| PC (34:2) + Na ⁺ | 780.5519 | 780.5533 | 2.8 ± 0.3 | 4.3 ± 0.5 | 3.5 ± 0.9 | 3.7 ± 0.9 |
| PC (34:1) + Na ⁺ | 782.5675 | 782.5680 | 62.0 ± 10.3 | 67.9 ± 4.3 | 73.0 ± 6.3 | 75.8 ± 6.8 |
| PC (36:3) | 784.5856 | 784.5748 | 6.6 ± 0.9 | 8.8 ± 1.0 | 9.5 ± 1.3 | 7.1 ± 1.1 |
| PC (36:1) | 788.6164 | 788.6171 | 5.6 ± 1.1 | 6.8 ± 1.3 | 6.6 ± 2.1 | 5.3 ± 1.1 |
| PC (34:2) + K ⁺ | 796.5259 | 796.5261 | 5.2 ± 0.9 | 6.0 ± 0.2 | 5.7 ± 0.8 | 5.5 ± 0.5 |
| SM (d18:1/20:0) | 797.5939 | 797.5943 | 12.4 ± 1.2 | 11.8 ± 1.7 | 11.8 ± 0.6 | 10.3 ± 1.2 |
| PC (36:1) + Na ⁺ | 810.5988 | 810.5993 | 15.3 ± 1.8 | 14.5 ± 1.4 | 16.4 ± 1.4 | 11.4 ± 1.3 |
| PC (34:6) + K ⁺ | 820.5259 | 820.5271 | 7.1 ± 1.9 | 6.9 ± 0.7 | 7.4 ± 1.0 | 5.7 ± 0.3 |
| PC (36:1) + K ⁺ | 826.5728 | 826.5733 | 18.3 ± 0.7 | 16.8 ± 0.8 | 18.1 ± 1.0 | 15.6 ± 1.0 |
| PC (38:4) + K ⁺ | 848.5572 | 848.5572 | 6.1 ± 1.6 | 6.1 ± 0.7 | 5.9 ± 0.8 | 4.2 ± 0.2 |

Data are mean ± SEM. The *p* values were calculated by the Kruskal-Wallis non-parametric test followed by Dunn's test. Control (*n* = 5), AD I–II (*n* = 5), AD III–IV (*n* = 5), AD V–VI (*n* = 5). Cal: calculated, Exp: experimental.

^a AD I–II vs control.

Matrix deposition on the tissue surface was carried out by sublimation prior to analysis, using a glass sublimator (ACE glass 8023). Sublimation was performed using 300 mg of 2-mercaptobenzothiazole (MBT), which has previously been reported as being an optimal chemical matrix for phospholipid detection [38]. We controlled the deposition time and temperature (30 min, 140 °C) and the thickness of the matrix layer, which optimized the signal/noise (*s/n*) ratio of the mass spectra, and avoided lipid migration due to the absence of organic solvents during the sublimation procedure. Finally, a recrystallization of the sample was performed using a normal glass Petri plate (100 mm diameter × 15 mm depth), and 1 ml of methanol (99%) was added to a piece of paper previously placed in the bottom of the Petri plate in order to create a vapor atmosphere using a hot plate (1 min, 40 °C). This step allowed us to achieve higher intensities in the detection of the peaks in the mass spectra [39].

2.4. MALDI imaging mass spectrometry

A MALDI LTQ-XL-Orbitrap (Thermo Fisher Scientific, Bremen, Germany) equipped with a nitrogen laser ($\lambda = 337$ nm, rep. rate = 60 Hz, spot size = 80 × 120 μm) was used for mass analysis. ImageQuest 1.0.1 software (Thermo Fisher Scientific, San Jose, CA, USA) was used for MALDI-IMS data acquisition. The images were acquired in positive ion mode in *m/z* range 500–1000 and in negative mode in *m/z* range 400–1100 (depending on their charge) with 10 laser shots per point at laser fluence of 15 μJ . However, from all the lipid species detected within those ranges, only the relative intensities of the most representative lipid species, or those showing significant modulations in patients are showed. The target plate stepping distance was set to 150 μm for both the *x*- and *y*-axes by the MSI image acquisition software. The mass resolution was 100,000 (full width at half maximum as defined at *m/z* 400). As there may be potential displacement of the masses on the tissue due to surface irregularities and other factors, it is necessary to normalize them using the total ion current (TIC) [40].

2.5. Image and spectra analysis of MALDI-IMS

The MALDI images were generated using ImageQuest software (Thermo Scientific, San Jose, CA). The software provided a *m/z* range that is plotted for signal intensity for each pixel (mass spectrum) across a given area (tissue section). The spectra normalization was performed by TIC with MSReader software [41]. The mean intensity of two regions of interest (ROI) in white and grey matter respectively was calculated using OriginPro 8 software of the five samples in each group. The most intense peak was considered to be 100% and the rest of the peaks were normalized to the most abundant species to compare the four groups. Differences between groups of patients were analyzed by using the Kruskal-Wallis non-parametric test followed by Dunn's *post-hoc* test with GraphPad Prism (San Diego, CA, USA) software. The results were considered significant when $p \leq 0.05$ [42].

2.6. Peak Assignment

The analysis of the lipid composition of tissue samples from *postmortem* human brain used in the present study is complex. A large number of different possible lipid species were detected and some of them share similar masses and had not been detected and/or assigned to a specific lipid molecule in previous studies. Nevertheless, the assignment of lipid species was facilitated by the use of databases such as Lipid MAPS (<http://www.lipidmaps.org/>), Madison Metabolomics (<http://mmcd.nmrfa.wisc.edu>) and previous reports [43–51]. For the assignment, 5 ppm mass accuracy was selected as the tolerance window. The glycerolipid species numbers (*x*:*y*) denote the total length and the number of double bonds of the acyl chains, while the sphingolipid and sulfatide species numbers correspond to the length and number of double bonds of the acyl chain added to those of the attached sphing-4-enine (d18:1) or sphinganine (d18:0) base.

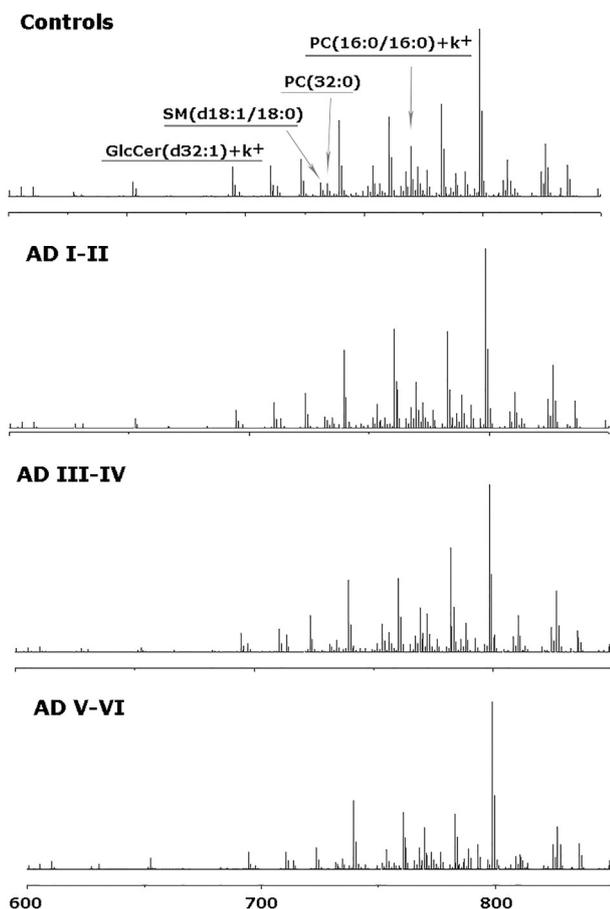


Fig. 6. Average mass spectra in positive ionization detection mode corresponding to the control group and the three groups of AD patients that were obtained selecting only white matter. The spectra parameters were 2 micro scans/step with 10 laser shots and a raster step size of 150 μm with the laser power at 15–20 μJ and using MBT matrix. The “y” axis represents the intensity of the measured MS signal as percentage of the maximal peak. The maximal peak is the most intense peak of the spectrum in this case 798.60 PC (34:1) + K^+ , which is set at 100%.

3. Results

The MALDI-IMS technique represents a useful approach to direct anatomical localization of lipids in tissue sections. In the present study this technique is applied to *postmortem* human brain sections of controls and AD patients in positive and negative ion detection mode to obtain anatomical images, which indicate the intensity of the signal for different lipid species. The results were obtained from serial sections of human brain samples of the frontal cortex area (Brodmann area 8). We compared the data obtained from control subjects with those of AD patients classified in three groups according to different Braak's stages. The mean data for each group of patients was obtained, showing the intensity of each lipid species in both white and gray matter separately by using a specific image software, as described in the methods section, thereby clearly differentiating the lipid composition of white and gray matter. The detection of different lipid molecules, calculations and statistical analysis were carried out for the molecular ions in negative and positive mode. The analyses were carried out by normalizing by TIC using the MSReader software, and setting the peak with the highest intensity at 100%, the rest of the peaks were normalized to the most abundant species indicating a percentage of relative intensity. In the case of negative ion mode in gray matter the maximal peak was the phosphatidylinositol (PI) (20:4/18:0), and in white matter it was ST (24:1). In contrast, in positive ion detection mode, the maximal peak was PC (34:1) + K^+ for both gray and white matter.

Firstly, MALDI-IMS analysis in negative ion detection mode demonstrated that ST content was reduced by more than 50% in gray matter of frontal cortex from the earliest clinically identifiable stage of AD, the group AD I–II (Fig. 1, Table 2).

In contrast, in white matter, there was a decrease in all ST species from the earliest stages, which did not become statistically significant until the advanced stages of the disease, and then only for some specific ST species (Fig. 2, Table 3).

Furthermore, we have observed an increase in ceramide-1-phosphoinositol (CPI) (40:2) in the earliest stages of the disease in both gray (Control $3.5 \pm 0.9\%$ vs AD I–II $20.5 \pm 5.8\%$, $p < 0.05$) and white matter (Control $0.2 \pm 0.1\%$ vs AD I–II $6.7 \pm 2.4\%$, $p < 0.05$). However, in the most advanced stages of AD, CPI (40:2) decreased to intensities comparable with those of control subjects. In addition, another ceramide, ceramide-1-phosphoethanolamine (CPE) (38:1) was increased only in white matter from the earliest stages and reached a statistically significant increase during moderate stages of AD (Control $3.3 \pm 0.8\%$ vs AD III–IV $8.1 \pm 0.6\%$, $p < 0.05$). The thionine stained sections from control subjects and AD patients showed the different structures in the tissue sections and differentiated the areas of white and gray matter selected for the IMS analysis (Fig. 3).

IMS method allowed us to obtain the anatomical distribution pattern of lipids in color coded images, and showed the intensity of the lipids that were modified in the cortical samples from AD patients (Figs. 4 and 7). The images showed lipid intensities both in gray and white matter and the differences in lipid intensities between control subjects and AD patients in different stages of the disease. These images have been obtained by using IMS in both negative (Fig. 4) and positive (Fig. 7) ion detection modes.

In respect of the results obtained in positive ion mode, the levels of only a few lipid species were significantly changed in AD patients. Moreover, these lipid species do not follow a clear pattern of specific types of molecules (as was the case of the decrease in ST species in negative mode). The intensity of the signal of two lipid species was modified in cortical gray matter: phosphatidic acid (PA) (36:2) + K^+ (Control $50.9 \pm 1.3\%$ vs AD I–II $33.4 \pm 3.6\%$, $p < 0.05$) and PA (38:2) + K^+ (Control $8.3 \pm 0.5\%$ vs AD I–II $2.2 \pm 0.3\%$, $p < 0.05$). Their lowest intensities were detected in the earliest stages of AD (Fig. 5, Table 4).

On the contrary, in cortical white matter we observed three lipid species with low intensities in AD patients using positive ion detection mode: PC (32:0) (Control $13.1 \pm 4.2\%$ vs AD V–VI $3.9 \pm 0.5\%$, $p < 0.05$), sphingomyelin (SM) (d18:1/18:0) (Control $7.4 \pm 1.3\%$ vs AD III–IV $2.3 \pm 0.8\%$, $p < 0.05$) and glucosylceramide (GlcCer) (d32:1) + K^+ (Control $18.1 \pm 1.1\%$ vs AD III–IV $10.7 \pm 1.3\%$, $p < 0.05$) (Fig. 6, Table 5).

4. Discussion

Lipid homeostasis is essential to good health and a deregulation of lipid metabolism plays an important role in many human diseases, including some neurological pathologies. In recent years, some experimental evidence indicates that impairments in some metabolic lipid pathways could be involved in the development of AD [3,52–54]. The analysis of complex lipid mixtures through the development of soft ionization techniques (MALDI and electrospray ionization, ESI), allow us to study in greater detail these lipid pathways. The advances made in mass spectrometry technologies during recent years have given rise to the increasingly important field of lipidomics, which provides us with new opportunities to understand the metabolism and signaling by lipids in different physiological conditions and diseases [55,56]. The present study uses IMS, to determine the involvement of changes in the lipid composition in the neurodegenerative processes, which occur in AD, and could indicate that there is degradation of structural cell membrane molecules. Therefore, we have tried to assess the differences in lipid species in human brain samples from prefrontal cortex of AD patients at different Braak's stages (I–II, III–IV, V–VI) by using the MALDI-IMS assay. The study was performed both in positive and negative ion

Table 5
Percentage of the relative intensity of representative lipids in white matter of *postmortem* human frontal cortex of controls and Alzheimer disease patients in positive ion detection mode. The maximal peak is the most intense peak of the spectrum, in this case 798.60 PC (34:1) + K⁺ which is set at 100%.

| Assignment | Cal m/z | Exp m/z | % intensity | | | |
|-----------------------------------|----------|----------|-------------|-------------|-------------------------|-------------------------|
| | | | Control | AD I–II | AD III–IV | AD V–VI |
| (–) | – | 610.4447 | 8.2 ± 2.3 | 3.4 ± 0.7 | 3.2 ± 0.2 ^a | 3.1 ± 0.3 |
| GlcCer (d32:1) + K ⁺ | 710.4968 | 710.4902 | 18.1 ± 1.1 | 10.7 ± 2.1 | 10.7 ± 1.3 ^a | 13.2 ± 1.1 |
| SM (d18:1/18:0) | 731.6067 | 731.6069 | 7.4 ± 1.3 | 4.1 ± 2.7 | 2.3 ± 0.8 ^a | 3.3 ± 0.4 |
| PC (32:0) | 734.5654 | 734.5688 | 13.1 ± 4.2 | 6.4 ± 1.3 | 6.4 ± 1.5 | 3.9 ± 0.2 ^b |
| PA (36:2) + K ⁺ | 739.4691 | 739.4683 | 49.9 ± 1.8 | 43.7 ± 5.6 | 41.9 ± 6.2 | 39.4 ± 1.8 |
| PA (38:2) + Na ⁺ | 751.5258 | 751.5248 | 4.9 ± 2.1 | 6.6 ± 0.8 | 4.9 ± 0.9 | 4.5 ± 0.6 |
| SM (d18:1/18:0) + Na ⁺ | 753.5886 | 753.5889 | 15.8 ± 3.4 | 14.6 ± 2.9 | 16.2 ± 3.6 | 12.3 ± 1.9 |
| PC (32:0) + Na ⁺ | 756.5519 | 756.5522 | 5.8 ± 0.5 | 7.3 ± 1.6 | 5.2 ± 1.6 | 6.5 ± 0.6 |
| PC (34:1) | 760.5856 | 760.5856 | 47.5 ± 11.2 | 56.6 ± 14.3 | 24.2 ± 2.9 | 35.5 ± 4.2 |
| PA (38:2) + K ⁺ | 767.4988 | 767.5004 | 15.1 ± 1.4 | 14.0 ± 2.6 | 38.6 ± 5.6 | 10.5 ± 1.9 |
| SM (d18:1/18:0) + K ⁺ | 769.5626 | 769.5629 | 30.1 ± 2.0 | 24.8 ± 2.3 | 11.5 ± 2.4 | 26.9 ± 1.2 |
| PC (32:0) + K ⁺ | 772.5259 | 772.5271 | 17.8 ± 2.2 | 15.5 ± 1.3 | 25.3 ± 2.2 | 10.6 ± 1.4 ^b |
| (–) | – | 776.5951 | 13.7 ± 4.1 | 12.5 ± 2.3 | 12.2 ± 1.3 | 10.5 ± 1.3 |
| PC (34:1) + Na ⁺ | 782.5675 | 782.5691 | 43.9 ± 9.4 | 61.0 ± 8.8 | 9.0 ± 1.8 | 49.7 ± 7.0 |
| PC (36:3) | 784.5856 | 784.5743 | 6.7 ± 1.2 | 7.1 ± 1.1 | 61.0 ± 9.1 | 5.6 ± 0.5 |
| PC (36:1) | 788.6157 | 788.6195 | 18.0 ± 2.8 | 18.3 ± 4.8 | 6.6 ± 0.7 | 9.2 ± 0.5 |
| (–) | – | 792.5689 | 13.9 ± 0.9 | 11.3 ± 2.6 | 111.9 ± 3.9 | 17.1 ± 2.6 |
| PC (34:2) + K ⁺ | 796.5259 | 796.5248 | 4.7 ± 0.6 | 5.2 ± 0.1 | 9.7 ± 1.5 | 5.0 ± 0.7 |
| PC (38:5) | 808.5851 | 808.5844 | 8.4 ± 2.6 | 10.0 ± 1.7 | 4.8 ± 0.5 | 7.6 ± 1.3 |
| PC (36:1) + Na ⁺ | 810.5988 | 810.6004 | 21.7 ± 3.9 | 20.7 ± 2.9 | 8.6 ± 2.4 | 14.1 ± 0.7 |
| PC (36:2) + K ⁺ | 824.5571 | 824.5576 | 14.5 ± 1.2 | 15.9 ± 0.6 | 2.16 ± 3.7 | 15.4 ± 1.5 |
| PC (36:1) + K ⁺ | 826.5728 | 826.5757 | 34.5 ± 0.9 | 35.7 ± 3.5 | 13.7 ± 2.8 | 25.1 ± 1.6 |
| SM (42:2) + Na ⁺ | 835.6663 | 835.6684 | 19.5 ± 3.7 | 16.0 ± 2.7 | 30.5 ± 4.6 | 15.8 ± 1.5 |
| SM (42:2) + K ⁺ | 851.6403 | 851.6411 | 30.9 ± 1.8 | 23.5 ± 2.6 | 14.3 ± 2.5 | 29.2 ± 3.4 |

Data are mean ± SEM. The p values were calculated by the Kruskal–Wallis non-parametric test followed by Dunn's test. Control (n = 5), AD I–II (n = 5), AD III–IV (n = 5), AD V–VI (n = 5). (–): unidentified lipid species; Cal: calculated, Exp: experimental.

^a AD III–IV vs control.

^b AD V–VI vs control.

mode, depending on the polarity used to record the different lipid species. Usually, the phospholipid species recorded in positive ion detection mode include PA, PC and SM, and in negative mode the most common lipids detected are PE, ST, PS and PI.

The main changes we have observed in the samples from AD patients were in ST species, a class of lipid molecules that are synthesized predominantly by oligodendrocytes. These are multi-functional molecules involved in the physiology of different tissues and systems including the peripheral and central nervous systems [57–59]. In the CNS, the ST are negative regulators for the differentiation and survival of the oligodendrocytes during myelination processes [60,61]. Studies in a ST deficient mouse model have shown the presence of structural abnormalities and loss of axonal conduction [62]. Other studies demonstrate the regulation of ST metabolism by apolipoprotein E (APOE) [47, 63], and the APOE ε4 allele is a risk factor for AD [6,64,65].

We report a significant reduction of relative intensity in different ST species in patients at different clinical stages of AD, in both cortical gray and white matter in comparison with the maximum intensity peak PI (38:4) and ST (24:1) respectively.

Nevertheless, as the observed ST relative intensity depletion was similar in all stages of the disease, this implies that there is a ST deficiency from the onset of AD. An important conclusion, which may be drawn from our study, is that ST depletion is a metabolic impairment possibly related to synaptic dysfunction and neurodegeneration present in AD from its earliest stages. The reduction of ST molecules in AD has previously been described by other research groups, and it has been demonstrated that this ST depletion specifically affects AD patients and does not appear in other neurodegenerative diseases, such as Parkinson's disease and Lewy body dementia [18,19,66]. Furthermore, ST has been used in plasma samples to distinguish between elderly control and AD patients [67]. In addition, a recent IMS study using experimental models exposed to cigarette smoke describes changes of ST species in the frontal lobe [68], thereby, supporting the possible relationship between cigarette smoking and the risk of developing AD [69]. A recent study reported no changes in the distribution of ST in temporal lobe brain

samples from three AD patients, but they do not discriminate between the different stages of the disease [70].

The metabolic mechanisms, which produce the reduction in specific ST species observed in the frontal cortex of AD patients are still unknown. It has been suggested that an accelerated degradation of ST could lead to the production of ceramides (Cer). Some authors have reported elevated levels of Cer in AD brains compared to non-affected subjects [22,71,72]. We observed an increase in CPI and CPE, but not in other Cer species previously reported. IMS allows anatomical detection without purification artifacts, but the ion suppression generated by MALDI-IMS could reduce the ability to detect molecular species of low abundance [73]. Moreover, the matrix (MBT) has a molecular weight (167.25) and can produce ionization products in a mass range similar to some Cer, thereby masking their detection. It has been shown that Cer species are able to stabilize the APP cleaving enzyme 1 (BACE1), promoting Aβ biogenesis [74,75]. On the other hand, a reduction of Cer levels leads to a reduced secretion of APP and Aβ in human neuroblastoma cells [76]. Thus, it has been suggested that Cer and Aβ could interact and induce neuronal death in AD [22]. In addition, in the APPsw transgenic mice model of familial AD, there is also ST depletion in cerebellum, but less than in cortex, supporting the idea that Aβ could be associated with ST deficiency, since Aβ accumulation does not occur to a significant extent in cerebellum [47,77].

In KO mice for the lipoprotein receptor-related protein 1 (LPR1), which is related to APOE in the transport and metabolism of plasma lipids and Aβ clearance, deficits in brain lipid metabolism have been described, characterized by decreased levels in cholesterol, ST and galactosylceramide. These lipid alterations positively correlate with synapse loss, neuroinflammation, memory loss and signs of neurodegeneration [78].

Due to the modifications observed in negative ion detection mode in AD patient's relative intensities, it is necessary to study ST during the earliest or even from prodromal stages of the disease. Specific lipid deregulation could have primary effects on the other neurodegenerative processes, including the formation of senile plaques and neurofibrillary

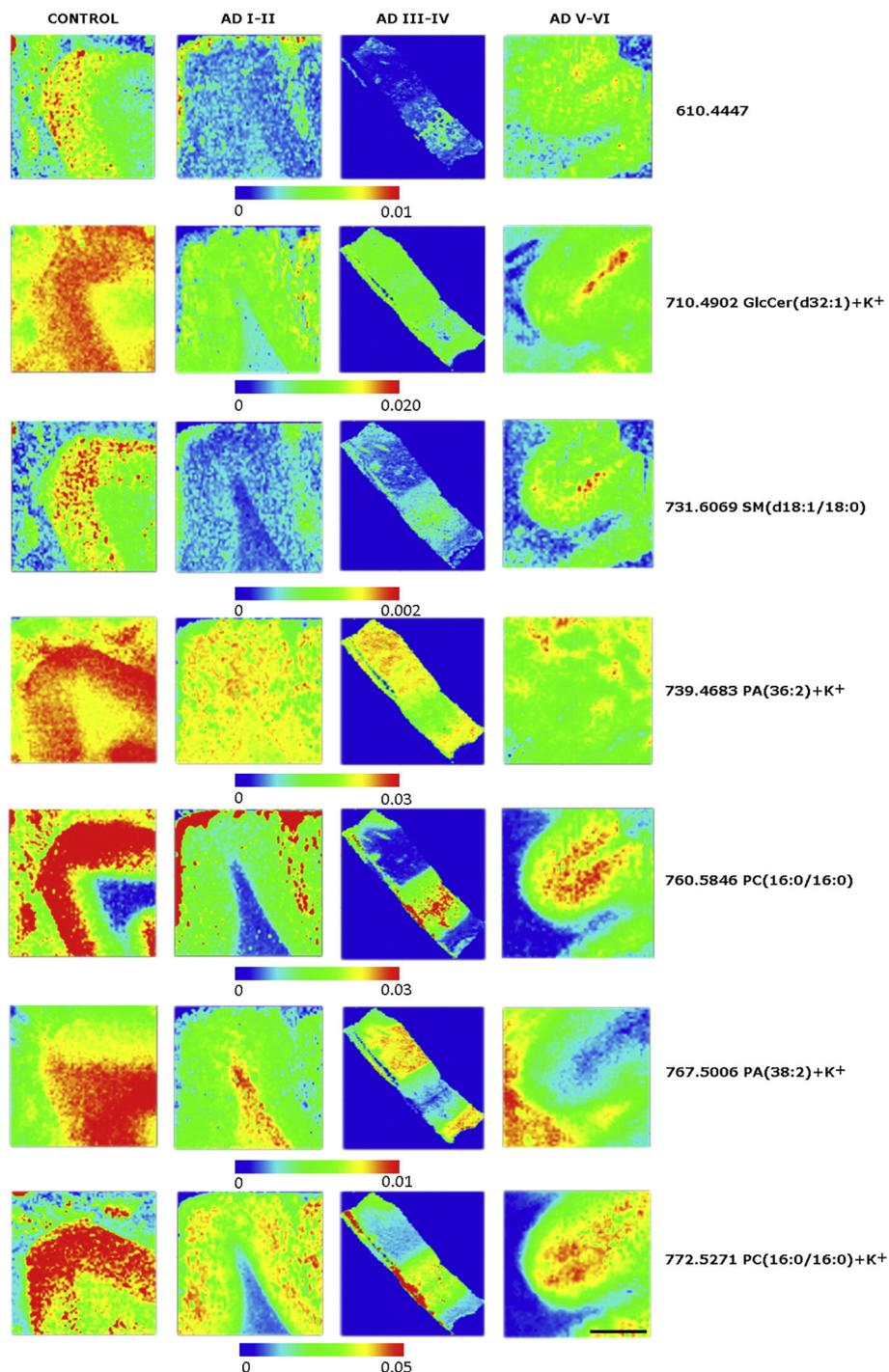


Fig. 7. The image shows the distribution of some representative lipid species that were found modified in gray or white matter areas of the frontal cortex from human brain tissue (controls and AD patients at three different stages of the disease). The spectra were recorded with 150 μm spacing. Ten shots were accumulated at each point with the spectrometer working in positive ion mode. Laser power was set at 15–20 μJ . Bar : 3 mm.

tangles. In addition, the levels of specific ST species could be considered as biomarkers for AD.

We also found other changes, such as the decrease of two PA species in gray matter in positive ion detection mode when normalized to PC (34:1) intensity (the most abundant species), which is in agreement with previous studies that reported a decrease of PA in temporal gyrus of AD patients [17]. Another study demonstrated that anionic phospholipids, predominantly PA, diphosphatidyl glycerol (DPG), PS, and PI, interact with A β peptide, in particular with A β (1–43) and A β (1–42), increasing the fibrillization of A β [79,80]. Interestingly, in our study, we found two PA species decreased in frontal cortex of AD patients,

but not the DPG, PS or PI lipid species. Accordingly, in early stages of AD, cerebral PE, PI and PS were unchanged in both white and gray matter [18]. Other authors have reported a decrease in PI in temporal and occipital cortex, and in cerebellum [17,81,82]. All these studies used traditional analytical techniques for phospholipid profiling, which require large brain samples and use tissue homogenates and extracts that could lead to cross contamination between different brain regions of gray and white matter. However, we minimized the sample size and the possible contamination between different brain regions, as MALDI-IMS was performed with tissue sections and gave anatomical localization of the lipids all over the tissue slice.

Finally, in this study we observed a decrease in GlcCer, SM and PC, when normalized to PC (34:1) (the most abundant species), in white matter. Recent studies revealed contradictory results about SM levels in AD depending on the brain areas analyzed and on the analytical technique used. Some authors have described unchanged SM levels in CSF, and extracts from cerebellum, middle frontal gyrus white matter of frontal cortex [18,22], whilst others have described an increase in CSF, middle frontal gyrus gray matter and cerebellum [17,64,82], or a decrease in SM levels in middle frontal gyrus [20]. We found that there is a decrease in one specific SM species with the progression of the disease, SM (d18:1/18:0). The oxidative stress induced by A β could lead to an increase in Cer levels, as described above. Many studies have claimed a decrease in PC in plasma, brain homogenates and brain tissue sections from AD patients [28,83,84], especially in PC containing docosahexaenoic acid (DHA). We have detected two different DHA-PC (38:6) and (40:6), but both of them without significant changes between controls and patients. However, we have observed a decrease of PC (32:0) relative intensity, a PC with two palmitic acids in its structure, in white matter of the frontal cortex. A recent study has stated that palmitoylation of APP regulates amyloidogenic processing [85]. The lower intensities of PC (32:0) could be an indication of the release of palmitic acids and the subsequent palmitoylation of APP during the progression of the disease.

In summary, the present study shows the anatomical localization of some lipids species that are modified during the progression of the AD in frontal cortex when expressed as relative intensity of the most abundant species. The identified ST species could be involved in the pathogenesis of the disease and be used as markers for AD.

A similar study of other brain areas affected by AD such as hippocampus, nucleus basalis of Meynert and amygdala would lead to a better understanding of lipid adaptations during the progression of the disease.

Transparency Document

The [Transparency document](#) associated with this article can be found, in online version.

Acknowledgements

Supported by grants from the regional Basque Government IT975-16 and ELKARTEK16/81 and Spanish Government, Ministry for Health, I. S. C. III PI 10/01202, partially financed by F.E.D.E.R.; European Union. Technical support provided by the analytical unit SGIKER (UPV/EHU, MICINN, GV, ESF) is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at <http://dx.doi.org/10.1016/j.bbamem.2017.05.009>.

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