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Review

High-throughput direct mass spectrometry-based metabolomics to characterize metabolite fingerprints associated with Alzheimer's disease pathogenesis

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Abstract: Direct mass spectrometry-based metabolomics has been widely employed in the last years to characterize metabolic alterations underlying to Alzheimer's disease development and progression. This high-throughput approach presents a great potential for fast and simultaneous fingerprinting of a vast number of metabolites, which can be applied to multiple biological samples such as serum/plasma, urine, cerebrospinal fluid and tissues. In this review article we present the main advantages and drawbacks of metabolomics based on direct mass spectrometry compared with conventional analytical techniques, and provide a comprehensive revision of the literature on the application of these tools in Alzheimer's disease research.

Keywords: Metabolomics, direct mass spectrometry, Alzheimer's disease, pathogenesis, biomarkers

1. The potential of direct mass spectrometry-based metabolomics

Metabolomics requires the use of powerful and versatile analytical techniques with the aim of covering the largest number of compounds comprising the great complexity of the metabolome, composed of metabolites with diverse molecular weights, polarities, acid-base properties, and other physicochemical characteristics. To this end, multiple metabolomic platforms have been proposed in the literature, including nuclear magnetic resonance (NMR), or mass spectrometry (MS) coupled to liquid chromatography (LC), to gas chromatography (GC), or to capillary electrophoresis (CE), each one of them having their own strengths and weakness. For this reason, the combination of several of these complementary techniques is emerging in the last years as the most suitable strategy to accomplish a comprehensive characterization of the metabolome [1–3]. Among these analytical tools, direct mass spectrometry (DMS)-based metabolomics has been usually relegated to the background due to its inherent drawbacks, such as the impossibility of resolving chemical isomers and problems associated with ion suppression due to the direct introduction of the whole sample into the mass spectrometer without a previous chromatographic or electrophoretic separation. However, some recently published review articles have also highlighted the great potentials of this metabolomic approach [4,5]. The most notable advantage of this tool is its high-throughput screening capability due to the absence of a previous time-consuming separation step, which considerably reduces the total analysis time, thus allowing the analysis of hundreds of samples per day. The elimination of this chromatographic/electrophoretic separation also prevents the

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introduction of biased and selective retention mechanisms, so that DMS can be used for the simultaneous determination of a huge number of metabolites, covering a wide physicochemical space. In this sense, it should be also noted that multiple instrumental configurations are available for performing DMS-based metabolomics, which can be combined to increase the metabolome coverage. For non-targeted metabolomics, direct infusion mass spectrometry (DIMS) is the simplest approach since only needs a syringe pump to constantly introduce the sample extract into the mass spectrometer. Complementarily, the sample can also be delivered by flow injection (FIMS) as a plug into a stream of solvent delivered by a LC pump. On the other hand, the multi-dimensional mass spectrometry-based shotgun lipidomic (MDMS-SL) approach developed by Han *et al.* allows the direct quantitation of hundreds of individual lipid species by means of a selective ionization of certain category of lipid classes at certain MS conditions [6]. In this context, simpler targeted metabolomic platforms are the AbsoluteIDQ™ kits developed by Biocrates Life Sciences AG (Innsbruck, Austria), focused on the FI-MS/MS-based quantification of multiple metabolite classes, including lipids (phospholipids, sphingolipids, acyl-carnitines, glycerolipids), amino acids, hexoses and biogenic amines [7]. In turn, most of these DMS-based configurations can be coupled with various complementary atmospheric pressure ionization sources. Electrospray ionization (ESI) is the most commonly employed source in non-targeted metabolomics, applicable for the simultaneous detection of compounds with very diverse polarities and molecular weights due to its sensitivity and versatility. Complementarily, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) sources can also be employed for the ionization of less polar compounds. Thus, the combination of complementary ion sources and ionization modes (i.e. positive and negative polarities), is recommended in order to maximize the metabolome coverage. To conclude, it is also worth noting that the lack of a separation step prior to MS detection facilitates the experimental design by avoiding common troubles associated with chromatography and electrophoresis, such as column/capillary clogging and deterioration, the need of complex data processing packages to align retention/migration times, as well as the minimization of the instrumental drift along batch analysis thanks to the reduced acquisition times usually employed in these approaches.

2. Alzheimer's disease, mild cognitive impairment and animal models

Alzheimer's disease (AD) is the most common neurodegenerative disorder worldwide in the elderly, which is primarily characterized by neuropathological alterations associated with the deposition of amyloid β plaques and the formation of intra-neuronal neurofibrillary tangles of hyperphosphorylated tau protein. Anyway, numerous authors have proposed that other multiple pathological processes can also play a pivotal role in the development of this disease, such as oxidative stress, mitochondrial dysfunction, neuroinflammatory mechanisms, abnormal metal homeostasis and many others [8–10]. The investigation of AD etiology involves a great challenge to the scientific community due to its great complexity and variability of clinical symptoms, its long pre-symptomatic period, and the impossibility of studying brain microscopic changes until the final stages of the disease. For these reasons, diagnosis of AD nowadays relies on the combination of various physical, neuropsychological and laboratory tests according to the clinical criteria of the National Institute of Neurological and Communicative Disorders and the Alzheimer`s Disease and Related Disorders Association (NINCDS-ADRDA) [11]. However, this diagnostic method is only effective at advanced stages of disease, which hinders the application of pharmacological interventions, and in addition suffers of low specificity against other dementias as demonstrated after *post mortem* histopathological verification [12]. Thus, the discovery of novel biomarkers for accurate diagnosis of AD is mandatory, especially for predicting the development of disease from pre-dementia phases, also known as mild cognitive impairment (MCI). MCI is a heterogeneous syndrome characterized by very mild symptoms of cognitive dysfunction, which is usually considered an intermediate stage in the development of Alzheimer's disease from normal aging. Although MCI shares many features with early AD, current data suggest that some patients may have a benign form of MCI as part of the normal aging process [13]. Therefore, there is a great need

to discover potential biomarkers for diagnosis and to investigate the pathological mechanisms associated with AD and MCI development and progression.

On the other hand, animal models are very useful tools for investigating the pathogenesis of AD and associated alterations in the central nervous system at different stages along the progression of disease [14], while studies in human cohorts are limited to *post-mortem* brain tissue, when disease is in its final stage. Transgenic mice, obtained by the over-expression of mutated forms of human genes associated with AD such as the amyloid precursor protein (APP), presenilin 1 (PS1), presenilin 2 (PS2) or apolipoprotein E (ApoE), are the most useful models since the neuropathology elicited by these animals is analogous to that observed in human AD, and furthermore biochemical routes in humans and rodents are very similar [15]. The most commonly transgenic mice employed in AD research are based on the over-expression of mutated forms of the APP, including the APP_{Tg2576}, APP_{V717F} and CRND8 transgenic lines, which usually show amyloid deposition in hippocampus and cortex and memory deficits, but not neuronal loss. In this vein, it has been demonstrated that the co-expression of mutated PS1, and to a lesser extent PS2, accelerates amyloid deposition, thus facilitating the appearance of the characteristic AD phenotype (APP×PS1, TASTPM). Taking into account that ε4 allele of the ApoE is the strongest risk factor for AD, several knock-in mice in which this protein is expressed have been developed, which show significant cognitive and synaptic plasticity impairments. On the contrary, only a few transgenic models expressing tauopathy have been developed to date due to the ignorance of genes involves in this process in AD (TAPP, 3×Tg).

3. Application of direct mass spectrometry-based metabolomics to AD research

Considering the multifactorial nature of AD etiology, the application of holistic metabolomic approaches has emerged in recent years for the investigation of pathological mechanisms underlying to this neurodegenerative disorder and for the identification of novel diagnostic biomarkers. In particular, DMS-based metabolomics has demonstrated a great potential to characterize the AD metabotype in a comprehensive manner, as discussed in this section.

Numerous non-targeted DMS-based metabolomic studies have been conducted in serum samples, which is a very useful biofluid in the clinical practice for the identification of diagnostic biomarkers in a non-invasive manner. González-Domínguez *et al.* employed a DIMS platform based on a two-step treatment of serum samples from AD patients to obtain a holistic snapshot of metabolite alterations associated to the early development of this neurodegenerative disorder [16,17]. The most notable findings could be associated with an abnormal homeostasis of neural membrane lipids, evidenced by reduced levels of circulating phospholipids containing polyunsaturated fatty acids (PUFAs) and increased content of lipid species composed of saturated fatty acids (SFAs) and some breakdown products (e.g. choline, glycerophosphocholine). Furthermore, significant impairments were also observed in biological pathways related to energy metabolism, neurotransmitter levels and fatty acid homeostasis. To complement this study, a FI-APPI-MS approach was subsequently applied to focus on the less polar metabolome, non-readily detectable by ESI-based metabolomics [18]. Increased serum levels of diacylglycerols and ceramides were detected in AD patients, indicative of up-regulated degradation of membrane phospholipids and sphingolipids by the action of phospholipases and sphingomyelinases, in line with results from DIMS analyses. Due to the central role that lipid dyshomeostasis seems to play in AD pathogenesis, serum samples from the same cohort of AD patients were subjected to DIMS-based lipidomics using a modification of the Bligh-Dyer extraction method [19]. Again, it was observed a reduced content of PUFA-containing phospholipids and increased levels of diacylglycerols, corroborating previous hypotheses. Furthermore, changes in other low molecular weight metabolites also evidenced severe impairments in the homeostasis of various neurotransmitter systems, nitrogen metabolism and oxidative stress. Taking into account this evidence about the major role that phospholipids play in AD etiology, a metabolomic multiplatform based on the combination of DIMS and LC-MS, this later coupled to both molecular (ESI) and elemental (inductively coupled plasma, ICP) mass spectrometry

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was employed to get a deeper understanding about the AD-associated phospholipidome [20]. Thus, results evidenced that multiple factors are involved in this abnormal phospholipid homeostasis, including the imbalance of PUFA/SFA contained in their structure, the over-activation of phospholipases, the implication of oxidative stress and peroxysomal dysfunction, among others. Complementarily, González-Domínguez *et al.* also employed the DIMS and FI-APPI-MS approaches previously described to investigate the AD-like pathology in various transgenic mice models. The analysis of serum samples from APP×PS1 mice revealed analogous metabolomic disturbances to those detected in previous studies with human cohorts, demonstrating the potential of these transgenic animals to model AD [21]. Additionally, DIMS-based fingerprinting has been also applied to the APP×PS1×IL4-KO transgenic model with the aim of investigating the role of inflammation induced by means of interleukin-4 depletion in AD pathology [22]. Alterations in serum levels of eicosanoids, amino acids and related compounds, and metabolites involved in the urea cycle demonstrated that depletion of interleukin-4 might potentiate AD pathology in the APP×PS1 model. It should be noted that all these results obtained by DMS analysis were subsequently validated by applying various orthogonal metabolomic techniques, including LC-MS, GC-MS and CE-MS [23–26], thus demonstrating the potential of MS-fingerprinting approaches to carry out fast and accurate screening of complex metabolic networks.

Other published studies on DMS-based metabolomics have focused on the characterization of metabolic impairments observed in brain from various transgenic mice models, a tissue of great interest in AD research since allows the *in situ* investigation of neuropathological processes associated with this neurodegenerative disorder. Lin *et al.* applied an optimized DIMS platform to look for characteristic metabolic impairments in hippocampus [27] and cerebellum [28] from the CRND8 mouse model. Major findings were observed with regards to an abnormal metabolism of amino acids and nucleotides, as well as the over-production of eicosanoids. In this line, DIMS-based analysis of various brain regions from the APP×PS1 mouse model (i.e. hippocampus, cortex, cerebellum, striatum, and olfactory bulbs) evidenced that hippocampus and cortex are the most affected areas by AD pathology [29]. Similarly to previous studies, significant differences were observed in levels of phospholipids, acyl-carnitines, fatty acids, nucleotides, amino acids and many other metabolites, results which were then confirmed by LC/GC-MS metabolomic analysis [30]. Recently, Wood *et al.* also employed a lipidomic approach based on DIMS to define potential biomarkers with the aim of distinguishing healthy controls from MCI and AD patients [31]. They analyzed frontal cortex grey, white matter and cerebrospinal fluid (CSF), an interesting biofluid that directly reflects the brain metabolic production, and detected abnormal levels of various lipid classes (e.g. plasmalogens, phosphatidylethanolamines, diglycerides), in agreement with previous studies. Alternatively, other peripheral organs from the APP×PS1 model have also been investigated to assess the possible systemic nature of AD, including the liver, kidneys, spleen and thymus [32]. In this work, authors found significant impairments associated with oxidative stress, lipid dyshomeostasis and imbalances in energy metabolism, among other processes, results which were subsequently validated by using a metabolomic multiplatform based on the combination of LC and GC coupled to MS [33,34]. Moreover, urine can also serve as a valid biological sample to study metabolomic perturbations associated with AD by using DIMS-based approaches, as demonstrated by González-Domínguez *et al.* [35]. For this purpose, various sample preparation methods and normalization strategies were tested, evidencing that ten-fold dilution of urine prior to MS-fingerprinting and subsequent statistical data normalization is enough to minimize ion suppression and to correct the inherent inter-individual variability of this matrix, respectively.

From a targeted perspective, the MDMS-SL platform optimized by Han *et al.* is a very interesting alternative for the comprehensive investigation of lipidomic alterations associated with AD, in samples coming from both human and animal models. The application of this tool to plasma and brain samples showed significant changes in the levels of plasmalogens [36], sulfatides [37–39], ceramides [37,40] and sphingomyelins [40], thus corroborating the pivotal role of lipid metabolism

in pathogenesis of AD. On the other hand, other authors proposed the use of AbsoluteIDQ™ kits to analyze blood, brain and CSF samples from AD and MCI patients, observing major changes in the content of phospholipids and acyl-carnitines [41–46]. However, it should be noted that this tool present a great drawback regarding its low metabolome coverage.

4. Conclusions

Metabolomic approaches based on DMS analysis are gaining great importance in the last years because of their high-throughput screening potential, reduced analysis time and wide metabolome coverage. Particularly, these platforms have been widely applied for studying complex and multifactorial disorders such as Alzheimer's disease, with the aim of elucidating pathological mechanisms underlying to disease development and progression and discovering potential diagnostic biomarkers. The analysis of multiple biological samples, including serum/plasma, urine, brain (hippocampus, cortex, cerebellum, etc.), cerebrospinal fluid and other organs (liver, kidney, spleen, thymus), has enabled obtaining a comprehensive snapshot of the major metabolic hallmarks associated with this neurodegenerative disorder, such as impairments in the homeostasis of membrane lipids, oxidative stress, inflammatory processes, imbalance in energy metabolism and neurotransmitter metabolism, among many others.

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