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·Review·

Metabolomics: a novel approach to identify potential diagnostic biomarkers and pathogenesis in Alzheimer's disease

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Abstract: Although the pathogenesis of Alzheimer's disease (AD) is still not fully understood, it is acknowledged that intervention should be made at the early stage. Therefore, identifying biomarkers for the clinical diagnosis is critical. Metabolomics, a novel "omics", uses methods based on low-molecular-weight molecules, with high-throughput evaluation of a large number of metabolites that may lead to the identification of new disease-specific biomarkers and the elucidation of pathophysiological mechanisms. This review discusses metabolomics investigations of AD and potential future developments in this field.

Keywords: metabolomics; biomarkers; Alzheimer's disease

1 Introduction

Alzheimer's disease (AD) is the most common cause of neurodegenerative dementia in the elderly, and accounts for 50%–75% of all dementia cases[1]. The current global prevalence of dementia, estimated at 24 million, is predicted double every two decades through to 2040, imposing a significant economic burden[2]. The current clinical diagnosis of AD relies mainly on medical history, physical examination, laboratory tests, neuroimaging and neuropsychological evaluation. This only results in an accuracy of ~80%[3] and may be established too late for successful disease modification[4]. The new AD clinical diagnosis criteria place emphasis on defining preclinical AD and mild cognitive impairment (MCI)[5], thus extending the traditional definition of the disease to very early stages, allowing more timely initiation of therapeutic interventions[4]. In addition, randomized controlled clinical trials have demonstrated that the symptom-relieving drugs such as acetylcholinesterase inhibitors are more effective at the early stage of AD rather than at the late stage[6]. Therefore, biomarkers for early diagnosis are needed to improve the prognosis and monitor the effects of medication. However, despite recent investments into the search for AD biomarkers in biofluid assays and neuroimaging, including amyloid imaging, structural and functional magnetic resonance imaging, unfortunately, ideal biomarkers that are accessible with minimal invasion, obtainable by simple operations and highly accurate have not been found[7-9].

Meanwhile, several strategies have been used to

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find novel biomarkers based on the two core pathological substrates of AD, amyloid plaques and neurofibrillary tangles[10-12]. Recently, several metabolic diseases such as diabetes, dyslipidemia and hypertension have been associated with AD and conversion from MCI to AD[13,14]. Moreover, several metabolic enzymes of the glycolytic, tricarboxylic acid cycle and oxidative phosphorylation pathways are impaired in AD, and pharmacological inhibition of metabolism further exacerbates AD pathology[15]. Therefore, identifying metabolic dysfunctions using an unbiased metabolomics approach could provide a more comprehensive picture of the pathways involved in their association with AD.

2 Metabolomics

2.1 Overview As a novel method with unbiased identification and state-specific quantification of all metabolites, metabolomics is based on low-molecular-weight molecules, which include lipids, amino-acids, peptides, nucleic acids, and organic acids. These are the end result of complex biological processes in a cell, a tissue or a whole organism, and thus are potential candidates to reflect disease phenotypes[16]. Together with the other more established 'omics' technologies, like genomics and proteomics, metabolomics could systematically reveal the functions of gene products and regulatory signaling pathways *in vivo*[17]. A typical metabolomics study includes four main steps (Fig. 1). First, research is taken from patients and controls (preferably by the same clinical coordinator) according to a standardized protocol, the collected samples are analyzed, then the output data are subjected to statistical analysis; finally, metabolite sets with significant changes are grouped, and new biomarkers/pathways reflecting the disease signature are found[18].

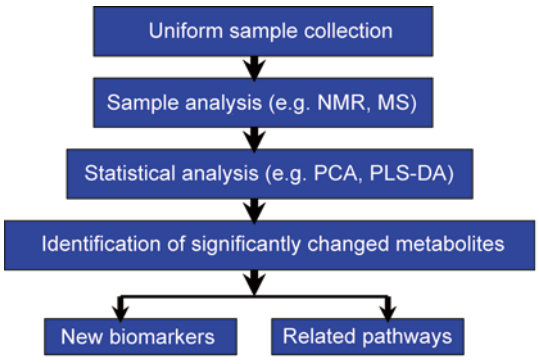

Fig. 1. Flowchart of a typical metabolomics study. MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PCA, principal component analysis; PLS-DA, partial least squares-discriminant analysis.

2.2 Analytical techniques A number of analytical techniques are used for metabolomics analyses and each has advantages and disadvantages. The choice of techniques depends on the type of problem to be evaluated. Most analyses employ one or two platforms, usually nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS).

2.2.1 NMR spectroscopy-based metabolomics This technique depends on the resonant frequencies of the nuclei present in the sample under a strong magnetic field, and as one of most common nuclei, ^{1}H-NMR is widely used. NMR can identify and quantify a broad range of metabolites in the micromolar range. NMR sample preparation is simple, the results are highly reproducible, and structural information is good, but, the major limitation is its relatively low sensitivity, making it inappropriate for the analysis of a large variety of low-abundance metabolites[19]. NMR is mainly used for liquids or tissue extracts, but recently, high-resolution magic-angle spinning NMR spectroscopy has been developed to improve the spectral resolution in intact tissues like cancers and grafts[20,21], combating the usual NMR weakness of the restricted vibration of molecules in the solid state.

2.2.2 MS-based methods for metabolomics MS requires the initial separation of metabolites, commonly by gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE) (Table 1). This is followed by ionization of metabolites and resolution according to the mass-to-charge ratio[22]. (1) GC separates compounds by exploiting the molecular interactions between the carrier gas and the column. Therefore, components need to be volatile and able to withstand elevated temperatures. In addition to extraction, derivatization is usually needed to improve volatility and stability, and reduce polarity. This

Table 1. Summary of mass spectrometry (MS)-based methods for metabolomics

Items/features	GC-MS	LC-MS	CE-MS
Universality of metabolite detection	Limited, volatile, nonpolar metabolites	Relatively wide	Limited, polar charged metabolites
Sample handling	Derivatization required	Relatively simple	Relatively simple
Separation efficiency	High	Especially high in UPLC-MS	High
Amount of sample used	Low μL range	Low μL range	Small, 1–20 nL
Time to collect basic data	Often in 1 h	Often in 30 min	Short, often in 10 min
Ease of molecular identification	Easy, compound libraries available	Compound libraries to be improved	Compound libraries to be improved
Analytical reproducibility	High	High	Relatively low

CE, capillary electrophoresis; GC, gas chromatography; LC, liquid chromatography; UPLC, ultra-performance liquid chromatography.

requires multiple steps of sample preparation and complicates comparisons across studies, as different reagents are used depending on the purpose of the derivatization in the GC-MS process[23]. Yet, some non-volatile, polar macromolecules are still unsuitable for analysis. In spite of these disadvantages, GC-MS is still a highly attractive analytical system because of its high separation efficiency, reproducible retention time and available compound libraries to identify metabolites based on both retention time in the column and the fragmentation pattern produced by the mass spectrometer[23]. (2) LC separates compounds by exploiting the molecular interactions between the carrier liquid and the column. Therefore, LC is better suited for the analysis of labile macromolecules and non-volatile polar and non-polar compounds in their native form. LC encompasses a range of systems including high performance liquid chromatography (HPLC) and ultra-performance liquid chromatography (UPLC). HPLC-MS has enjoyed growing popularity as the platform for metabolomics studies due to its high throughput, soft ionization, and good coverage of metabolites, as well as its sensitivity and quantitative reproducibility[24]. Similar to HPLC, UPLC allows greater separation and resolution of compounds by decreasing the column size and increasing chromatography pressures[25]. Unlike GC, however, the main drawback of LC-MS is that its commercially available libraries are poorly developed, making specific metabolite characterization using LC-MS more difficult than with GC-MS[24]. (3) CE separates molecules based mainly on charge and size, and is particularly suitable for the separation of polar and charged metabolites. Combining CE with MS offers high resolution, with short analysis times (often in 10 min) and very small sample requirements (injection volumes ranging from 1 to 20 nL)[26]. Thus, CE-MS is a powerful and promising technique for high-throughput metabolomics of polar charged metabolites.

In fact, none of these techniques can capture the whole network of the metabolome alone. The large number of endogenous metabolites in biological samples makes the application of multi-dimensional separation techniques (e.g., 2D-NMR and 2D-GC) very attractive for separating as many metabolites as possible[27,28]. Moreover, combinations of techniques (e.g., HPLC-NMR, capillary electrochromatography and CE-NMR) can be used to augment separation and/or expand the acquisition of analytic information[29].

3 Metabolomics and AD

Metabolic differences have been found in neurological and psychological disorders, such as Parkinson's disease, depression and schizophrenia, using a focused metabolomics approach[30-32], indicating that it could also be a promising tool to assist in the discovery of suitable biomarkers and in revealing the detailed pathogenesis of AD.

3.1 Metabolomics studies on animal models Using ^{1}H-NMR spectroscopy and two multivariate statistical methods, principal component analysis and projection to latent structures by partial least squares discriminant analysis, Salek and co-workers[35] compared the metabolomic profiles of eight brain regions (cerebral cortex, frontal cortex, cerebellum, hippocampus, olfactory bulb, pons, midbrain and striatum) in the wild-type and the TgCRND8 mice, an aggressive early-onset model of amyloidosis[33]. The metabolomic profiles discriminated controls from APP695 mice that did not yet display early AD-like histopathology[34]. The affected regions were the hippocampus, cerebral cortex, frontal cortex, midbrain and cerebellum, with hippocampal and cortical regions being the most extensively involved, demonstrating widespread metabolic disturbances in AD, even involving the cerebellum and midbrain[35]. Furthermore, one of the most notable metabolic changes in these regions was a decrease in N-acetyl-*L*-aspartate (NAA), which was apparent as early as 2–3 months, prior to any histologically detectable neuronal loss and clinical symptoms in transgenic mice. Therefore, NAA may reflect the energy status of neurons as a marker of their dysfunction, but is not a marker of healthy neurons or total neuronal density[35]. These results suggest that metabolomic disturbances occur at an early stage of AD, even before detectable neuropathology. These changes can be readily compared in elderly patients with MCI and those with AD.

3.2 Metabolomics studies on AD patients

3.2.1 Monoamine metabolism in AD using LC-electrochemical array (ECA) Previous studies have demonstrated that dopamine and serotonin pathways play an important role in the pathogenesis of AD[36,37]. Kaddurah-Daouk and colleagues collected post-mortem ventricular cerebrospinal fluid (CSF) from 15 autopsy-confirmed AD patients and 15 controls, and quantified 33 known metabolites within the dopamine, serotonin and oxidative stress pathways by LC-ECA. They found that norepinephrine (NE), as well as its metabolites, were significantly depleted in patients with AD, suggesting that enzymes within the pathway of NE monoamine oxidase, such as alcohol dehydrogenase and catechol-o-methyl transferase, might be modified in AD[38]. This was a pilot study using postmortem ventricular CSF, so further studies using more readily available samples, such as lumbar CSF and plasma are needed to confirm the biochemical pathways, understand the mechanisms, and identify potential biomarkers.

3.2.2 Lipidomics in AD using UPLC-MS/multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL) Previous studies with post-mortem brain tissue samples have demonstrated altered lipidomes at different stages of AD[39]. A relationship between altered plasma ceramide levels and hippocampal volume loss in MCI highlights a potential role of lipids as biomarkers for AD[40]. Recently, another study showed nine potential biomarkers for AD, including lysophosphatidyl cholines (LPCs), tryptophan, dihydrosphingosine, phytosphingosine and hexadecasphinganine, using UPLC-MS and principal component pattern recognition methods to analyze plasma samples from 20 AD patients and 20 healthy controls. Among the nine potential candidates, LPCs, sphingosine and tryptophan remained decreased in AD upon further testing[41].

Lipidomics, a branch of metabolomics, is derived from technology, most notably MS for the detection and characterization of lipids and their biosynthetic enzymes in living cells[42]. Given the importance of lipidomic disorders in AD, Han and co-workers[43] analyzed over 800 molecular species of lipids in plasma from 26 AD patients (mild to moderate dementia) and 26 normal controls by MDMS-SL. The results demonstrated that eight sphingomyelin (SM) species, particularly those containing long aliphatic chains (22 and 24 carbon atoms), were significantly lower in AD, and two ceramide species (N16:0 and N21:0) were significantly higher[43]. Moreover, altered mass levels of both N20:2 SM and OH-N25:0 ceramides were correlated with mini-mental state examination scores in AD[43]. These findings are consistent with those described above[40,44], but also provide a deeper and broader description of the lipid species involved and demonstrate their association with the severity of dementia.

3.3 Metabolomics studies on MCI patients Accumulating evidence supports the hypothesis that elevated serum cholesterol levels increase the risk of developing AD[45].

Three types of samples, lipoprotein lipids, low-molecular-weight metabolites and lipid extracts, were measured using ^{1}H-NMR in serum from subjects with MCI and controls. Statistically significant changes in serum metabolite levels were found between control and MCI by holistic self-organizing map analysis based on the combination of the three spectra rather than by analysis at individual time points, suggesting that the combined changes of several metabolites can be descriptive while the changes in individuals metabolites are not[46]. In addition, the data set also reflects the complexity of serum biochemistry and cognitive decline, as well as various biochemical pathways underlying MCI[46]. The analyses also address the role of elevated glycoproteins in the risk for AD, supporting the view that the coexistence of inflammation and metabolic syndrome forms a high risk condition for cognitive decline[47].

These preliminary studies (Table 2), including targeted and ultra-targeted approaches, collectively support the notion that metabolomics occupies an important position in AD research. Overall, as a high-throughput research tool, ultra-targeted approaches are particularly suited to obtain detailed information on metabolites, which is essential to discover novel biomarkers and reveal the complex metabolic disturbances behind the pathogenesis of AD. However, key issues currently limit its application to AD research: (1) the current analytical and data-handling techniques are not satisfactory for reliable feature-detection, and it is necessary to develop new feature-detection algorithms for high-resolution data sets; and (2) in clinical trials, we need to standardize and reach a consensus on guidelines for experimental procedures to ensure that the results from different studies can be compared.

4 Summary and perspective

Metabolomics is one of the important 'omics' methodologies, and is a promising tool in AD research. Using

Table 2. Summary of metabolomics studies in Alzheimer's disease (AD) and mild cognitive impairment (MCI)

Subject	Sample type	Analytical technique	Results
Transgenic *vs* wild-type mouse	Brain tissue	^{1}H-NMR	Decreased NAA, glutamate, glutamine, taurine, γ-amino butyric acid, choline and phosphocholine, creatine, phosphocreatine and succinate; and increased lactate, aspartate, glycine and other amino-acids including alanine, leucine, iso-leucine, valine and water-soluble free fatty-acids (0.8–0.9 and 1.2–1.3 ppm) in six affected regions[35]
AD patients *vs* controls (n = 15)	Ventricular CSF	LC-ECA	Alterations in tyrosine, tryptophan, purine, and tocopherol pathways; reductions in norepinephrine and its metabolites[38]
AD patients *vs* controls (n = 20)	Plasma	UPLC-MS	Nine potential diagnostic biomarkers (LPCs, tryptophan, dihydrosphingosine, phytosphingosine and hexadecasphinganine) identified for AD[41]
AD patients *vs* controls (n = 26)	Plasma	MDMS-SL	Eight sphingomyelin species significantly lower in AD; two ceramide species (N16:0 and N21:0) increased in AD; ratios of ceramide to sphingomyelin species differed significantly between groups; both N20:2 SM and OH-N25:0 ceramides were correlated with MMSE in AD[43]
MCI patients (n = 54) *vs* controls (n = 126)	Serum	^{1}H-NMR	Omega-3 fatty acids indicative of MCI; elevated glycoproteins may be a risk for AD[46]

CSF, cerebrospinal fluid; LC-ECA, liquid chromatography-electrochemical array; LPC, lysophosphatidyl choline; MDMS-SL, multi-dimensional mass spectrometry-based shotgun lipidomics; MMSE, mini-mental state examination; MS, mass spectrometry; NAA, N-acetyl-*L*-aspartate; NMR, nuclear magnetic resonance spectroscopy; SM, sphingomyelin; UPLC, ultra-performance liquid chromatography.

this approach, disturbances in many metabolic pathways including amino-acid metabolism, neurotransmitter systems (dopamine and serotonin), sphingolipids such as SMs and ceramides have been reported to be related to AD. However, the study of metabolomics in AD is still in its infancy, and many aspects need to be improved. First, most studies have been based on small sample sizes of cases *versus* controls, and after these pilot studies, further validation of candidate biomarkers is usually missing, although Kaddurah-Daouk and colleagues[38] have estimated that their model of NE, tryptophan and indoleacetic acid would be 90% accurate in discriminating AD from control. Besides, none of these studies has yet included other dementia subtypes, and the metabolomic disturbances in AD may not be specific. Thus, larger well-designed validation tests, including subjects with MCI, early AD and other dementia subtypes are urgently needed. Second, valid comparisons across studies seem impossible at present. We have noted various designs of specimen collection, cohort constitution and analytical methods across studies. Differences in analytical methodology like the derivatization reagent used in the extraction method, could make comparisons complex even using the same type of instrument. Meanwhile, the development of spectral libraries may not keep pace with the new techniques. Therefore, a unified methodology and multi-center-based libraries should be established as soon as possible. It is inspiring to hear that The Human Metabolome Database, offering metabolite data such as structural information, reference NMR and MS spectra, biofluid concentrations and pathway information of all detectable metabolites (>1 mmol/L) in the human body, is freely available on-line[48]. Furthermore, no single platform is able to capture the entire metabolome at present. Combinations of different methods, such as HPLC-NMR and GC×GC, may increase the coverage of the metabolome of AD. In addition, as metabolite profiles reflect both environmental effects and genetic influences in patients, multiple factors, such as diet, physical exercise, stress, common genetic polymorphisms, and medications, need to be evaluated at the same time. Metabolomics is "downstream" from genomics transcriptomics and proteomics. Upstream changes finally give rise to particular disease-specific metabolic profiles. It is attractive to apply this end-stage technology of metabolomics to study AD in a systemic way, and to identify potential therapeutic targets and AD-specific biomarkers in future.

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