Targeted Metabolomic Analysis in Alzheimer’s Disease Plasma and Brain Tissue in Non-Hispanic Whites

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Abstract.
Background: Metabolites are biological compounds reflecting the functional activity of organs and tissues. Understanding metabolic changes in Alzheimer’s disease (AD) can provide insight into potential risk factors in this multifactorial disease and suggest new intervention strategies or improve non-invasive diagnosis.

Objective: In this study, we searched for changes in AD metabolism in plasma and frontal brain cortex tissue samples and evaluated the performance of plasma measurements as biomarkers.

Methods: This is a case-control study with two tissue cohorts: 158 plasma samples (94 AD, 64 controls; Texas Alzheimer’s Research and Care Consortium – TARCC) and 71 postmortem cortex samples (35 AD, 36 controls; Banner Sun Health Research Institute brain bank). We performed targeted mass spectrometry analysis of 630 compounds (106 small molecules: UHPLC-MS/MS, 524 lipids: FIA-MS/MS) and 232 calculated metabolic indicators with a metabolomic kit (Biocrates MxP® Quant 500).

Results: We discovered disturbances (FDR ≤ 0.05) in multiple metabolic pathways in AD in both cohorts including microbiome-related metabolites with pro-toxic changes, methylhistidine metabolism, polyamines, corticosteroids, omega-3 fatty acids, acylcarnitines, ceramides, and diglycerides. In AD, plasma reveals elevated triglycerides, and cortex shows altered amino acid metabolism. A cross-validated diagnostic prediction model from plasma achieves AUC = 82% (CI95 = 75–88%); for females specifically, AUC = 88% (CI95 = 80–95%). A reduced model using 20 features achieves AUC = 79% (CI95 = 71–85%); for females AUC = 84% (CI95 = 74–92%).

Conclusion: Our findings support the involvement of gut environment in AD and encourage targeting multiple metabolic areas in the design of intervention strategies, including microbiome composition, hormonal balance, nutrients, and muscle homeostasis.

Keywords: Alzheimer’s disease, antioxidants, bacterial toxins, biomarkers, human microbiome, hyperlipidemia, lipidomics, metabolic pathways, metabolomics, polyamines

INTRODUCTION

Every year, over 6 million people worldwide develop Alzheimer’s disease (AD) [1]. AD is a complex disorder, multifactorial in its origin. There are known genetic, environmental, and sociodemographic factors and risks for AD linked to other
diseases [2]. Underlying mechanisms eventually lead to accumulation of amyloid plaques, neurofibrillary tangles, and neuronal death [3]. When exactly neuronal homeostasis becomes pathological is unclear.

Following the success of genomic studies, other omic techniques applied to AD have shed light on disease mechanisms that can potentially identify biomarkers for early detection [4]. One of them is metabolomics, which allows the quantitative analysis of physiological metabolites in numerous classes of compounds present in biofluids and tissues. Metabolites are the high-level mediators of biochemical processes in the human body and are of important diagnostic value.

Broad metabolomic studies in AD are limited, generally lacking validation and sometimes yielding contradictory results [5]. To date, no metabolomic model in AD has reached sufficient performance to serve as a reliable clinical diagnostic biomarker. Replication efforts are hindered by differences in assay coverages and participant characteristics including AD stage. Among the larger studies, Varma et al. [6] used a targeted assay and compared two serum cohorts and one brain cohort in terms of metabolite association with multiple AD pathology related scores and produced a list of 25 metabolites ranked by the number of associated scores. Proitsi et al. [7] performed untargeted analysis and list several compounds different in AD, only two of which are identified by chemical name. Barupal et al. [8] found 15 metabolites different in AD without considering covariates. Recently, Lim et al. [9] recorded 152 metabolites different in AD, but included several summary lipid measures as covariates, effectively comparing relative proportions of individual lipid species rather than absolute values. Studies rarely consider such confounding factors as comorbidities, medication, and sample handling. Protection against violations of model assumptions is often neglected, e.g., silently assuming homoscedasticity, which can lead to incorrect results.

In this case-control study, we performed a comprehensive targeted metabolomic analysis in plasma and in tissue from the frontal cortex to identify metabolic changes characteristic of AD, which could extend our understanding of the disease and potentially serve as a diagnostic biomarker or target for intervention. The analysis covers most important metabolic pathways. Targeted analysis generally offers higher accuracy compared to untargeted approaches and a more reliable identification of the compounds. We included a range of possible confounders and performed a statistically robust analysis with a series of regression models and explored possible plasma biomarkers with a cross-validated diagnostic prediction model.

METHODS

Participants and samples

Plasma samples (200 μl) were obtained from 94 AD cases and 64 control subjects, which were part of a longitudinal study conducted by the Texas Alzheimer’s Research and Care Consortium (TARCC) [10]. The study was performed in 2005–2018 with participants, older than 55 years, recruited at dementia clinics of the TARCC member institutions. Controls included volunteers and family members (although we do not have access to the familial pairing). We selected a large subset with the following characteristics: consistent diagnosis during subsequent follow-up visits with at least 3 annual follow-ups, no other major disorder of central nervous system (CNS), equal sex representation, and controls with matched distributions of age, sex, and apolipoprotein E (APOE) ε4 allele carriers. The diagnosis follows a clinical examination with a neuropsychological battery using NINCDS-ADRDA criteria [11], with our AD cases classified as “probable AD”, while controls showed no cognitive impairment and achieved a zero on the Clinical Dementia Rating (CDR) scale [12]. The selected samples were collected in 2007–2014 at 4 sites: Baylor College of Medicine (Houston, TX), University of Texas Southwestern Medical Center (Dallas, TX), Texas Tech University Health Sciences Center (Lubbock, TX), and University of North Texas Health Science Center (Fort Worth, TX). All 4 centers followed a harmonized collection protocol and we adjusted for a possible site-related effect in the statistical analysis.

Postmortem frontal cortex samples (500 mg) from 35 AD cases and 36 controls, collected in 2004–2018 and continuously stored at −80°C, were obtained from the Banner Sun Health Research Institute brain bank [13] (Sun City, AZ). The diagnosis followed histopathological examination and NIA-Reagan classification [14], with the AD cases classified as “high likelihood of AD”. Controls had no history of clinical diagnosis of cognitive impairment. The participants had no other major CNS pathology.

We excluded several non-White or Hispanic subjects because there were not enough observations to reliably estimate the effect of other races or
ethnicities. Presence of such effects is plausible due to cultural differences in diet and lifestyle as well as genetic variations and not accounting for them would negatively impact statistical power of the analysis. Additional details on sample filtration are provided in the Supplementary Methods.

We also accessed and analyzed available data on non-CNS diagnoses and current medication use as listed in the Supplementary Methods. For the cortex cohort, these data were updated during pre-mortem visits.

**Chromatography and mass spectrometry**

Targeted metabolomic analysis was based on triple quadrupole ultra-high-performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using Shimadzu Nexera chromatography platform (Shimadzu Corporation, Kyoto, Japan) coupled to Sciex QTrap 5500 mass spectrometer (AB Sciex LLC, Framingham, MA, USA). We applied the Biocrates MxP® Quant 500 targeted kit (Biocrates Life Sciences AG, Innsbruck, Austria), potentially quantitating 106 small molecules in chromatography mode and 524 lipids in flow-injection mode (FIA-MS/MS), covering the most important metabolic pathways. Annotations for the individual metabolites with identifiers to external databases are provided in Supplementary File 1. Additionally, 232 metabolites with identifiers to external databases are metabolic pathways. Annotations for the individual metabolites with identifiers to external databases are provided in Supplementary File 1. Additionally, 232 metabolic indicators were calculated from sums or ratios of relevant metabolites according to Biocrates MetaboINDICATOR™ formulas [15]. We refer to the whole set of metabolites and metabolic indicators as analytes. The indicators can be regarded as physiologically-relevant derived measures and are statistically analyzed separately from metabolites. The indicators denoted as “X synthesis” are computed as a ratio of metabolite X and its main precursors in attempt to reflect the conversion ratio. Since there are multiple explanations why such an indicator could be altered, the interpretation needs to be done cautiously in context of the individual metabolites.

Brain cortex samples were extracted with 85% ethanol in phosphate buffered saline at concentration 3 µl/mg, homogenized with sonicator, and centrifuged for 20 minutes. The clear extract (30 µl) was transferred onto a kit plate (with pre-injected internal standards) and dried down. For plasma samples, we added 10 µl directly to the plate. In brief, the rest of the assay includes derivatization with 5% phenylisothiocyanate in pyridine, ethanol and water (1:1:1), and subsequent extraction with 5 mM ammonium acetate in methanol. Chromatography was done with 0.2% formic acid in acetonitrile (organic mobile phase) and 0.2% formic acid in water (inorganic mobile phase). Flow-injection analysis was performed with methanol and Biocrates MxP® Quat 500 proprietary additive of undisclosed composition. As proprietary knowledge, Biocrates prefers to keep mass transitions of individual metabolites undisclosed.

Sample handling was done on dry ice to avoid multiple freeze-thaw cycles. We randomized the samples across plates already prior to their processing to avoid any accidental bias toward one of the diagnostic groups. Plates included blanks to calculate limits of detection and repeats of a quality control sample to monitor the coefficient of variation (presented in the Limitations section in Discussion; also see Supplementary Methods for more details).

**Data preprocessing**

Chromatographic peaks were identified and integrated in Biocrates MetIDQ™ Oxygen-DB110-3005 and manually reviewed. Areas of metabolite peaks were divided by areas of their respective internal standards. Further processing was done in R v3.6.1 [16] with RStudio [17] and details are provided in the Supplementary Methods. Briefly, we performed median scaling plate normalization to account for possible batch effects, calculated limits of detection from blanks to filter out low-abundant metabolites with poor detection, transformed the data via Box-Cox transformation through R package car [18] to better approximate normal distributions, and adjusted outliers via Tukey’s fencing [19] to avoid skewing the means by extreme values. The values were then standardized with respect to control samples to facilitate comparison of regression coefficients in the statistical analysis. We imputed several cases of missing sociodemographic values (BMI, education) by manual review of other visits (plasma cohort) or conditioned on diagnosis group and sex (cortex cohort).

**Statistical methods**

The statistical analysis was done in R v3.6.1 [16] with RStudio [17] and package ggplot2 [20] for visualization. We provide more details in the Supplementary Methods. Briefly, differential analysis was performed with a series of multivariable bootstrapped de-sparsified L1-regularized linear regression models via R package hdi [21], checked for collinearity with
Here, postmortem interval. A group comparison was performed with Welch’s t-test [22] and adjusted for heteroscedasticity when suspected with Breusch-Pagan test [22] (R package lmtest [23]). This type of model has been shown suitable for accurate high-dimensional inference with many covariates, estimating reliable confidence intervals thanks to de-sparsification and bootstrapping [24]. The covariates include sociodemographic attributes, disorders, medication, and supplementation as well as sample collection and handling attributes. The unit of regression coefficients is 1 standard deviation (SD) of the distribution of controls. False discovery rate (FDR) is controlled from raw values to final tables) as Supplementary Dataset files (separately for both cohorts and for metabolites and metabolic indicators). The largest differences are in Mini-Mental State Examination (MMSE) score [32] (p < 0.001), with mild-moderate impairment (mean = 23, SD = 4) in the plasma cohort and AD and mild-severe impairment (mean = 15, SD = 8) in the cortex cohort AD (based on MMSE to dementia severity mapping [33]). Education of AD participants in the plasma cohort is slightly lower compared to controls (–1.3 y, p = 0.002). Postmortem intervals are short, close to 3 hours on average, with no difference between the groups. Duration of storage in the freezer since the collection is somewhat higher for the control cortex samples, as we had to go further in time and obtain “older” samples to get an adequate number of controls. Nevertheless, it does not pose an issue: Any tissue degradation would be reflected in higher levels of choline and amino acids, but the observed values do not conform to this pattern. Choline levels of longer stored control samples are indistinguishable from control samples with shorter freezer storage (Welch’s t-test p = 0.92 for upper versus lower tertile). We also control for a potential freezer storage effect in the regression alongside other covariates.

Brief association analysis further shows a link between AD and diagnosis of depression (OR = 4.1, 95% confidence interval (CI95) = 1.4–14.5, FDR = 0.044), hypercholesterolemia (OR = 2.3, CI95 = 1.2–4.5, FDR = 0.044), and hypertension (OR = 2.2, CI95 = 1.2–4.2, FDR = 0.044), in the plasma cohort, and between AD and cerebral amyloid angiopathy (OR = 33, CI95 = 9.1–208, FDR < 0.001), APOE e4 allele (OR = 5.6, CI95 = 2.5–13, FDR < 0.001),

| Vehicle | AD (n = 94) | Controls (n = 64) | p^a |
|---------|-------------|------------------|-----|
| Sex, male, No. (%) | 47 (50%) | 32 (50%) | 1 |
| Age, y, mean (SD) | 71 (8) | 71 (8) | 0.78 |
| Race | All non-Hispanic White | 1 |
| Education, y, mean (SD) | 15 (3) | 16 (3) | 0.002 |
| BMI, kg/m², mean (SD) | 27 (4) | 28 (5) | 1.000 |
| APOE, e4 carrier, No. (%) | 43 (46%) | 27 (42%) | 0.74 |
| e4/e4 carrier, No. (%) | 6 (6%) | 2 (3%) | 0.47 |
| MMSE, mean (SD) | 23 (4) | 29 (1) | <0.001 |
| Fasting, h, mean (SD) | 5 (4) | 4 (3) | 0.09 |
| PMI, h, mean (SD) | NA^c | NA^c | 3.4 (0.8) |
| Storage, y, mean (SD) | 10 (2) | 10 (2) | 0.56 |

Table 1. Participant Demographics and Sample Characteristics

AD, Alzheimer’s disease; APOE, apolipoprotein E; BMI, body mass index; MMSE, Mini-Mental State Examination; NA, not available; PMI, postmortem interval. Group comparison was performed with Welch’s t-test (continuous variables) and Fisher’s exact test (count variables).

^a Here, e4/e4 carriers are a subgroup of e4 carriers. ^b Fasting status is unknown for deceased donors. Postmortem interval is not applicable for living plasma donors.

RESULTS

Participant characteristics are summarized in Table 1. We also present all metadata and data tables (from raw values to final tables) as Supplementary Dataset files (separately for both cohorts and for metabolites and metabolic indicators). The largest differences are in Mini-Mental State Examination (MMSE) score [32] (p < 0.001), with mild-moderate impairment (mean = 23, SD = 4) in the plasma cohort and AD and mild-severe impairment (mean = 15, SD = 8) in the cortex cohort AD (based on MMSE to dementia severity mapping [33]). Education of AD participants in the plasma cohort is slightly lower compared to controls (–1.3 y, p = 0.002). Postmortem intervals are short, close to 3 hours on average, with no difference between the groups. Duration of storage in the freezer since the collection is somewhat higher for the control cortex samples, as we had to go further in time and obtain “older” samples to get an adequate number of controls. Nevertheless, it does not pose an issue: Any tissue degradation would be reflected in higher levels of choline and amino acids, but the observed values do not conform to this pattern. Choline levels of longer stored control samples are indistinguishable from control samples with shorter freezer storage (Welch’s t-test p = 0.92 for upper versus lower tertile). We also control for a potential freezer storage effect in the regression alongside other covariates.
and cerebral white matter rarefaction (OR = 4.4, CI95 = 1.9–11, FDR = 0.010) in the cortex cohort.

**Metabolomic analysis in plasma**

**Differentially altered metabolites and metabolic indicators**

We found 353 metabolites (including lipid species) and 26 metabolic indicators altered in AD plasma. Among small molecules (Table 2), the most intriguing findings are changes related to microbial activity. There is increased 5-aminovaleric acid (+0.72, CI95 = 0.36–1.09, FDR = 0.001), para-cresol sulfate (+0.35, CI95 = 0.02–0.70, FDR = 0.017) and trimethylamine N-oxide (+0.45, CI95 = 0.00–0.87, FDR = 0.020). Indole derivatives are shifted towards more abundant indoxyl sulfate (+0.49, CI95 = 0.18–0.82, FDR = 0.001) and 3-indoleacetic acid (+0.36, CI95 = 0.02–0.70, FDR = 0.013), whereas 3-indolepropionic acid is decreased (–0.35, CI95 = –0.67–0.05, FDR = 0.012). Bile acid profile is altered by decreased cholic acid (–0.32, CI95 = –0.71–0.01, FDR = 0.023) and its increased 7α-dehydroxylation (+0.53, CI95 = 0.13–0.92, FDR = 0.037).

Further, methylhistidine metabolism (methylhistidines, carnosine, β-alanine) is upregulated. Polyamine metabolism is disrupted with increased spermine. Steroids cortisol and cortisone are elevated. Levels of omega-3 fatty acids (docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)) are decreased. Several carboxylic acids are elevated: 3-hydroxyglutaric acid, succinic acid, and aconitic acid. Betaine synthesis (ratio betaine/choline) is decreased. There is a mild increase in amino acids, with alanine, arginine, threonine, and valine reaching a statistically significant FDR level. Volcano plot (Fig. 1A) shows the most significantly altered small molecules in plasma: 5-aminovaleric acid, carnosine, indoxyl sulfate, β-alanine, DHA, EPA, 3-indoleacetic acid, 3-indolepropionic acid, and trans-4-hydroxyproline.

In lipid metabolism (Table 3; individual lipid species are listed in Supplementary Table 1), we see a general elevation in multiple lipid classes: Several saturated acyl carnitines are elevated (3:0, 8:0, 10:0, 18:0). Phosphatidylcholines are increased, most significantly polyunsaturated and diacyl phosphatidylcholines. Lyso phosphatidylcholines are increased regardless of saturation or size, possibly indicating higher activity of phospholipase A2. Ceramides are increased, including some glycosylceramides. Unsaturated triglycerides, diglycerides, and several cholesterol species are also elevated. Volcano plot of lipid species (Fig. 1B) captures the upregulated lipid metabolism. See Supplementary Figure 1 for visualization of AD effects across lipid classes and Supplementary Figure 2 for illustrative boxplots of several of the altered analytes.

Additionally, we report on observed sex-specific metabolic changes (Supplementary Table 2): Compared to females, males have higher levels of several amino acids (only glycine is lower), increased tryptophan metabolism including serotonin, muscle-related metabolites, spermidine, betaine as well as dehydroepiandrosterone sulfate (DHEAS), but lower EPA. Among lipids, males have consistently lower ceramides, glycosylceramides, sphingomyelins, hydroxysphingomyelins, cholesteryl esters, phosphatidylcholines, lysophosphatidylcholines, and higher diglycerides and triglycerides.

**Differentially altered pathways**

Pathway analysis identified several KEGG pathways (Table 4) dysregulated in AD: steroid biosynthesis, branched-chain amino acid biosynthesis, 3 pathways related to omega-6 and omega-3 fatty acids, metabolism of glycerolipids and glycerophospholipids, and metabolism of sphingolipids. The custom set of metabolites linked to microbial activity is also significant.

These results are repeated with SMPDB pathways (Table 5), which provide rather better coverage, and extend the set of significant results by several pathways related to methylhistidine metabolism (histidine metabolism, β-alanine metabolism, carnosine synthesis), homocysteine metabolism (betaine metabolism, glycine, and serine metabolism), spermidine and spermine biosynthesis, and multiple pathways related to biosynthesis of triglycerides and phosphatidylethanolamines. Note that both KEGG and SMPDB pathway databases are incomplete and do not contain majority of lipid pathways.

**Diagnostic prediction model**

Using all analytes and basic sociodemographic data (sex, age, education, BMI, APOE ε4), the cross-validated model achieves AUC 82% (CI95 = 75–88%), significantly more (p < 0.001) than a model with the basic sociodemographic data alone (AUC 58%, CI95 = 48–67%), which in turn is not significantly better than a random model (AUC 54%, CI95 = 45–63%) as expected (since the controls have matched distributions of sex, age, and APOE ε4). Interestingly, the model seems to
Table 2
Regression Coefficients of Small Molecules and Indicators Altered in AD Plasma or Frontal Cortex

| Metabolite or indicator                          | Plasma    |          |       | Frontal cortex |          |       |
|------------------------------------------------|-----------|----------|-------|----------------|----------|-------|
|                                                  | Effecta   | CI95     | FDRb  | Effecta        | CI95     | FDRb  |
| Microbiome-related metabolites                   |           |          |       |                |          |       |
| 5-Aminovaleric acid                              | 72%       | (36% – 109%) | 0.001 | 61%            | (23% – 101%) | 0.015 |
| para-Cresol sulfate                              | 35%       | (2% – 70%) | 0.017 | 50%            | (3% – 95%) | 0.07  |
| Trimethylamine N-oxide                           | 45%       | (0% – 87%) | 0.020 | 9%             | (–29% – 51%) | 0.43  |
| 3-Indoleacetic acid                              | 36%       | (2% – 70%) | 0.013 | 34%            | (–17% – 79%) | 0.16  |
| 3-Indolepropionic acid                           | –35%      | (–67% – –5%) | 0.012 | NAc            | NAc      | NAc   |
| Indoxyl sulfate                                  | 49%       | (18% – 82%) | 0.001 | 58%            | (14% – 100%) | 0.027 |
| Sum of indoles                                   | 48%       | (16% – 82%) | 0.009 | 55%            | (16% – 100%) | 0.020 |
| Cholic acid                                      | –32%      | (–71% – 1%) | 0.023 | –1%            | (–41% – 37%) | 0.54  |
| Deoxycholic acid                                 | 28%       | (–6% – 62%) | 0.044 | 7%             | (–35% – 46%) | 0.48  |
| 7α-Dehydroxylation of cholic acid                | 53%       | (13% – 92%) | 0.037 | 12%            | (–27% – 49%) | 0.29  |
| Methylhistidine metabolism                       |           |          |       |                |          |       |
| 1-Methylhistidine                                | 33%       | (–2% – 67%) | 0.024 | 50%            | (10% – 90%) | 0.034 |
| 3-Methylhistidine                                | 36%       | (1% – 67%) | 0.020 | –5%            | (–43% – 32%) | 0.46  |
| 1-Methylhistidine synthesis                      | –38%      | (–74% – –3%) | 0.10  | 47%            | (6% – 91%) | 0.038 |
| β-Alanine                                        | 46%       | (12% – 81%) | 0.004 | 34%            | (–12% – 80%) | 0.15  |
| Anserine                                         | NAc       | NAc      |       | NAc            | –80%     | (–145% – –13%) | 0.049 |
| Anserine synthesis                               | NAc       | NAc      |       | NAc            | –85%     | (–121% – –50%) | 0.003 |
| Carnosine                                        | 64%       | (27% – 99%) | 0.001 | 38%            | (–2% – 78%) | 0.09  |
| Carnosine synthesis                              | 65%       | (27% – 105%) | 0.009 | 6%             | (–30% – 46%) | 0.35  |
| Homocysteine metabolism                          |           |          |       |                |          |       |
| Homocysteine                                     | 21%       | (–9% – 55%) | 0.06  | 71%            | (23% – 117%) | 0.027 |
| Betaine                                         | –25%      | (–62% – 13%) | 0.06  | –62%           | (–110% – –14%) | 0.034 |
| Betaine synthesis                                | –44%      | (–81% – –18%) | 0.019 | –13%           | (–54% – 27%) | 0.28  |
| Sum of betaine and related metabolites           | –33%      | (–66% – –6%) | 0.046 | –64%           | (–109% – –15%) | 0.022 |
| Choline                                         | 12%       | (–22% – 45%) | 0.13  | –58%           | (–93% – 23%) | 0.007 |
| Sarcosine synthesis from choline                 | –1%       | (–34% – 28%) | 0.62  | 65%            | (25% – 109%) | 0.007 |
| Cystine                                         | –4%       | (–30% – 37%) | 0.21  | 70%            | (28% – 110%) | 0.007 |
| Cysteine synthesis                               | 4%        | (–33% – 38%) | 0.60  | 51%            | (5% – 97%) | 0.039 |
| Glutathione constituents                         | 16%       | (–19% – 46%) | 0.44  | 100%           | (47% – 158%) | 0.003 |
| Taurine synthesis                                | –8%       | (–44% – 18%) | 0.44  | –85%           | (–131% – –36%) | 0.003 |
| Polyamines                                       |           |          |       |                |          |       |
| Putrescine                                       | –9%       | (–42% – 26%) | 0.17  | –52%           | (–90% – –16%) | 0.015 |
| Putrescine synthesis                             | –22%      | (–57% – 9%) | 0.24  | –76%           | (–121% – –29%) | 0.003 |
| Spermidine                                       | 8%        | (–28% – 39%) | 0.20  | 63%            | (26% – 102%) | 0.007 |
| Spermidine synthesis                             | 22%       | (–9% – 50%) | 0.23  | 75%            | (33% – 115%) | 0.003 |
| Spermine                                         | 37%       | (0% – 72%) | 0.021 | 12%            | (–25% – 55%) | 0.41  |
| Spermine synthesis                               | 38%       | (7% – 68%) | 0.08  | –51%           | (–88% – –13%) | 0.003 |
| Sum of polyamines                                | 10%       | (–32% – 43%) | 0.59  | 60%            | (26% – 97%) | 0.007 |
| Purines                                          |           |          |       |                |          |       |
| Xanthine synthesis                               | NAc       | NAc      |       | NAc            | 71%      | (32% – 109%) | 0.003 |
| Steroids                                         |           |          |       |                |          |       |
| Cortisol                                         | 41%       | (–1% – 81%) | 0.023 | 80%            | (42% – 117%) | 0.007 |
| Cortisone                                        | 35%       | (–2% – 73%) | 0.025 | NAc            | NAc      | NAc   |
| Omega-3 fatty acids                              |           |          |       |                |          |       |
| DHA                                              | –42%      | (–78% – –14%) | 0.003 | –33%           | (–67% – 0%) | 0.07  |
| EPA                                              | –37%      | (–71% – –6%) | 0.009 | –44%           | (–83% – –5%) | 0.05  |
| Ratio of DHA to ARA                               | –55%      | (–93% – –16%) | 0.030 | –33%           | (–68% – 2%) | 0.06  |

(Continued)
| Metabolite or indicator | Plasma | Frontal cortex |
|-------------------------|--------|---------------|
|                         | Effect | CI95          | FDR  | Effect | CI95          | FDR  |
| **Carboxylic acids**    |        |               |      |        |               |      |
| Aconitic acid           | 26%    | (-7% – 61%)   | 0.044| 10%    | (-29% – 49%)  | 0.41 |
| 3-Hydroxyglutaric acid  | 37%    | (2% – 71%)    | 0.017| 53%    | (7% – 104%)   | 0.06 |
| Lactic acid             | 25%    | (-10% – 62%)  | 0.06 | 73%    | (27% – 118%)  | 0.015|
| Succinic acid           | 27%    | (-7% – 62%)   | 0.046| 53%    | (7% – 96%)    | 0.049|
| Sum of carboxylic acids | 26%    | (-11% – 63%)  | 0.23 | 79%    | (37% – 124%)  | 0.003|
| **Amino acids**         |        |               |      |        |               |      |
| Alanine                 | 32%    | (1% – 63%)    | 0.020| 118%   | (72% – 171%)  | 0.007|
| Arginine                | 33%    | (2% – 64%)    | 0.017| 73%    | (30% – 111%)  | 0.007|
| Cysteine                | 15%    | (-19% – 54%)  | 0.11 | 106%   | (54% – 157%)  | 0.007|
| Glutamine               | 9%     | (-19% – 43%)  | 0.13 | 61%    | (22% – 103%)  | 0.022|
| Histidine               | -2%    | (-33% – 29%)  | 0.22 | 87%    | (42% – 133%)  | 0.007|
| Isoleucine              | 26%    | (-6% – 59%)   | 0.05 | 67%    | (22% – 109%)  | 0.022|
| Leucine                 | 20%    | (-13% – 53%)  | 0.07 | 67%    | (25% – 110%)  | 0.015|
| Lysine                  | 24%    | (-13% – 60%)  | 0.06 | 56%    | (12% – 101%)  | 0.048|
| Methionine              | 25%    | (-9% – 61%)   | 0.05 | 63%    | (20% – 106%)  | 0.007|
| Phenylalanine           | 4%     | (-25% – 35%)  | 0.19 | 109%   | (60% – 159%)  | 0.007|
| Serine                  | 1%     | (-37% – 37%)  | 0.23 | 91%    | (43% – 144%)  | 0.007|
| Threonine               | 30%    | (-6% – 64%)   | 0.032| 118%   | (59% – 177%)  | 0.007|
| Tryptophan              | 3%     | (-34% – 38%)  | 0.22 | 118%   | (63% – 169%)  | 0.007|
| Tyrosine                | -1%    | (-33% – 32%)  | 0.22 | 105%   | (57% – 158%)  | 0.007|
| Valine                  | 24%    | (-9% – 58%)   | 0.050| 94%    | (46% – 139%)  | 0.007|
| Dihydrolipoamide dehydrogenase deficiency | 11% | (-25% – 46%) | 0.48 | -65% | (-106% -- -24%) | 0.007|
| Fischer ratio           | 34%    | (-2% – 67%)   | 0.15 | -49%   | (-93% -- -5%) | 0.045|
| Glycine synthesis       | 10%    | (-26% – 43%)  | 0.48 | -49%   | (-105% -- -24%) | 0.020|
| Sum of AAs              | 25%    | (-8% – 61%)   | 0.23 | 116%   | (62% – 168%)  | 0.003|
| Sum of aromatic AAs     | 1%     | (-31% – 35%)  | 0.61 | 111%   | (60% – 160%)  | 0.003|
| Sum of branched-chain AAs | 24% | (-9% – 56%)  | 0.24 | 78%    | (37% – 123%)  | 0.003|
| Sum of essential AAs    | 22%    | (-14% – 59%)  | 0.30 | 99%    | (50% – 146%)  | 0.003|
| Sum of non-essential AAs | 26% | (-9% – 61%)  | 0.23 | 109%   | (58% – 158%)  | 0.003|
| Sum of solely glucogenic AAs | 29% | (-4% – 65%) | 0.15 | 112%   | (57% – 165%)  | 0.003|
| Sum of solely ketogenic AAs | 24% | (-11% – 59%) | 0.24 | 65%    | (22% – 110%)  | 0.007|
| Sum of sulfur-containing AAs | 28% | (-8% – 65%) | 0.23 | 101%   | (49% – 150%)  | 0.003|
| Others amino acid related |      |               |      |        |               |      |
| α-Aminoadipic acid      | 9%     | (-27% – 39%)  | 0.17 | 131%   | (75% – 185%)  | 0.007|
| α-Aminobutyric acid     | 26%    | (-14% – 66%)  | 0.06 | 55%    | (15% – 110%)  | 0.034|
| Acetylornithine         | NAc    | NAc           | NAc | 53%    | (7% – 102%)   | 0.049|
| Creatinine              | 20%    | (-14% – 53%)  | 0.08 | 74%    | (30% – 118%)  | 0.007|
| Histamine               | NAc    | NAc           | NAc | 54%    | (11% – 101%)  | 0.042|
| Homoarginine            | 13%    | (-20% – 41%)  | 0.13 | 50%    | (13% – 88%)   | 0.031|
| trans-4-Hydroxyproline  | 52%    | (10% – 91%)   | 0.009| 92%    | (45% – 134%)  | 0.007|
| Kynurenine              | -9%    | (-43% – 27%)  | 0.15 | 99%    | (47% – 147%)  | 0.007|
| Methionine sulfoxide    | -13%   | (-45% – 17%)  | 0.12 | 54%    | (12% – 97%)   | 0.039|
| Methionine oxidation    | -27%   | (-58% – 5%)   | 0.19 | -44%   | (-84% – -4%)  | 0.039|
| SDMA                    | 9%     | (-30% – 45%)  | 0.18 | 50%    | (16% – 85%)   | 0.022|
| Sum of dimethylated arginine | 9% | (-30% – 48%) | 0.52 | 50%    | (14% – 85%)   | 0.010|

(Continued)
AUC 88% (CI 95% = 80–95%) versus AUC 75%. A model with all analytes but without basic sociodemographic data achieves almost identical performance as the full model.

Step-wise reduction of the model features (Fig. 2A) shows a stable performance up to around 120 features with AUC 83% (slight increase comparing to the full model, since feature selection reduces noise), after which the performance slowly decreases down to AUC 79% at around 20 features, followed by a rapid deterioration in performance down to AUC 70% with 2 features.

Importance plot (Fig. 2B) captures the distribution of average feature importance with respect to the feature rank during the reduction process. The top 30 features are detailed in Fig. 2C, with 5-aminovaleric acid (+ 0.61, CI95 = 0.23–1.01, FDR = 0.06) with p-values of multivariable de-sparsified L1-regularized linear regression models. FDR ≤ 0.05 is rounded to 3 decimal places and highlighted in red (upregulated) and blue (downregulated). 3 Value not available when the metabolite was not sufficiently detected (in at least 50% of samples in either group above the limit of detection). In case of indicators, this happens if a) all metabolites of a sum indicator were not sufficiently detected, b) all metabolites in a nominator or denominator of a ratio indicator were not sufficiently detected, or c) the remaining sufficiently detected metabolites in the indicator completely overlap with another more specific indicator (e.g., Sum of triglycerides and Sum of unsaturated triglycerides).

**Neurotransmitters**

|                        | Plasma               | Frontal cortex       |
|------------------------|----------------------|----------------------|
| DOPA synthesis         | NA c                 | −130% (−191% − −68%)|
| GABA synthesis         | −6% (−35% − 30%)     | 0.59                 |
| Serotonin synthesis    | −2% (−32% − 32%)     | −73% (−120% − −29%)  |

Metabolic indicators: Anserine synthesis, anserine/carnosine; Betaine synthesis, betaine/choline; Carnosine synthesis, carnosine/histidine; Cysteine synthesis, cysteine/(serine + methionine); 7α-Dehydroxylation of cholic acid, deoxycholic acid/cholic acid; Dihydrolipoamide dehydrogenase deficiency, proline/phenylalanine; DOPA synthesis, DOPA/tyrosine; Fischer ratio, sum of branched-chain amino acids/sum of aromatic amino acids; GABA synthesis, GABA/glutamate; Glutathione constituents, glutamate + glycine + cysteine; Glycine synthesis, glycine/serine; Methionine oxidation, methionine sulfoxide/methionine; 1-Methylhistidine synthesis, 1-methylhistidine/(carnosine + anserine); Putrescine synthesis, putrescine/ornithine; Sarcosine synthesis from choline, sarcosine/choline; Serotonin synthesis, serotonin/tryptophan; Spermide synthesis, spermide/pentosine; Spermine synthesis, spermine/spermidine; Taurine synthesis, taurine/cysteine; Xanthine synthesis, xanthine/hypoxanthine. AAs, amino acids; ARA, arachidonic acid; BC, branched-chain; CI95, 95% confidence interval; DHA, docosahexaenoic acid; DOPA, 3,4-dihydroxyphenylalanine; EPA, eicosapentaenoic acid; FDR, false discovery rate; GABA, γ-aminobutyric acid; NA, not available; SDMA, symmetric dimethylarginine. AD regression coefficient in units of 1 standard deviation of the distribution of controls. 3FDR control with q-values following bootstrapped p-values of multivariable de-sparsified L1-regularized linear regression models. FDR ≤ 0.05 is rounded to 3 decimal places and highlighted in red (upregulated) and blue (downregulated). 3Value not available when the metabolite was not sufficiently detected (in at least 50% of samples in either group above the limit of detection).

**Metabolomic analysis in brain frontal cortex**

**Differentially altered metabolites and metabolic indicators**

We found 103 metabolites and 66 indicators altered in AD brain cortex. Several of the small molecules (Table 2) are related to microbial activity: 5-aminovaleric acid (+ 0.61, CI95 = 0.23–1.01, FDR = 0.015) and indoxyl sulfate (+ 0.58, CI95 = 0.14–1.00, FDR = 0.027), while para-cresol sulfate is close to FDR significance (+ 0.50, CI95 = 0.03–0.95, FDR = 0.07).

Methylhistidine metabolism is disrupted with elevated 1-methylhistidine and decreased anserine. Homocysteine metabolism exhibits upregulated transsulfuration pathway and decreased betaine and choline. Polyamines are dysregulated by decreased putrescine and elevated spermidine, but unchanged spermine. Cortisol is elevated. Purine metabolism exhibits increased xanthine synthesis (ratio xanthine/hypoxanthine), which may indicate increased activity of xanthine oxidoreductase. Decrease in omega-3 fatty acids is close to FDR significance (EPA 0.05, DHA 0.07). Among carboxylic acids, lactic acid and succinic acid are elevated, and elevation of 3-hydroxyglutaric acid is close to FDR significance (0.06). Synthesis of multiple neurotransmitters is decreased (serotonin, γ-aminobutyric acid (GABA), dopamine precursor dihydroxyphenylalanine (DOPA)). There is a general increase across most
of amino acids and their related compounds, including trans-4-hydroxyproline, kynurenine, methionine sulfoxide, α-aminoadipic acid, creatinine, and symmetric dimethylarginine (SDMA).

Among lipids (Table 3; individual lipid species are listed in Supplementary Table 1), we found elevated acylcarnitines, regardless of their saturation or length. Acyl-alkyl phosphatidylcholines are increased, whereas lysophosphatidylcholines show the opposite trend (FDR < 0.1). Several sphingomyelins are elevated as well as ceramides, especially glycosylceramides. Certain unsaturated triglycerides and diglycerides are increased. Saturated cholesteryl esters are increased, whereas polyunsaturated cholesteryl esters are decreased.

**Differentially altered pathways**

On the pathway level, we see alterations across many amino acid groups in KEGG (Table 4). Next, there are changes in several pathways reflecting transsulfuration, β-alanine metabolism, purine metabolism, glyoxylic and dicarboxylate metabolism, nicotinate and nicotinamide metabolism, and from lipids glycerolipid and glycerophospholipid.
Table 3
Regression Coefficients of Lipid Indicators Altered in AD Plasma or Frontal Cortex

| Indicator                  | Plasma                  | Cortex                  |
|----------------------------|-------------------------|-------------------------|
|                            | Effect$^a$ | CI95 | FDR$^b$ | Effect$^a$ | CI95 | FDR$^b$ |
| Acylcarnitines             |            |      |        |            |      |        |
| Ratio of ACs to fatty acids| 17%  (-15% – 50%) | 0.36 | 85% (42% – 131%) | 0.003 |
| Ratio of hydroxylated ACs to ACs | NA$^c$  | NA$^c$ | NA$^c$ | -70% (-110% – -31%) | 0.003 |
| Ratio of SCFA ACs to MCFA ACs | -27% ( -61% – 6%) | 0.21 | 61% (12% – 114%) | 0.018 |
| MCAD deficiency screen (C8/C2) | 58% (17% – 101%) | 0.019 | NA$^c$ | NA$^c$ | NA$^c$ |
| SBCAD deficiency screen (C5/C0) | -40% (-78% – 0%) | 0.13 | 50% (12% – 90%) | 0.018 |
| Sum of ACs                  | 11% (-25% – 42%) | 0.48 | 92% (48% – 136%) | 0.003 |
| Sum of MUFA ACs             | 15% (-16% – 49%) | 0.38 | 63% (21% – 104%) | 0.010 |
| Sum of PUFA ACs             | NA$^c$ | NA$^c$ | NA$^c$ | 70% (9% – 125%) | 0.039 |
| Sum of SFA ACs              | 9% (-25% – 42%) | 0.51 | 91% (50% – 135%) | 0.003 |
| Sum of LCFA ACs             | 10% (-25% – 46%) | 0.47 | 63% (10% – 113%) | 0.027 |
| Sum of MCFA ACs             | 29% (-5% – 67%) | 0.21 | 65% (26% – 105%) | 0.003 |
| Sum of SCFA ACs             | 20% (-11% – 50%) | 0.29 | 90% (52% – 127%) | 0.003 |
| Phosphatidylcholines        |            |      |        |            |      |        |
| Ratio of PCs to choline     | 19% (-13% – 52%) | 0.30 | 62% (23% – 99%) | 0.003 |
| Ratio of diacyl PCs to choline | 20% (-12% – 50%) | 0.29 | 60% (20% – 101%) | 0.003 |
| Ratio of acyl-alkyl PCs to choline | 11% (-17% – 41%) | 0.38 | 66% (35% – 99%) | 0.003 |
| Ratio of acyl-alkyl to diacyl PCs | -20% (-53% – 14%) | 0.30 | 44% (4% – 81%) | 0.039 |
| Sum of PCs                  | 51% (20% – 85%) | 0.009 | 5% (-51% – 64%) | 0.37 |
| Sum of diacyl PCs           | 53% (22% – 87%) | 0.019 | -9% (-65% – 48%) | 0.34 |
| Sum of acyl-alkyl PCs       | 32% (2% – 61%) | 0.12 | 44% (8% – 76%) | 0.025 |
| Sum of PUFA PCs             | 56% (26% – 90%) | 0.009 | 5% (-48% – 55%) | 0.38 |
| Sum of lysoPCs              | 68% (35% – 100%) | 0.009 | -34% (-76% – 7%) | 0.09 |
| Sum of MUFA lysoPCs         | 57% (20% – 90%) | 0.009 | -41% (-92% – 3%) | 0.07 |
| Sum of PUFA lysoPCs         | 81% (47% – 112%) | 0.009 | -32% (-66% – 4%) | 0.08 |
| Sum of SFA lysoPCs          | 58% (24% – 90%) | 0.009 | -28% (-65% – 7%) | 0.11 |
| Sum of LCFA lysoPCs         | 69% (36% – 104%) | 0.009 | -34% (-74% – 6%) | 0.09 |
| Sum of choline lipids       | 58% (26% – 93%) | 0.009 | -51% (-88% – -15%) | 0.020 |
| Phospholipase A2 activity (over PC aa C38:6) | 51% (21% – 81%) | 0.009 | -17% (-73% – 36%) | 0.29 |
| Sphingomyelins              |            |      |        |            |      |        |
| Ratio of hydroxylated SMs to non-hydroxylated SMs | -23% (-53% – 7%) | 0.23 | 49% (16% – 83%) | 0.010 |
| Sum of hydroxylated SMs     | 11% (-13% – 43%) | 0.37 | 58% (19% – 94%) | 0.003 |
| Sum of VLCFA SMs            | 14% (-12% – 43%) | 0.32 | 42% (5% – 77%) | 0.039 |
| Ceramides                  |            |      |        |            |      |        |
| Ratio of glycosylCer to Cer | -47% (-77% – -14%) | 0.037 | 37% (-3% – 76%) | 0.07 |
| Ratio of hexosylCer to Cer  | -49% (-80% – -13%) | 0.019 | 36% (-3% – 75%) | 0.07 |
| Sum of Cer                  | 60% (24% – 92%) | 0.019 | 41% (8% – 78%) | 0.039 |
| Sum of glycosylCer          | 16% (-17% – 51%) | 0.34 | 39% (6% – 73%) | 0.039 |
| Sum of hexosylCer           | 10% (-21% – 41%) | 0.47 | 38% (4% – 72%) | 0.039 |
| Sum of dihexosylCer         | 19% (-14% – 52%) | 0.32 | 39% (4% – 73%) | 0.038 |
| Sum of trihexosylCer        | 20% (-10% – 49%) | 0.25 | 47% (3% – 94%) | 0.049 |
| Sum of LCFA Cer             | 60% (26% – 95%) | 0.019 | 46% (9% – 86%) | 0.022 |
| Sum of LCFA glycosylCer     | 15% (-16% – 46%) | 0.37 | 42% (6% – 75%) | 0.027 |
| Sum of VLCFA Cer            | 58% (27% – 92%) | 0.009 | 35% (0% – 69%) | 0.06 |
| Sum of VLCFA glycosylCer    | 15% (-17% – 45%) | 0.36 | 37% (3% – 71%) | 0.039 |
| Sum of VLCFA dihydroCer     | 81% (44% – 117%) | 0.009 | NA$^c$ | NA$^c$ | NA$^c$ |

(Continued)
metabolism and sphingolipid metabolism. These results are repeated with SMPDB pathways (Table 5) and further extended with betaine metabolism, spermidine and spermine biosynthesis, urea cycle and ammonia recycling, and biosynthesis of multiple triglycerides and phosphatidylethanolamines.

**Diagnostic prediction model**

A model with all analytes and basic sociodemographic data (sex, age, education, BMI, *APOE e4*) achieves cross-validated performance of AUC 80% (CI$_{95}$ = 69–90%), which is significantly ($p = 0.018$) more than a random model (AUC 53%, CI$_{95}$ = 39–66%). A model with the basic sociodemographic data alone now yields AUC 64% (CI$_{95}$ = 51–77%), mainly owing to the *APOE e4* genotype, and the full model outperforms it less significantly with $p = 0.09$. A model with all analytes but without basic sociodemographic data achieves almost identical performance as the full model. Again, the full model tends to classify females (AUC 88%, CI$_{95}$ = 71–99%) better than males (AUC 74%, CI$_{95}$ = 58–88%), but the difference is not statistically significant ($p = 0.27$).

Step-wise reduction of the model features (Fig. 3A) shows a stable performance up to around 100 features with AUC 81%, after which the performance slowly decreases down to AUC 75% at around 20 features, and then further down to AUC 70% with 2 features. Importance plot (Fig. 3B) captures the distribution of average feature importance with respect to the feature rank during the reduction process. The top 30 features are detailed in Fig. 3C, with *APOE e4* and acylcarnitine C3:0 being most important. We can see multiple metabolites or indicators already detected in the differential analysis: lipid species and indicators including cholesterols, triglycerides, acylcarnitines, several amino acids and related products, serotonin, trans-4-hydroxyproline, 5-aminovaleric acid as well as indicators of anserine and spermidine synthesis. Several triglycerides and lysophosphatidylcholine C18:1 show a negative association with AD, which we did not capture in the statistical analysis due to lower FDR significance.

**DISCUSSION**

We found alterations in multiple metabolites and metabolic pathways, often overlapping in plasma and brain. The diagnostic prediction models achieve moderate performance, with the top results exploiting around 100 metabolites and indicators, but with most power coming from the top
Table 4

| Pathway                          | Plasma       | Cortex       |
|---------------------------------|--------------|--------------|
|                                 | Effect\(^a\) | FDR\(^b\)   | Effect\(^a\) | FDR\(^b\) |
| Steroid biosynthesis            | 27%          | <0.006       | 13%          | 0.86     |
| Arginine biosynthesis           | 15%          | 0.58         | 49%          | <0.001   |
| Purine metabolism               | NA\(^c\)     | NA\(^c\)     | 35%          | 0.047    |
| Alanine, aspartate and glutamate metabolism | 16% | 0.54 | 58% | <0.001 |
| Glycine, serine and threonine metabolism | 13% | 0.62 | 71% | <0.001 |
| Cysteine and methionine metabolism | 16% | 0.47 | 72% | <0.001 |
| Valine, leucine and isoleucine biosynthesis | 25% | 0.007 | 87% | <0.001 |
| Lysine degradation              | 29%          | 0.25         | 66%          | <0.001   |
| Arginine and proline metabolism | 25%          | 0.24         | 54%          | <0.001   |
| Histidine metabolism            | 25%          | 0.19         | 50%          | <0.001   |
| Tryptophan metabolism           | 12%          | 0.67         | 65%          | 0.045    |
| beta-Alanine metabolism         | 26%          | 0.23         | 47%          | 0.002    |
| Taurine and hypotaurine metabolism | 12%   | 0.79     | 77%          | 0.008    |
| D-Amino acid metabolism         | 21%          | 0.19         | 72%          | <0.001   |
| Glutathione metabolism          | 16%          | 0.62         | 52%          | <0.001   |
| Glycerolipid metabolism         | 50%          | <0.001       | 21%          | 0.016    |
| Glycerophospholipid metabolism  | 32%          | <0.001       | 26%          | <0.001   |
| Arachidonic acid metabolism     | 29%          | <0.001       | 22%          | 0.05     |
| Linoleic acid metabolism        | 28%          | <0.001       | 22%          | 0.05     |
| alpha-Linolenic acid metabolism | 30%          | <0.001       | 22%          | 0.045    |
| Sphingolipid metabolism         | 28%          | <0.001       | 42%          | <0.001   |
| Glyoxylate and dicarboxylate metabolism | 14% | 0.63 | 51% | 0.003 |
| Nicotinate and nicotinamide metabolism | 16% | 0.47 | 38% | 0.021 |
| Sulfur metabolism               | 14%          | 0.62         | 69%          | 0.001    |
| Aminoacyl-tRNA biosynthesis     | 15%          | 0.39         | 77%          | <0.001   |
| Microbial metabolites\(^d\)     | 30%          | 0.012        | 30%          | 0.11     |

FDR, false discovery rate. \(^a\)Average magnitude (absolute value) of AD regression coefficients (from bootstrapped multivariable de-sparsified L1-regularized linear regression models) for assigned metabolites. \(^b\)FDR control with q-values following set enrichment using Kolmogorov-Smirnov test. FDR \(\leq 0.05\) is rounded to 3 decimal places and highlighted in gold. \(^c\)Pathway excluded due to not passing the threshold of at least 4 assigned metabolites. \(^d\)A custom-defined set of metabolites related to microbial activity, as they are not included in human KEGG pathways.

20 spread across various metabolic pathways. The performance diminishes faster when using fewer features. This supports the idea that the metabolic changes act as independent risk factors, each contributing to the outcome. Presence of APOE e4 allele remains among the most important factors. Top 30 features for the plasma and brain models intersect on 5-aminovaleric acid, methylyhistidine metabolism, serotonin, trans-4-hydroxyproline, and on involvement of acylcarnitines, triglycerides, lysophosphatidylcholines, and cholesteryl esters. The models tend to better classify females in both tissue cohorts and given the incidence of AD is higher among females [34], this increase seems to be reflected in metabolism, although further study is needed to confirm this trend.

In AD plasma, there are a number of microbiome-related metabolites changed. 5-Aminovaleric acid is a degradation product derived from various bacterial genera, e.g., Clostridium, Escherichia, and Corynebacterium [35, 36], but can also be produced endogenously. It has not previously been associated with AD or cognitive impairment. Paracresol sulfate is a microbial uremic toxin inducing neuroinflammation in mice [37]. Indoles are derived from gut bacteria, by sequestering tryptophan, and indoxyl sulfate is another uremic toxin with neurotoxic effects [38]. 3-indoleacetic acid is a pro-apoptotic compound negatively associated with cognition [39]. In contrast, 3-indolepropionic acid is neuroprotective [40] and we found it decreased. Other changes include elevated trimethylamine N-oxide, which has been linked to
Table 5
SMPDB Pathways Significantly Altered in AD Plasma or Frontal Cortex

| Pathway                                           | Plasma | Cortex |
|---------------------------------------------------|--------|--------|
| Glycine and Serine Metabolism                     | 18%    | 71%    |
| beta-Alanine Metabolism                           | 27%    | 47%    |
| Ammonia Recycling                                 | 7%     | 57%    |
| Glutathione Metabolism                            | 16%    | 78%    |
| Alpha Linolenic Acid and Linoleic Acid Metabolism | 21%    | 50%    |
| Arginine and Proline Metabolism                   | 20%    | NAc    |
| Oxidation of Branched-Chain Fatty Acids           | 22%    | 62%    |
| Valine, Leucine, and Isoleucine Degradation        | 21%    | 68%    |
| Methionine Metabolism                             | 14%    | 62%    |
| Sphingolipid Metabolism                           | 29%    | 44%    |
| Histidine Metabolism                              | 31%    | 51%    |
| Purine Metabolism                                 | 11%    | 37%    |
| Urea Cycle                                        | 17%    | 58%    |
| Tryptophan Metabolism                             | 15%    | 72%    |
| Aspartate Metabolism                              | 17%    | 47%    |
| Glutamate Metabolism                              | 16%    | 62%    |
| Betaine Metabolism                                | 21%    | 63%    |
| Spermidine and Spermine Biosynthesis              | 21%    | 46%    |
| Carnitine Synthesis                               | 21%    | 47%    |
| Warburg Effect                                    | 18%    | 58%    |
| PE Biosynthesis PE(14:0/18:1(9Z))                 | NAc    | 55%    |
| PE Biosynthesis PE(16:0/16:1(9Z))                 | NAc    | 55%    |
| PE Biosynthesis PE(16:0/18:1(11Z))                | 24%    | 56%    |
| PE Biosynthesis PE(16:0/18:1(9Z))                 | 24%    | 56%    |
| PE Biosynthesis PE(16:0/18:2(9Z,12Z))             | 32%    | 62%    |
| PE Biosynthesis PE(16:0/20:3(8Z,11Z,14Z))         | NAc    | 53%    |
| PE Biosynthesis PE(16:1(9Z)/18:1(9Z))             | 29%    | NAc    |
| PE Biosynthesis PE(18:0/20:4(5Z,8Z,11Z,14Z))      | NAc    | 59%    |
| PE Biosynthesis PE(18:1(11Z)/18:1(11Z))           | 27%    | 57%    |
| PE Biosynthesis PE(18:1(9Z)/16:0)                 | 24%    | 56%    |
| PE Biosynthesis PE(18:1(9Z)/18:1(9Z))             | 27%    | 57%    |
| PE Biosynthesis PE(18:1(9Z)/18:2(9Z,12Z))         | 27%    | 47%    |
| PE Biosynthesis PE(18:1(9Z)/20:3(8Z,11Z,14Z))     | NAc    | 53%    |
| PE Biosynthesis PE(18:1(9Z)/20:4(5Z,8Z,11Z,14Z))  | NAc    | 48%    |
| PE Biosynthesis PE(18:2(9Z,12Z)/18:2(9Z,12Z))     | 29%    | 46%    |
| PE Biosynthesis PE(18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z)) | NAc    | 52%    |
| De Novo TG Biosynthesis TG(16:0/16:1(9Z)/18:0)    | 43%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/20:0)    | 45%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/20:1(11Z)) | 44%    | 32%    |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/18:2(9Z,12Z)) | 48%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/20:4(5Z,8Z,11Z,14Z)) | 54%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/20:1(11Z)) | 52%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z)) | 56%    | <0.001 |
| De Novo TG Biosynthesis TG(16:1(9Z)/18:1(9Z)/18:2(9Z,12Z)) | 45%    | <0.001 |
| De Novo TG Biosynthesis TG(16:1(9Z)/18:1(9Z)/20:4(5Z,8Z,11Z,14Z)) | 57%    | <0.001 |
| De Novo TG Biosynthesis TG(18:1(9Z)/18:2(9Z,12Z)/20:4(5Z,8Z,11Z,14Z)) | 53%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/20:3(5Z,8Z,11Z)) | 52%    | <0.001 |
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/22:5(4Z,7Z,10Z,13Z,16Z)) | 53%    | <0.001 |

(Continued)
atherosclerosis and AD [41], and decreased cholic acid with increased 7α-dehydroxylation into deoxycholic acid, mediated by gut bacteria and previously associated with cognitive decline [42], indicating higher microbial activity converting primary bile acids into pro-inflammatory secondary bile acids. The changes in microbiome-related metabolites are consistently in the pro-toxic direction and we hypothesize that they contribute to the disease etiology. Indeed, recent research reveals direct inflammatory effect of microbiota in AD [43]. We found some of these metabolites are elevated even in the AD brain; i.e., 5-aminovaleric acid, indoxyl sulfate, and with FDR = 0.07 also para-cresol sulfate. Whether the presence of microbial metabolites in brain is due to blood-brain barrier crossing or microbial invasion of CNS, it highlights the importance of studying the gut-brain interaction.

We found two dietary compounds with anti-inflammatory effects decreased in AD: omega-3 fatty acids and betaine. Reduced omega-3 fatty acids have been implicated in AD [44], possibly due to less efficient synthesis in liver [45], which can affect the microbiome composition [46]. Betaine regulates signaling pathways involved in oxidative stress and inflammation [47] and facilitates re-methylation of homocysteine, linked to atherosclerosis [48] and AD [49]. The betaine pathway may be stressed due to impaired metabolism of folate and vitamin B12 deficiency, commonly associated in AD [50]. Interestingly, betaine supplementation has been reported to provide clinical benefit in AD [51]. Altered methylation cycle flux can result in hypomethylation [52, 53] or affect polyamines by enhanced decarboxylation of S-adenosylmethionine, promoting aminopropylation.

Table 5 (Continued)

| Pathway                                           | Plasma                | Cortex                |
|--------------------------------------------------|-----------------------|-----------------------|
| De Novo TG Biosynthesis TG(16:0/18:1(9Z)/22:5(7Z,10Z,13Z,16Z,19Z)) | 53% < 0.001 NA c      | NA c                  |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/20:3(5Z,8Z,11Z)) | 55% < 0.001 NA c      | NA c                  |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/18:3(6Z,9Z,12Z)) | 57% < 0.001 29% 0.50  | 29% 0.50              |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/18:3(9Z,12Z,15Z)) | 57% < 0.001 29% 0.50  | 29% 0.50              |
| De Novo TG Biosynthesis TG(16:0/18:2(9Z,12Z)/22:6(4Z,7Z,10Z,13Z,16Z,19Z)) | 29% 0.036 NA c      | NA c                  |
| De Novo TG Biosynthesis TG(18:1(9Z)/18:2(9Z,12Z)/20:5(5Z,8Z,11Z,14Z,17Z)) | 38% 0.001 18% 0.48  | 18% 0.48              |
| Microbial metabolites d | 30% 0.002 30% 0.12   | 30% 0.12              |

FDR, false discovery rate; PE, phosphatidylethanolamine; TG, triglyceride. aAverage magnitude (absolute value) of AD regression coefficients (from bootstrapped multivariable de-sparsified L1-regularized linear regression models) for assigned metabolites. bFDR control with q-values following set enrichment using Kolmogorov-Smirnov test. FDR ≤ 0.05 is rounded to 3 decimal places and highlighted in gold. cPathway excluded due to not passing the threshold of at least 4 assigned metabolites. dA custom-defined set of metabolites related to microbial activity, as they are not included in human SMPDB pathways.

Increased polyamine spermidine in AD brain has been previously reported [54], which we also observed. In plasma, however, we found higher spermine, perhaps due to lower abundance of spermine synthase in cerebral cortex. Polyamines generally have a protective effect [55] although increased levels can promote tau condensation and neurotoxicity [56, 57], potentially contributing to AD.

Some evidence implicates spermine oxidase activity (ratio spermine/spermidine) in muscle pathophysiology [58]. Indeed, AD is associated with muscle reduction [59, 60], and we found elevated amino acids (strongly in cortex) and methylhistidine metabolites (mainly in plasma, especially 3-methylhistidine, a myofibrillar compound), indicative of muscle degradation. Other amino acid related metabolites are increased, including 3-hydroxyglutaric acid with excitotoxic properties on N-methyl-D-aspartate (NMDA) receptors [61]. Muscle activity can affect the microbiome and in turn influence neurodegeneration [62]. Musculature is also affected by androgens, known to be decreased in AD [63, 64], and negatively impacted by corticosteroids [65]. Consistent with the hypothesis of hormonal link to AD muscle loss, we found increased levels of corticosteroids and decreased DHEAS (in the diagnostic prediction model), validating previous reports [66, 67]. This increase is sometimes explained by chronic stress response or dysregulated circadian cycle and sleep disorders, a common theme in AD, negatively impacting glymphatic clearance and hippocampal volume [68]. In connection with brain function, we found decreased synthesis of
neurotransmitters (serotonin, GABA, DOPA), reflecting impairment associated with depression, a common comorbidity in AD [69].

Another possible reason for neuromuscular degeneration occurring in AD is related to mitochondrial dysfunction [70–72], promoted by oxidative stress, inflammatory environment, and insulin resistance [73], which is considered to be related to AD [74]. We observed elevated acylcarnitines, key elements in mitochondrial beta-oxidation, which accumulate during mitochondrial impairment [75] and correlate with ICU-acquired muscle loss [76]. Further, aconitic acid (plasma only) and succinic acid are elevated, suggesting a disturbed mitochondrial tricarboxylic acid (TCA) cycle, possibly due to inefficient succinate dehydrogenase, which is involved in neurodegeneration and lipid accumulation [77]. Brain energy metabolism includes production and consumption of

\[ \text{Fig. 2. Predictive Performance and Most Important Features For Plasma Samples. A) AUC of a diagnostic prediction model for AD versus controls in the plasma cohort in dependence on the number of selected features (analytes+basic sociodemographic profile: sex, age, education, BMI, APOE e4). Dashed gold line shows AUC of a reference model using only basic sociodemographic profile. Dashed black line shows AUC of a reference random model. Shaded areas illustrate 95% confidence intervals. The } p \text{-value was obtained with DeLong’s test between the full model and basic model. B) Average importance (magnitude of feature contribution to the model decision) of the model features plotted against the feature rank (as ranked by consecutive feature elimination, } 0 = \text{best}. \) The green line denotes a threshold of top 30 features, which are detailed in (C). The color scale corresponds to the importance weight. Positive value: increased in AD; negative: decreased in AD. This figure presents results averaged over 20 randomizations to reduce random noise. Metabolic indicators: Asymmetrical arginine methylation, asymmetrically dimethylated arginine (ADMA)/arginine; Carnosine synthesis, carnosine/histidine; MCAD deficiency screen, C8/C2; 1-Methylhistidine synthesis, 1-methylhistidine/(carnosine + anserine); Proline hydroxylation, hydroxyproline/proline; SBCAD deficiency screen, C5/C0. ACs, acylcarnitines; AUC, area under receiver operating characteristic curve; 5-A VA, 5-aminovaleric acid; CE, cholesteryl ester; Cer, ceramide; Cn, acylcarnitine Cn:0; DHA, docosahexaenoic acid; DHEAS, dehydroepiandrosterone sulfate; 2MBG, 2-methylbutyrylglycinuria; MCAD, medium-chain acyl-CoA dehydrogenase; OH, hydroxylated; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acid; SBCAD, short/branched-chain acyl-CoA dehydrogenase; TG, triglyceride; VLCFA, very long-chain fatty acid. \]
Fig. 3. Predictive Performance and Most Important Features For Cortex Samples. A) AUC (area under receiver operating characteristic curve) of a diagnostic prediction model for AD versus controls in the cortex cohort in dependence on the number of selected features (analytes + basic sociodemographic profile: sex, age, education, BMI, APOE e4). Dashed gold line shows AUC of a reference model using only basic sociodemographic profile. Dashed black line shows AUC of a reference random model. Shaded areas illustrate 95% confidence intervals. The p-value was obtained with DeLong’s test between the full model and basic model. B) Average importance (magnitude of feature contribution to the model decision) of the model features plotted against the feature rank (as ranked by consecutive feature elimination, 0 = best). The green line denotes a threshold of top 30 features, which are detailed in (C). The color scale corresponds to the importance weight. Positive value: increased in AD; negative: decreased in AD. This figure presents results averaged over 20 randomizations to reduce random noise. Metabolic indicators: Anserine synthesis, anserine/carnosine; MC deficiency screen, C16/C3; Serotonin synthesis, serotonin/tryptophan; AOP, acylcarnitines; ae, acyl-alkyl; alpha-AAA, α-aminoadipic acid; AUC, area under receiver operating characteristic curve; 5-AVA, 5-aminovaleric acid; CE, cholesteryl ester; Cer, ceramide; Cn, acylcarnitine Cn:0; OH, hydroxylated; PC, phosphatidylcholine; MC, multiple carboxylase; ACs, acylcarnitines; ae, acyl-alkyl; alpha-AAA, α-aminoadipic acid; AUC, area under receiver operating characteristic curve; 5-AVA, 5-aminovaleric acid; CE, cholesteryl ester; Cer, ceramide; Cn, acylcarnitine Cn:0; OH, hydroxylated; PC, phosphatidylcholine; MC, multiple carboxylase; MUFA, monounsaturated fatty acid; t4-OH-pro, trans-4-hydroxyproline; TG, triglyceride.

lactic acid [78], which is formed in cytosol via a pathway alternative to the TCA cycle, and we found it elevated in cortex. Lactate accumulates in inflamed tissues [79], in traumatic brain injury [80] and is implicated in accelerated production of β-amyloid peptide [81], thus possibly linked to AD.

Besides acylcarnitines, we found other lipid classes elevated in AD. Ceramides are linked to oxidative stress and mitochondrial dysfunction [82] and might directly contribute to AD [83]. Increased plasma dihydroceramides are predictive of future diabetes mellitus [84]. Glycosylceramides are abundant in neuronal tissue and their elevation has also been previously associated with AD [85]. Increased plasma lysophosphatidylcholines may reflect phospholipase A2 activity and are linked to inflammation and immunomodulation [86]. Interestingly, cortex lysophosphatidylcholines show the opposite trend, regardless of their saturation, but specifically those with long chains, whereas those with very long chains (VLCFA) all trend towards upregulation. This might be due to inefficient transport to brain via Mfsd2a
transporter, which is also the main route for omega-3 fatty acids uptake, but VLCFA lysophosphatidylcholines are synthesized in brain tissue by elongases and has been shown to be unaffected in MFSD2A knock-out mice [87].

Elevated diglycerides, triglycerides, and cholesteryl esters in AD correspond to results of other studies [88, 89], although the decrease in polyunsaturated cholesteryl esters in AD brain has not been reported yet. Triglycerides are associated with xanthine oxidoreductase activity [90] and we found it similarly increased in AD brain. Overall, hyperlipidemia is considered a risk factor for AD [91], reflecting inefficient lipid degradation in lysosomes and autophagosomes [92, 93]. Lipotoxicity is further implicated in liver disease and microbiome dysbiosis [94], and in turn, liver disease is sometimes considered a contributor to AD [95].

Our findings align well with the metabolites identified by Varma et al. [6] as an intersection of associations with several AD pathology scores (ranging from future conversion to AD to histopathology markers, amyloid-β, and tau proteins). They performed a targeted study using Biocrates AbsoluteIDQ p180, a predecessor of Biocrates MxP® Quant 500 which we applied in this study. Out of 20 metabolites that they link to AD in brain, we discovered 11 (8 lipid species + 3 small molecules), 3 more lipids achieved slightly lower statistical significance (FDR ≤ 0.06), and additional 6 lipids all clearly show a correct trend of downregulation although further from FDR significance. In great consistency, none of the metabolites show an opposite trend than they predict. For plasma, they list 16 metabolites (excluding two with inconsistent association direction), out of which we identified 6 (5 lipids +1 small molecule) and 4 more lipids with slightly lower FDR significance. For 5 metabolites we did not see any trend and 1 metabolite we identified in the opposite direction than they predict. This particular metabolite is acylcarnitine C3:0 and we found it elevated in both AD brain and plasma, whereas they found it positively associated in brain but negatively associated in plasma. Collectively, this is a good overlap considering we analyzed only clinical AD diagnosis in the plasma cohort but not other markers of AD pathology or progression as described by Varma et al.

In summary, we identified multiple metabolic changes in AD with frequent similarities between plasma and cortex indicating increased microbial toxins, oxidative stress, pro-inflammatory environment, and mitochondrial dysfunction on the one hand, and decreased protective and anti-inflammatory potential on the other hand. While the changes are often small, they provide some insight into the disease complexity and potential risk factors. For example, plasma level of 5-aminovaleric acid higher than 90% of controls poses OR = 3.5 (CI95 = 1.5–9.1). Considering the prevalence of AD in general elderly population around 10% [96], the estimated relative risk (RR) would be 2.9 (CI95 = 1.9–4.3), more than that of diabetes mellitus (RR = 1.5, CI95 = 1.2–1.8) in a meta-analysis [97]. Similarly for cortex, 5-aminovaleric acid higher than 80% of controls leads to the estimated RR = 3.0 (CI95 = 1.6–5.7). Moreover, some of the metabolic changes are modifiable, with several studies showing positive effects of specific diets on cognitive decline and risk of AD [98]. Our results encourage the design of more complex interventional strategies, targeting multiple metabolic areas simultaneously, ideally tailored to one’s metabolic profile, e.g., microbiome composition, hormonal balance, nutrients, and muscle homeostasis.

Strengths and limitations

This is the first study to date to apply the metabolomic kit Biocrates MxP® Quant 500 to study AD. The assay explores a broad spectrum of metabolic pathways together with hundreds of lipid species in 12 lipid classes. The brain samples were collected within a short postmortem interval averaging 3 h, reducing any postmortem effect. We applied advanced statistical methods to maximize statistical power of the analysis while satisfying underlying assumptions and adjusting for multiple hypothesis testing. The model controls for various clinical information as well as tissue handling factors, which are often not considered in other studies. Further, we applied a machine learning approach to test for biomarkers with a nested cross-validation for a reliable estimation of the performance. Efforts were made to minimize any potential bias.

While our analysis is broad and encompasses most areas of the human metabolome, it does not cover all metabolites within the pathways, which only narrowly-focused specialized single-pathway methods can do. This, however, does not affect the validity of our results. Second, the lipid part is performed via flow-injection with a limited number of internal standards (only several per lipid class). This is common in lipidomics and this might result in slightly higher noise and less precise quantification. Based
on quality control samples, the average coefficient of variation was 14% (plasma, across 4 plates) and 10% (cortex, 2 plates) in the UHPLC-MS/MS method, and 19% (plasma) and 20% (cortex) in the FIA-MS/MS method. This is reasonable although the noise might negatively impact the predictive performance. The identification of lipid molecules provides lower resolution due to existence of potential isobaric and isomeric forms inseparable by mass spectra only. Another limitation is unavailability of any information regarding dietary regime of the subjects, which would allow to adjust for its contribution to their metabolic profiles.

Conclusion

We present evidence that AD is associated with multiple pro-toxic changes in microbial metabolites and several metabolic pathways, often overlapping in plasma and brain tissue, including metabolism of methylhistidine, homocysteine, polyamines, corticosteroids, omega-3 fatty acids, carboxylic acids, amino acids, and various lipid classes. We validated multiple previously published studies and discovered newly associated metabolites, e.g., 5-aminovaleic acid, advancing our knowledge of metabolism in AD. Overall, the results highlight the disease complexity and a plausible role of the detected metabolic dysfunctions as individual risk factors for AD due to their pathological nature.

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SUPPLEMENTARY MATERIAL

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