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Metabolic correlates of prevalent mild cognitive impairment and Alzheimer’s disease in adults with Down syndrome

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Abstract

Introduction: Disruption of metabolic function is a recognized feature of late onset Alzheimer’s disease (LOAD). We sought to determine whether similar metabolic pathways are implicated in adults with Down syndrome (DS) who have increased risk for Alzheimer’s disease (AD).

Methods: We examined peripheral blood from 292 participants with DS who completed baseline assessments in the Alzheimer’s Biomarkers Consortium–Down Syndrome (ABC-DS) using untargeted mass spectrometry (MS). Our sample included 38 individuals who met consensus criteria for AD (DS-AD), 43 who met criteria for mild cognitive impairment (DS-MCI), and 211 who were cognitively unaffected and stable (CS).

Results: We measured relative abundance of 8,805 features using MS and 180 putative metabolites were differentially expressed (DE) among the groups at false discovery rate.
1 | INTRODUCTION

Down syndrome (DS) is the most common neurodevelopmental disorder, affecting more than 250,000 individuals in the United States. The syndrome is characterized by triplication of the 21st chromosome (Ch21) which contains the APP gene encoding the amyloid precursor protein (APP). Overexpression of APP results in accelerated amyloid beta (Aβ) accumulation, leading to the development of the hallmark neuropathology of Alzheimer’s disease (AD) in most individuals with DS by age 40. Despite the same genetic risk for AD neuropathology from birth, age at onset of AD clinical symptoms varies across individuals with DS, with some showing cognitive impairment as early as the mid 30s while others maintain cognitive stability until their early 70s. This strongly suggests that factors in addition to increasing Aβ accumulation are involved in individual trajectories of AD in DS-AD.

Several metabolic pathways have been implicated as modifiers of AD risk in the neurotypical population including inflammation and immune response, oxidative stress, lipid metabolism, and single carbon metabolism. Alterations in these same metabolic pathways are also characteristic of people with DS and there is growing appreciation of the role of inflammation, oxidative stress, and lipid metabolism. Several genes on Ch21 including S100a, SOD1, PIGP, and BACH1 are linked to these metabolic pathways and may also contribute to DS-AD risk beyond that directly due to Aβ accumulation. Further, early accumulation of Aβ may lead to dysregulation of these and other pathways, which may modify individual DS-AD trajectories in ways to accelerate or delay symptom onset. Deep metabolomic analysis of people with DS can capture alterations in these and other metabolic pathways that serve to modify trajectories of risk and resilience.

There is now great interest in developing early biomarkers of preclinical AD to identify those at highest risk for developing late onset Alzheimer’s disease (LOAD) and to determine when to intervene when effective therapies become available. To date, most fluid DS-AD biomarker investigations have focused on traditional biomarkers obtained from blood or cerebrospinal fluid (CSF) that characterize the proteopathy required for neuropathological diagnosis, namely Aβ42, total tau, and phospho-tau. However, other studies in DS have shown promising results for novel biomarkers such as the neurofilament light chain (NF-L) protein and markers of inflammation. Regarding Aβ, results from blood biomarker studies generally show higher Aβ levels in people with DS compared to neurotypical controls, but the relationship between Aβ and DS-AD is inconsistent. Many studies show higher Aβ levels in DS-AD relative to non-demented individuals with DS but others show lower levels. With the overexpression of APP from birth, Aβ levels may be as informative for diagnosis or predicting onset of clinical symptoms in DS as in the neurotypical population. Given that most people with DS will develop AD, the main role of a biomarker (or combination of biomarkers) is to inform when symptoms are most likely to begin.

Metabolomic analysis may be ideally suited to answering this question in the case of DS-AD because the time course of biological processes measured by metabolomic analysis is much shorter than proteomic, transcriptomic, and genomic or epigenomic levels and thus may be a more accurate representation of the current phenotypic state. Furthermore, because small molecule end products of metabolism are most proximate to the clinical phenotype, they may more accurately reflect small changes in pathobiology that affect the phenotype.

To date, there have been some metabolomic biomarker studies applied to DS and most have focused on characterizing the metabolome of younger individuals with DS, including children. Cara-cusai et al. found increased levels of key metabolites involved in mitochondrial metabolism including pyruvate, succinate, fumarate, lactate, and formate, that were generally consistent with the 3:2 gene dosage model of trisomy21. These findings in a pediatric DS sample are
provocative because they suggest chronic alterations of cellular energy metabolism in DS, which may contribute to Aβ accumulation and produce individual trajectories of DS-AD over the lifespan.

In a cross-sectional study, Borelli et al. found overall hypogalactosylation in adults with DS (age range: 10–58 years, median age = 26) compared to non-DS controls (age range 12–52 years, median age = 31), but an age-related increase in several glycomic features suggesting accelerated aging in DS. These results suggest that glycosylation may be associated with an aging phenotype in DS that only partially overlaps with aging in neurotypical individuals. They also suggest that altered glycosylation may be associated with DS-AD, given the age-related risk of DS-AD. However, this study did not examine this directly.

In a third study by our group, we used untargeted metabolomics to examine metabolic pathways in adults with DS and DS-AD. We found higher levels of lactate, pyruvic acid, and methyladipic acid in addition to lower levels of uridine in the participants with DS-AD relative to participants with DS, but without AD. We consider these findings in adults with DS-AD an extension of those from Caracausi et al. in children with DS. Together, both studies suggest that disruption of energy metabolism, particularly mitochondrial metabolism, is a prominent feature of DS and DS-AD.

Here, we sought to examine the metabolic differences that characterize stages of cognitive and functional impairment over the course of DS-AD, from cognitively stable, to mild cognitive impairment (MCI), to DS-AD. We hypothesized that the pathobiology of DS-AD and LOAD overlap significantly in a final common pathway that includes cellular energy metabolism. We predicted that metabolic alterations of DS-AD progression would be similar to those seen in LOAD.

2 | METHODS

2.1 | Participants

All participants for this study were enrolled in Alzheimer’s Biomarkers Consortium-Down Syndrome (ABC-DS; https://www.nia.nih.gov/research/abc-ds), a multi-site, longitudinal cohort study of adults with DS over age 25. The ABC-DS is designed to discover biomarkers of DS-AD at eight clinical performance sites including The University of California Irvine, Columbia University Irving Medical Center, The Institute for Basic Research in Developmental Disabilities, Massachusetts General Hospital, The University of Wisconsin-Madison, The University of Pittsburgh, Barrow Institute, and Cambridge University (UK). All ABC-DS participants undergo deep clinical and cognitive phenotyping, neuroimaging including positron emission tomography (PET) amyloid and tau and structural and functional magnetic resonance imaging (MRI), and CSF and blood collection at 16-month intervals. All ABC-DS protocols and procedures are approved by the respective local clinical performance site Institutional Review Boards. Informed consent is obtained from all participants where possible, otherwise assent is obtained from the participant and informed consent obtained from the participant’s proxy or legally authorized representative.

RESEARCH IN CONTEXT

1. Systematic review: The authors reviewed the literature on the relationship between markers of metabolism found in peripheral blood and dementia status in adults with Down syndrome (DS) using traditional sources including PubMed, GoogleScholar, and ResearchGate. We found very few published studies directly addressing the metabolic characteristics of mild cognitive impairment and clinical Alzheimer’s disease (AD) in adults with DS. Several studies examined metabolism in children with DS, but did not directly address AD. We felt this was a neglected area of study given the strong risk for AD in adults with DS.

2. Interpretation: We found strong evidence of fatty acid and cellular energy metabolic differences which might underlie AD-related clinical and cognitive decline in people with DS.

3. Future directions: These findings call for a prospective study to examine within-individual longitudinal changes in metabolism that may be useful for understanding the evolution of AD in people with DS.

A total of 353 adults with DS were enrolled in ABC-DS at the time of the Wave I data freeze (January 15, 2019) and 329 had (1) sufficient data to make a clinical consensus decision and (2) a blood sample for analysis. The 329 DS participants were classified into four non-overlapping groups by expert ABC-DS clinicians who considered all available clinical and cognitive measures.

We classified participants into four groups, generally consistent with the recommendations of the AAMR-IASSID Working Group for the Establishment of Criteria for the Diagnosis of Dementia in Individuals with Developmental Disability. Participants were classified as cognitively unaffected and stable (CS) if there was no evidence of clinically significantly cognitive or functional decline from baseline based on caregiver report and medical record review. We classified participants as mild cognitive impairment (DS-MCI) if they had shown some cognitive and/or functional decline over and above what would be expected with aging per se, but not severe enough to indicate the presence of DS-AD. Participants were classified as DS-AD if there was evidence of substantial decline in cognitive function and activities of daily living compared to caregiver-reported baseline and medical record review. Finally, we classified individuals with cognitive and functional impairment that could be better explained by changes in life circumstance (eg, staff changes, bereavement) or medical conditions unrelated to AD (eg, severe sensory loss, chronic pain, psychiatric diagnosis, seizure disorder) as “unable to determine” and these participants were excluded from this study. The consensus conference was able to confidently classify 312 participants and unable to confidently classify 17 DS participants.
DNA samples were genotyped for two apolipoprotein (APOE) single-nucleotide polymorphisms (SNPs; rs429358 and rs7412) with the competitive allele specific PCR (KASP) genotyping system (LGC Genomics). Genotype data for these two SNPs were used to define APOE ε2, ε3, and ε4 alleles. Participants with at least one copy of the APOE ε4 allele were classified as APOE ε4 carriers. A total of 20 participants were missing APOE data and were excluded from the analysis. In total, 292 DS participants had a consensus clinical classification, an available blood sample, and APOE status for inclusion in this study. Characteristics of the participants can be found in Table 1.

|TABLE 1  Participant characteristics|
|-------------------------------------|
|                                      | N (M/F) | Age (SEM) | BMI (SEM) | % with ApoE ε4 |
|-------------------------------------|---------|-----------|-----------|----------------|
|Cognitively unaffected and stable (CS)| 211 (109/102) | 42.16 (0.59) | 31.99 (0.5) | 21.3% (45/211) |
|Mild cognitive impairment (Down syndrome [DS]-MCI) | 43 (30/13) | 52.21 (1.06) | 28.89 (0.95) | 34.9% (15/43) |
|Alzheimer’s Disease (DS-AD) | 38 (18/20) | 54.39 (0.93) | 30.15 (1.1) | 36.8% (14/38) |

*The groups differed in proportion with ε4 (χ^2 = 6.52, P < .05).

2.2 | Blood collection and processing

All blood collection and processing methods were harmonized across all ABC-DS sites. Venous blood was collected from the median cubital vein using standard venipuncture technique into a 10 mL ethylenediaminetetraacetic acid (EDTA; Lavender top) vial. The EDTA tube was the first tube collected, followed by Sodium Heparin, Serum Separator, and PAXgene tubes. All blood collection and processing methods were harmonized across all ABC-DS sites. Venous blood was collected from the median cubital vein using standard venipuncture technique into a 10 mL ethylenediaminetetraacetic acid (EDTA; Lavender top) vial. The EDTA tube was the first tube collected, followed by Sodium Heparin, Serum Separator, and PAXgene tubes. Given the general considerations of working with people with DS, we did not attempt to standardize blood collection procedures with regard to prescribed medication administration, prandial state, or time of day. After venipuncture, collection tubes were gently inverted 8 to 10 times, placed on wet ice, then centrifuged to separate the plasma component. Centrifugation took place in a chilled (4°C) centrifuge for 10 minutes at a site-specific RPM equivalent to 2000 × g. The plasma fraction was aliquoted in 250 µL units to individual 500 µL siliconized cryovials, and stored at −80°C at local ABC-DS clinical performance sites. Time from blood draw to storage at −80°C was estimated to be <3 hours. The vials were shipped from the local ABC-DS sites on dry ice via overnight courier to the National Cell Repository for Alzheimer’s Disease at Indiana University, where they were stored at −80°C until sent in one shipment to the Lombardi Cancer Center Shared Resource Facility Metabolomics Core at Georgetown University for mass spectrometry (MS) analyses. The time in storage from blood draw to MS for all samples was <2 years.

2.3 | Untargeted UPLC-ESI-QTOF-MS metabolomics

We used ultra-performance liquid chromatography electro-spray ionization-quadrupole-time of flight-mass spectrometry (UPLC-ESI-QTOF-MS; Xevo-G2 QTOF, Waters Corporation) based data acquisition for untargeted metabolomic profiling as described in our previous work.9,37,40,41 Briefly, we prepared plasma samples for MS by solvent extraction and resolved using reverse phase chromatography on an Acquity UPLC (Waters Corp.) online with a QTOF-MS in the positive and negative electrospray mode with optimized run parameters. A total of 8805 features (4790 ESI+- detection mode; 4015 ESI- detection mode) representing putative metabolites were identified by mass-to-charge ratio (m/z) and retention time (RT) after processing of raw spectral data using XCMS software.42 We submitted these putative metabolites to differential expression (DE) analysis. A subset of relevant metabolites were identified using tandem MS.

3 | STATISTICAL METHODS

3.1 | Differential expression analysis of metabolite features

We used R version 3.6.1 and the limma package to assess DE of individual metabolites as a function of clinical status (CS/DS-MCI/DS-AD). Individual F-statistics were derived for each metabolite feature passing quality control thresholding as we have implemented previously.37,43 Because metabolomic abundance measurements are often positively skewed, we logarithm transformed abundance values prior to analysis consistent with other applications of surrogate variable analysis (SVA).33,44 Resulting F-test statistic P-values were false discovery rate (FDR)-corrected to reduce the multiple testing burden, resulting in a corresponding q-value.45 An FDR q < 0.05 was considered significantly DE. We quantified the magnitude of group-wise DE as the logarithm of the group-wise fold change provided by the limma function topTableF(). Because fold change is only capable of representing pairwise comparisons, we explicitly make note of the groups compared for the remainder of this article when necessary. We also explicitly indicate the reference condition (eg, CS, DS-MCI) from which fold-change comparisons are made.

3.2 | Parametrization of nuisance variability in the peripheral metabolome

In addition to metabolite variability attributable to disease progression, the plasma metabolome is likely to reflect physiological processes and technical artefacts unrelated to neurological disease. This is a challenge because (1) these confounding factors have the
potential to limit reproducibility of differential expression findings and (2) the identity and number of these variables may be incompletely known a priori. We have previously proposed and employed SVA to approximate these confounders (eg, due to sample age, analytical batch, medication status) in high-dimensional metabolomic data.37,43,46

We used the “be” method47 to estimate a non-zero number of surrogate variables which we included as covariates in further DE analyses conducted using limma (SVs = 25). Because APOE ε 4 carrier status was significantly disproportionate among the three groups (χ2 = 6.52, P < .05), and ε 4 may impact peripheral metabolic processes related to dementia in DS,48 we explicitly directed SVA to consider the presence of this allele in fitting the 25 SVs obtained here.

3.3 Metabolite set enrichment analysis of LC-MS metabolites

We used the Mummichog software library to generate statistical inferences regarding metabolic pathway alterations in the DS-AD participants.49 To minimize false positives, we used the most recent Mummichog 2 software, which enforces RT matching across putatively related mass features (personal communication; Dr. Shuzhao Li 9/2018). We specified instrument accuracy as 7 ppm. Significance thresholds for DE calling were selected over the list of all nominal P-values generated by the preceding DE analysis.49 In all comparisons for metabolite set enrichment analyses, we considered a nominal P-value < .05 to be DE.

3.4 Machine learning classifier models for group prediction

We submitted all metabolite features found to be significantly DE at the q < .05 level per pairwise contrast to predictive modelling. To integrate surrogate variable derived information into our modelling analysis and maximally leverage available data, we took an empirical Bayesian approach (ie, empiricalBayesLM in the R package WGCNA) and residualized all metabolite abundances by their corresponding sample-level loadings on each SV. This resulted in an augmented metabolite expression matrix free of nuisance variability attributable to each of the 25 SVs parametrized here. These data were further log transformed, quantile normalized, and adjusted to have unit variance and zero mean (ie, z-scaled, autoscaled).9,40,43

It is possible that choices in machine learning algorithm and resampling scheme selection may affect model performance in a way unrelated to underlying disease-associated signal in the plasma metabolome. To address this, we evaluated several resampling schemes (ie, 10-fold cross validation [CV], Monte Carlo CV) and algorithmic approaches (ie, least absolute shrinkage and selection operator [LASSO]-regularized logistic regression, linear support vector machine [SVM]) in parallel. We evaluated classification performance quantitatively using receiver operating characteristic area under the curve (ROC AUC) statistics and qualitatively according to the distribution of prediction accuracy (Figure S1 in supporting information). In all cases, plotted ROC curves reflect testing/validation performance, as opposed to classification over the training data set, which is likely to yield overoptimistic estimates of model strength.43 In the case of our modeling efforts contrasting DS-MCI and DS-AD, we found the FDR-significant feature space to be small (ie, 17 features) and thus unsuitable for LASSO-regularized variable selection. In this case, we prioritized a subset of these features using a linear SVM-based approach.50

3.5 Targeted LC-MS/MS metabolomics

We used multiple reaction monitoring mass spectrometry (MRM MS/MS) targeted analysis of the putatively identified metabolites selected by LASSO and SVM feature selection algorithms. Briefly, we mixed plasma samples (25 μl) with 300 μl of methanol: chloroform (2:1). To this, we added 100 μl of water and chloroform, separately. The samples were vortexed and incubated on ice for 10 minutes. We centrifuged the samples at 13,000 rpm at 60°C for 15 minutes. The upper aqueous layer was transferred to a separate vial and dried under a stream of nitrogen. We reconstituted the samples in ACN:water (50:50) containing 1 μg/mL of internal standard (tyrosine-15N). The supernatant was transferred to an MS vial and 5 μl of sample was used for analysis. The LC and MRM methods used in this study were optimized in the metabolomics core at Georgetown in collaboration with Sciex. The sample queue was randomized and solvent blanks were injected to assess sample carryover using four biological replicates for each comparative group.

4 RESULTS

4.1 Plasma metabolites are differentially expressed as a function of cognitive status

We identified 8,805 putative metabolites across negative and positive mode LC-MS analyses. We found no DE features comparing the CS and DS-MCI groups under the multiple testing correction (all FDR q > .05; Figure 1A). This contrast was consequently not considered a candidate for further classification modeling. However, a total of 180 features were DE at the FDR q < .05 level across the pairwise DS-MCI versus DS-AD, and CS versus DS-AD contrasts (Figure 1 B&C).

4.2 Differentially expressed metabolite features predict cognitive status

In the case of the DS-AD versus CS contrast, 163 features FDR q < .05 were submitted to LASSO-regularized regression modelling using 10-fold CV. Considering only the subset of features selected over at least 50% of resampling epochs, the algorithm selected nine metabolites
FIGURE 1 Differentially expressed metabolite features. Volcano plots showing differential expression (DE) of individual features for each of the three comparisons: cognitively unaffected and stable (CS) versus Down syndrome-mild cognitive impairment (DS-MCI; A), DS-MCI versus Down syndrome-Alzheimer’s disease (DS-AD; B), and CS versus DS-AD (C). We enforced false discovery rate (FDR) \( q < 0.05 \), but no fold change criterion for DE. There were no DE features for the CS versus DS-MCI comparison, 17 DE features for the DS-MCI versus DS-AD comparison, and 163 DE features for the CS versus DS-AD comparison. The red horizontal line represents the cut-off for FDR and red circles represent DE features in each plot.

4.3 Differentially expressed LC-MS metabolites underscore role of energy and lipid metabolism in DS-AD progression

Although we were able to form effective, robust classifiers of cognitive status for (1) DS-AD versus CS and (2) DS-AD versus DS-MCI comparisons, the underlying biochemical perturbations driving this discriminability could only be inferred from the few definitively identified metabolites included in the classifier models. Furthermore, it was not clear whether semantic biological meaning could be distilled from the DS-MCI versus CS contrast in spite of a lack of FDR \( q < 0.05 \) significant features. To evaluate this, all DE features on the basis of group-wise DE nominal \( P \)-values were putatively identified based on \( m/z \) and mobile phase column RT. We submitted these features to metabolic set enrichment analysis for high-level semantic characterization and systems biochemical interpretation. Corroborating earlier reports from our group in an independent cohort of DS adults, we again find that known metabolic pathways involved in fatty acid/lipid metabolism and central carbon/carbohydrate metabolism appear perturbed in peripheral blood plasma with dementia progression (Table 3). Interestingly, metabolic pathways characterizing contiguous phenotype transitions (ie, CS vs DS-MCI and DS-AD vs DS-MCI) appear particularly enriched with metabolites involved in fatty acid metabolism. Fatty acid metabolism in the brain has been increasingly linked to neuropathology and cognitive integrity in LOAD; however, the overall metabolic contributions to AD progression remain unclear broadly. That fatty acid metabolism involving both biosynthesis and oxidation may index these transitions specifically is an intriguing idea implicating well-understood genetic programs and metabolic regulatory mechanisms. In addition to their role as biomarker candidates, these findings may consequently suggest novel therapeutic targets in DS-AD, and perhaps AD broadly, that are
FIGURE 2  Features selected by the machine learning algorithms. This figure shows the group distributions of the nine features selected by the least absolute shrinkage selection operator (LASSO) feature selection algorithm for the cognitively unaffected and stable (CS) versus Down syndrome-Alzheimer’s disease (DS-AD) comparison (A) and the five features selected by the support vector machine (SVM) for the Down syndrome-mild cognitive impairment (DS-MCI) versus DS-AD comparison. The boxplots show the distribution of metabolite abundances for each group with each participant represented as a solid circle. The solid line in each box represents the median while the lower and upper boundaries of the box reflect the first and third quartiles. The whiskers reflect the minimum and maximum values. The horizontal red line in each panel represents the optimum cut-off for sensitivity and specificity in a univariate receiver operating characteristic area under the curve (ROC AUC). Panels with red outlines are the metabolites definitively identified by MS/MS and are listed by name in Table 2. Panels without red outlines could not be definitively identified by MS/MS.
**FIGURE 3** Classification performance using putative metabolites. Receiver operating characteristic area under the curve (ROC AUC) for the classification models using the nine unidentified features for the cognitively unaffected and stable (CS) versus Down syndrome-Alzheimer’s disease (DS-AD) comparison (A) and the five features for Down syndrome-mild cognitive impairment (DS-MCI) versus DS-AD comparison (B). For the CS versus DS-AD comparison, the left panel shows strong classification using a logistic regression model with 10-fold cross validation (ROC AUC = 0.868), the middle panel shows similar performance for the same model using a more rigorous 100-fold Monte Carlo cross-validation procedure (ROC AUC = 0.855), and the right panel shows consistent classification performance using an alternate support vector machine (SVM) classification algorithm (ROC AUC = 0.859). In the DS-AD versus DS-MCI comparison (B) the left panel shows strong classification performance using the logistic regression model with 10-fold cross validation (ROC AUC = 0.891), the middle panel shows similar performance with 10-fold Monte Carlo resampling approach (ROC AUC = 0.881) and the right panel shows strong SVM performance (ROC AUC = 0.885) currently better understood in other types of metabolic pathology (eg, type 2 diabetes; non-alcoholic fatty liver disease, NAFLD).

5 | DISCUSSION

This is one of the first, large-scale blood-based investigations of metabolic factors associated with aging and cognitive status in adults with DS-AD. We used untargeted metabolomics to consider a broad scope of metabolic alterations which can be followed up in subsequent targeted metabolomic studies. We find strong evidence of metabolic perturbations which characterize groups of people with DS who were cognitively unaffected with stable cognition, DS-MCI, and DS-AD. In general, changes in lipid metabolism including fatty acid biosynthesis and degradation pathways characterize these phenotypic state changes, with evidence of broad, cellular energy metabolic decline present in adults with DS, from unimpaired to manifest DS-AD. It is not clear from this study if these energy alterations begin in adulthood or are present from birth, but this is an interesting avenue for future research.

A valid concern with classification models derived from a large number of parameters and a significantly fewer number of cases (here, 8805 metabolites and 292 participants) is the risk of model overfitting and lack of generalizability from the experimental sample to the population of interest. This remains a challenge in biomarker development across many fields. Here, we attempted to mitigate model overfitting by using machine learning algorithms for feature selection to eliminate biases for particular metabolites or classes of metabolites. Further, the logistic regression classification models were refined using multiple resampling schemes (10-fold CV and 100-fold Monte Carlo cross-validation) and cross-checked using a separate SVM model. Finally, we attempted to minimize the effects of other known variables (eg, APOE, sex, medications, age, etc) or unknown variables which might produce false positive results or complicate interpretation using...
| Comparison          | Ion mode | Precursor m/z (RT) | Metabolite | Compound name                                                                 |
|---------------------|----------|-------------------|------------|-------------------------------------------------------------------------------|
| CS versus DS-AD     | NEG      | 1100.522 (8.99)   | Metabolite 1 | CDP-DG(22:5)                                                                  |
| CS versus DS-AD     | POS      | 732.6076 (10.02)  | Metabolite 2 | 1-Oleoyl-2-myristoyl-sn-glycero-3-phosphocholine                              |
| CS versus DS-AD     | POS      | 723.4909 (10.01)  | Metabolite 3 | 1,2-Dioleoyl-sn-glycero-3-phosphate                                           |
| CS versus DS-AD     | NEG      | 721.4582 (6.97)   | Metabolite 4 | PA(15:0/20:2)                                                                 |
| CS versus DS-AD     | POS      | 459.1245 (2.09)   | Metabolite 5 | 3,4,5-trihydroxy-6-(2-hydroxy-1,2-diphenylethoxy)oxane-2-carboxylic acid      |
| CS versus DS-AD     | NEG      | 840.6669 (0.3)    | Metabolite 6 | PE-Nme(18:1)                                                                   |
| CS versus DS-AD     | NEG      | 593.4771 (7.55)   | Metabolite 7 | DG(15:0/18:4)                                                                  |
| CS versus DS-AD     | NEG      | 1102.75 (0.3)     | Metabolite 8 | -                                                                              |
| CS versus DS-AD     | NEG      | 493.3887 (7.35)   | Metabolite 9 | -                                                                              |
| DS-MCI versus DS-AD | POS      | 840.521 (10.09)   | Metabolite 2 | PS(20:0/20:3(8Z,11Z,14Z))                                                     |
| DS-MCI versus DS-AD | POS      | 518.4922 (8.09)   | Metabolite 3 | -                                                                              |
| DS-MCI versus DS-AD | POS      | 628.4103 (7.28)   | Metabolite 4 | -                                                                              |
| DS-MCI versus DS-AD | POS      | 536.4959 (8.12)   | Metabolite 5 | -                                                                              |

Abbreviations: AD, Alzheimer’s disease; CS, cognitively unaffected and stable; DS, Down syndrome; LASSO, least absolute shrinkage selection operator; MCI, mild cognitive impairment; SVM, support vector machine

**FIGURE 4** Classification performance using definitively identified metabolites. These receiver operating characteristic (ROC) plots show the classification model performance using the seven MS/MS definitively identified metabolites for the cognitively unaffected and stable (CS) versus Down syndrome-Alzheimer’s disease (DS-AD) comparison (A) and the two definitively identified metabolites for the DS-AD versus Down syndrome-mild cognitive impairment (DS-MCI) comparison (B). The classification performance from these reduced set of metabolites is not significantly different from the larger sets used in Figure 3. The consistency of the ROC AUC across the resampling schemes (10-fold CV and 100-fold Monte Carlo CV) and classification models (logistic regression and support vector machine) shows the overall stability of the models and argues against overfitting.
### TABLE 3  Metabolic set enrichment analysis

| KEGG Pathway | Pathways | CS versus DS-MCI | DS-MCI versus DS-AD | CS versus DS-AD |
|--------------|----------|------------------|---------------------|----------------|
| Fatty acid metabolism | Fatty acid oxidation, peroxisome | 0.04025 | | |
| Fatty acid metabolism | Leukotriene metabolism | 0.03823 | | |
| Fatty acid metabolism | De novo fatty acid biosynthesis | 0.04537 | 0.00067 | |
| Fatty acid metabolism | Fatty acid activation | 0.00445 | 0.00067 | |
| Fatty acid metabolism | Fatty acid oxidation | 0.00462 | 0.00622 | |
| Fatty acid metabolism | Omega-3 fatty acid metabolism | 0.00975 | 0.01874 | |
| Lipid metabolism | Glycerophospholipid metabolism | 0.02183 | 0.01244 | |
| Amino acid metabolism | Arginine and proline metabolism | 0.03748 | 0.00857 | |
| Metabolism of xenobiotics by cytochrome P450 | Xenobiotics metabolism | 0.03487 | | |
| Fatty acid metabolism | Linoleate metabolism | | 0.01218 | |
| Fatty acid metabolism | Saturated fatty acids beta-oxidation | | 0.02823 | |
| Fatty acid metabolism | Omega-6 fatty acid metabolism | | 0.02823 | |
| Fatty acid metabolism | Carnitine shuttle | | 0.0037 | |
| Lipid metabolism | Phosphatidylinositol phosphate metabolism | | 0.00639 | |
| Nucleotide metabolism | Purine metabolism | | 0.00302 | |
| Neuroactive ligand-receptor interaction | Dynorphin metabolism | 0.02773 | 0.04016 | 0.04949 |
| Lipid metabolism | Limonene and pinene degradation | 0.03445 | | |
| Lipid metabolism | Bile acid biosynthesis | | 0.04655 | |
| Lipid metabolism | Glycosylphosphatidylinositol (GPI)-anchor biosynthesis | | 0.04193 | |
| Lipid metabolism | Vitamin D3 metabolism | | 0.04865 | |
| Lipid metabolism/Carbohydrate metabolism | Glycosphingolipid metabolism | | 0.04672 | |
| Carbohydrate metabolism | Hexose phosphorylation | | 0.04193 | |
| Carbohydrate metabolism | Pentose phosphate pathway | | 0.00588 | |
| Carbohydrate metabolism | Galactose metabolism | | 0.04193 | |
| Carbohydrate metabolism | Fructose and mannose metabolism | | 0.04193 | |
| Nucleic acid/Purine metabolism; Carbohydrate metabolism | Vitamin B1 (thiamin) metabolism | | 0.04193 | |
| Carbohydrate metabolism/Glycan biosynthesis and metabolism | Aminosugars metabolism | | 0.04193 | |
| Glycan biosynthesis and metabolism | Heparan sulfate degradation | | 0.04193 | |
| Glycan biosynthesis and metabolism | Keratan sulfate degradation | | 0.04193 | |
| Glycan biosynthesis and metabolism | Chondroitin sulfate degradation | | 0.04193 | |
| Glycan biosynthesis and metabolism | Sialic acid metabolism | | 0.04672 | |

*KEGG, Kyoto Encyclopedia of Genes and Genomes (https://www.genome.jp/kegg/). Contrasts highlighted in red involved fatty acid metabolism pathways. Contrasts highlighted in green involve lipid metabolic pathways. Contrasts highlighted in yellow involve cellular energy metabolic pathways. * P values reflect significant enrichment of metabolites on the relevant KEGG pathways.*

The surrogate variable approach. In aggregate, the models produced consistent and robust classification results with ROC AUC’s ranging from 0.84 to 0.90 and accuracies around 78%. These classification models need to be replicated in additional cohorts, but appear robust and consistent with previous literature from our group and others implicating lipid- and energy-related metabolites as possible markers of DS-AD progression. While large-scale external replication is the current gold standard for biomarker validation, multiple independent observations of the same phenomena on a smaller scale are also useful for validating a set of results and we would consider other attempts to replicate these findings in small to medium sized cohorts valuable.
The metabolite enrichment set analysis also produced findings that are consistent with the extant literature regarding the involvement of lipid metabolism in LOAD.53–55 These lipid alterations may not be independent from the hallmark neuropathology of LOAD. With respect to plaque formation, it has been observed that membrane lipid compositional changes may increase amyloidogenic processing of APP. The mechanism for this is thought to involve increased proximity of the β-secretase (BACE1) enzyme and its substrate APP via lipid rafts, which overall serves to promote BACE1 activity and amyloidogenesis.56 It has also been proposed that the tauopathy and neurofibrillary tangle (NFT) formation that are hallmarks of LOAD may be subject to lipid-dependent mechanisms; tau paired helical filament aggregation may initiate at the plasma membrane and lipids (ie, phospholipids, cholesterol, sphingomyelins) have been isolated from paired helical filaments derived from LOAD brain.57,58

There is also a growing appreciation of lipid metabolic alterations in DS. For example, individuals with DS have peripheral lipid metabolism that differs from euploid siblings independent of body weight.59 Furthermore, our group has recently reported evidence of lipid and small-molecule metabolic alterations in the peripheral blood of adults with DS as a function of cognitive impairment.37 Although it is possible that our peripheral metabolomic analyses reported here index DS-AD associated pathology directly at the cellular or subcellular level within the CNS, there is increasing reason to suspect that peripherally evident metabolic alterations (eg, of lipids, bioenergetically relevant small molecules) may co-occur with cognitive decline and reflect disease progression in a manner not entirely dependent on CNS metabolism.55,60–64 It is challenging to contextualize lipid physiological findings in both the dementing and pre-symptomatic brain across multiple levels of organization (eg, single neuron/glial cell, autonomously considered CNS tissue, cross-organ-system communication).65–68 We are therefore encouraged by the fact that, within the ABC-DS cohort, we have recapitulated a previously identified, disease-associated metabolic signature of dementia in an independent, expanded, and rigorously prospectively collected cohort of adults with DS.37 Consistent with these earlier findings, we find lipid/fatty acid metabolism and central carbon pathway metabolites to be principally affected.

5.1 Lipid metabolism in AD: shared vulnerabilities in LOAD and DS-AD?

Lipid alterations may impact plaque formation via amyloid-processing genes (presenilin 1-2; PSEN1-2) implicated in rare, heritable forms of AD (familial Alzheimer’s disease [FAD]); however, there is substantial reason to think that aberrant lipid metabolism may impact sporadic LOAD directly.69 Furthermore, the pattern of lipid alteration in LOAD (versus other dementias such as frontotemporal, vascular, Lewy body) may be relatively specific, suggesting a potential role of these molecules in differential diagnosis.70 Importantly, the APOE ε4 allele represents one of the earliest discovered and strongest common risk variants predisposing individuals to LOAD specifically,71 although it is only now being considered a therapeutic target in AD.72 apoE serves to clear amyloid plaques within the aging brain, but is also crucially important for intercellular lipid transport.73 APOE is expressed in metabolically relevant peripheral tissues such as the liver and is primarily produced in the brain by glial cells (eg, astrocytes, microglia).74,75 Within the CNS, lipoprotein complexes (1) facilitate axonal remodeling and survival in neurons through their uptake by the low-density lipoprotein receptor (LDLR) and (2) modulate microglial inflammatory phenotype by signaling through LDLRs and triggering receptor expressed on myeloid cells 2 (TREM2).74,76,77 The rare, recently described R47H mutation in TREM2 represents another genetic, lipid-associated risk factor impacting LOAD specifically and underscores the role of neuroinflammatory homeostasis and microglia in AD.78–80

That glia disproportionately express APOE is an intriguing finding, given that these cells have substantial capacity for lipid synthesis and metabolic plasticity,81–85 whereas neurons routinely avoid lipid catabolism for bioenergetic purposes under physiologic circumstances.86 This relationship may instead highlight the non-metabolic (ie, bioactive signaling) roles of certain lipids (polyunsaturated fatty acids [PUFAs]) which are produced substantially in astrocytes, but exert neuroprotective and immunomodulatory roles within neurons when taken up by these latter cells.87,88 Although the role of glia in AD has become substantially better understood,89–91 the degeneration of neurons remains the factor most proximally driving cognitive decline in AD.92–95

It is also in substantial part through lipidation of apoE that cholesterol efflux is facilitated within the CNS. Although the brain represents 2% of total body mass, it contains ≈ 25% of the total unesterified cholesterol.96 This may be of importance in AD given findings that individuals experiencing cognitive impairment (either due to MCI or manifest AD) demonstrate up to 30% reduction in ABCA1-mediated cholesterol efflux into the CSF compared to cognitively intact controls.97 Interestingly, this parallels earlier reports suggesting that patients experiencing AD or vascular dementia demonstrate elevated levels of the plasma oxygenated 24S-hydroxycholesterol (cerebrosterol). This may suggest that enhanced flux of this metabolite across the blood-brain barrier is associated with dementia severity.98 Crucially, oxysterols of CNS origin can cross the blood-brain barrier and enter the plasma, and studies in humans have confirmed that most of the oxysterol that appears in peripheral circulation comes from the brain.96,99 Underscoring our systems-level approach to AD metabolomics, it is worth noting that oxysterols such as cerebrosterol represent a key biosynthetic intermediate bridging sterol and primary bile acid synthesis. Bile acids may be altered in AD as a function of cognitive impairment, reflect the role of brain-gut axis dyshomeostasis in the disease, and suggest a broader association of this metabolite class with dementia-associated neuroinflammatory processes.100,101

Although peripheral dyslipidemia and poor metabolic control are associated with dementia risk,102 there is paradoxically little correspondence between and relatively low turnover of CNS relative to peripheral cholesterol (ie, most CNS cholesterol is synthesized de novo).99 Interestingly, APOE genotype is itself associated with efficiency of cholesterol efflux, with the APOE ε4 AD risk allele producing
the greatest reduction and promoting the formation of lipid-depleted apoE lipoprotein complexes in CSF.55,97,103 Although it is possible that APOE-driven LOAD risk is principally mediated through effects on amyloid processing and deposition,104 it is also feasible that direct effects of apoE-associated lipid trafficking may impact the demencing brain.105 This is perhaps particularly relevant, given that CNS tissues are metabolically heterogeneous (both macro-anatomically and with respect to cell type) and may experience suboptimal provisioning of lipids (eg, cholesterol, phospholipids) with AD progression in a cell-type or regionally dependent manner. It is thus possible that constraints upon lipoprotein (eg, apoE, apoJ/Clusterin) mediated lipid trafficking are of substantial importance within the demencing brain, perhaps compounding other core metabolic deficits (ie, brain glucose hypometabolism) well established as features of early AD in both euploid and DS populations.15,16,106–109 Moving forward, it will be of great importance to better understand how core AD neuropathology such as plaques and NFTs are differentially impacted by lipid dyshomeostasis and metabolic/bioenergetic stress.110

5.2 Systemic metabolic alterations in DS-AD: evidence beyond the brain

One limitation of the present study is that the untargeted metabolomic (ie, LC-MS) approaches used do not permit unambiguous chemical identification of individual dementia-associated metabolites in peripheral blood.49,111 Nonetheless, our statistical approaches to better delineate disease-associated signals within the plasma metabolome and characterize systems-level biochemical alterations revealed several metabolic pathways (ie, lipid/fatty acid and central carbon metabolism) which demonstrated a significantly overrepresented number of differentially expressed LC-MS features. Potentially representing high-value druggable targets, these findings remain challenging to interpret.

It is noteworthy that recent work in AD metabolomics has attempted to link disease-associated alterations within the peripheral blood metabolome to corresponding alterations in the CNS metabolome,55,112 thereby grounding peripheral blood biochemical alterations as surrogate biomarkers for CNS lipid abnormalities. Due to the extensive, "connective" role of the cardiovascular system, it is, however, unclear if these blood plasma perturbations uniquely reflect brain pathology, as opposed to pathological alterations of other organ systems. In recent years, AD has been increasingly understood as a metabolic disease,63 necessarily implicating structures beyond the CNS. This is certainly applicable to DS, which is marked by substantially elevated, genetically penetrant risk for both AD and type 1 diabetes.113–115 It is similarly reflected in the variety of biomolecules recently implicated in AD via metabolomics approaches. In many cases, while these molecules possess currently underappreciated roles within the CNS, their conventional roles in peripheral, metabolically active tissues remain physiologically better understood. It will be important for future work in adults with DS-AD to better contextualize the heterogeneity of these molecules and implicated physiological processes with respect to core AD pathophysiology. Rather than simply reflecting an imperfect signal of brain metabolism, the peripheral plasma metabolome may instead index broader metabolic derangement associated with disease progression.116–128 To the extent that this suggests interventional targets and effective biomarkers, this may prove to be an advantage rather than a shortcoming of peripheral metabolomic approaches within DS-AD.

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CONFLICTS OF INTEREST

MM and AKC are listed as inventors on issued and pending patents related to material in this manuscript and assigned to Georgetown University. The terms of this arrangement have been reviewed and approved by the University of California, Irvine in accordance with its conflict of interest policies. Remaining authors declare no competing financial interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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