Biochimica et Biophysica Acta 1842 (2014) 1232–1239

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Review

Recent advances in the application of metabolomics to Alzheimer's Disease ☆

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ARTICLE INFO

Article history:
Received 7 April 2013
Received in revised form 18 June 2013
Accepted 19 June 2013
Available online 29 June 2013

Keywords:
Alzheimer's Disease
Biomarkers
Metabolomics
Plasma
Cerebrospinal fluid
Animal models

ABSTRACT

The pathophysiological changes associated with Alzheimer's Disease (AD) begin decades before the emergence of clinical symptoms. Understanding the early mechanisms associated with AD pathology is, therefore, especially important for identifying disease-modifying therapeutic targets. While the majority of AD clinical trials to date have focused on anti-amyloid-beta (Aβ) treatments, other therapeutic approaches may be necessary. The ability to monitor changes in cellular networks that include both Aβ and non-Aβ pathways is essential to advance our understanding of the etiopathogenesis of AD and subsequent development of cognitive symptoms and dementia. Metabolomics is a powerful tool that detects perturbations in the metabolome, a pool of metabolites that reflects changes downstream of genomic, transcriptomic and proteomic fluctuations, and represents an accurate biochemical profile of the organism in health and disease. The application of metabolomics could help to identify biomarkers for early AD diagnosis, to discover novel therapeutic targets, and to monitor therapeutic response and disease progression. Moreover, given the considerable parallel between mouse and human metabolism, the use of metabolomics provides ready translation of animal research into human studies for accelerated drug design. In this review, we will summarize current progress in the application of metabolomics in both animal models and in humans to further understanding of the mechanisms involved in AD pathogenesis. This article is part of a Special Issue entitled: Misfolded Proteins, Mitochondrial Dysfunction, and Neurodegenerative Diseases.

1. Introduction

Alzheimer's Disease (AD) currently affects more than 5 million Americans, with numbers expected to grow dramatically as the population ages [1]. AD is a devastating neurodegenerative disorder characterized by progressive memory loss and impairment in behavior, language, and visuospatial skills [2]. Definitive diagnosis of AD requires post-mortem examination of the brain, and is based on the presence of Aβ plaques and neurofibrillary tangles, the intracellular fibrillar aggregates of the microtubule-associated protein tau that exhibits hyperphosphorylation (p-tau) and oxidative modifications [3]. As evidenced by the many failed clinical trials for AD to date, with the majority focusing on Aβ, there appears to be no treatment benefit in the fully symptomatic stage of the disease. One explanation for this lack of efficacy is that treatment may be administered too late in the disease process. Indeed, AD pathophysiology is thought to begin several years, probably decades, before the emergence of clinical symptoms [4–10]. Amyloid pathology associated with decreased levels of Aβ in cerebrospinal fluid (CSF) and increased accumulation in the brain is thought to precede neuronal injury by up to 20 years. Moreover, neuronal injury and neurodegeneration also precede cognitive decline, but are temporally closer [4]. Thus, there is a critical need to identify the early pathological

Abbreviations: AD, Alzheimer's Disease; Aβ, amyloid-beta; CSF, cerebrospinal fluid; p-tau, phospho-tau; FDG-PET, [18F]-fluorodeoxyglucose positron emission tomography; MRI, magnetic resonance imaging; NIA, National Institute on Aging; AA, the Alzheimer's Association; MCI, mild cognitive impairment; NMR, nuclear magnetic resonance spectroscopy; MS, mass spectroscopy; LC, liquid chromatography; GC, gas chromatography; LCECA, liquid chromatography electrochemistry array; FAD, familial Alzheimer's Disease; APP, amyloid precursor protein; PS1, presenilin 1; PS2, presenilin 2; NAA, N-acetylaspartate; m-In, myo-inositol; GABA, gamma-amino butyric acid; Cre, creatine; pCre, phosphocreatine; PLS-DA, partial least squares discriminant analysis; NTG, non-transgenic; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; NAD, nicotinamide adenine dinucleotide; HR-MAS, ^{1}H high resolution magic angle spectroscopy; SPE-LC-MS/MS, solid phase extraction-liquid chromatography-mass spectrometry/mass spectrometry; CN, control subjects; CE–MS, capillary electrophoresis–mass spectrometry; LDA, linear discriminant analysis; UPLC–MS, ultra-performance liquid chromatography–mass spectrometry; 2D GC–TOF-MS, two-dimensional gas chromatography–time-of-flight mass spectrometry; CNS, central nervous system; MDMS-SL, multi-dimensional mass spectrometry-based shotgun lipidomics; MMSE, Mini-Mental State Examination; FDA, The Food and Drug Administration; NE, norepinephrine; GCA, glycocholate; GCDCA, glycochenodeoxycholate; GDCA, glycodeoxycholate; PC, phosphatidylcholine; SCI, subjective cognitive impairment

☆ This article is part of a Special Issue entitled: Misfolded Proteins, Mitochondrial Dysfunction and Neurodegenerative Diseases.

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http://dx.doi.org/10.1016/j.bbadis.2013.06.014

mechanisms that contribute to the development of AD pathology, as well as to the emergence of cognitive symptoms and dementia.

While Aβ is essential for a diagnosis of AD (including preclinical AD for research purposes based on the National Institute of Aging–the Alzheimer's Association (NIAAA) criteria), it is not sufficient to ultimately cause cognitive impairment and dementia. Approximately 30% of individuals aged 70 and older do not develop cognitive impairment or dementia despite having significant amyloid load detected with magnetic resonance imaging (MRI) [11,12]. Therefore, in order to better understand the etiopathogenesis of AD, it is necessary to focus on *both* Aβ and non-Aβ pathways to advance our understanding of early disease mechanisms and to develop efficient therapeutic approaches. Previous studies have suggested that non-Aβ mechanisms including calcium dysregulation, mitochondrial dysfunction, altered cell signaling, oxidative stress, inflammation, and lipid homeostasis are perturbed in AD [13,14]. However, the temporal relationship between these mechanisms, AD pathology (i.e., Aβ plaques, neurofibrillary tangles, and neurodegeneration), and clinical symptoms is not clear. Novel approaches are needed to monitor global changes in multiple biochemical in order to reveal molecular mechanisms and associated biomarkers that can lead to the development of new therapeutic strategies and early diagnosis in the preclinical and early clinical (i.e., mild cognitive impairment (MCI)) stages of AD, when treatment is likely to be most effective.

Metabolomics allows monitoring the perturbations in a pool of metabolites that reflects changes downstream of genomic, transcriptomic and proteomic fluctuations. Metabolomics represents an accurate biochemical profile of an organism in health and disease aiding to further understanding of alterations in complex biological networks involved in AD [15,16]. The strength of metabolomics is in its ability to identify dynamic qualitative and quantitative changes in a large number of individual metabolites representing multiple functional networks and pathways. An inherent advantage of metabolomics, compared to the use of proteomics or genomics, is the ability to directly translate data across species, especially with regards to drug development. Since metabolic pathways are conserved through evolution, and are essentially similar in rodents and humans, the metabolic signatures identified in mechanistic and therapeutic studies of AD animal models could be directly translated into human studies. Moreover, metabolomic profiling is inexpensive, time-efficient, and can be done in a variety of easily accessible sources such as CSF, plasma and peripheral tissue, thus highlighting the clinical utility of this approach. Here, we will review current data generated using metabolomics in animal models of AD and in clinical studies.

2. Metabolomic platforms

The size of the metabolome has not been defined and could range from a few thousand to tens of thousands of metabolites [17,18]. The ability to simultaneously measure dynamic changes in many molecules in biological samples became available only recently through the utilization of such advanced analytical technologies as high resolution nuclear magnetic resonance (NMR) and mass spectroscopy (MS) coupled with either high or ultrahigh resolution liquid (LC) or gas (GC) chromatography, and the development of sophisticated methods of data analysis. An excellent, detailed description of the analytical platforms available for multiple metabolomic applications has been published previously [19]. We will briefly describe the metabolomic platforms utilized to date in AD research (Table 1).

A number of early metabolomic studies employed ^{1}H NMR, also called Magnetic Resonance Spectroscopy (MRS), to determine changes associated with disease progression in AD animal models and AD patients [16]. An advantage of ^{1}H MRS is the rapid detection of a large number of molecules, with excellent quantitative precision, in a high throughput manner (Table 1). This method is also non-invasive and provides an in vivo opportunity to study metabolites in living organisms [16]. However, a disadvantage of ^{1}H MRS is in its high cost and relatively low sensitivity.

MS is the most utilized technique for the identification and quantification of known metabolites, both for the detection of molecules with low abundance signals (e.g., hormones) and also for the detection of reproducible, but unidentified, molecules. The coupling of MS with either gas chromatography (GC) or liquid chromatography (LC) has been successfully applied for targeted metabolomics to analyze changes in lipids (lipidomics) or other metabolites (e.g., catecholamines). These methods are also used to detect global changes in biochemical networks (non-targeted metabolomics). Compared to NMR, MS is more

Table 1
Analytical platforms utilized in metabolomic research in animal models of AD and AD patients.

Analytical platforms	Advantages	Disadvantages
Nuclear magnetic resonance (NMR) spectroscopy	• universal detection • excellent quantitative precision • high throughput (>100 samples/day) • rigorous structural analysis of many metabolites in crude extracts, cell suspensions, intact tissues, or whole organisms • structural determinations including the atomic positions of isotopic labels (e.g., 13C, 15N, or 2H) generated during stable isotope tracer studies • speed • detection of unidentified metabolites	• relatively poor sensitivity • high initial cost of NMR; instruments (over one million dollars)
Mass spectroscopy (MS)	• most important technique for known metabolite identification • high sensitivity for low abundance signals • detection of molecules that are not yet identified	
Gas chromatography–mass spectroscopy (GC–MS)	• structural information • quantitative precision • high throughput • higher sensitivity compared to NMR • excellent tool for targeted studies	• inability to study molecules that cannot be readily volatilized • low mass accuracy • increased mass accuracy is associated with higher initial cost and reduced throughput.
Liquid chromatography-mass spectroscopy (LC-MS)	• measures metabolites without derivatization • could be tailored for specific class of compounds • detection of broad range of compounds; • stable isotope/flux experiments • excellent tool for broad non-targeted studies • detection of unidentified metabolites with the exact mass immediately known	• lack of consistent quantitative precision
Liquid chromatography–electrochemistry array (LCECA)	• small molecule detection based on oxidation/reduction • high reproducibility • high sensitivity	• lack of structural information • low throughput

sensitive and allows for the measure of a broader array of metabolites (Table 1). However, one of the disadvantages of MS is that it typically requires chemical manipulation in order to produce ionic species that are more readily separated.

LC-electrochemistry array metabolomics platform (LCECA) is another method utilized for both targeted and non-targeted applications to detect changes in neurotransmitter pathways and pathways involved in oxidative stress [20,21]. This method has high sensitivity and reproducibility. However, it does not allow generation of structural information and has relatively low throughput (Table 1).

3. Metabolomics in animal models of AD

The familial form of AD (FAD), which has an early-onset (<65 years of age), is caused by mutations in the genes encoding amyloid precursor protein (APP) and presenilins 1 and 2 (PS1 and PS2) [22]. There is a number of well-characterized transgenic animal models of FAD that closely recapitulate the onset and progression of human disease. These models offer an outstanding opportunity to investigate the early pathologic disease mechanisms in order to identify novel therapeutic targets and relevant biomarkers. Further, the utilization of FAD animals allows establishing the direct correlations between metabolomic signatures in affected brain regions, plasma or peripheral tissue and the extent of disease progression including levels of amyloid deposition and cognitive decline, which cannot be accomplished in humans.

Neurochemical profiles in the brain of living FAD mice were defined using ^{1}H MRS-based metabolomics (Table 2) [23–26]. Despite the fact that animal models in these studies differed in the origin of FAD mutations, age, and examined brain regions, all studies reported a decrease in the levels of N-acetylaspartate (NAA) and glutamate (Table 2). NAA is synthesized under normal conditions in the mitochondria of neurons, and is considered a marker of neuronal density and integrity [27]. However, the details of its biological function in the brain are not well understood, and there is evidence that NAA could be involved in lipid synthesis into myelin, in osmoregulation, in bioenergetics of neuronal mitochondria, and axon-glial signaling [28]. It is interesting to note that changes in NAA and glutamate were identified in the brain of adult APP and PS2APP mice examined at 19 and 20 months of age, respectively, when the amyloid toxicity was wide spread (Table 2) [23–26], as well as in the brain of young APP/PS1 animals of 2.5 months of age, prior to the development of neurological and memory phenotype and amyloid deposits (Table 2) [24,29]. In addition to the reduction in NAA and glutamate levels, APP/PS1 mice showed a significant increase in myo-inositol (m-In), which correlated with age and severity of AD in these animals (Table 2) [24]. Since m-In is expressed to the greater extent in glial cells compared to neurons, its levels are considered to represent the extent of gliosis [30,31]. Similar to the results in FAD animals, levels of NAA, m-In and glutamate were found to fluctuate in the similar way with progression of AD in humans suggesting that FAD animals accurately recapitulate metabolic changes associated with human disease [32,33]. Moreover, along with the decreased levels of NAA indicative of mitochondrial dysfunction, metabolic profiles in the brains of PS2APP mice also showed progressive age-dependent development of brain hypometabolism [25]. This data is consistent with the decrease in brain metabolism observed in human carriers of the ApoE4 mutation that predisposes to late-onset AD [34–36]. Furthermore, these spectroscopic measures in PS2APP mice in vivo correlated well with the progressive formation of Aβ plaques in the frontal cortex. A diagnostic test, based on the changes in NAA and glutamate levels in PS2APP mice, reached 92% sensitivity and 82% specificity at age 20 months [25], demonstrating the robustness of the metabolomic approach in disease diagnosis.

Additional study conducted in TgCRND8 mouse model using ^{1}H NMR examined metabolic changes in the extracts from eight brain regions including cortex, frontal cortex, cerebellum, hippocampus, olfactory bulb, pons, midbrain and striatum (Table 2) [26]. This study, similar to the experiments conducted by Marjanska and colleagues [24], assessed metabolic changes in relationship to disease progression starting with animals 2–3 months of age and continuing with mice 12–13 months old. Analysis of the NMR spectra discriminated control from TgCRND8 tissues in most of the examined brain regions, with hippocampus and cortex being affected to a greater extent. In a good agreement with the data reported for other FAD animals (Table 2, ^{1}H MRS), the authors found a decrease in NAA, glutamate, glutamine, taurine, gamma-amino butyric acid (GABA), choline, phosphocholine,

Table 2
Summary of metabolomic studies conducted in animal models of AD.

Animal model	Age	Examined tissue	Analytical platform	Changes in most important metabolites	References
APP (Tg 2576, K670N/M671L)	19 months	Cerebral cortex (in vivo and in vitro)	MRS	Increase: taurine Decrease: NAA, glutamate, glutathione	[23]
APP/PS1 (APP K670N/M671L + PS1 M146L)	2.5–30 months	Cortex and hippocampus	^{1}H MRS	Increase: *myo*-inositol Decrease: NAA and glutamate	[24]
PS2APP (PS2 N141I + APP K670N/M671L)	4–24 months	Frontal cortex	^{1}H MRS	Decrease: NAA and glutamate were significantly reduced in the older animals No changes in myo-inositol	[25]
TgCRND8 (APP KM670/671NL + V717F)	2–3 months 12–13 months	Cortex, frontal cortex, cerebellum, hippocampus, olfactory bulb, pons, midbrain and striatum	^{1}H NMR	Increase: free fatty acids, leu-ile/val, lactate, Leu/lys/Arg, alanine, glycine, aspartate, serine Decrease: GABA, NAA, glutamate, glutamine, creatine/P-creatine, taurine, NAAG, myo-inositol, choline/P-choline, succinate, malonate	[26]
APP/PS1 (APP K670N/M671L + PS1 M146L)	16 weeks	Hippocampus	GC/MS	Increase: threonic acid, ethanolamine, alanine, mannitol, glycerol 3-P, pyroglutamic acid, NAA, creatinine, lactic acid, succinic acid, methylglutamate, NAD, adenosine, adenine, citric acid	[37]
APP (Tg 2576, K670N/M671L)	36 weeks	Hippocampus	GC/MS	Increase: adenosine, AMP, Adenine, Decrease: NAA, Myo-inositol, pantothenic acid, Pi, threose, creatinine, malonic acid, ATP, Glycerol, Inosine, Citric acid, ADP	[37]
PS1 (M146L)	36 weeks	Hippocampus	GC/MS	Decrease: ADP, ATP, NAD, Pi, fumaric acid, myo-inositol, malonic acid, lysine, GDP, threose, glycerol, GTP, NAA, glutamic acid, GMP	[37]
APP/PS1 (APP K670N/M671L + PS1 M146L)	12–20 weeks old animals treated with Donepezil	Striatum cortex Hippocampus	^{1}H MRS	Increase: glutamate/creatine ratio choline/creatine ratio Decrease: taurine/creatine ratio	[38]

creatine (Cre), phosphocreatine (pCre) and succinate in hippocampus, cortex, frontal cortex and midbrain of TgCRND8 animals. Metabolites that discriminated between old and young mouse tissue were lactate, alanine, lysine and N-acetyl-aspartyl-glutamate, which decreased with age and glutamine, Cre, m-In, and malonate, which increased as mice aged. The authors also reported an increase in lactate, aspartate, glycine and other amino acids including alanine, leucine, iso-leucine, valine and water-soluble free fatty acids. It is interesting to note that no sex differences were observed in metabolomic profiles in TgCRND8 mice. Overall, this study, for the first time, demonstrated that the perturbations in metabolism caused by expression of FAD mutations are widespread and include the cerebellum and midbrain. Furthermore, disease-related changes were associated with a wide range of metabolites providing a justification for metabolomics as a valuable tool for diagnosis and monitoring the AD progression [26]. Taken together, studies using ^{1}H MRS analytical platform confirmed that metabolic changes correlate with AD progression in multiple animal models of AD, and these changes resemble alterations observed in human AD patients. However, the major limitation of these animal studies was a relatively limited number of metabolites detected with ^{1}H MRS.

Using GC/MS-based metabolomic approach we have investigated metabolic changes in the brain of APP, PS1 and APP/PS1 FAD mice prior to the development of amyloid plaques and cognitive deficit (Table 2) [37]. Analysis of metabolomic profiles in the hippocampal tissue using partial least squares discriminant analysis (PLS-DA) revealed that PS1, APP and APP/PS1 mice have metabolomic signatures that were distinct from non-transgenic (NTG) littermates and also from each other. Moreover, for the first time in the animal metabolomic study, we demonstrated that metabolic signatures in APP/PS1 mice had significant sex differences. Thus, NTG female and male mice had very similar metabolomic profiles, while profiles of APP/PS1 female mice were affected to a greater extent than in male APP/PS1 animals [37]. Metabolites in the separate pair comparison revealed presence of characteristic signatures of mitochondrial toxicity with altered tissue levels of energy metabolites ATP, ADP, AMP, nicotinamide adenine dinucleotide (NAD), adenosine, fumaric acid, adenine, Cre and β-alanine. Increased levels of adenosine, AMP and fumaric acid and decreased levels of NAA indicated altered ability to maintain oxidative phosphorylation leading to mitochondrial stress and energetic dysfunction. Metabolic pathway analyses revealed that in all three FAD mouse models there were significant alterations in the levels of metabolites involved in energy metabolism including nucleotide metabolism, mitochondrial Krebs cycle, energy transfer, carbohydrate, neurotransmitter and amino acid metabolic pathways. However, along with the pathways equally affected in all three FAD mouse models, we identified metabolic pathways and metabolites that were specific to the mutation. Thus, alteration in neurotransmitter metabolism and energy transfer pathway was affected to a greater extent in APP and PS1 animals. Synergistic effect of both mutations in APP/PS1 mice resulted in significantly stronger alterations in glycolytic pathway that involved Krebs cycle, and neurotransmitter and amino acid metabolism. Using a battery of biochemical and cell biology techniques, we demonstrated that mitochondrial dynamics, distribution, and function, including the integrity of synaptic mitochondria were affected in the brain tissue from all three FAD animal models early in AD progression [37]. Therefore, our data suggest that metabolomics accurately detects early disease-related changes associated with mitochondrial dysfunction in presymptomatic FAD animals justifying the application of this approach for diagnostic purposes.

In addition to the identification of altered pathways and a panel of affected metabolites associated with AD progression, metabolic signatures could be used to monitor efficacy of therapeutic intervention. The effect of donepezil administration in APP/PS1 mice was monitored in vivo using ^{1}H MRS (Table 2) [38]. Donepezil is an acetylcholine-esterase inhibitor, which enhances the life of the neurotransmitter acetylcholine in the synapse and increases cholinergic neurotransmission [39,40]. The FDA approved the use of donepezil for symptomatic treatment of AD patients [41]. Indeed, application of metabolomics to analyze changes in the brain metabolites in donepezil-treated APP/PS1 mice revealed significant decrease in the ratio of taurine/Cre and increase in choline/Cre and glutamate/Cre indicative of an improved cholinergic activity [38].

Taken together, data generated to date using multiple metabolomic platforms suggest that animal models of AD closely mimic changes in metabolic networks involved in disease progression in humans. Metabolomics could distinguish age and sex-specific changes associated with early disease mechanisms and could be utilized to monitor therapeutic efficacy of experimental drugs. Taking into consideration that biochemical pathways are essentially conserved between humans and rodents, metabolomics could provide translational biomarkers for accelerated dug development.

4. Metabolomic studies in AD patients

Multiple human studies have used metabolomics to establish disease-related plasma or CSF metabolite differences between cognitively normal (CN) individuals, mild cognitive impairment (MCI), and AD patients as predictors of AD progression. However, direct comparison of results is difficult due to the utilization of diverse analytical platforms, different sample mediums (CSF or plasma) collected either from living individuals or post-mortem, and varied distributions in age, disease severity and sex. Since a comprehensive overview of application of the NMR spectroscopy-based metabolomics in AD is provided elsewhere [16], we will focus on studies (grouped by the sample medium) that have utilized MS and LCECA analytical platforms (Table 3).

4.1. Post-mortem ventricular CSF

Using LCECA, Kaddurah-Daouk and colleagues [42] examined post-mortem ventricular CSF from 15 AD patients and 15 age- and sex-matched CN individuals. While hundreds of metabolites were identified, the authors restricted their analyses to 33 known molecules within key neurotransmitter pathways (e.g., dopamine and serotonin) and pathways involved in oxidative stress. Norephinephrine (NE) levels were significantly decreased in AD cases compared to controls. There were also non-significant trends for group differences in tyrosine, tryptophan, purine, and tocopherol pathways. Notably, a model that included tryptophan, NE, and indoleacetic acid provided complete separation of the groups. A limitation of that study is that metabolite levels in ventricular CSF may be impacted by the death process and post-mortem intervals. However, the authors also reported significant correlations between several metabolites and both plaque and tangle pathology, providing further credence to the findings. Specifically, depletion of NE, methionine, alpha-tocopherol, 3-methoxytyramine, and metabolites within the purine pathway were associated with neurofibrillary tangle formation and/or Aβ deposition. In contrast, higher levels of 5-hydroxytryptophan were associated with greater neurofibrillary tangle and Aβ burden (Table 3).

4.2. In vivo CSF

Since each metabolomic platform has limitations (Table 1), the utilization of multiple analytical platforms should help to obtain more accurate metabolic signatures of disease process in biofluids and tissues [43]. Czech and colleagues [44] combined GC–MS and LC-MS/MS for broad profiling and solid phase extraction-LC-MS/MS (SPE-LC-MS/MS) to establish differences in catecholamines and steroids in the CSF of 51 CN participants, 53 mild AD, and 26 moderate AD patients (Table 3). Among the 343 detected metabolites, 80 compounds were unambiguously identified and absolute quantification was determined. Notably, the number of metabolite differences between AD and CN subjects was much larger for women compared to men, in agreement with findings in FAD mice [37]. This observation highlights the importance of sex-specific research using metabolomics, particularly in

relationship to the development of neurodegenerative diseases. Further, the mild AD group had significantly more metabolic changes compared to the moderate AD group. This might be expected as the accumulating pathology with advanced AD leads to the loss of neurons and metabolic function. The combination of increased cysteine and decreased uridine levels best separated AD and CN groups, with approximately 75% sensitivity and 75% specificity. Uridine is a nucleoside and one of the precursors of phosphatidylcholine, a major component of cell membranes. Thus, decreased uridine levels could be indicative of neurodegeneration. The authors suggested that the observed increase in cysteine could reflect an imbalance in the homocysteine metabolism. Interestingly, levels of NE were also increased in the CSF of AD patients, which is in contrast to the findings reported by Kaddurah-Daouk et al. [42] supporting the notion that the death process could have affected the metabolic profiles in that study.

In addition to examining changes in metabolites for diagnostic purposes, a recent study used capillary electrophoresis–mass spectrometry (CE–MS) to identify metabolic changes predictive of AD progression (Table 3) [45]. Compared to other MS techniques, CE is particularly suited for the rapid separation of ionic and highly polar metabolites. Study participants were followed for 2 years and categorized into the following groups based on their baseline diagnosis and progression: subjective memory complaints that remained stable over the two-year follow-up period (SCI-nonAD), MCI that remained stable (MCI-nonAD), MCI that progressed to AD (MCI-AD), and AD. Samples were divided into a training dataset consisting of 73 CSF samples across the diagnoses and a test set consisting of 12 samples. Ten metabolites were identifies that allowed 90.1% correct classification for the four groups by linear discriminant analysis (LDA). These metabolites included choline, valine, arginine, suberylglycerine, carnitine, creatine, serine, and histidine. Using this same LDA model, 83% of the 12 CSF test samples, initially blinded to diagnosis, were correctly classified. It will be important to confirm whether these molecules can be used as predictors of disease progression in larger cohorts. Future research is also needed to examine the relation between changes in identified metabolites and pathological features including Aβ plaques and neurofibrillary tangles.

4.3. Blood

While CSF metabolites are thought to be most reflective of brain changes compared to blood, the collection of CSF is invasive and is not ideal for screening purposes in the general population or for repeated follow-up visits to assess the efficacy of medications on disease progression. Therefore, the identification of blood-based biomarkers would be ideal for clinical use and, as a result, metabolomic studies have also focused on profiling in blood. Using ultra-performance liquid chromatography (UPLC) coupled with MS, Greenberg and colleagues [46] examined metabolic changes in plasma in 10 CN elderly individuals, 12 MCI, and 16 AD patients (Table 3). Partial least squares discriminant (PLSD) analysis demonstrated a clear separation of subject groups. Two

Table 3
Summary of metabolomic studies conducted in AD patients.

Analytical platform	Samples	Findings	References
• LCECA • Focused on neurotransmitter and oxidative stress pathways	Post-mortem ventricular CSF from 15 AD and 15 CN	• Norepinephrine (NE) levels lower in AD vs. CN • NE, methionine, and alpha-tocopherol negatively correlated with tangle formation and/or amyloid deposition • 5-hydroxytryptophan and tyramine positively correlated with tangle formation and/or amyloid deposition	[42]
• GC–MS and LC-MS/MS for broad profiling • SPE-LC-MS/MS for catecholamine and steroids	CSF from 79 AD (53 mild and 26 moderate) and 51 CN	• Greater metabolic changes in female vs. male AD • Greater metabolic changes in mild vs. moderate AD • Cortisol positively correlated with AD severity • The combination of cysteine and uridine had 75% sensitivity and specificity for separating mild AD from CN	[44]
• CE–MS for non-targeted metabolomics	*Test set*: CSF from 19 SCI, 22 stable MCI, 9 MCI progressors, and 23 AD. *Validation set*: 4 SCI, 2 stable MCI, 4 MCI progressors, 2 AD.	• Based on LDA, correct classification of training set for 94.7% of SCI, 85.7% of MCI-stable; 88.9% of MCI progressors; and 90.9% of AD • For the test set, 83% of the samples were correctly assigned to their corresponding group • Choline, valine, carnitine, serine, and a tripeptide significantly differed by group in targeted analyses	[45]
• UPLC-MS	Plasma from 16 AD, 12 MCI, and 10 CN.	• High-influence variables for AD included glycerophosphocholine and D-glucosamininde • GCA, GCDCA, and GDCA were increased in MCI and AD vs. CN	[46]
• UPLC-MS for global lipidomics • 2D GC x GC-TOFMS for global profiling of small polar metabolites	Serum from 46 CN, 37 AD, 52 MCI who progressed to AD, and 91 stable MCI.	• AD patients had decreased concentrations of several lipid classes, including phosphatidylcholine, plasmalogens, sphingomyelins, and sterols • Dihydroxybutanoic acid best separated MCI patients who did and did not progress to AD over 2 years	[52]
• UPLC-ToF-MS	Plasma and CSF from 15 CN, 15 MCI, and 15 AD.	• The number of affected pathways in CSF and plasma increased with disease severity • In both the CSF and plasma there were significant group differences in pathways related to energy metabolism and mitochondrial function; lipid biosynthesis, trafficking and metabolism; amino acid biosynthesis and metabolism; neurotransmitter biosynthesis and metabolism; and hormone biosynthesis and metabolism	[55]
• LC-MS • Analyses focused on phospholipids	Plasma from 10 CN, 10 MCI, and 10 AD.	• AD and CN had complete group separation • Lysophospolipid 18:1 decreased incrementally with increasing disease severity (CN > MCI > AD)	[59]
• Liquid chromatography–atmospheric pressure chemical ionization-mass spectroscopy • Analyses focused on sterol-related compounds	*Test set*: Plasma and CSF from 10 AD and 10 CN. *Validation set*: Plasma from 42 CN, 26 MCI, and 41 AD	• Plasma and CSF desmosterol was decreased in AD vs. controls • Notable sex differences in desmosterol levels and in the sensitivity/specificity of group separations (e.g., MCI vs. AD and MCI vs. CN) • Demonstrated importance of methodology as there were no differences in sterol levels when GC–MS was used	[60]
• Non-targeted approach using MDMS-SL	Plasma from 26 AD and 26 CN	• Long-chain sphingomyelin species were lower in AD patients compared to controls • Levels of 2 ceramide species (N16:0 and N21:0) were higher in AD than CN • Ratios of ceramide to sphingomyelin species better discriminated between AD cases and CN compared to either lipid species alone	[63]

high-influence variables for AD included glycerophosphocholine and D-glucosaminide. While no relationship between the later metabolite and AD mechanisms has previously been reported, research conducted in both post-mortem CSF and CSF from living subjects demonstrated that glycerosphophocholine levels were elevated in AD patients relative to controls [47–49]. The replication of these findings in the blood suggests this metabolite may indeed be indicative of changes in the brain and could serve as a potential diagnostic marker for AD. Another interesting result in this study was the demonstration that levels of three bile acids (glycocholate, glycodeoxycholate, and glycochenodeoxycholate) were elevated in both MCI and AD patients relative to controls (Table 3). While there is dearth of data examining the role of bile acids in neurodegenerative diseases, recent studies have suggested that bile acids are present in the brain [50] and that they may attenuate APP processing and Aβ deposition in APP/PS1 mice [51].

In another study, Oresic and colleagues [52] used UPLC-MS and 2D GC–ToF-MS to determine predictors of conversion from MCI to AD over a 2-year period (Table 3). The authors examined serum from 46 CN individuals, 91 who had stable MCI at baseline and follow-up, 52 MCI who progressed to dementia, and 37 AD cases. The main metabolite that separated MCI cases that did and did not progress was 2,4-dihydroxybutanoic acid, which was higher in those that progressed to AD. While little is known about this molecule in serum, it is over-produced under conditions of low oxygen [53]. Thus, this marker may represent hypoxic pathways involved in AD [54].

4.4. CSF and blood

As blood represents a non-invasive, inexpensive, and acceptable source for repeated measures and CSF most closely reflects brain-specific changes, the identification of metabolomic differences in both mediums within the same subjects would validate the use of specific blood-based metabolites as diagnostic or prognostic biomarkers of AD. We therefore examined metabolomic differences, using UPLC-ToF-MS, in the CSF and plasma of 15 CN, 15 MCI, and 15 AD subjects [55] (Table 3). There were distinct group differences in multiple metabolites and pathways in both the plasma and CSF. Approximately 30% of the metabolic pathways were altered in the CSF in MCI patients versus CN, and 60% in AD patients versus CN, were *also* affected in the plasma from the same individuals, showing the consistency between mediums. The number of affected pathways in the CSF and plasma increased with disease severity. For example, while only L-arginine and tryptophan pathways were altered in both plasma and CSF of MCI patients, the number of pathways equally affected in the CSF and plasma of AD patients considerably increased and included beta-alanine, aspartate and aspargine, alanine, L-cysteine, L-methionine, methionine–cysteine–glutamate along with L-arginine and lysine metabolic pathways. Further, compared to CN, bile acid biosynthesis and metabolism was significantly affected in the plasma of AD patients, which is similar to the results of Greenberg and colleagues [46]. Our data demonstrate that CSF and plasma have significant overlap in affected pathways, and most of the pathways affected early in MCI continue to be altered in AD subjects. In both the CSF and plasma of MCI and AD groups, the perturbed canonical pathways included those related to energy metabolism and mitochondrial function; lipid biosynthesis, trafficking and metabolism; amino acid biosynthesis and metabolism; neurotransmitter biosynthesis and metabolism; and hormone biosynthesis and metabolism.

4.5. Lipidomics

Perturbations in lipid metabolism have been noted since Alois Alzheimer's first reported case of AD [56], but understanding of their relationship and contribution to AD pathology has been hampered by inadequate methodology. Lipidomics is a newer metabolomic approach that can be used to conduct system-level analyses of lipids in biological samples [57]. Lipids have several important roles in the CNS and periphery including the maintenance of membrane structure, the formation of lipid rafts, and the involvement in signaling pathways. Several lines of evidence suggest that the dysregulation of cholesterol, sphingolipid, and fatty acid metabolism may initiate or accelerate AD pathology and, therefore, be important in the etiopathogenesis of AD and future therapeutic strategies [58].

While conventional metabolomic methods can identify some lipids such as cholesterol and fatty acids, other lipids will not be identified because they have polarities that will partition them into the aqueous phases during the extraction procedures and make them "invisible" for detection. As a result, specific lipidomic methods have evolved to conduct both global (non-targeted) lipid analyses as well as targeted measures of specific lipid classes; both methodologies have been utilized in AD research. Sato and colleagues [59] measured phospholipids and sterols in plasma from 10 CN, 10 MCI, and 10 AD patients (Table 3). Using PLS analyses and a supervised clustering method, the authors demonstrated a complete separation between the AD and CN groups. Among the MCI individuals, half had lipid profiles similar to the AD patients and half similar to the CN. This might be expected, since MCI represents a heterogeneous group that includes individuals who will progress to AD, who will progress to other dementias, and individuals who will not progress but remaining MCI. While determination of the specific lipids that were unique to each group was ongoing, lysophospholipid 18:1 was found to have incremental decreases such that CN > MCI > AD.

Using similar methodology, Sato et al. extended their research to examine group differences in plasma sterols [60] (Table 3). In an initial test sample of plasma from 10 AD cases and 10 CN, several peaks were identified that differentiated the groups. Levels of desmosterol, a precursor to cholesterol, were significantly ($p < 0.009$) decreased in AD patients versus CN. To validate this finding, the authors examined samples from 42 elderly CN, 26 MCI, and 41 AD patients and confirmed that levels of desmosterol were sequentially decreased with disease severity (CN > MCI > AD). There were also negative correlation between plasma desmosterol levels and the Mini-Mental State Examination (MMSE), with the findings strongest among females. Two important aspects of this study provoke further discussion. First is the specific identification of desmosterol. It is well established that in late-life, within 10 years of dementia onset, lipid levels begin to decrease [61]. This is likely due to changes in dietary habits, weight loss, and frailty. Thus, changes in desmosterol levels could most closely represent the pathological progression of AD. Second, a previous study using GC–MS suggested there were no differences in desmosterol levels between control and AD patients [62]. The authors therefore rerun their samples with GC–MS and also found that levels of desmosterol did not differ [60]. This finding highlights differences in methodologies and suggests that LC-MS represents a more sensitive analytical platform capable of separating desmosterol from metabolites with similar structures.

Another lipidomic study [63] utilized a non-targeted multi-dimensional mass spectrometry-based shotgun lipidomic (MDMS-SL) approach to measure levels of over 800 species of phospholipids, phosphatidylinositol, sphingomyelin, ceramide, triacylglycerol, cholesterol, and cholesterol esters (Table 3). An advantage of the shotgun approach is the speed, robustness, and the capacity for automation. However, identification of low abundance lipids and the complication with strong ion suppression can be problematic. Using plasma from 26 AD (17 with mild and 9 with moderate AD) and 26 CN, it was found that sphingolipid levels were significantly lower, and ceramide levels were higher in AD patients compared to controls. The ratio of ceramides to sphingomyelins for specific carbon chain lengths (e.g., C22:0) more robustly discriminated between AD cases and CN compared to either lipid type alone. Further, among AD cases, there were strong correlations ($p < 0.004$) between the rank of the changed mass levels of sphingomyelin and ceramides and the rank MMSE score. While this study was the first to examine a shotgun sphingolipidomic approach, the findings are in line with previously published data generated with targeted methods where AD patients were found to have higher

levels of ceramides in the middle frontal cortex [64], white matter [65], and CSF [66] compared to normal controls. Targeted studies of plasma sphingolipids have also highlighted the importance of blood ceramides. High blood ceramide levels predicted cognitive impairment and AD among CN individuals [11,67]; memory decline and hippocampal volume loss among amnestic MCI patients [68]; and faster rates of cognitive decline among AD patients [69]. Thus, the robustness of the findings across methods and sample severities (brain tissue, CSF, plasma) suggests that the sphingolipid pathway is perturbed in AD and warrants additional research relevant to identification of potential biomarkers and therapeutic targets.

5. Conclusion

Metabolomics provides a novel approach to identify alterations in multiple biochemical networks over the course of AD. Application of metabolomics allowed identification of both expected and non-expected changes in biochemical pathways related to AD pathology in both animal models of AD and in human samples. These findings highlight the translational strength of metabolomics since there is considerable parallel between mouse and human metabolism. Metabolomic profiling allows establishing dynamic changes in metabolites that correlate with disease severity in CSF and plasma, where changes in plasma accurately mimic changes in CSF, making it attractive for the clinical application. Since metabolic changes associated with AD progression occur prior to the development of clinical symptoms, metabolomics by itself or in conjunction with the additional currently available biomarkers for AD diagnosis (CSF and plasma levels of Aβ, tau and p-tau along with the advanced imaging techniques) could serve as an additional tool to increase the accuracy of diagnostic, to predict the disease progression, and to monitor the efficacy of therapeutic intervention. However, very few studies to date included both test and sample validation. Therefore, future work will be critical for confirming current findings in the larger cohorts of patients.

Acknowledgements

We would like to thank Ms. Jennifer Scott for help with manuscript preparation. Research reported in this publication was supported by the National Institute of Environmental Health Sciences of the National Institutes of Health under Award Number R01ES020715; BrightFocus Foundation Grant A2011084 as well as the Mayo Clinic Stimulus Award (Grant UL1 TR000135 from the National Center for Advancing Translational Science) (to ET). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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