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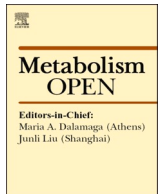
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## Characterising Alzheimer's disease through integrative NMR- and LC-MS-based metabolomics

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### ABSTRACT

**Background:** Alzheimer's Disease (AD) is a complex and multifactorial disease and novel approaches are needed to illuminate the underlying pathology. Metabolites comprise the end-product of genes, transcripts, and protein regulations and might reflect disease pathogenesis. Blood is a common biofluid used in metabolomics; however, since extracellular vesicles (EVs) hold cell-specific biological material and can cross the blood-brain barrier, their utilization as biological material warrants further investigation. We aimed to investigate blood- and EV-derived metabolites to add insights to the pathological mechanisms of AD.

**Methods:** Blood samples were collected from 10 AD and 10 Mild Cognitive Impairment (MCI) patients, and 10 healthy controls. EVs were enriched from plasma using 100,000×g, 1 h, 4 °C with a wash. Metabolites from serum and EVs were measured using liquid chromatography-mass spectrometry (LC-MS) and nuclear magnetic resonance (NMR) spectroscopy. Multivariate and univariate analyses were employed to identify altered metabolites in cognitively impaired individuals.

**Results:** While no significant EV-derived metabolites were found differentiating patients from healthy individuals, six serum metabolites were found important; valine ( $p = 0.001$ , fold change, FC = 0.8), histidine ( $p = 0.001$ , FC = 0.9), allopurinol riboside ( $p = 0.002$ , FC = 0.2), inosine ( $p = 0.002$ , FC = 0.3), 4-pyridoxic acid ( $p = 0.006$ , FC = 1.6), and guanosine ( $p = 0.004$ , FC = 0.3). Pathway analysis revealed branched-chain amino acids, purine and histidine metabolisms to be downregulated, and vitamin B6 metabolism upregulated in patients compared to controls.

**Conclusion:** Using a combination of LC-MS and NMR methodologies we identified several altered mechanisms possibly related to AD pathology. EVs require additional optimization prior to their possible utilization as a biological material for AD-related metabolomics studies.

**Abbreviations:** Aβ, Amyloid-β; ACE, Addenbrooke's cognitive examination; AD, Alzheimer's Disease; AUC, Area under the curve; BBB, Blood-brain barrier; BCAA, Branched-chain amino acid; CNS, Central nervous system; CSF, Cerebrospinal fluid; CV, Cross-validation; EVs, Extracellular vesicles; FAQ, Functional activities questionnaire; FDR, False discovery rate; MMSE, Mini-mental state examination; MCI, Mild cognitive impairment; PCA, Principal component analysis; p-tau, Phospho-tau; ROC, Receiver operating characteristics; sPLS-DA, Sparse partial least squared discriminant analysis; t-tau, Total-tau.

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## 1. Introduction

Alzheimer's Disease (AD) is a chronic neurodegenerative disease and comprises the largest part of dementia subtypes [1]. The amyloid hypothesis has for a long time been the main focus in AD research and the development of therapeutic interventions [2]. However, clinical trials are continuously failing [3], which has led researchers on new paths to uncover the complexity of this multifactorial disease [4]. Furthermore, with the current diagnostic methods, such as positron emission tomography and cerebrospinal fluid (CSF) proteins, some limitations hinder their use as first-line diagnostic or even screening tools. CSF provides for a biological fluid in close contact with the brain parenchyma, allowing identification of brain-related molecules in higher concentrations compared to that in peripheral blood samples. This biofluid may be useful for the analysis of brain metabolic alteration [5], but limitations associated with scanning methods and CSF sampling could be circumvented by blood-based biomarkers, as a minimally invasive diagnostic tool [6]. Blood is a versatile body fluid being in close connection with every organ, thus potentially reflecting their state [7]. The blood-brain barrier (BBB) becomes disrupted during AD pathogenesis, potentially allowing brain metabolites to be reflected in a blood sample [8].

With blood providing a complex matrix for biomarker investigations and AD being a multifactorial disease, integration of in-depth technologies and large data structures are needed. The term "systems biology" describes the understanding of the biological system as a whole, rather than paying attention to single factors in disease pathologies. This analytical power was realized by the omics-era [9]. Metabolomics is one of the newest fields in the omics family [9] being the study and exploration of all metabolites (<1,500 Da) in a cell, organ, or organism, and comprises lipids, amino acids, vitamins, peptides, and minerals, among others [10]. Since metabolites are the endpoints of genes, transcripts, and protein regulations, minor changes in the level of the upstream molecules can cause significant alterations in metabolites [11]. Not only disease progression can cause such changes, but also medication, nutrition, and environmental factors can affect metabolites [12]. Lastly, metabolic pathways are evolutionarily conserved across species, making them ideal targets for clinical studies [13].

The two most common methods for metabolomics studies are mass spectrometry (MS)-based metabolomics and nuclear magnetic resonance (NMR) spectroscopy [14]. Utilizing these two methods in combination can overcome several of their limitations, thus providing greater coverage of the metabolome, with MS-based metabolomics identifying low abundance metabolites and NMR identifying core metabolites in key metabolic pathways [12,13]. Interestingly, perturbations in metabolic pathways have been shown to be one of the first measurable changes to occur before manifestations of clinical symptoms [15]. Several studies have also examined metabolic alterations in AD, presenting different metabolic panels specific for AD [16–18].

Extracellular vesicles (EVs) are nano-sized particles, surrounded by a lipid-bilayer, and packed with active biomolecules such as proteins, lipids, and genetic material [19]. They are released by all cell types, including cells present within the central nervous system (CNS) [20], and hence, are important players in intercellular communication in physiological and pathological conditions. EVs can be identified in a wide variety of biofluids, including blood [19]. Several studies have investigated their role in AD in relation to the spreading [21] and clearing [22] of amyloid- $\beta$  (A $\beta$ ). In addition, EVs have been shown to be able to bypass the BBB through various suggested mechanisms [23]. These features of EVs as potential sources of biomarkers have included them in the term "liquid biopsies" [24]. With both metabolomics and EVs being relatively new fields, the combination of the two in search for biomarkers is therefore also scarce. Few studies have examined the metabolome of EVs, mostly focused on biomarkers for various cancer types [25]. To the knowledge of the authors, no study has yet explored EV-derived metabolites in AD.

Therefore, this study aimed to explore metabolic perturbations

related to pathological changes in AD, through the combined effort of MS- and NMR-based metabolomics approaches. Both serum and EV-derived metabolites were examined from patients with AD or Mild Cognitive Impairment (MCI) and compared to that of healthy controls. We identified several interesting metabolites distinguishing between diseased and healthy individual samples. Also, serum seemed to be a more suitable biological matrix for studying metabolic changes in AD; however, further optimization is needed for EVs in order to become a biological matrix of choice for future metabolomics studies.

## 2. Methods

### 2.1. Study participants

A total of 30 participants were enrolled for this study, distributed in three groups with 10 AD patients, 10 MCI patients, and 10 healthy controls. The patient groups were consecutively included from the Department of Neurology at Aalborg University Hospital. Study inclusion was at their time of diagnosis and blood samples were drawn prior to initiation of treatment. The diagnosis of mild to moderate AD patients was based on the International Classification of Diseases and Related Health Problems 10th Edition (ICD<sub>10</sub>) criteria [26] and the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [27]. For MCI patients, the diagnosis was made based on the Petersen criteria [28]. Paraclinical measurements of patients were performed when necessary for diagnostic certainty and included the Mini-Mental State Examination (MMSE), Addenbrooke's Cognitive Examination (ACE), Function Activities Questionnaire (FAQ), CSF A $\beta$ , CSF phospho-tau (p-tau), and CSF total-tau (t-tau).

For comparison with patient groups, age- and sex-related donors were recruited from the blood bank at Aalborg University Hospital. Donors were required to be of age 65 or older and complete a questionnaire regarding physical and mental health, stating information about i.e. fatigue, chest pain, and memory impairment. All included participants signed a consent form prior to study inclusion. The study was conducted in accordance with the Declaration of Helsinki and approved by the local North Denmark Region Committee on Health Research Ethics (N-20150010).

### 2.2. Sample collection and routine analyses

Collection and handling of blood samples were performed as previously described [29]. Briefly, using the median cubital vein as access point and a 21-gauge needle, blood samples (plasma and serum) were collected in 9 mL 0.105 M (3.2%) trisodium citrate (Vacuette, Greiner Bio-One, Austria) and 10 mL clot activator tubes (BD Vacutainer, UK). After blood collection, samples were subjected to double centrifugation at 2,500 $\times$ g for 15 min at room temperature to obtain either platelet-free plasma or serum. Plasma and serum were aspirated until 1 cm above the buffy coat or pellet. Aliquots of plasma and serum samples were snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further processing.

In addition, routine analyses were applied as previously described to ensure that study participants presented with no co-morbidities [29]. Briefly, measurements of normal system functioning and markers of organ function and damage were investigated by; alanine transaminase, albumin, carbamide, cholesterol, creatinine, C-reactive protein, glucose, high and low-density lipoprotein, haemoglobin, lactate dehydrogenase, and triglycerides.

### 2.3. Enrichment of extracellular vesicles

For MS, EVs were enriched from 1 mL plasma. A two-step centrifugation process at 100,000 $\times$ g for 1 h at  $4^{\circ}\text{C}$  was performed using an Avanti J-30i centrifuge together with a J A-30.50 fixed angle rotor, k-

factor 280 (Beckman Coulter, Brea, CA, USA). In-between centrifugations pellets were washed with 1 mL 0.22 µm filtered buffer (10 mM ammonium acetate in HPLC grade water). The resulting final EV pellets were resuspended in 100 µL of the same buffer. For NMR analysis, EVs were enriched from 1 mL plasma in triplicates for one sample per group. A two-step centrifugation process at 100,000×g for 1 h at 4 °C was performed using a LKB 2331 Ultraspinn 70 (LKB, Bromma, Sweden). In-between centrifugations pellets were washed with 1 mL 0.22 µm filtered phosphate-buffered saline. The resulting final EV pellets were resuspended in 150 µL of the same buffer, and due to the dilution of samples and relatively high metabolite concentration requested for NMR analysis (>1 µM), samples were pooled resulting in three samples in total, one EV isolate from each group. Furthermore, we have previously characterised pellets of EVs from the same enrichment, thus confirming their presence in our samples [30].

#### 2.4. Mass spectrometry analysis

For MS-based metabolomics analysis, both serum and EV samples were investigated. Samples were thawed on ice and four times volume extraction solvent was added, followed by vigorous vortexing. For serum, the extraction solvent comprised of methanol/acetonitrile/H<sub>2</sub>O (5:3:2), and for EVs methanol/acetonitrile (5:3) was used. Samples were then centrifuged at 16,000×g for 15 min at 4 °C. The supernatant was aspirated, lyophilized, and stored at -20 °C. Prior to liquid chromatography-mass spectrometry (LC-MS) analysis, samples were dissolved in 0.1% formic acid (30 µL) and centrifuged at 16,000×g for 5 minutes at room temperature.

The samples were analysed as in Dall et al. [31]. In brief, 5 µL was injected using a 400 µL/min and the following composition of eluent A (0.1% formic acid) and eluent B (0.1% formic acid, acetonitrile) solvents: 3% B from 0 to 1.5 min, 3%–40% B from 1.5 to 4.5 min, 40%–95% B from 4.5 to 7.5 min, 95% B from 7.5 to 10.1 min and 95%–3% B from 10.1 to 10.5 min before equilibration for 3.5 min with the initial conditions. The flow from the UPLC was coupled to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific) for mass spectrometric analysis in both positive and negative ion modes. The raw data were also processed as in Dall et al. [31] using MZmine (v 2.53) [32].

#### 2.5. Nuclear magnetic resonance spectroscopy analysis

NMR spectroscopy was used to investigate serum and EV-derived metabolites. Samples were thawed for 30 min at 4 °C, vortexed, and centrifuged at 12,100×g for 5 min at 4 °C using a multifuge 3 S-R centrifuge (Heraeus, Hanau, Germany). A total of 400 µL serum supernatant or EV isolate was mixed with 200 µL buffer (0.2 M NaPO<sub>4</sub>, 99% D<sub>2</sub>O, pH 7.4), as previously described [33,34]. Samples were aliquoted in 5 mm NMR tubes and kept on ice until analysed. A PULCON sample consisting of glucose and buffer was used as an internal standard.

A Bruker AVANCE 800 MHz NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) equipped with a cryogenically cooled, triple-resonance (<sup>1</sup>H, <sup>13</sup>C, <sup>15</sup>N) CPP-TCI probe, and operated at 298.1 K (25 °C) was used to record <sup>1</sup>H NMR spectra. T<sub>2</sub> filtered Carr-Purcell-Meiboom-Gill (CPMG) experiments with water presaturation were obtained using the following parameters; 65536 data points covering a spectral width of 20 ppm using 256 scans for serum and 128 scans for plasma (EV) samples, with 32 dummy scans, a fixed receiver gain of 203, and a relaxation delay (D1) of 4 s. Presaturation of the water resonance was achieved during D1 by continuous irradiation at γB<sub>1</sub>/2π = 25 Hz. T<sub>2</sub> filtering was then performed with a τ-180°-τ (τ = 300 µs) pulse sequence, which was repeated 256 times for 80 ms. The TopSpin 3.1 software (Bruker BioSpin, Rheinstetten, Germany) was used for spectral acquisition and processing, including enhancement of spectral resolution using artificial zero-filling by adding digital data points to the free induction decays, line broadening (0.3 Hz), Fourier transformation, phase and baseline correction, and calibration to the L-alanine methyl

**Table 1**

**Demographics and clinical data of study groups.** The presented values are shown as mean ± standard deviation. Abbreviations; Aβ: amyloid-β, ACE: Addenbrooke's Cognitive Examination, AD: Alzheimer's Disease, Con: healthy controls, CSF: cerebrospinal fluid, FAQ: Functional Activities Questionnaire, MCI: Mild Cognitive Impairment, MMSE: Mini-Mental State Examination, p-tau: phospho-tau, t-tau: total-tau. \*Ages 51–70. Interval <500 for ages 71–90.

	Con (n = 10)	MCI (n = 10)	AD (n = 10)	p-value	Reference interval
<b>Demographics</b>					
Female/male (n)	6/4	8/2	6/4	–	–
Age (years)	65 ± 0.5	72 ± 5	70 ± 5	0.005	–
<b>Cognitive performance</b>					
ACE	–	85.0 ± 5.6 (n = 6)	58.7 ± 16.5 (n = 3)	0.007	–
FAQ	–	4.0 ± 2.0 (n = 3)	10.4 ± 4.6 (n = 5)	0.066	–
MMSE	–	27.4 ± 2.3	23.6 ± 4.6	0.041	–
<b>Paraclinical measurements</b>					
CSF Aβ	–	998.5 ± 428.6 (n = 4)	626.3 ± 260.9 (n = 6)	0.148	> 500
CSF t-tau	–	563.0 ± 363.9 (n = 4)	628.2 ± 288.9 (n = 6)	0.760	< 61
CSF p-tau	–	98.0 ± 61.3 (n = 4)	80.5 ± 29.5 (n = 6)	0.556	< 450*

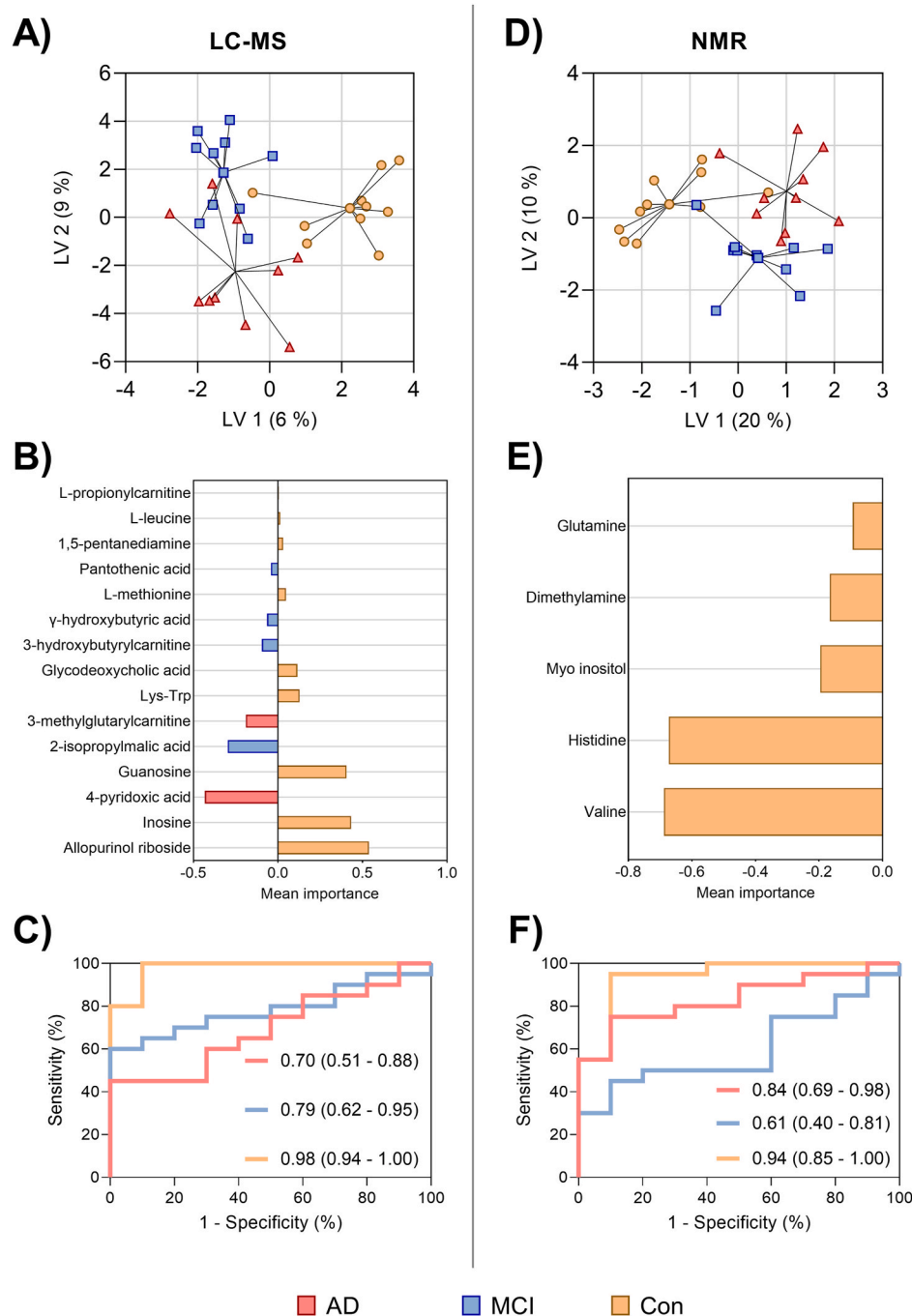
peak (1.48 ppm), as previously described [35]. Metabolite annotation was performed using 2D <sup>1</sup>H–<sup>1</sup>H total correlation spectroscopy and <sup>1</sup>H–<sup>13</sup>C heteronuclear single-quantum correlation spectra, the Human Metabolome Database (HMDB) [36], and literature [33,37,38], while quantification was based on integrating the sum of all points within a signal of interest, as previously described [38].

#### 2.6. Data analysis

For LC-MS, in serum and EV samples a total of 130 and 65 features were annotated at MS2 level, respectively and were subsequently corrected for signal drift using the *statTarget* R package [39]. For NMR spectroscopy a total of 38 metabolites were identified. Prior to multivariate analysis, data were generalized log-transformed and auto-scaled using MetaboAnalyst 5.0 (Xia Lab, Quebec, Canada) [40]. Supervised sparse-partial least squared discriminant analysis (sPLS-DA) was used to detect metabolites related to cognitive impairments. A five-fold cross-validation (CV) repeated 100 times was employed. Scores plots of sample grouping and loadings plots of selected metabolites are presented. Receiver operating characteristics (ROC) curves for group discrimination were created based on the CV adjusted models. The area under the curve (AUC) and 95% confidence intervals (CI) were used to report the sensitivity and specificity of the models. Multivariate analysis was performed using the *mixOmics* R package [41]. R script and files for sPLS-DA can be accessed in Supplementary Materials File S1 and Table S1 – S3.

Correlations were performed for metabolites identified by both MS and NMR to assess methods' compatibility using Pearson's ρ (Supplementary Material File Fig. S1). Data are presented as means ± standard deviations (SD). Group comparisons were performed using analysis of variance (ANOVA) with Tukey's honestly significant difference (HSD) post hoc test in IBM SPSS Statistics 27 (SPSS, Chicago, IL, USA). Benjamini-Hochberg false discovery rate (FDR) was applied for multiple correction. Fold changes (FC) were calculated for between-group comparisons. A p-value < 0.05 was considered statistically significant. GraphPad Prism 9.1.1 (GraphPad Software, La Jolla, CA, USA) was used for data visualization.

Network analysis was conducted using MetScape version 3.1.3 [42] for serum metabolites differentiating AD patients and healthy



**Fig. 1.** Metabolic signatures related to cognitive impairment through MS- and NMR-based approaches. Sparse-partial least squared discriminant analysis (sPLS-DA) models for serum metabolites, together with receiver operating characteristics (ROC) curves for each of the models. For LC-MS serum samples (A) scores plot, (B) loadings plot, and (C) ROC curves were shown. For NMR serum samples (D) scores plot, (E) loadings plot, and (F) ROC curves were shown. Each score represents a sample and the loadings represent the variation in a specific metabolite. The size of the bars indicates their importance for the sample grouping, and the color-coding indicate their importance for one of the groups. Based on the selected metabolites, the ROC curves compare their ability to distinguish the three study groups from each other for the data-sets. The area under the curve (AUC) and the respective 95% CI are presented for each of the ROC curves. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

individuals. Networks were build using KEGG IDs. Log<sub>2</sub> FC was used to indicate whether the affected metabolic pathways were altered in diseased compared to healthy individuals. Tables for MetScape analysis can be accessed in Supplementary Material File [Table S4](#).

### 3. Results

#### 3.1. Subject characteristics

The measured biochemical parameters, test results of cognitive performances, and paraclinical measurements have been presented in earlier studies by Nielsen et al. [29,30]. Briefly, most biochemical measurements were within standard reference intervals, however, with few individuals presenting elevated levels of LDL cholesterol and

triglycerides. A small but significant difference in age was found between healthy and diseased individuals ( $p = 0.005$ ). AD patients presented with significantly lower scores of cognitive testing based on MMSE ( $p = 0.04$ ) and ACE ( $p = 0.007$ ) tests, and higher scores on the FAQ test, compared to MCI patients. For paraclinical measurements of CSF markers, AD patients were observed to have slightly lower levels of A $\beta$  and p-tau and higher levels t-tau ([Table 1](#)).

#### 3.2. Metabolic signatures of cognitive impairments

For this study, serum samples were measured by both LC-MS and NMR, while EVs only by LC-MS due to samples being too diluted for NMR, resulting in few signals obtained on the NMR spectrum (Supplementary Material File [Fig. S2](#)).



**Table 2**

21 differentially altered metabolites in serum samples measured by mass spectrometry comparing all groups. Abbreviations; AD: Alzheimer's Disease, ANOVA: analysis of variance, Con: healthy controls, FC: fold change, FDR: false discovery rate, MCI: Mild Cognitive Impairment.

Metabolites	Mean $\pm$ SD [Intensities]			ANOVA	FDR	AD  Con		MCI  Con		AD  MCI	
	Con	MCI	AD			FC	p	FC	p	FC	p
1-Pentadecanoyl-sn-glycero-3-phosphocholine	970.2 $\pm$ 282.5	1249.8 $\pm$ 478.9	507.9 $\pm$ 210.0	0.0004	0.020	0.5	0.021	1.3	0.213	0.4	0.0003
Allopurinol riboside	1829.6 $\pm$ 1293.6	296.5 $\pm$ 193.8	444.4 $\pm$ 330.8	0.0004	0.020	0.2	0.002	0.2	0.001	1.5	0.915
Inosine	1626.3 $\pm$ 1014.0	404.7 $\pm$ 258.1	496.4 $\pm$ 361.9	0.0005	0.020	0.3	0.002	0.2	0.001	1.2	0.950
Guanosine	1809.1 $\pm$ 1069.5	401.2 $\pm$ 387.7	590.5 $\pm$ 556.7	0.0006	0.020	0.3	0.004	0.2	0.001	1.5	0.848
1-Palmitoyl-sn-glycero-3-phosphocholine	997.1 $\pm$ 246.8	1109.8 $\pm$ 283.7	625.4 $\pm$ 222.1	0.001	0.025	0.6	0.011	1.1	0.615	0.6	0.001
13-cis-Retinol	876.4 $\pm$ 207.2	1244.9 $\pm$ 467.0	590.3 $\pm$ 293.7	0.001	0.032	0.7	0.194	1.4	0.073	0.5	0.001
4-Pyridoxic acid	815.4 $\pm$ 119.9	1234.5 $\pm$ 309.8	1288.6 $\pm$ 393.5	0.004	0.074	1.6	0.006	1.5	0.016	1.0	0.921
1-Myristoyl-sn-glycero-3-phosphocholine	1170.2 $\pm$ 527.6	1198.8 $\pm$ 534.9	548.9 $\pm$ 271.9	0.009	0.139	0.5	0.021	1.0	0.991	0.5	0.016
2-Isopropylmalic acid	769.4 $\pm$ 209.3	1164.1 $\pm$ 319.1	1135.1 $\pm$ 311.0	0.011	0.147	1.5	0.029	1.5	0.017	1.0	0.974
Leu-Leu	842.5 $\pm$ 223.6	946.4 $\pm$ 319.7	1366.4 $\pm$ 488.3	0.011	0.147	1.6	0.013	1.1	0.816	1.4	0.051
7-Methylguanine	1021.0 $\pm$ 141.6	1040.4 $\pm$ 165.1	1308.5 $\pm$ 298.9	0.013	0.156	1.3	0.021	1.0	0.980	1.3	0.033
2-Phenylethanol,sulfate	724.1 $\pm$ 390.3	845.6 $\pm$ 503.7	1570.6 $\pm$ 882.6	0.017	0.164	2.2	0.021	1.2	0.912	1.9	0.054
1-Oleoyl-sn-glycero-3-phosphocholine	971.3 $\pm$ 289.9	1070.1 $\pm$ 270.7	674.4 $\pm$ 289.6	0.017	0.164	0.7	0.086	1.1	0.742	0.6	0.017
L-Lysine	1138.1 $\pm$ 312.3	795.2 $\pm$ 150.8	1085.9 $\pm$ 280.5	0.019	0.164	1.0	0.904	0.7	0.023	1.4	0.060
5-Androsten-3 $\beta$ ,17 $\beta$ -diol-3-sulfate	777.0 $\pm$ 490.1	696.2 $\pm$ 513.6	1478.9 $\pm$ 775.1	0.020	0.164	1.9	0.053	0.9	0.957	2.1	0.028
1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	892.4 $\pm$ 397.8	1094.5 $\pm$ 336.2	633.7 $\pm$ 217.4	0.020	0.164	0.7	0.230	1.2	0.399	0.6	0.015
9(10)-Epoxy-12Z-octadecenoic acid	694.8 $\pm$ 412.9	1342.5 $\pm$ 685.4	768.1 $\pm$ 379.0	0.023	0.179	1.1	0.950	1.9	0.032	0.6	0.061
3-Methylglutaryl carnitine	600.8 $\pm$ 190.9	995.2 $\pm$ 420.7	1214.7 $\pm$ 695.4	0.037	0.251	2.0	0.031	1.7	0.211	1.2	0.604
D-erythro-Sphingosine-1-phosphate	905.0 $\pm$ 247.9	1156.4 $\pm$ 454.3	732.4 $\pm$ 255.8	0.039	0.251	0.8	0.523	1.3	0.263	0.6	0.031
Glycodeoxycholic acid	1370.1 $\pm$ 869.5	837.6 $\pm$ 691.7	527.0 $\pm$ 314.6	0.039	0.251	0.4	0.032	0.6	0.226	0.6	0.590
Lys-Trp	827.9 $\pm$ 106.5	736.1 $\pm$ 47.4	774.3 $\pm$ 47.9	0.041	0.251	0.9	0.278	0.9	0.032	1.1	0.515

**Table 3**

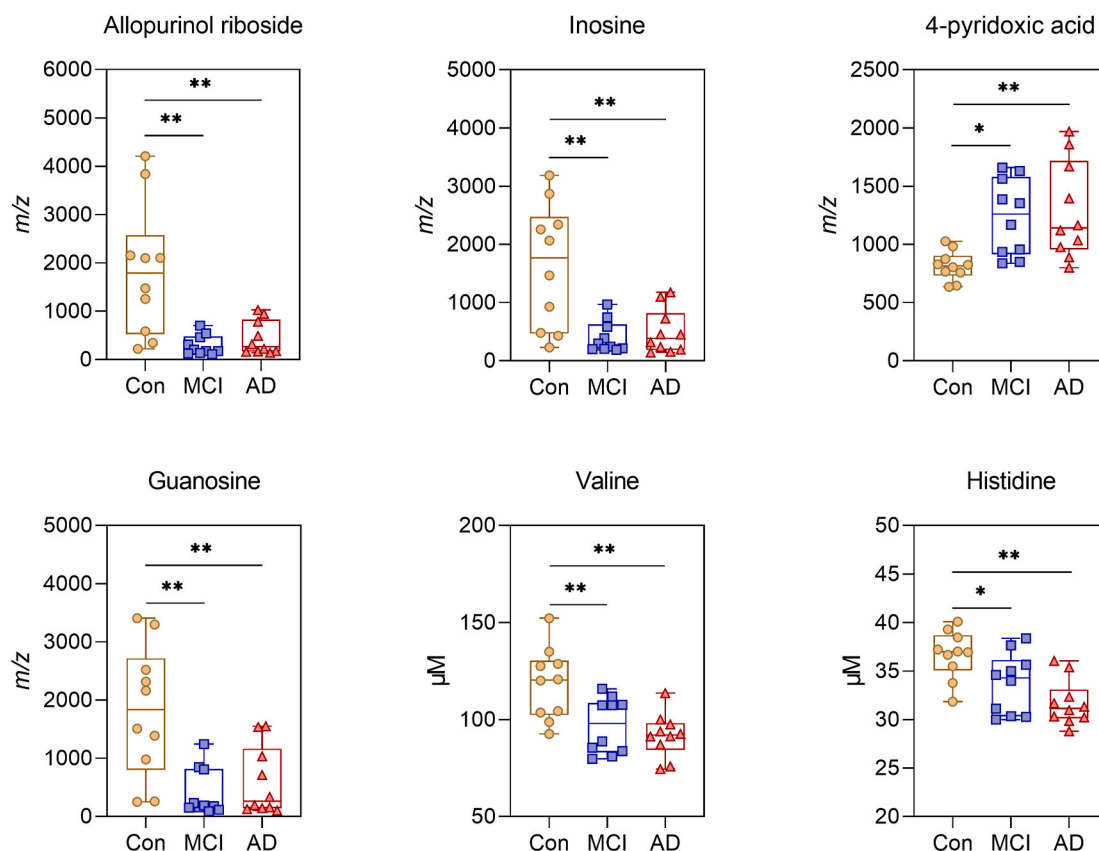
Seven altered metabolites in serum samples measured by nuclear magnetic resonance spectroscopy comparing all groups. Abbreviations; AD: Alzheimer's Disease, ANOVA: analysis of variance, Con: healthy controls, FC: fold change, FDR: false discovery rate, MCI: Mild Cognitive Impairment.

Metabolites	Mean $\pm$ SD [ $\mu$ M]			ANOVA	FDR	AD  Con		MCI  Con		AD  MCI	
	Con	MCI	AD			FC	p	FC	p	FC	p
Valine	118.4 $\pm$ 17.6	96.6 $\pm$ 13.5	91.8 $\pm$ 10.7	0.001	0.016	0.8	0.001	0.8	0.009	0.9	0.730
Histidine	36.7 $\pm$ 2.4	33.7 $\pm$ 3.0	31.7 $\pm$ 2.2	0.001	0.016	0.9	0.001	0.9	0.048	0.9	0.232
Formate	4.6 $\pm$ 0.9	3.0 $\pm$ 0.8	4.1 $\pm$ 0.8	0.001	0.016	0.9	0.415	0.7	0.001	1.4	0.026
Myo-inositol	40.2 $\pm$ 5.9	33.6 $\pm$ 3.8	34.6 $\pm$ 4.2	0.013	0.121	0.9	0.044	0.8	0.016	1.0	0.902
Glutamine	308.4 $\pm$ 33.3	277.0 $\pm$ 18.0	283.0 $\pm$ 14.9	0.020	0.154	0.9	0.075	0.9	0.023	1.0	0.852
Dimethylamine	5.6 $\pm$ 2.2	4.0 $\pm$ 1.3	3.6 $\pm$ 0.8	0.025	0.159	0.6	0.027	0.7	0.092	0.9	0.834
Isoleucine	44.8 $\pm$ 8.9	37.3 $\pm$ 7.0	36.5 $\pm$ 6.0	0.046	0.248	0.8	0.062	0.8	0.096	1.0	0.974

To facilitate the identification of possible metabolites associated with cognitive impairment sPLSDA was performed. A small overlap across the groupings could be observed for the serum samples measured by LC-MS and NMR (Fig. 1A – F). Among the 130 measured metabolites by LC-MS, 15 were found discriminating between groups by using 2 latent variables, with a classification error of 0.56 (Fig. 1A and B) and an AUC = 0.70 (95% CI = 0.51–0.88) for AD patients compared to MCI patients and controls, an AUC = 0.79 (95% CI = 0.62–0.95) for the MCI group compared to AD patients and controls, and an AUC = 0.98 (95% CI = 0.94–1.00) for healthy controls compared to patient groups (Fig. 1C). For NMR, five metabolites were found significantly

contributing to sample grouping (Fig. 1D and E), with a classification error rate of 0.50, and an AUC = 0.84 (95% CI = 0.69–0.98) for AD compared to the MCI and control groups, an AUC = 0.61 (95% CI = 0.40–0.81) for MCI compared to the AD and healthy individuals, and an AUC = 0.94 (95% CI = 0.85–1.00) for healthy controls compared to patient groups (Fig. 1F). A less distinct separation was observed for the EV samples with a greater classification error rate of 0.62 (Supplementary Material File Fig. S3), indicating lower accuracy of EV-based metabolites in distinguishing patients from healthy individuals.

Several serum metabolites were found to be significantly altered, with 6 of these being significant after FDR correction (Table 2) for LC-



**Fig. 2.** Important serum metabolites identified for discrimination of cognitively affected and healthy individuals. Boxplots representation with medians, interquartile ranges, and whiskers for minimum and maximum measurements. Significance is indicated by \*\*  $<0.01$  and \*  $<0.05$ . Allopurinol riboside, inosine, 4-pyridoxic acid, and guanosine were measured by LC-MS, while valine and histidine were measured by NMR.

MS analysis. For NMR, seven metabolites were significantly altered, with three of them being significant after FDR correction (Table 3). In contrast, EV samples only showed 5 significantly altered metabolites, and none of them being significant after FDR correction (Supplementary Material File Table S5). Fig. 2 depicts the most important serum metabolites found to differentiate between groups, including allopurinol riboside, inosine, 4-pyridoxic acid, guanosine, valine, and histidine.

### 3.3. Dysregulated pathway analysis related to cognitive impairment

To extrapolate metabolic changes possibly related to disease pathology, pathway analysis was performed (Fig. 3). 4-pyridoxic acid involved in vitamin B6 metabolism ( $\log_2 FC = 0.7$ ,  $p = 0.003$ ) was found to be the most elevated metabolite, while inosine ( $\log_2 FC = -1.7$ ,  $p = 0.006$ ) and guanosine ( $\log_2 FC = -1.6$ ,  $p = 0.007$ ), both from the purine metabolism were the most decreased metabolites. Other impaired pathways included histidine and branch-chained amino acids (BCAAs, valine, leucine, and isoleucine) metabolisms.

## 4. Discussion

In this study, we investigate serum and EV-derived metabolites possibly related to cognitive impairments, including MCI and AD. Using multivariate and univariate statistics, we identified altered metabolic signatures in serum able to differentiate cognitive affected from healthy individuals.

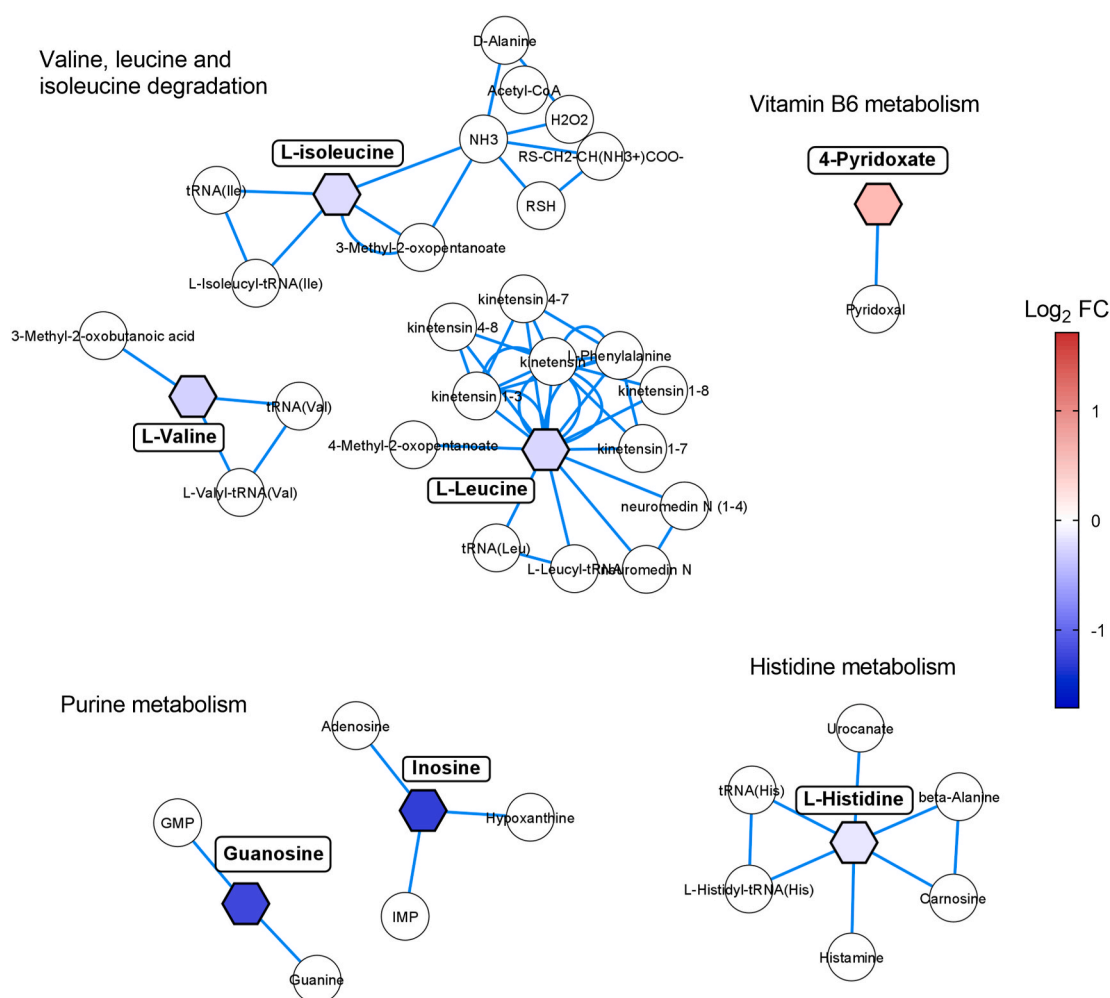
For serum samples, we investigated the metabolome using both LC-MS and NMR to obtain a broader coverage of the metabolome. Although LC-MS has a higher sensitivity and coverage (130 identified metabolites), the reproducibility and robustness of NMR present great clinical applicability [43] since the 38 identified metabolites could be

quantified ( $\mu M$  concentration).

Important serum metabolites revolved around BCAAs (valine, leucine, and isoleucine), purine (inosine and guanosine), and histidine metabolisms, which were found decreased, while 4-pyridoxic acid was increased in AD patients. BCAAs have previously been associated with increased risk for AD and dementia [44], hence, our study confirms previous findings. Valine is the most extensively studied of the BCAAs in relation to AD, and its levels were previously been found decreased in AD patients [45]. Also, valine levels showed correlation with the cognitive decline in patients [17]. A function of BCAAs is part of the glutamate metabolism [46]. Glutamine is converted to glutamate, acting as principal excitatory neurotransmitters in the CNS [47]. Decreased levels of BCAAs, as we observed, could affect this conversion of glutamine and glutamate, thereby decreasing neurotransmission. In line with this observation, we found lower levels of glutamine in the cognitively affected individuals, and a previous study has also reported decreased levels of glutamate in AD patients [48]. In addition, a study found a correlation between peripheral and CSF glutamine levels [49].

Guanosine possesses protective effects for neurons by i.e. modulating neurochemical processes, reducing oxidative stress, and regulate glutamate excitotoxicity and inflammation [50]. Inosine also provides beneficial effects on the CNS, by improving memory and learning, as well as providing anti-inflammatory effects [51]. We also found decreased levels of allopurinol riboside, a metabolite which inhibits the effects of purine nucleoside phosphorylase on inosine [52]. This could be a response to the already observed lower levels of the purines inosine and guanosine, thereby partially preventing the conversion of inosine to hypoxanthine and guanosine to guanine.

Histidine, a precursor to an important component in the immune response histamine, was found decreased in AD in our study. Histidine has been shown to possess multiple neuroprotective functions in relation



**Fig. 3.** Dysregulated metabolic pathways related to AD pathology. Hexagonal nodes indicate altered metabolites in the study and circular nodes represent metabolites involved in the pathway not identified in the study. Color codes represent the  $\log_2$  FC values, with red representing upregulated metabolites and blue representing downregulated metabolites. Mapped metabolites are based on KEGG IDs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to cerebral hypoperfusion, promoting neurogenesis and BBB integrity post disruption [53]. Furthermore, treatment with this amino acid reduced glial scarring and promoted migration of astrocytes towards the core, thus providing long-term neuroprotection [54]. Hence, our findings may indicate possible derangements in the overall immune response mechanisms.

Hyperhomocysteinaemia is a modifiable risk factor of AD and dementia. Studies have shown that dietary supplementation with B vitamins, such as folate, B12, and B6 have the ability to lower homocysteine levels, improve upon cognition, and decrease the progression of MCI and AD [55]. 4-pyridoxic acid is the catabolic product of vitamin B6 [56], and thus, our observations of an increased level in AD and MCI patients could be due to preventive measurements by the study participants taking these dietary supplements.

As part of their biogenesis, EVs are packed with molecular components from their parental cell, including metabolites at possibly sub-nanomole concentrations. Thus, even with the use of the more sensitive MS methodology, adequate detection levels might still be difficult to achieve [57]. This was evident in our study, both through a more limited number of metabolites measured by MS when compared to serum, but also when performing NMR analysis, since we were unable to recover adequate metabolite information not even after pooling of several samples per measurement. According to Gézi and co-workers [58], the detection limit, as well as an adequate volume for analysis complicates

the usage of EVs in omics studies. Despite our limited findings, few studies in cancer research have investigated the EV metabolome, confirming that EVs are able to modify the metabolome of the recipient cell [59]. Hence, several challenges remain to be resolved, including pre-analytical and analytical steps related to enrichment procedure and global consensus and standard operating procedures, before their usage in metabolomics can be harnessed [25,60]. Thus, in terms of usage for metabolic investigations, our study indicates serum as preferable compared to EVs.

Although our study showed promising indications of metabolic signatures in blood for AD, some limitations are to be mentioned. Firstly, our study population consisted of small cohorts of patients and healthy controls. However, even with small sample subset, clear differences between diseased and healthy individuals were observed. Secondly, although patient groups were verified clinically, not all patients had paraclinical measurements or neuropsychological tests, as it was deemed unnecessary for the diagnosis made by the physician. Thirdly, the healthy individuals were on average younger compared to AD and MCI patients, since recruitment of older blood donors was not feasible. The difference in age was found to be significant, however, this was possibly also due to the narrow age span in the control group, compared to that of patients, although their age spans overlapped. In addition, including CSF samples for disease characterisation using metabolomics alongside blood samples could possibly strengthen the identification of



CNS-related affected pathways reflected in the peripheral system, but CSF sampling was not available in most of the patients and controls. Lastly, characterising metabolic changes might provide targets for the implicated molecular processes, which in turn could aid in alleviating AD-related pathological processes such as neuro-inflammation and –transmission.

Our findings aided in the search for molecular signatures related to AD using untargeted metabolomics strategies, although, for such discovery-based approaches, further validations are needed to confirm these candidates in larger independent cohorts.

## 5. Conclusions

In this study, we identified several serum metabolite alterations in AD and MCI patients related to BCAAs, purine, histidine, and 4-pyridoxate metabolisms. Also, serum provides a more suitable matrix for investigating metabolic alterations in relation to AD pathology compared to that of EVs, however, additional optimization is needed for EVs to confirm this finding.

## Data availability

Data is available upon request.

## CRediT authorship contribution statement

**Jonas Ellegaard Nielsen:** Conceptualization, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Raluca Georgiana Maltesen:** Formal analysis, Investigation, Resources, Data curation, Writing – review & editing. **Jesper F. Havelund:** Formal analysis, Investigation, Resources, Data curation, Writing – review & editing. **Nils J. Færgeman:** Resources, Writing – review & editing. **Charlotte Held Gotfredsen:** Formal analysis, Investigation, Data curation, Writing – review & editing. **Karsten Vestergård:** Resources, Writing – review & editing. All authors have read and agreed to the published version of the manuscript. **Søren Risom Kristensen:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration. **Shona Pedersen:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.metop.2021.100125>.

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