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Metabolomic study of lipids in serum for biomarker discovery in Alzheimer's disease using direct infusion mass spectrometry

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ABSTRACT

In this study, we demonstrated the potential of direct infusion mass spectrometry for the lipidomic characterization of Alzheimer's disease. Serum samples were extracted for lipids recovery, and directly analyzed using an electrospray source. Metabolomic fingerprints were subjected to multivariate analysis in order to discriminate between groups of patients and healthy controls, and then some key-compounds were identified as possible markers of Alzheimer's disease. Major differences were found in lipids, although some low molecular weight metabolites also showed significant changes. Thus, important metabolic pathways involved in neurodegeneration could be studied on the basis of these perturbations, such as membrane breakdown (phospholipids and diacylglycerols), oxidative stress (prostaglandins, imidazole and histidine), alterations in neurotransmission systems (oleamide and putrescine) and hyperammonaemia (guanidine and arginine). Moreover, it is noteworthy that some of these potential biomarkers have not been previously described for Alzheimer's disease.

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1. Introduction

Multiple pathological disorders have been associated with Alzheimer's disease (AD), involving abnormal protein aggregation in brain (amyloid β plaques and tangles of hyperphosphorylated τ protein) [1] and other processes such as oxidative stress [2], mitochondrial dysfunction [3], neurotransmission changes [4], and others. In this context, the importance of metabolites for studying the pathogenesis of diseases has been demonstrated, since the metabolome is the biological level closer to phenotype [5]. Particularly, lipids are very useful targets since play important roles in biological systems, so the global characterization of these compounds in a large-scale, or lipidomics, has a high potential in health survey [6]. In AD, lipids can be linked to several hallmarks of disease [7], principally dysregulation of membrane lipids, oxidative stress and vascular changes. Breakdown of cellular membranes is one of the most characteristic features of neurodegeneration, associated with abnormal metabolism of membrane lipids [8]. In this sense, alterations in two families of compounds have been described; (i) phospholipids, such as phosphocholines, phosphoethanolamines and plasmalogens [9], and (ii) sphingolipids and related compounds, such as sphingomyelins, ceramides or sulfatides [10]. On the other hand, brain is particularly susceptible to oxidative damage because of the high concentration of polyunsaturated fatty acids (PUFAs) and high oxygen consumption rates. Thus, the contribution of oxidative stress to AD also has consequences on the lipidomic profile, leading to the accumulation of typical markers of lipid oxidation. An important group are the eicosanoids, oxidation products of araquidonic acid through different enzymatic pathways [11]. Furthermore, the attack of reactive oxygen species (ROS) causes lipid peroxidation, generating isoprostanes (free radical peroxidation of araquidonic acid) [12], neuroprostanes (from docosahexaenoic acid) or aldehydes such as 4-hydroxynonenal and malondialdehyde [13]. Finally, AD has been also associated with several vascular risk factors, such as the epsilon 4 allele of the apolipoprotein E (ApoE), elevated homocysteine levels, hyperlipidemia, obesity or diabetes. These vascular defects could cause abnormalities in the vascular system, specifically in cerebrovascular system (atrophy, structural changes in the blood–brain barrier and inflammation), which result in decreased cerebral blood flow that finally involves neuronal loss [14]. In this sense, the contribution of high

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levels of triglycerides, cholesterol, lipoproteins or fatty acids has been previously reported as one of the most important vascular factors in AD. For all these reasons, the characterization of global changes in lipids and their metabolites can be interesting in order to understand the role of these compounds in physiopathology of AD.

The study and identification of lipidomic biomarkers requires analytical techniques with high sensitivity and selectivity, and wide range of applicability to analyze the large number of molecules existing, with very different structures and functions. In this sense, mass spectrometry with soft ionization technologies as electrospray (ESI) or atmospheric pressure chemical ionization (APCI) is emerging in this field [15]. This platform offers capability for both quantitative and qualitative analyses and it may be coupled to separation techniques, principally chromatography and capillary electrophoresis. On the other hand, direct infusion of samples into the spectrometer is also possible, providing faster analysis and higher reproducibility, but it presents the disadvantage of isobaric interferences. For this, the analysis of complex samples requires the use of high resolution and accuracy instruments such as time of flight (TOF-MS), Fourier transform ion cyclotron resonance (FTICR-MS) or Orbitrap [16]. Moreover, the hybrid system Q-TOF-MS, which allows more accurate mass measurement than single TOF instrument and structural elucidation by MS/MS experiments [17], is gaining great importance in recent years in metabolomics [18,19], and particularly in lipidomics on the basis of multi-dimensional mass spectrometry-based shotgun lipidomics, or MDMS-SL [20–22].

The present work represents a lipidomic approximation to Alzheimer's disease based on direct infusion mass spectrometry analysis. Metabolic changes in blood serum samples of AD patients respect to healthy controls were evaluated by ESI-Q-TOFMS fingerprinting, demonstrating the involvement of different classes of lipids and individual molecular species of these compounds, as well as low molecular mass metabolites.

2. Material and methods

2.1. Reagents and samples

Methanol and chloroform (HPLC-grade) were purchased from Aldrich (Steinheim, Germany), and ammonium acetate was supplied by Merck (Darmstadt, Germany). Blood samples were obtained by venipuncture of the antecubital region after 8 h of fasting, from 22 patients (10 male and 12 female, medium age 78.5 ± 5 y) newly diagnosed of sporadic Alzheimer's disease (AD), according to the criteria of NINCDS-ADRDA [23], and 18 matched healthy controls, HC (7 male and 11 female, medium age 70.7 ± 4.1 y). All samples were collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system, previously cooled in a refrigerator. The samples were immediately cooled and protected from light for 30 min to allow clot retraction to obtain serum after centrifugation (3500 rpm for 10 min). The serum was divided into aliquots in Eppendorf tubes and frozen at −80° C until analysis. The study was performed in accordance with the principles contained in the Declaration of Helsinki. All persons gave informed consent for the extraction of peripheral venous blood and controls subjects were studied by neurologists to confirm the absence of neurological and cognitive disease.

2.2. Sample treatment

Extraction of serum samples was performed following a procedure derived from the method proposed by Bligh and Dyer [24], employing a mixture of chloroform and methanol. In addition, since neutral lipids are not readily ionized by ESI, addition of ammonium ions was selected for analysis in positive ion mode. In the case of negative ionization, any additive was employed. For extraction, 50 μL of serum are mixed with 150 μL of methanol, containing 30 mM ammonium acetate for ESI(+) and pure methanol for ESI(−) analysis. After stirring during 1 min in vortex, which causes the precipitation of proteins, the extract is combined with 200 μL of chloroform and again stirred for another minute. Finally, sample is centrifuged at 10,000 rpm and 4 °C during 10 min, and organic phase is taken for analysis.

2.3. Instrumentation

The experiments were performed in a QSTAR XL Hybrid system (Applied Biosystems, Foster City, CA, USA) using an electrospray (ESI) source. The samples were introduced into the mass spectrometer using an integrated apparatus pump and a 1000 μL volume Hamilton syringe at flow rate 5 μL min^{-1}. Data were obtained both in positive and negative ion mode, acquiring full scan spectra for 0.2 min in the m/z range 50–1100 with 1.005 s scan time. In positive mode, the ion spray voltage (IS) was set at 3300 V, the curtain gas flow at 1.13 L min^{-1} and the nebulizer gas flow at 1.56 L min^{-1}. The source temperature was fixed at 60 °C, with a declustering potential (DP) of 60 V and a focusing potential (FP) of 250 V. In ESI(−), only few parameters were modified respect ESI(+) method, with an ion spray voltage at −4000 V, a declustering potential (DP) of −100 V and a focusing potential (FP) of −250 V. To acquire MS/MS spectra, nitrogen was used as collision gas.

2.4. Data analysis

To carry out statistical analysis, spectra were submitted to peak picking and matching of peaks across samples in order to reduce the results into a two-dimensional data matrix of spectral peaks and peak intensities, by using Markerview™ software (Applied Biosystems). Then, SIMCA-P™ software (version 11.5, published by UMetrics AB, Umeå, Sweden) was employed for statistical processing. Partial least squares discriminant analysis (PLS-DA) was performed to build predictive models in order to find differences between the groups of study (AD patients and healthy controls) and further study of potential biomarkers. Quality of the model was assessed by the R^2 and Q^2 values, provided by the software (indicative of class separation and predictive power of the model, respectively).

2.5. Compounds identification

Identification of significant compounds was made matching the experimental accurate mass and tandem mass spectra with those available in metabolomic databases (HMDB, METLIN, KEGG and LIPIDMAPS), using a mass accuracy of 50 ppm. Moreover, different classes of lipids were confirmed based on characteristic fragmentation patterns reported in literature. Phosphatidylcholines and plasmenylethanolamines presented characteristic ions in negative ionization mode at m/z 168.04 and 196.07, respectively [25]. In addition, the fragmentation in the glycerol backbone and release of the fatty acyl substituents enabled the identification of individual species of phospholipids [26]. Finally, diacylglycerols [27], fatty acid amides [28] and eicosanoids [29] were also confirmed with characteristic fragments described in the literature.

3. Results

3.1. Metabolomic profiles

Mass spectra of serum extracts provided abundant biochemical information, considering the high number of signals that

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