Review

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Bioanalysis

Next-generation biomarker discovery in Alzheimer's disease using metabolomics – from animal to human studies

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Alzheimer's disease (AD) is a complex disease driven mainly by neuronal loss due to accumulation of intracellular neurofibrillary tangles and amyloid β aggregates in the brain. The diagnosis of AD currently relies on clinical symptoms while the disease can only be confirmed at autopsy. The few available biomarkers allowing for diagnosis are typically detected many years after the onset of the disease. New diagnostic approaches, particularly in easily-accessible biofluids, are essential. By providing an exhaustive information of the phenotype, metabolomics is an ideal approach for identification of new biomarkers. This review investigates the current position of metabolomics in the field of AD research, focusing on animal and human studies, and discusses the improvements carried out over the past decade.

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Neurodegenerative disorders include a broad range of diseases characterized by progressive loss of neurological functions, such as Parkinson's disease (PD), Alzheimer's disease (AD) and amyotrophic lateral sclerosis (ALS), showing the highest incidence in the worldwide population [1,2]. AD, the most prevalent neurodegenerative disorder, is the best-known form of late-life mental failure in humans, and its incidence is expected to be triplicated over the next 50 years [3]. This complex disorder involves progressive conditions such as memory loss, behavioral disorder, impairment of cognitive and motor functions, and a complete loss of self-sufficiency in the final stage [4]. AD affects central nervous system (CNS) leading to atrophy of brain cortex, especially in the neocortex and hippocampus areas which are related to superior mental functions. The pathophysiological changes in patients suffering from AD begin many years before the onset of dementia and cognitive decline, and the speed of mental deterioration varies among patients. The hallmarks of AD include the presence of intraneuronal neurofibrillary tau tangles (NFTs) composed of hyper-phosphorylated and aggregated tau proteins; extracellular β-amyloid plaques formed by amyloid β (Aβ) peptide aggregates; and synapses loss. These structures are the cause of biochemical alterations in AD brain, leading to neuronal dysfunction and death [3]. Oxidative stress, lipid dysregulation and inflammation also play essential roles in AD brain alterations. Altogether, these biochemical alterations lead to unspecific symptoms that start with cognitive deficits to remember autobiographical events specific to a place and time [5–9], followed by damages in long-term memory and alteration of ordinary capacities of attention and semantic memory as the illness progresses to other brain areas, leading to dementia [10].

Disease classification

The majority of AD cases, up to 95%, are classified as sporadic (sAD), with no familial genetic risk factors. sAD typically begins after the age of 60 with a linear increase of the risk of developing it with age, therefore referred to as late-onset AD [11]. Late-onset AD, also known as type 2 AD, is characterized by complex interactions between genetics and environment with a strong genetic association with polymorphisms in the ε4 allele of the apolipoprotein

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E (APOE) gene [11]. The human *APOE* gene exists as three polymorphic alleles, namely, ε2, ε3 and ε4. ApoE protein is highly expressed in the liver and the brain, although lower levels can be found in several other organs. In the brain, *APOE* is mostly expressed in astrocytes, that transport cholesterol to neurons via ApoE receptors, which are members of the low-density lipoprotein receptors [12]. ApoE lipoprotein plays a crucial role in regulation of triglyceride metabolism throughout the body and cholesterol metabolism in the brain. It is implicated in amyloid trafficking and plaque clearance [13]. ApoE ε4 allele is the strongest genetic risk factor for sAD with an increased frequency (up to 40%) of the ε4 allele in AD patients and, although the mechanisms underlying ApoE influence on AD progression remain not fully understood yet, this allele enhances Aβ deposition into plaques and reduces clearance of Aβ from the brain in a gene dose-dependent manner [14]. AD frequency has also shown to be higher in APOE ε4 homozygous subjects compared with heterozygous ones [15]. Furthermore, APOE ε4 homozygous subjects present an earlier mean age of clinical onset of AD compared with heterozygous cases (68 years of age vs 76) [15], which might be explained by an impaired delivery of cholesterol from astrocytes to neurons, causing an increment of cholesterol concentration in neurons leading to an induction of $A\beta_{42}$ accumulation through the β-secretase pathway [16]. It has been also recently shown in mice that APOE ε4 also affects tau pathogenesis and tau-mediated neurodegeneration independently of Aβ pathology [17]. On the contrary, the ε2 allele decreases the risk of developing the disease via protective effects against both late-onset and familial AD [18–20].

About 1–5% of AD cases have a genetic disposition following Mendelian disease transmission patterns, referred to as autosomal-dominant familial AD (fAD). In contrast to sAD, this early-onset AD typically occurs earlier in life with some cases diagnosed at 35 years old [11]. In humans, the genetic factors associated with fAD so far identified are the presence of mutations in the β-amyloid precursor protein (*APP*) gene or in genes involved in APP processes, such as presenilin 1 (*PSEN1*) or presenilin 2 (*PSEN2*) [21]. Mutations in these genes cause a dysfunctional metabolism of APP in the brain, leading to an accumulation of Aβ and soluble APP, and causing amyloid plaques formation. Depending on the gene mutated, fAD can be divided into three different types. Type 1 AD is caused by mutations in the *APP* gene and is responsible for 15% of all early-onset fAD cases. Mutations in *PSEN1* or *PSEN2* account for type 3 AD and type 4 AD, representing 70 and 5% of all early-onset AD cases, respectively.

Causes of AD

Even though research in AD field has grown exponentially in the past two decades, the causes of this disease are not yet fully known, making difficult the development of optimal treatments to delay the onset and progression of the disease [22]. The two most prominent hypotheses to explain AD are the amyloid cascade hypothesis [23] and the neuronal cytoskeleton degeneration hypothesis involving tau hyperphosphorylation [24]. According to Hardy and Higgens [23], accumulation of Aβ peptide in the brain is the primary cause of AD. Aβ is a product of the APP metabolism. Different Aβ species exist, notably the ones ending at position 40 ($A\beta_{40}$) and 42 ($A\beta_{42}$). The longer forms of Aβ, particularly $A\beta_{42}$, are more hydrophobic and fibrillogenic, therefore prone to deposit in the brain. Mutations in the *APP* gene favor the proteolytic processing of APP by β- and γ-secretases, increasing the concentration of Aβ in brain [25]. Aβ tends to self-aggregate forming senile plaques, known as well as neuritic plaques or Aβ plaques, which lead to neural injury prior to neuronal death [26]. Asides of the Aβ plaque formation, other factors may also account for AD progression, such as dysfunctions in tau phosphorylation, vascular risk factors and blood–brain barrier (BBB) dysfunctions, which impair Aβ clearance from the brain [27]. According to Braak and Braak [24], cytoskeletal changes such as the abnormal phosphorylation of the microtubule-associated protein tau precede the deposition of amyloid plaques. Under pathological conditions, the amount of hyperphosphorylated tau increases, leading to tau aggregation. In consequence, tau affinity for microtubules is reduced, affecting neuronal plasticity and causing neurodegeneration [28].

In more recent years, increased evidence points to the concomitant action of tau and soluble forms of Aβ toward diseased neurons. Furthermore, characteristic toxic properties of the Aβ peptide require the presence of tau. Brain Aβ accumulation (amyloidosis) is an upstream event of tauopathy in the AD pathway and Aβ is responsible for the conversion from normal tau to hyperphosphorylated tau, which is the toxic form of this protein [29]. However, some studies demonstrate that the toxic state of tau enhances Aβ toxicity via a feedback loop. Some cellular processes such as neuron death or synaptic dysfunction are caused by soluble Aβ and depend on soluble tau. Soluble forms of these proteins can aggregate and self-propagate throughout the brain by prion-like mechanisms [30].

Stages of AD

In AD pathology, a series of biological processes begin years ahead of the emergence of clinically evident impairment in cognition. This relatively long asymptomatic period is referred to as preclinical phase and is divided into three stages [31]. Not all the individuals included in these three preclinical stages will progress to dementia [31]. Indeed, some individuals may present all the neuropathological features of AD at autopsy without having ever expressed clinical dementia symptoms during their life. This highlights the existence of possible factors that give resistance to further progression of AD, such as protective genetic factors, environmental influences, and cognitive or brain reserve. Brain reserve represents the capacity of the brain to withstand pathological insult, whereas cognitive reserve is the ability to engage alternate brain networks to cope with the effects of encroaching pathology [31].

The link between the pathophysiological process of AD and the emergence of the clinical symptomatology has not been elucidated yet. Nevertheless, several studies have already provided preliminary evidence that abnormalities consistent with AD pathophysiological processes are detectable before the emergence of clinical symptomatology via detection of specific biomarkers, which might therefore be predictive of subsequent cognitive decline. Recent studies have typically focused on the accumulation of Aβ before the appearance of clinical symptoms, which is essential but not sufficient to produce clinical symptoms. Also, the presence of one or two APOE ε4 alleles is being studied as a genetic biomarker since ε4 alleles are linked to an increased risk of developing AD dementia [31].

Mild cognitive impairement (MCI) is an intermediate phase in the development of AD compared with normal aging, but not severe enough to interfere notably in daily-life activities to fulfill the criteria of dementia. This phase is defined by cognitive impairment, mainly in memory functions, and subjects suffering from MCI appear more like AD patients than subjects with memory failure as part of aging [32]. The conversion rate of MCI patients toward AD is difficult to estimate and prone to large variability depending on the studies, also because different diagnostic criteria have been applied for MCI in the literature [33].

Moreover, AD is not the only possible evolution of MCI, since several outcomes, including recovery to normal cognition, are possible [34]. No valid method for estimating the conversion rate of MCI to AD is currently available, logically representing one of the major focuses of MCI research today [35]. Finally, the amount of clinical trials performed over the last years with patients at MCI stage has substantially increased as it is believed that pharmacological intervention at an earlier AD stage is a key factor to possibly delay or even prevent the evolution of MCI to dementia [36,37].

Diagnosis of AD

The current diagnosis of AD is mainly based on clinical investigation, including physical and neurological criteria, medical history and laboratory tests for potential biomarkers. These clinical parameters allow for a prediction of AD with an accuracy rate of >90%. However, a definitive diagnosis can only be assessed post-mortem, based on the presence of tangles and plaques in the brain [38]. The current clinical diagnostic approach remains time-consuming and is based on the exclusion of other neurologic and psychiatric disorders. Current diagnosis biomarkers include the ratio between total Aβ peptide and tau protein in cerebrospinal fluid (CSF) combined with brain imaging techniques, which represents a very costly approach and is not sensitive enough at the earlier stages of the disease [27].

An important challenge in AD diagnosis is that the current approaches are only capable of identifying patients at advanced stages of the disease, once the dementia is already present. This highlights the need for innovative approaches allowing for the diagnosis of AD at earlier stages of the disease, before appearance of the symptoms. The discovery of such biomarkers would also certainly help in enabling a reliable determination of the conversion rate from MCI to AD in affected patients [39]. In this context, omics-based approaches, including genomics, transcriptomics, proteomics and metabolomics can play an essential role. Notably, metabolomics-based techniques are considered promising in the determination of novel biomarkers or metabolic pathways altered in AD, and for a better understanding of the mechanisms underlying AD pathophysiology.

Genome- & proteome-derived biomarkers

Biomarkers in CSF

CSF plays an important protective role in the CNS and is responsible for transporting nutrients to maintain homeostasis of neuronal cells. CSF composition closely reflects the brain extracellular space composition and is therefore related to metabolic processes occurring in the brain [40]. Hence, CSF is very attractive for the discovery of new biomarkers in AD research, where various compounds, mostly proteins or polypeptides, have been already

shown to be associated to AD. Notably, increased levels of total tau and phospho-tau-181 (pTau) in CSF, measured by ELISA, currently represent the most accepted and validated biomarkers so far [38].

Tau protein, a brain-specific microtubule-associated protein, is important in the progression of AD as it is responsible for the formation of NFTs. In healthy subjects, CSF-tau levels increase with age, being approximately 130 pg/mL in 21–50 years-old subjects, increasing up to threefold until approximately 340 pg/mL in subjects older than 71 years old. In AD patients, tau levels are significantly increased, with a cut-off concentration of ≥600 pg/mL [38,41]. Moreover, CSF-tau levels might also be relevant to determine the progression of MCI patients into dementia, as 90% of MCI patients that finally progressed to dementia have shown significantly higher tau levels in CSF compared with MCI-stable subjects [42].

Phosphorylated tau (pTau) is a fraction of total tau, and thus a characteristic component of NFTs. In AD, tau protein can be hyperphosphorylated in 39 possible sites, resulting in a loss of protein functionality that eventually leads to a dysfunction of axonal transport. Tau phosphorylation at residue 181 is one of the mainly occurring tau isoforms found in NTFs, with levels significantly higher in patients with AD compared with nondemential subjects [43].

In addition to tau and phospho-tau levels, reduced levels of $A\beta_{42}$ in CSF determined using ELISA have also been recognized as a well-established analysis [44]. Levels of $A\beta_{42}$ in CSF AD are typically lower than 500 pg/mL, whereas non-dementia subjects present higher levels (ca. 800 pg/mL) [41]. The decrease in $A\beta_{42}$ levels is mainly due to enhanced aggregation and plaque formation in AD brain, as well as reduced clearance of Aβ from the brain to the CSF [38]. It is worth mentioning that only the combination of these three CSF biomarkers significantly increases the efficacy and accuracy of the diagnosis of sAD [45].

Despite the abundant research the has been carried out over the last years to find new biomarkers in CSF, no other protein or polypeptide has been validated for clinical use. For instance, CSF levels of nerve growth factor (NGF), affecting cholinergic transmission, have been shown to be significantly higher in AD patients compared with healthy subjects, indicating accumulation of NGF in the brain of AD patients [46]. However, NGF levels are too low to be detected with sufficient sensitivity with current techniques, hampering its use as biomarker [47].

Blood-derived biomarkers

Compared with CSF, blood is easily collected and therefore represents the matrix of choice for the discovery of new and easily-accessible biomarkers. Indeed, despite the presence of the BBB, the peripheral circulation is also expected to transport compounds that may reflect the brain (patho)physiology.

Logically, the first attempts in finding blood-based biomarkers were focused on $A\beta_{42}$ levels [48]. Different conclusions have been drawn on the evolution of $A\beta_{42}$ plasma concentration, where some studies showed an increase in $A\beta_{42}$ levels in familial AD patients with *APP* or presenilin mutations [49] while others did not find any significant differences in Aβ plasma concentrations among groups, rendering the use of plasma Aβ levels inaccurate for the diagnosis of sAD [48]. Multiple hypotheses have been raised to explain the diversity in $A\beta_{42}$ levels, in other words, the influence of therapies, such as insulin treatments, which considerably influence Aβ expression; the fact that Aβ levels might differ in the different stages of AD and among subjects; other potential sources of Aβ production, such as platelets, contribute to the total plasma Aβ levels; and Aβ binds to other proteins, rendering its detection as a free form not straightforward [38].

Similar to Aβ levels, enzymes related to tau pathology have been also evaluated as possible AD plasma biomarkers. For instance, glycogen synthase kinase-3 (GSK-3), involved in tau hyperphosphorylation, has been reported to be significantly increased in both AD and MCI white blood cells compared with controls, suggesting that GSK-3 dysregulation occurs in early stages of AD pathogenesis [50]. In contrast, reduced GSK-3 levels in peripheral-blood mononuclear cells have been reported in patients with MCI. These opposite conclusions highlight the difficulty in using GSK-3 as a biomarker for AD diagnosis [51].

APOE ε4 allele is known to be the main risk factor for sAD and is associated with increased risk of amyloid plaques. However, no consistent correlation between AD and plasma apoE levels has been reached so far, since some studies have documented reduced levels [52,53], elevated levels [54], or no difference compared with controls [55]. When studying the levels of ApoE in CSF, results are also inconclusive. On one side, a study carried by Hesse *et al.* showed that higher levels of ApoE in CSF were found in individuals that possessed the ApoE ε4 alleles, both in the AD and in the control group [56]. On the other side, Cruchaga *et al.* found that ApoE levels in CSF were correlated with $A\beta_{42}$ levels and clinical dementia rating, but were independent of the APOE genotype [55].

Table 1. Most investigated and characterized biomarkers for Alzheimer's disease.

Biomarker	Sample	Levels compared with controls	Use in clinics	Ref.
Tau	CSF	Increased	Yes	[42]
pTau	CSF	Increased	Yes	[43]
$A\beta_{42}$	CSF	Decreased	Yes	[41]
$A\beta_{40}$ and $A\beta_{42}$	Plasma	Increased in fAD/no changes in sAD	No	[48,49]
GSK-3	Plasma	Increased in AD and MCI/reduced in MCI	No	[50,51]
apoE	Plasma	Inconsistent	No	[52–55]
IL-6	Plasma	Inconsistent	No	[57]
IL-12, IFN-α and IFN-β	Plasma	No changes	No	[57]
Plaques (Aβ)	Post-mortem tissue	Increased	Yes	[38]
NFT (tau)	Post-mortem tissue	Increased	Yes	[38]

Only CSF biomarkers and post-mortem analysis are currently used in clinics.
Aβ: Amyloid β; CSF: Cerebrospinal fluid; NFT: Neurofibrillary tau tangle.

Finally, AD has a strong inflammatory component. Aβ deposition in the AD brain triggers a broad range of inflammatory responses which probably contribute to neuronal cell death, explaining why possible inflammation markers in plasma or serum have been also investigated [5]. However, many of these proteins have difficulties in crossing the BBB, limiting the relevance of their plasma levels. Indeed, inflammatory markers such as C-reactive protein, IL-6, TGF-β,or IL-1β, among others, show inconsistent results in the literature. On the other hand, IL-12, IFN-α and IFN-β blood concentrations have shown to remain unaffected in AD patients relative to control groups [57].

Post-mortem analysis

To date, the post-mortem analysis of brain tissue currently remains the only approach available to obtain an accurate and definitive diagnostic of AD. In this case, Aβ and tau are stained by immunohistochemistry in order to detect the amyloid plaques and NFTs, respectively. Post-mortem histology can also be used to detect pathological changes in an AD brain or the therapeutic effect of an administered drug against AD progression [38].

Genome- & proteome-derived biomarkers: limitations

Even though the number of biomarkers investigated for AD has significantly increased over the past years, altogether with the amount of literature published on this subject, only few of them are used in routine to help for AD diagnosis, as listed in Table 1.

Commonly, routine AD diagnosis still relies on the criteria established by the National Institute for Neurological and Communicative Disorders and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS/ADRDA, now known as Alzheimer's disease Association), which states that only samples obtained by autopsy of the patient can be used for definitive diagnostic [58]. Therefore, the in vivo analysis of CSF biomarkers is increasingly considered in routine, focusing on the analysis of $A\beta_{42}$, total tau and phosphor-tau-181, which altogether increase the confidence in the clinical diagnosis. This approach, however, presents some relevant drawbacks. First, CSF collection remains very invasive and prone to risks for the patients, which also renders follow-up analysis of a patient over time very challenging [38]. Moreover, no consensus is currently available concerning the cutoff values for $A\beta_{42}$, tau and pTau CSF levels. Indeed, the diagnostic sensitivities and specificities reported in the literature are strongly dependent on the subjects included and the experimental design, making the definition of standard criteria for the diagnosis of AD very difficult [44]. Typically, considerable difference in CSF concentrations has been observed between laboratories, especially for $A\beta_{42}$ levels, even when using the same kit [59]. Standardization in clinical and analytical procedures for measuring $A\beta_{42}$, tau and pTau levels is therefore essential to ensure reproducibility between laboratories and understand the sources of this variability (e.g., inclusion criteria, definition of MCI, sample collection frequency, etc.) [60,61]. Finally, as previously mentioned, increased evidence shows that only the combination of these three biomarkers can lead to sufficient sensitivity and specificity – but not separately [27].

Blood, despite easier to collect compared with CSF, leads to additional challenges since protein-based or polypeptide biomarkers do not easily cross the BBB. This highlights the needs for the discovery of new biomarkers based

on low molecular weight compounds or lipophilic metabolites, which can more easily pass through the BBB, and, therefore, be detectable in blood at sufficient concentrations [5].

It is worth mentioning that the BBB has shown to be altered in AD subjects. On one side, the expression of the proteins in charge of transporting Aβ into blood, named low-density LRP1 and P-glycoprotein (P-gp), are reduced in AD subjects leading to Aβ accumulation due to its poor clearance from the brain. Moreover, Aβ damages its own LRP1-mediated transport by oxidating LRP1 and $A\beta_{42}$ downregulates the expression of P-gp and other Aβ-transporters, intensifying the accumulation of Aβ in the brain and accelerating the neurodegeneration in AD. On the other side, expression of the receptor for advanced glycation end products mRNA, a receptor responsible for the influx of circulating Aβ into the brain, has been found to be significantly reduced in mice treated with $A\beta_{42}$. In plasma, the major transport protein for peripheral Aβ is a soluble form of LRP1, which maintains the homeostasis of Aβ levels by inhibiting the reentry of free plasma Aβ into the brain. Thus, Aβ levels can be detected in the blood but are not correlated to the CSF ones [62].

Finally, two important challenges in AD research are the detection of pre-dementia states and the identification of MCI patients who are at risk to progress to dementia. As already mentioned, MCI is an heterogeneous group and it is currently difficult to evaluate the possible conversion from one stage to the following one [63]. Accurate diagnosis of AD in early stages also remains difficult because a variety of neurodegenerative disorders share the same early symptoms, which makes it complicated to discriminate them. Therefore, ideal biomarkers will be able to distinguish between AD and other neurodegenerative disorders, such as vascular dementia, MCI, or Lewy body dementia, which require different treatment approaches [38].

Next-generation biomarker discovery in AD: metabolomics-based strategies

Metabolomics is defined as the comprehensive analysis of all metabolites (i.e., compounds with a mass lower than 1000 Da) within a cell, tissue or organism and represents an attractive approach to study alterations of the metabolism upon physiological stimuli, genetic alteration, pathogenic factors (immune response) or lifestyle (e.g., diet, physical exercise, smoking habits, gut microbiome, etc.) [13,34]. Together with other omics approaches, metabolomics is expected to play an essential role in implementation of personalized medicine and provide a better understanding of biomolecular mechanisms underlying important diseases, including AD [64,65]. Particularly, metabolomics shows promises in discovering biomarkers that might predict disease progression since the metabolome is evolving along with a specific pathological status [66]. This highlights the importance of such discipline in AD research, where the drive has recently switched towards identifying biomarkers that can predict the rates of cognitive decline and conversion from MCI to AD, rather than identifying markers that distinguish cases versus cognitively normal healthy controls [67]. Finally, metabolomics-based studies are also of relevant help for revealing the pharmacokinetics and pharmacodynamics of drugs in both patients and *in vitro* models [68].

Another useful feature of metabolomics is the possibility of mapping the metabolome in multiple bio-samples, including blood-derived matrices, urine, CSF or tissues. Such diversity is of high relevance in AD research, enabling the comparison of the metabolic profiles between compartments [39]. Ideally, this approach would also allow to decipher if peripheral molecular changes (measured for instance in plasma) are related to alterations in the CNS, or to peripheral metabolism [6].

Untargeted versus targeted metabolomics

The detection of biomarkers for AD can be accomplished via two different approaches, namely, untargeted and targeted metabolomics [69]. Targeted metabolomics is an *a priori*-based approach relying on the analysis of a restricted number of metabolites that are involved in a specific metabolic pathway or reaction. This approach allows for (semi-)quantitation of known metabolites, for instance already reported in the literature such as cholesterol, lipids or homocysteine [3], but is not suited for the discovery of potential biomarker candidates which remain to be discovered [70].

In contrast to the targeted strategy, untargeted metabolomics approaches allow for the discovery of novel unreported biomarkers through the study of as many metabolites as possible in a biological system [3]. This unbiased approach is particularly interesting in AD, due to the relatively poor knowledge of pathophysiological and metabolic processes occurring in AD brain. As an example, the evolution of the metabolic profile over time can lead to relevant information that can lead to the generation of novel hypotheses about the causes, origin and progression of AD, or the support of previous investigations [70].

Both approaches are typically used sequentially, where molecules of interest are first selected using untargeted metabolomics, followed by more targeted approaches to quantify and confirm the role of the potential biomarker candidates. It is worth mentioning that both strategies also differ significantly in the data analysis workflows. The latter is particularly important in untargeted approaches which typically deal with large number of variables/metabolites in large scale studies, where an adequate data pre-processing and analysis strategy is essential to draw significant conclusions. The differences between targeted and untargeted metabolomics and their respective challenges have been extensively reviewed, for instance by Kohler *et al.* [71].

In AD research, a lot of the reported results have been gathered using targeted approaches that allow for the (semi-)quantification of specific compounds suspected to play a role in the disease, mostly oxidative stress markers, biogenic amines, oxylipins and other lipid mediators, and this based on the previous knowledge available on the pathophysiological mechanisms involved in AD. On the other hand, the untargeted approaches reported so far did not lead to the discovery of novel biomarkers, and failed in highlighting the connectivity between the different biochemical pathways that drive AD pathology [70].

State-of-the-art analytical techniques

State-of-the-art metabolomics relies on two complementary analytical strategies, namely, nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS)-based techniques [72]. Due to multiple technological development carried out in the past decades in both techniques together with the development of advanced data analysis methods, the coverage of the metabolome has been significantly extended [73].

NMR is a largely used technique which allows for simultaneous identification and quantitation of metabolites from complex mixtures [13], since the peak area measured on an NMR spectrum is linearly related to the number of nuclei in the sample, and thus the metabolite concentration [71]. NMR presents some advantages, such as nondestruction of the samples, the requirement of a minimal sample preparation before the analysis, short analytical run times and detection of metabolites presenting a large diversity in their physicochemical properties within a single run [72]. The main limitation of NMR is its relatively low sensitivity compared with MS–based techniques, which can be improved by using strong superconductive magnets and cryoprobes to reduce the electronic noise [71]. Other common drawbacks include high costs, low performance in lipid analysis, pH-dependent chemical shifts and signal suppression in the presence of water [71,73]. Up-to-date, the number of NMR studies in AD is still rather limited, as listed in Tables 2–6.

MS is commonly coupled to separation techniques such as liquid chromatography (LC) or gas chromatography (GC). Due to its high resolution and mass accuracy, especially in the most recent state-of-the-art mass analyzers, MS is very powerful in untargeted metabolomics, allowing for both a large coverage of the metabolome as well as identification of unknown compounds [72]. MS is much more sensitive than NMR and allows for the analysis of a large number of metabolites. However, MS is a destructive technique, less reproducible than NMR, and requires adequate sample preparation to avoid potential matrix effects [73]. Historically, GC–MS has been the first technique used in metabolomics, leading to very efficient separations and allowing for straightforward compounds identification thank to the multiple databases commercially available. GC–MS shows some limitations, such as the potential loss of thermolabile compounds, a rather cumbersome sample preparation involving a two-step derivatization of nonvolatile compounds, and a higher variability than the one observed with LC–MS [71,72]. LC–MS has therefore gained importance in metabolomics, allowing for the direct analysis of metabolites presenting a large physicochemical diversity with high throughput, selectivity and sensitivity. In the past two decades, several technological improvements have been carried out in the field of LC–MS, such as the development of alternative chromatographic modes, the comeback of supercritical fluid chromatography and the design of new sizes of porous particles, leading to significant improvements in separation efficiency, resolution, selectivity and speed of analysis. These developments have fostered the use of LC–MS in modern clinical metabolomics, where it is now considered the gold standard approach [121]. Clinical metabolomics will also certainly and significantly benefit from the latest developments in LC, for instance the use of nano-columns equipped with micropillar array instead of the conventional columns packed with particles, which represents a breakthrough in the world of chromatography and allows for unprecedented results in terms of separation efficiency and resolution [122,123]. Micropillar array columns have notably demonstrated to enable separation of lipid isomers, showing their promises in lipidomics [124].

Besides chromatography, MS can also be coupled to capillary electrophoresis (CE), allowing for separation of polar and/or charged metabolites with high efficiency and selectivity, with significantly lower amount of sample

Table 2. Summary of metabolomics studies conducted in animal models of Alzheimer's disease.

Animal model	Age	Matrix	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
				Increased levels	Decreased levels	
APPtg2576	19 months	Frontal cortex	NMR	Taurine	NAA, Glu, GSH	[74]
APPtg2576	30 months	Frontal cortex, rhinal cortex, hippocampus, midbrain and cerebellum	NMR	Glu, creatine, taurine (6 and 11 months)	NAA, Glu, myo-inositol, creatine, phosphocholine, GABA (3 months)	[75]
APP-PS1	66–904 days	Frontal cortex	NMR	Myo-inositol	NAA, Glu	[76]
PS2APP	4–24 months	Frontal cortex	NMR	–	NAA, Glu	[77]
CRND8	2–3 months 12–13 months	Cortex, frontal cortex, cerebellum, hippocampus, pons, olfactory bulb, midbrain and striatum	NMR	–	NAA, Glu, Gln, taurine, GABA, choline, phosphocholine, creatine, phosphocreatine, succinate, lactate, Asp, Gly, Ala, Leu, Ile, Val, water-soluble FFAs	[26]
APP-PS1	3, 5 and 8 months	Hippocampus	NMR	Myo-inositol (3 months)	NAA, Glu (5 and 8 months)	[78]
CRND8	20 weeks	Hippocampus	FT-ICR LC–MS	Hippurate, salicylurate, L-gulonolactone, glucose 1-phosphate, 6-phosphogluconic acid, arachidonic acid	–	[79]
APP-PS1	6 months	Hippocampus, cortex, striatum, cerebellum and olfactory bulbs	GC–MS LC–MS	Adenosine, guanosine, inosine, Ser, lactose, myo-inositol, phosphocholine and phosphoserine	AMP, cAMP, cGMP, Glu, Gly, His, lactic acid, malic acid, creatinine, PPi, citric acid, G6P, 2-hydroxyglutaric acid, DHA, urea and dopamine	[80]
APP-PS1	6 months	Hippocampus, cortex, striatum, cerebellum and olfactory bulbs	DIMS	FFA, G3P, glycerophosphocholine, phosphocholine, choline, glycerophosphoinositol, HEPE, HETE, docosapentaenoic acid, docosatetraenoic acid, pyruvate and N1-acetylspermidine	DHA, PE, PPE, L-carnitine, AMP, cholesterol, cholesterol sulfate, DCA, TCA organic acids, Tyr, dopamine and Asp	[81]
TASTPM	50 weeks	Brain tissue and plasma	GC–MS	L-Val, L-Ser, L-Thr, DHEA and D-fructose	Zymosterol, linoleic acid, arachidonic acid, D-Glu, D-Gal, palmitic acid and D-gluconic acid	[82]
APPtg2576 PS1 APP-PS1	36 weeks 36 weeks 16 weeks	Brain tissue	GC–MS	Adenosine, AMP and adenine	NAA, inorganic phosphates, glycerol, malonic acid, ATP, ADP, myo-inositol, GDP, threose, GTP, glutamic acid and malic acid	[83]
APP-PS1	6 months	Serum	DIMS	Diacylglycerols, triacylglycerols, eicosanoids, inosine, choline and glycerophosphoethanolamine	Cholesteryl esters, FFAs, lysophosphocholines, phosphoethanolamine, urea, Ser, Val, Thr, pyroglutamate, His, Tyr, Trp, glucose, carnitine and creatine	[84]
TAPP	4, 10 and 15 months	Urine	NMR	Allantoin, homogentisate and 3-hydroxykynurenine	–	[85]
APPtg2576 APPLd2 CRND8	3–16 months 4–23 months 2–8 months	Urine	GC–MS	Phenylacetone and 1-octen-3-ol	–	[86]
CRND8	15–17 weeks 25–28 weeks 30–31 weeks	Urine	LC–MS	Methionine and desaminotyrosine	N^1-acetylspermidine and 5-hydroxyindoleacetic acid	[87]
CRND8	12 weeks 18 weeks	Urine	LC–MS	*N*-acetylvanilalanine, 3-methoxytyrosine, N-actylserotonin, N-methylserotonin, N-methyltryptamine and α-ketoglutaric acid	Anthranilic acid and xanthurenic acid	[88]

Ala: Alanine; AMP: Adenosine monophosphate; Asp: Aspartate; cAMP: Cyclic adenosine monophosphate; cGMP: Cyclic guanosine monophosphate; CSF: Cerebrospinal fluid; DCA: Dichloroacetic acid; DHA: Docosahexanoic acid; DIMS: Direct infusion mass spectrometry; FFA: Free fatty acids; FT-ICR/MS: Fourier-transform-ion cyclotron resonance/mass spectrometry; GABA: γ-Aminobutyric acid; GC–MS: Gas chromatography–mass spectrometry; GDP: Guanosine diphosphate; GTP: Guanosine triphosphate; Gln: Glutamine; Glu: Glutamic acid; Gly: Glycine; GSH: Glutathione; G3P: Glyceraldehyde 3-phosphate; G6P: Glucose-6-phosphate; HEPE: Hydroxy-eicosapentaenoic; HETE: Hydroxyeicosatetraenoic acid; His: Histidine; Ile: Isoleucine; IMP: Inosinic acid; LC–MS: Liquid chromatography–mass spectrometry; Leu: Leucine; NAA: Naphthaleneacetic acid; NMR: Nuclear magnetic resonance; PE: Phosphatidylethanolamines; PPE: Plasmenylethanolamines; PPi: Inorganic pyrophosphate; Ser: Serine; TCA: Tricholoroacetic acid; Thr: Threonine; Trp: Tryptophan; Tyr: Tyrosine; Val: Valine.

Table 3. Summary of metabolomics studies carried out using cerebrospinal fluid samples in mild cognitive impairment and/or Alzheimer's disease patients.

Study population	Metabolomics approach	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
			Increased levels	Decreased levels	
Control (n = 38) MCI (n = 36) AD (n = 40)	Targeted	LC–ECA	Met, 5-HIAA and hypoxanthine (MCI); Met, 5-HIAA, vanillylmandelic acid, xanthosine and glutathione (AD)	–	[89]
Control (n = 15) MCI (n = 15) AD (n = 15)	Non-targeted	LC–TOF-MS	Acetoacetic acid, pyruvic acid, 2-methyl-3-ketovaleric acid, 2-furoic acid, N-acetyl-a-neuraminic acid, dehydroascorbic acid, 5-(hydroxymethyl)- 2-furancarboxylic acid, diacetyl, diethanolamine, Ne-methyl-L-lysine, lecanoric acid, ethopropazine and 8-amino caprylic acid (MCI and AD); 2-methylbutyrylglycine and pyrimethamine (MCI); Pro (AD)	Deoxyadenosine, isoniazid, fumaric acid, pyroglutamic acid, testosterone sulfate, lactaldehyde, pirenzepine, methylglyoxal, (S)-2-hydroxyglutarate, L-Aspartic acid b-semialdehyde, cotarnine, 4-hydroxy-L-threonine, acetazolamide, imidazolone, Met, Ala, Glu, Lys and His (MCI and AD); Pro (MCI); 2-methylbutyrylglycine and pyrimethamine (AD)	[90]
Control (n = 33) MCI stable (n = 26) MCI progressors (n = 13) AD (n = 27)	Targeted	CE-LIF	L-Arg, L-Glu, L-Asp and L-Lys	GABA	[91]
Control (n = 19) Stable MCI (n = 22) MCI progressors (n = 9) AD (n = 23)	Non-targeted	CE-MS	Choline and Ser (MCI); Val and dimethylarginine (AD)	His (MCI and AD); Arg, creatine, carnitine and suberylglycine (AD)	[92]
Control (n = 21) Stable MCI (n = 21) MCI progressors (n = 12) AD (n = 21)	Non-targeted	LC–TOF-MS	Xhantine, uracil, nonanoylglycine, hydroxyphophinyl-piruvate, taurine and sphingosine-1-phosphate (MCI); uridine, methylsalsolinol, dopamine-quinone, caproic acid and 5′-methylthioadenosine (MCI and AD)	Vanylglycol and uracil (AD); serinyl-tyrosine, pipecolic acid, His and Trp (MCI); creatinine (MCI and AD)	[93]
Control (n = 51) AD (n = 79)	Targeted	GC–MS LC–MS	Cortisol, cysteine and NE	Uridine and dopamine	[94]
Control (n = 17) AD (n = 12)	Targeted	NMR	Glu, Ala, Leu, Val, Tyr, Ile, Lys, lactate, pyruvate and inositol	–	[95]
Control (n = 34) Subjects with typical AD marker profile (n = 10)	Non-targeted	NMR	Creatinine	–	[96]

Information in brackets refers to the case group.
5-HIAA: 5-hydroxyindoleacetic acid; AD: Alzheimer's disease; Ala: Alanine; Arg: Arginine; Asn: Asparagine; Asp: Aspartate; CE-LIF: Capillary electrophoresis-laser-induced fluorescence, CE-MS: Capillary electrophoresis-mass spectrometry; Cys: Cysteine; GABA: γ-Aminobutyric acid; GC–MS: Gas chromatography–mass spectrometry; GC–TOF-MS: Gas chromatography–time-of-flight-mass spectrometry; Glu: Glutamic acid; His: Histidine; Ile: Isoleucine; LC–ECA: Liquid chromatography-electrochemical array; LC–MS: Liquid chromatography–mass spectrometry; LC–TOF-MS: Liquid chromatography–time-of-flight-mass spectrometry; Leu: Leucine; Lys: Lysine; MCI: Mild cognitive impairment; Met: Methionine; NE: Norepinephrine; NMR: Nuclear magnetic resonance; Pro: Proline; Ser: Serine, Trp: Tryptophan; Val: Valine.

and solvents consumed [92,125]. However, CE–MS remains little used in metabolomics due to poor reproducibility and instrumental constraints which limit high-throughput techniques [71,121].

In AD research, LC–MS remains by far the method of choice, even though some direct infusion mass spectrometry (DIMS) approaches have been reported [81,84,126,127]. DIMS relies on the direct infusion of prepared samples in the MS without prior chromatographic separations, allowing for high throughput analyses and large coverage of metabolites, but is more prone to matrix effects and ion suppression [128,129]. DIMS is particularly popular in lipidomics, where an additional separation dimension can be used based on ion mobility mass spectrometry, which enables the separation of lipid isomers and/or lipid classes. Ion mobility combined to DIMS has been for instance already used to study amyloid formation in AD, and will certainly be increasingly considered in AD research to better understand the role of some lipids in the disease pathology [130].

Potential of metabolomics in AD research & biomarker discovery

Omics technologies aim at investigating the composition of a biological sample to better understand the pathophysiological processes occurring in an organism. While traditional experimental models only offer a temporal picture

Table 4. Summary of metabolomics studies conducted in blood-derived samples of Alzheimer's disease patients.

Study population	Metabolomics approach	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
			Increased levels	Decreased levels	
Plasma: – Control (n = 15) – MCI (n = 15) – AD (n = 15)	Nontargeted	LC–TOF/MS	Val, Ser and Arg (MCI and AD); Trp and Phe (MCI); cholesterol and myo-inositol (AD)	Gly, Phe, Met, His, Asp, Cys (MCI and AD); Trp and Phe (AD); cholesterol (MCI)	[90]
Plasma: – Control (n = 8) – AD (n = 8)	Nontargeted	LC–MS	L-DOPA, anserine, ornithine, Ile, Ala, Lys, Cys, alloleucine and Asp	Carnosine, dopamine, Arg and citrulline	[97]
Serum: – Control (n = 99) – AD (n = 93)	Targeted	FIA-MS/MS LC–MS/MS	No difference	No difference	[98]
Plasma: – Control (n = 10) – MCI (n = 12) – AD (n = 16)	Targeted	LC–MS	GCA, GDCA and GCDCA	–	[99]
Plasma: non-demented participants (n = 2067)	Targeted	LC–MS	Anthranilic acid, glutamic acid, taurine and hypoxantine	–	[100]

AD: Alzheimer's disease; Ala: Alanine; Arg: Arginine; Asn: Asparagine; Asp: Aspartate; Cys: Cysteine; DOPA: Dihydroxyphenylalanine; FIA-MS/MS: Flow injection analysis/tandem mass spectrometry; GABA: γ-Aminobutyric acid; GCA: Glycocholic acid; GCDCA: Glycochenodeoxycholic acid; GDCA: Glycodeoxycholic acid; Glu: Glutamic acid; His: Histidine; Ile: Isoleucine; LC–MS: Liquid chromatography–mass spectrometry; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LC–TOF/MS: Liquid chromatography–time-of-flight-mass spectrometry; Lys: Lysine: MCI: Mild cognitive impairment; Met: Methionine; Phe: Phenylalanine; Pro: Proline, Ser: Serine, Trp: Tryptophan.

Table 5. Summary of metabolomics studies conducted in post-mortem samples of Alzheimer's disease patients.

Study population	Metabolomics approach	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
			Increased levels	Decreased levels	
Entorhinal cortex: – Control (n = 4) – AD (n = 16)	Nontargeted	LC–MS	Guanine	Deoxyguanosine, IDP, xanthosine, Gly, dGMP	[101]
Hippocampus, cerebellum and superior frontal gyrus: – Control (n = 23) – AD (n = 12)	Targeted	LC–MS HPLC	L-Arg	L-ornithine, agmatine, spermidine, putrescine, spermine, Glu	[102]
Frontal cortex: – Control (n = 19) – AD (n = 21)	Nontargeted	LC–MS	NAA, lactate, serine, pyruvate, Glu and malate	Asp and citrate	[70]
Frontal, parietal and occipital lobes: – Control (n = 10) – AD (n = 10)	Nontargeted	LC–TOF/MS	Spermine, spermidine and putrescine	–	[103]
Frontal cortex: – AD (n = 8) – ALS (n = 11)	Nontargeted	NMR	Acetate, Ala, Glu and glutamine	Creatine and lactate	[104]
Brain homogenates: – Control – AD	Nontargeted	LC–MS/MS	L-Phe	L-lactic acid	[105]

AD: Alzheimer's disease; Ala: Alanine; ALS: Amyotrophic lateral sclerosis; Arg: Arginine; Asp: Aspartate; Gly: Glycine; Glu: Glutamic acid; dGMP: Deoxyguanosine monophosphate; HPLC: High-pressure liquid chromatography; IDP: Inosine diphosphate; LC–MS: Liquid chromatography–mass spectrometry; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LC–TOF/MS: Liquid chromatography–time-of-flight/mass spectrometry; MCI: Mild cognitive impairment; NAA: Naphthaleneacetic acid; NMR: Nuclear magnetic resonance; Phe: Phenylalanine.

of the complex and dynamic biological networks of human diseases, these technologies can show relationships between different pathways and molecules in a biological system. Hence, omics approaches have attracted a lot of attention in AD for the discovery of novel biomarkers candidates that may lead to improved and earlier diagnosis, as well as better therapies and discovery of new therapeutic targets [131]. This growing attention is underlined in the large number of studies using metabolomics and other omics approaches that have been published in the last couple of years.

Metabolomics plays a crucial role in biomarker discovery and drug development since metabolites reflect the final effect of genes- and protein-based (patho)physiological processes. Moreover, metabolites are closely associated

Table 6. Summary of lipidomics studies conducted in Alzheimer's disease patients.

Study population and matrix	Lipidomics approach	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
			Increased levels	Decreased levels	
Plasma: – Control (n = 10) – MCI (n = 10) – AD (n = 10)	Nontargeted	LC–MS	–	LPE 1:18	[106]
Plasma: – Control (n = 42) – MCI (n = 26) – AD (n = 41)	Nontargeted	LC–MS	–	Desmosterol	[68]
Brain tissue: – Control (n = 10) – AD (n = 9)	Targeted	LC–MS NMR	–	Desmosterol	[107]
Plasma: – Control (n = 20) – AD (n = 20)	Nontargeted	LC–MS	–	Trp, phytosphingosine, dihydrosphingosine, hexadecasphinganine and lysoPC	[108]
Cortex, cerebellum and hippocampus: – Control (n = 17) – AD (n = 37)	Nontargeted	DIMS	Nervonic acid, oleic acid, stearic acid, palmitic acid, ximenic acid, palmitoleic acid and mead acid	DHA and cholesteryl esters	[109]
Serum: – Control (n = 45) – MCI (n = 17) – AD (n = 75)	Nonttargeted	LC–MS	Acylcarnitine, phenylacetylglutamine, phosphocholines, phosphoethanolamines and sphingomyelins	Phospholipids, sphingolipids, oleamide, monoglycerides, arachidonic acid, DHA, linoleic acid, plasmalogens and histidine	[66]
Serum: – Control (n = 46) – Stable MCI (n = 91) – MCI progressors (n = 52) – AD (n = 47)	Nontargeted	GC–TOF/MS LC–MS	2,4-dihydroxybutanoic acid, pentose phosphate and histamine	Phospholipids, phosphatidylcholines, sterols, sphingomyelins and plasmalogens	[34]
Plasma: – Control (n = 152) – AD (n = 148)	Nontargeted	NMR	PC 40:4, PC 36:3 and VLCTs	Desmosterol	[110]
Plasma: – Control (n = 35) – MCI (n = 33) – AD (n = 43)	Targeted	LC–MS/MS FIA-MS/MS	LysoPC a C18:1 and lysoPC a C18:2	PC aa C34:4, C36:6, C38:3, C40:5 and C40:6	[27]
Plasma: – Control (n = 49) – MCI (n = 50) – AD (n = 42)	Nontargeted	LC–MS NMR	–	PC 16:0/20:5, 16:0/22:6 and 18:0/22:6	[111]
Plasma: – Control (n = 51) – MCI (n = 77) – LOAD (n = 90)	Targeted	MS	DAG 34:2 and DAG36:2	PlsE 38:6 (16:0/22:6) and PlsE 40:6 (18:0/22:6)	[112]
Brain tissue: – Control (n = 46) – AD (n = 193)	Targeted	^{31}P NMR	SPH, plasmalogen	PtdEtn, PtdIns, DPG and PtdA	[113]
Brain tissue: – Control (n = 7) -AD (n = 7)	Targeted	DIMS	Ceramide 24:0, galactosylceramide, free cholesterol and HNE	SPH	[114]
Hippocampus and cerebellum: – Control (n = 6) – AD (n = 6)	Targeted	LC–MS/MS	HFA-Cer(d18:1/24:0)	GalCer	[115]
Plasma: – Control (n = 26) – AD (n = 26)	Nontargeted	MDMS–SL	Ceramide N16:0 and N21:0	SPH 22:0 and 24:0	[116]
Serum: – Control (n = 17) – AD (n = 19)	Targeted	DIMS LC–MS	–	PCs, LPPCs, PtdEtn, LPEs and plasmalogens	[117]

AD: Alzheimer's disease; DAG: Diacylglycerol; DHA: Docosahexaenoic acid; DIMS: Direct-infusion mass spectrometry; DPG: Diphosphatidyl glycerol; EDI-MS: Electrospray droplet impact-mass spectrometry; FA: Fatty acid; FIA-MS/MS: Flow-injection analysis/tandem mass spectrometry; GalCer: Galactosylceramide; GC–TOF/MS: Gas chromatography–time-of-flight/mass spectrometry; HFA-Cer: Hydroxy fatty acids-ceramide; HNE: Hydroxynonenal; HPTLC: High-performance thin-layer chromatography; LC–MS: Liquid chromatography–mass spectrometry; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LPE: Lysophosphatidylethanolamine; LPPC: Lysopalmitoylphosphatidylcholine; LysoPC: Lysophosphatidylcholines; MCI: Mild cognitive impairment; MDMS–SL: Multi-dimensional mass spectrometry-based shotgun lipidomics; MS: Mass spectrometry; NMR: Nuclear magnetic resonance: PCs: Phosphatidylcholines; PlsE: Ethanolamine plasmalogen; PtdEtn: Phosphatidylethanolamine; PtdA: Phosphatidic acid phosphatase; PtdIns: Phosphatidylinositol; PUFA: Polyunsaturated fatty acids; SPH: Sphingomyelin; Trp: Tryptophan; VLCT: Very long chain triglycerides.

Table 6. Summary of lipidomics studies conducted in Alzheimer's disease patients (cont.).

Study population and matrix	Lipidomics approach	Analytical platform	Potential biomarkers in case group compared with control group		Ref.
			Increased levels	Decreased levels	
Plasma and brain tissue: – Control (n = 12) – MCI (n = 12) – AD (n = 12)	Targeted	LC–UV	α-linolenate	Linoleic acid, oleic acid, ω-6 FAs, ω-3 FAs, DHA, palmitate, eicosapentaenoic acid (plasma); DHA (brain)	[118]
Frontal brain cortex: – Control (n = 20) – AD (n = 10)	Targeted	HPTLC	–	22:6n-3 PUFA, DHA, 18:1n-9 FA and oleic acid	[119]
Serum: – Control (n = 26) – MCI (n = 19)	Targeted	NMR	Cholesterol	ω-3 FAs, sphingomyelin and PC	[120]

AD: Alzheimer's disease; DAG: Diacylglycerol; DHA: Docosahexaenoic acid; DIMS: Direct-infusion mass spectrometry; DPG: Diphosphatidyl glycerol; EDI-MS: Electrospray droplet impact-mass spectrometry; FA: Fatty acid; FIA-MS/MS: Flow-injection analysis/tandem mass spectrometry; GalCer: Galactosylceramide; GC–TOF/MS: Gas chromatography–time-of-flight/mass spectrometry; HFA-Cer: Hydroxy fatty acids-ceramide; HNE: Hydroxynonenal; HPTLC: High-performance thin-layer chromatography; LC–MS: Liquid chromatography–mass spectrometry; LC–MS/MS: Liquid chromatography–tandem mass spectrometry; LPE: Lysophosphatidylethanolamine; LPPC: Lysopalmitoylphosphatidylcholine; LysoPC: Lysophosphatidylcholines; MCI: Mild cognitive impairment; MDMS–SL: Multi-dimensional mass spectrometry-based shotgun lipidomics; MS: Mass spectrometry; NMR: Nuclear magnetic resonance: PCs: Phosphatidylcholines; PlsE: Ethanolamine plasmalogen; PtdEtn: Phosphatidylethanolamine; PtdA: Phosphatidic acid phosphatase; PtdIns: Phosphatidylinositol; PUFA: Polyunsaturated fatty acids; SPH: Sphingomyelin; Trp: Tryptophan; VLCT: Very long chain triglycerides.

with changes in the phenotype [68]. Metabolomics offers several advantages in biomarkers discovery, including information on molecular mechanisms by monitoring dynamic changes inside biological systems; suitability for clinical application as well as translational medicine; and identification of novel molecular biomarkers and affected pathways, allowing for a better understanding of the pathological mechanisms [73].

The use of metabolomics has significantly increased in the recent years in AD research. Large-scale prospective studies, such as the Alzheimer's Disease Neuroimaging Initiative (ADNI), the Framingham study and the Rotterdam study, have strongly contributed to this phenomenon, showing their importance in supporting the identification of possible molecular changes related to AD pathology. These large-scale studies provide adequate experimental designs for metabolomics due to the number of subjects included in the cohorts. Moreover, since these studies also focus on a population before development of symptoms, they provide key biological insights that could lead to a targeted preclinical prevention. For instance, using the Framingham cohort, the investigation of 217 plasma metabolites levels in 2067 dementia-free participants using LC–MS demonstrated higher plasma levels of three amines (taurine, glutamic acid and anthranilic acid) and one purine (hypoxanthine), all associated with greater risks of developing dementia [6]. The Rotterdam study cohort was used to highlight 26 metabolites which showed different plasma levels in AD patients compared with control subjects [6]. Using network visualization, five main hub compounds (i.e., metabolites of high regulatory importance, located in the center of the network) were identified, including glycylglycine, lysophosphatic acid C18:2, tyrosine, glutamine and platelet-activating factor C16:0. Adding genetic information to these networks (i.e., *APOE* ε4 positive versus negative status) suggested alternative biochemical dysregulations depending on the genotype, also highlighting the usefulness of omics data integration. Finally, targeted and nontargeted metabolomics were used to analyze samples from the Alzheimer Disease Metabolomics Consortium (ADMC) in partnership with ADNI, mapping metabolic pathways to create a comprehensive biochemical database for AD. The latest results of ADNI1 cohort showed alterations in different metabolites depending on the AD stage. Alterations in the levels of ether-containing phosphatidylcholines and sphingomyelins were found in preclinical biomarker-defined AD stages, whereas levels of several amines and acylcarnitines changed in symptomatic stages [7,132].

It is worth mentioning that the availability and diversity of such cohorts is also very beneficial for replication studies. Indeed, it is now widely accepted that potential biomarkers candidates highlighted in the discovery phase in a specific cohort should be independently replicated in another cohort. Replication studies are crucial to ensure the validity of the results obtained during the discovery phase, which is essential in omics field due to multiple testing challenges [133].

Besides a better understanding of pathophysiological mechanisms of a disease, the potential of metabolomics also relies in identifying possible biomarker candidates which can predict drug response and evaluate the (off-target) effects of a drug on the metabolome. This was for instance highlighted using a transgenic mouse model of AD (APPswe/PS1ΔE9) and a novel drug candidate called CAD-31 [134]. Metabolomics analysis revealed that the major effect of CAD-31 is related to inflammation and lipid metabolism, with a shift in the metabolic profile of fatty

acids toward the production of ketone bodies, which are known to be neuroprotective and are used as a potent energy source for the brain when the glucose levels are low, such as in AD. Moreover, CAD-31 increased the levels of sphingolipids, which are also dysregulated in AD [134]. Administration of bile acids may also be an interesting therapeutic approach in AD. Indeed, some bile acids have anti-inflammatory and protective effects on the brain and show altered plasma levels in AD patients [135]. Novel therapeutic approaches based on bile acids are therefore very promising, as demonstrated by the effect produced by administration of tauroursodeoxycholic acid in APP/PS1 mice models after disease onset, which led to attenuation of Aβ deposition in the brain, and concomitant decrease of $A\beta_{40}$ and $A\beta_{42}$ levels [136].

Overall, these selected clinical examples show the relevance of metabolomics in AD research for a better understanding of the molecular mechanisms underlying the disease pathology, or for prediction of a drug response/off target effects.

Metabolomics in AD: from animal to human studies

This section presents and discusses the studies that have been carried out in animal models and humans using metabolomics and in different types of biosamples, including blood-derived material, CSF and post-mortem samples.

Animal studies

Several transgenic models are available to study AD which carry the mutations in *APP*, *PSEN1* or *PSEN2*, and Tau genes. Animal models allow for the investigation of not only peripheral fluids such as blood or urine, but also brain tissues. Moreover, longitudinal studies can also be carried out with animal models to track the disease progression, which remains much more challenging in humans. Metabolic pathways are evolutionarily relatively well conserved between mice and humans. Thus, the metabolic changes identified in animal research closely mimic changes in human patients, making the translation into human studies easier.

Table 2 lists the studies that have reported metabolic changes in animal models of AD. This table illustrates the large diversity of studies performed with AD animal models, using either NMR or MS-based techniques. Most of the studies have focused on brain tissues, typically hippocampus and frontal cortex, while few studies have been reported in urine or plasma samples. Multiple metabolites have shown to be altered versus control samples or along with the time, typically amino acids, some organic acids, fatty acids, lipids and neurotransmitters. Figure 1 summarizes the results obtained in animal models of AD using metabolomics-based approaches in brain tissue, plasma and urine, showing the altered pathways as well as possible pathophysiological consequences.

Human studies

Pre-mortem brain tissues being not available compared with mice models, most of the human studies focus on the analysis of blood-derived samples, CSF and post-mortem brain samples.

Table 3 lists all the studies performed in human using CSF samples using untargeted and targeted approaches. Despite requiring more invasive methods for sample collection compared with plasma, CSF represents an attractive study matrix in AD research due to the close connection with brain events. Changes in CSF levels of tau protein and $A\beta_{42}$ peptide are well-known, leading to the hypothesis that the CSF metabolome might also be altered upon MCI, dementia or AD. More than 400 metabolites have been already reported in healthy CSF [92], a number expected to grow with the large amount of metabolomics studies currently focused on CSF analysis. In the field of AD research, the number of metabolomics-based studies investigating CSF composition is also expected to grow, remaining currently relatively low as shown in Table 3. Supplementary Figure 1 illustrates the main results that have been found using LC–MS, GC–MS or CE–MS for the analysis of CSF samples.

Despite the presence of the BBB, peripheral effects of AD might also be captured beyond the CSF, in other words, in blood-derived samples. Potential biomarkers candidates in such matrix show the main advantages to be easily accessible. However, changes in the plasma metabolome are then difficult to interpret and to link with AD pathophysiology. Table 4 lists all the studies performed in human blood samples using metabolomics approaches, both targeted and untargeted. Numerous efforts have been made in the last years to discover potential biomarkers of AD in peripheral samples different from CSF, such as blood samples, as highlighted in Table 4. Figure 2 illustrates the main results that have been found using LC–MS, GC–MS or CE–MS for the analysis of blood-derived samples.

Finally, brain tissue represents the closest matrix to study the pathophysiology of AD. Post-mortem samples have indeed brought about the main theories underlying the origin of AD. The metabolite composition varies

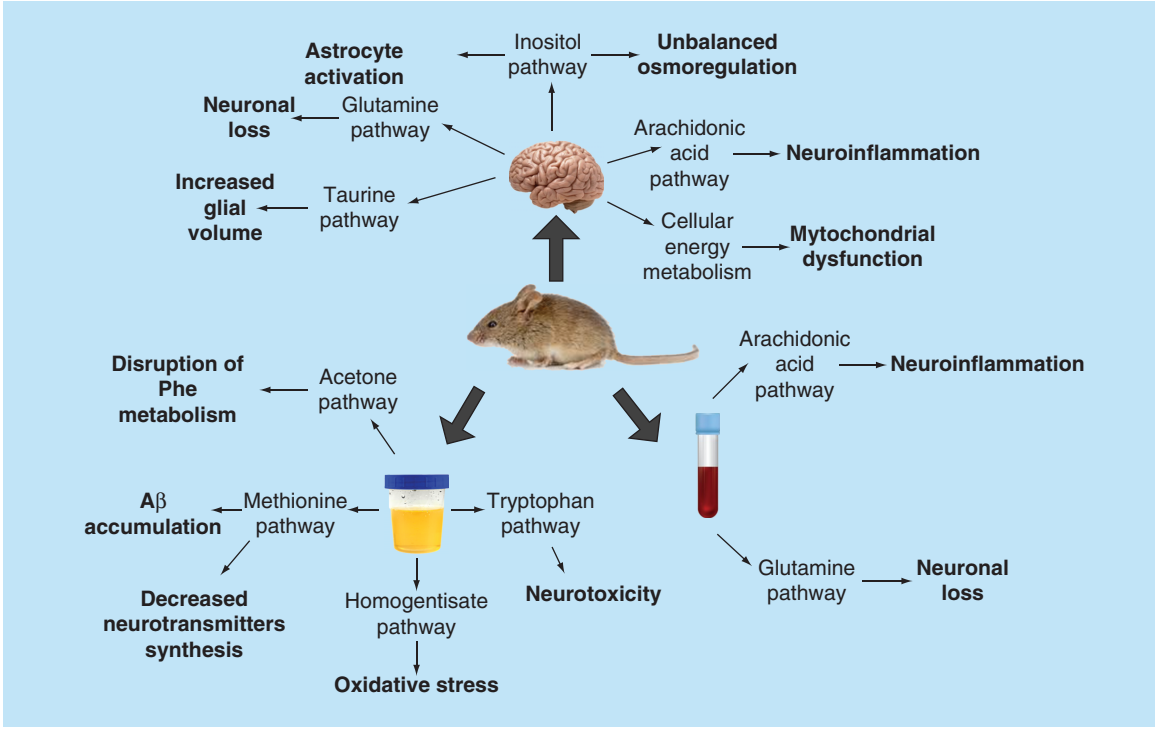

Figure 1. Main pathways altered in mouse models of Alzheimer's disease. Principal pathways altered in blood, urine and brain tissue of Alzheimer's disease mouse models and the biologic consequences of these alterations.

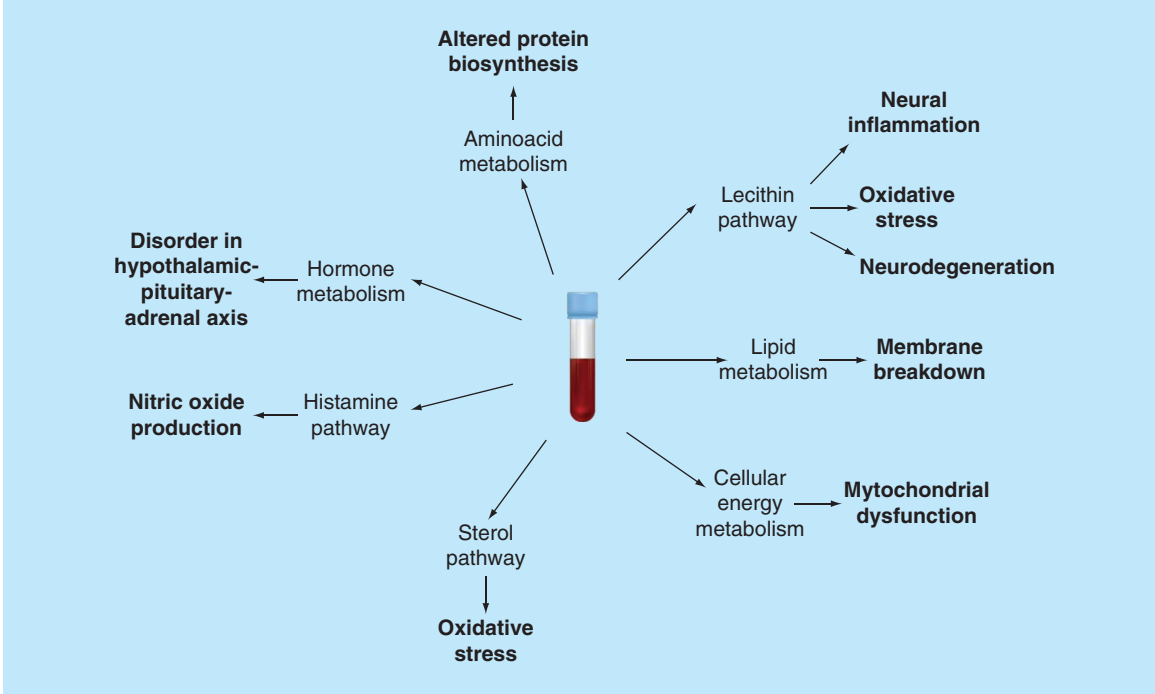

Figure 2. Main metabolic classes altered in the blood of Alzheimer's disease patients. Several metabolic classes are altered in blood-derived samples of Alzheimer's disease patients, highlighting different biological consequences (in bold).

extensively depending on the brain areas, highlighting the careful selection of the section of interest. The majority of metabolomics-based studies have therefore been performed on several brain sections within the same study. Table 5 list the studies and findings associated to post-mortem brain analysis. Supplementary Figure 2 illustrates the main results that have been found using LC–MS, GC–MS or CE–MS for the analysis of post-mortem tissue samples.

It is worth mentioning that besides metabolites, the latest research has been also focusing on even smaller compounds, in other words, metal ions (referred to as 'ionomics'). Indeed, metal ions are important as they act as essential cofactors for many proteins and play an important role in preserving neuronal function. An increasing body of literature suggests a critical role for metal ions in AD. On one hand, certain metal ions, such as zinc, copper and iron, have been found to be enriched in brain Aβ plaques, leading to a reduction in their bioavailability and thereby causing an imbalance in metal homeostasis. Metal ions can be neurotoxic and induce epigenetic changes, therefore aggravating the progression of AD. Moreover, alterations in metal ions concentrations in the brain can lead to increase of Aβ and tau aggregation along with an increase in APP processing [137]. Finally, iron dyshomeostasis is an early event in AD pathology. Impaired iron homeostasis causes formation of toxic oxidative species and increased production of Aβ proteins [138]. Supplementary Table 1 gives an overview of relevant studies that have focused on metal ions in the context of AD [139–142].

Next-generation biomarker discovery: the role of lipidomics

Lipidomics, in other words, the large-scale study of lipid species and networks in biological systems, has been capturing much more attention in the past few years. Lipids are not only the major constituents of cell membranes and critical to maintain the characteristics of membranes, such as mobility or fluidity, but they also play an essential role in cell signaling and multiple other physiological processes, including inflammation. Investigations on the structure and function of diverse lipid species may not only provide insights on the roles of lipids in numerous human diseases, but identify potential biomarkers for diagnosis and reveal the mechanisms underlying cellular lipid homeostasis [34]. Lipidomics has recently become a field of interest in the discovery of biomarkers for AD and numerous AD metabolic profiling studies of blood samples have been carried out using lipidomics-based approaches, focusing on different lipid classes and lipid mediators.

Table 6 lists the studies that have focused on lipid analysis in the context of AD research, using blood-derived or brain tissues samples. Multiple lipid classes have been investigated, either in a targeted or untargeted fashion, including free fatty acids, sphingolipids, glycerophospholipids, glycerolipids and sterol lipids. Desmosterol, a precursor of cholesterol, has gained a lot of attention in AD research due to the role of cholesterol and neurosteroids in brain pathology. It has been for instance reported that the local synthesis of cholesterol was decreased in specific brain areas due to decreased expression of the gene *DHCR24* which governs the conversion of desmosterol to cholesterol. The enzyme 24-dehydrocholesterol reductase (DHCR24) has also shown to counteract the β-secretase cleavage of APP and formation of Aβ [107]. Polyunsaturated fatty acids (PUFAs), such as docohexaenoic acid (DHA), linoleic acid and eicosapentaenoic acids (EPA) are also attracting attention due to their possible role in AD pathology. Together with arachidonic acid, DHA (C22:6), an omega-3 fatty acid, is the major PUFA component of brain lipids and is essential for normal brain development in early life, formation of neuronal synapses and membrane fluidity. DHA levels in blood have shown to be associated with higher general cognitive ability in a large-scale cross-sectional study (more than 20'000 individuals) [8]. High intakes of DHA and fish (oil) in AD prevention have also been investigated, since DHA plasma levels are increased by eating fat fish. Further research is needed since the literature reporting the effects of DHA supplementation in AD leads to different and sometimes inconsistent results [143]. Supplementary Figure 3 illustrates the main altered lipid classes that have been reported using lipidomics approaches, using post-mortem tissue and plasma samples.

Conclusion

Despite the multiple efforts carried out in the last decade in AD research, the pathophysiological mechanisms underlying the disease remain poorly understood. In this context, metabolomics-based approaches, in combination with other omics, are believed to expand this knowledge and allow for an earlier diagnosis of sAD as well as a better understanding of the biomolecular mechanisms, ultimately leading to the discovery of new therapeutic targets.

Due to its closeness to the brain, CSF remains the most studied body fluid in AD research. However, even though CSF is essential for a better understanding of the biomolecular mechanisms involved in the disease, its collection is invasive and prone to high risks. There is now a clear trend toward the investigation of other bio-fluids which

can be easily collected, such as blood or urine, where the disease progression could be tentatively captured at the metabolite level, allowing for large-scale screenings and monitoring of disease progression, and this at lower costs. Currently, only CSF and post-mortem brain tissues are used, since no blood-based biomarker has been shown to have sufficient predictive value to be used in clinical practice.

Future perspective

Metabolomics-based studies using large-scale prospective cohorts will probably help to gain a better understanding of the disease. Indeed, multiple findings reported over the last decades in a rather small population could not be replicated in larger cohorts. Indeed, metabolite levels are prone to relatively high intra- and interindividual variabilities, showing the importance of large-scale studies for the discovery of possible biomarker candidates. Such large cohorts also allow for replication of preliminary findings obtained in the discovery phase. Moreover, most of these cohorts also include collection of other omics information (genomics, proteomics, epigenomics) and/or brain imaging measurements. Integrating all omics approaches will certainly lead to the discovery of novel biomolecular pathways in AD pathophysiology. One essential requirement of such cohorts is the standardization of clinical and operating procedures. Indeed, different cohorts might not use the same criteria for the diagnosis of MCI or AD, and/or have different sample collection and pre-analytical procedures, which will hamper the comparison and replication of findings between cohorts.

A large diversity of metabolites has been reported to potentially play a role in AD pathology, belonging to multiple biochemical pathways. This strongly supports the new paradigm observed in metabolomics-based research, where the initial idea of finding one single specific metabolite is being replaced by the quest for larger metabolic biomarker profiles, encompassing dynamic interactions between metabolic pathways. Among the metabolites believed to play a crucial in AD pathophysiology, (signaling) lipids appear very promising. Lipids and signaling lipids have already shown to be involved in multiple (patho)physiological processes. Therefore, there is no doubt that lipidomics will be an essential part of future AD research to better understand the causes of the disease.

Executive summary

- Current clinical approaches for the diagnosis of Alzheimer's disease (AD) rely on proteomics and genomics-derived biomarkers, including cerebrospinal fluid Tau and $A\beta_{42}$ levels, as well as *APOE*, *PSEN1*, *PSEN2* and *APP*. Post-mortem analysis is used for definitive diagnosis of the disease.
- In recent years, metabolomics has become a promising tool to better understand the underlying mechanisms of AD and provide new strategies for the discovery of new biomarkers candidates.
- Several metabolomics-based studies have reported alterations in metabolite levels in different populations, for example, mild cognitive impairment and AD patients, showing the diversity of metabolites that might play a role in AD pathophysiology.
- Lipids are involved in several physiological processes; some (signaling) lipids may be putative and promising biomarker candidates for AD.

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