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FEATURED ARTICLE

Predictive metabolic networks reveal sex- and *APOE* genotype-specific metabolic signatures and drivers for precision medicine in Alzheimer's disease

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Abstract

Introduction: Late-onset Alzheimer's disease (LOAD) is a complex neurodegenerative disease characterized by multiple progressive stages, glucose metabolic dysregulation, Alzheimer's disease (AD) pathology, and inexorable cognitive decline. Discovery of metabolic profiles unique to sex, apolipoprotein E (*APOE*) genotype, and stage of disease progression could provide critical insights for personalized LOAD medicine.

Methods: Sex- and *APOE*-specific metabolic networks were constructed based on changes in 127 metabolites of 656 serum samples from the Alzheimer's Disease Neuroimaging Initiative cohort.

Results: Application of an advanced analytical platform identified metabolic drivers and signatures clustered with sex and/or *APOE* ε4, establishing patient-specific

A complete listing of ADNI investigators can be found at: http://adni.loni.usc.edu/wp-content/uploads/how_to_apply/ADNI_Acknowledgement_List.pdf

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biomarkers predictive of disease state that significantly associated with cognitive function. Presence of the *APOE* ε4 shifts metabolic signatures to a phosphatidylcholine-focused profile overriding sex-specific differences in serum metabolites of AD patients.

Discussion: These findings provide an initial but critical step in developing a diagnostic platform for personalized medicine by integrating metabolomic profiling and cognitive assessments to identify targeted precision therapeutics for AD patient subgroups through computational network modeling.

KEYWORDS
Alzheimer's Disease Neuroimaging Initiative, apolipoprotein E ε4, computational systems biology, late-onset Alzheimer's disease, metabolic biomarkers, metabolic network, metabolomics, precision medicine, sex-specific metabolic changes

Abbreviation

alpha-AAA	alpha-amino adipic acid
Aβ	amyloid beta
$A\beta_{1-42}$	amyloid beta peptide 1-42
AD	Alzheimer's Disease
ADAS-Cog	The Alzheimer's Disease Assessment Scale–Cognitive Subscale
ADNI	Alzheimer's Disease Neuroimaging Initiative
ADNI_MEM	Alzheimer's Disease Neuroimaging Initiative Memory Test Score
ADNI_EF	Alzheimer's Disease Neuroimaging Initiative Executive Function Test Score
aMCI	amnestic mild cognitive impairment
ANOVA	Analysis of variance
APOE	apolipoprotein E
APOEε4	ε4 allele of the Apolipoprotein E (APOE)
APOEε4-	APOEε4 negative/non-carrier
APOEε4+	APOEε4 positive/carrier
AUC	area under the curve
BCAA	branched chain amino acid
BMI	Body mass index
BN	Bayesian network
C7-DC	Pimelylcarnitine
CI	confidence interval
CN	cognitively normal
CSF	cerebrospinal fluid
DE	Differential Expression
Dx	disease diagnosis
FDR	false discovery rate
FDG-PET	fluorodeoxyglucose-positron emission tomography
LOAD	Late-onset Alzheimer's disease
lyso-PC	Lysophosphatidylcholine
MCI	mild cognitive impairment
p-tau	hyperphosphorylated-Tau protein
PC	phosphatidylcholine
SM	sphingomyelin
XGBoost	eXtreme Gradient Boosting

1 | BACKGROUND

Alzheimer's disease (AD) is a progressive neurodegenerative disorder without a cure. Recent clinical trial failures targeting amyloid beta (Aβ) or hyperphosphorylated tau protein (p-tau) underscore the importance of understanding disease-driving mechanisms. The primary risk factors of late-onset AD (LOAD), the predominant form, include age, female sex, and the presence of the apolipoprotein E (*APOE*) ε4 allele.[1,2] LOAD is a multifactorial disorder with perturbations in glucose and insulin signaling, energy and lipid homeostasis, mitochondrial function, oxidative stress, inflammation, and neurotransmission.[3,4] Recent progress in dissecting sex-specific mechanisms of AD has become possible through the implementation of systems-level approaches and availability of clinically characterized samples including Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort tissue and biofluids samples from AD patients and cognitively normal (CN) individuals enabling large-scale multi-omics studies. Metabolomics is the newest omics that measures thousands of metabolites reflecting alterations in genetic, transcriptomic, proteomic profiles, and influences from the environment.[5,6] A large number of studies using metabolomics and lipidomics platforms has provided new biochemical insights about disease mechanisms and early changes in disease, and provided support that peripheral metabolic changes inform about central changes and ATN (cerebrospinal fluid [CSF] $A\beta_{1-42}$ [A]; CSF p-tau [T]; fluorodeoxyglucose positron emission tomography [FDG-PET; N]) markers of disease.[7–20]

We recently conducted stratified linear regression analyses of serum metabolites from 1517 ADNI participants to determine the association of metabolic signatures with disease diagnosis (Dx) and ATN biomarkers.[14] Changes in metabolites associated with the Dx or ATN were influenced by sex and *APOE* and related to altered energy homeostasis.[14] We applied a recently developed computational predictive network model[21–24] to construct sex- and *APOE*-specific metabolic networks of CN and LOAD patients from the ADNI cohort with respect to sex and genotype and to clinical diagnosis cognitive parameters. We confirmed previous findings, demonstrated that metabolic panels associate with cognitive assessment, and identified metabolic drivers of LOAD. These findings further support the application of blood-based metabolomics as a precision medicine

tool for disease stage profiling, prognosis, and identification of novel therapeutic targets.

2 | METHODS

2.1 | Participants

Figure 1 and Table 1 summarize information on ADNI participants used in this study.

2.2 | Metabolomics data acquisition, normalization, and covariate adjustment

Metabolomics data normalization followed a six-step procedure[11] (Figure 1B, Method-1 in supporting information). The final 127 metabolites are listed in Table S1 in supporting information, and the final 362 CN and 294 AD samples were stratified into eight groups based on sex and *APOE* genotype (Table S2 in supporting information).

2.3 | Predictive network modeling

For each patient group, metabolites of AD and CN subjects were integrated with Dx into the predictive network modeling pipeline.[22–25] The network model consists of metabolites and Dx as nodes and causal interactions between them (Figure 2, Method-2 in supporting information).

2.4 | Empirical non-parametric bootstrap and consensus network analysis

For each patient group, the 95% confidence interval (CI) of each edge was evaluated with the empirical bootstrap method (Method-3 in supporting information). To derive the patient-specific consensus metabolic networks, we included top 10% of edges with 95% confidence per patient group (Table S3 in supporting information). The metabolic signature was extracted as the three-step upstream subnetwork of Dx in the patient-specific network (Table S4 in supporting information).

2.5 | Evaluation of heterogeneity of key drivers

The heterogeneity of key drivers was evaluated by calculating the significance of robustness and confidence of patient-specific key driver in each patient group (Table S5 and Method-4 in supporting information).

2.6 | Differential expression analysis

After covariate adjustment, metabolites were subjected to *t*-test using Limma R package[26] between AD and CN samples in each group (Figure 3; Figure S1 and Table S6 in supporting information).

RESEARCH IN CONTEXT

1. **Systematic Review**: Literature was reviewed using PubMed. Current findings on the effects of sex and apolipoprotein E (*APOE*) genotype on metabolic signature in Alzheimer's disease (AD) are heterogeneous. Replication using mouse models are missing. As metabolic data are susceptible to systemic perturbation and random noise, regression methods are often underpowered to detect significant associations with AD in a stratified analysis limited by small sample size.
2. **Interpretation**: By applying a systems biology approach to Alzheimer's Disease Neuroimaging Initiative data, we discovered clear metabolic differences in sex, *APOE* genotypes, and their interactions. We identified upstream metabolic drivers (potential therapeutic targets) to shift disease trajectory in each patient subgroup. We discovered *APOE* ε4 genotype shifts metabolic signature and drivers to a phosphatidylcholine-focused profile that overrides sex-specific differences in serum metabolites of AD patients. We identified patient-specific metabolic panels associated with diagnosis and cognitive performance. Our findings provide an initial but critical step in developing personalized precision medicine for AD.
3. **Future Directions**: Further validation on independent cohorts and translational models are necessary to incorporate metabolic drivers and biomarker panels into clinical practice and the drug discovery pipeline.

2.7 | Machine learning model and feature selection

To derive a biomarker panel for each patient group, we used a two-step machine learning procedure consisting of quantifying the feature importance in the first step followed by training elasticnet and XGBoost models to select a subset of features from input features (Method-5 in supporting information). To evaluate the prediction accuracy of every patient-specific panel, we performed five-fold cross-validation in each group and repeated 100 times. The prediction performance was evaluated by calculating the averaged area under the curve (AUC).

2.8 | Biomarker association with clinical features

For each biomarker panel, we extracted principal components that explained > 90% of the variance in data. The response variables (clinical cognitive test scores) were regressed on these principal components, and analyses of variance with F-statistics were used to calculate the fitness of regression. Multiple testing was adjusted by calculating the false discovery rate (FDR) value and significance reported based on FDR < 0.05 (Table S8 in supporting information).

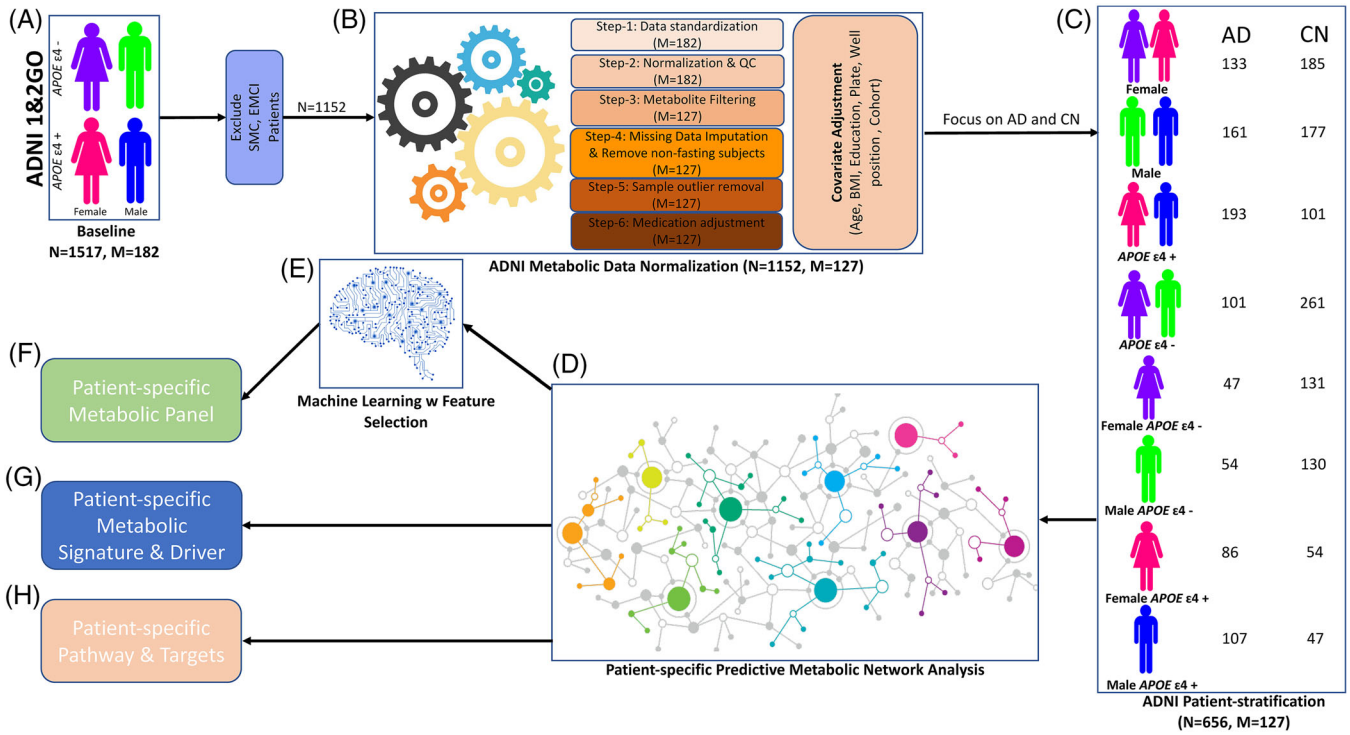

FIGURE 1 Analytical pipeline used in the study. The analytical pipeline included 1152 samples from the ADNI cohort. Patients with SMC and EMCI were removed leaving 1152 samples from AD, LMCI, and CN (A). Data were normalized; the residuals were obtained after covariate adjustment (B). Three hundred sixty-two CN and 294 AD samples were stratified into eight groups based on sex and *APOE* genotype (C). A predictive network model was built (D) to derive patient-specific metabolic signatures and drivers of progression from CN to AD in each group (G). The DE analysis identified significant changes in metabolites. Metabolic biomarker panels (F) were derived using machine learning models (E). Patient-specific pathways were identified based on metabolic signatures and drivers (H). AD, Alzheimer disease; ADNI, Alzheimer's Disease Neuroimaging Initiative; ADNI 1&2GO, phase 1 and phase 2/GO of ADNI; *APOE*, apolipoprotein E; BMI, body mass index; CN, cognitively normal; DE, differential expression; EMCI, early mild cognitive impairment; LMCI, late mild cognitive impairment; M, the number of metabolites; N, the number of participants; QC, quality control; SMC, subjective memory complaint

3 | RESULTS

3.1 | Sex- and *APOE*-specific consensus metabolic networks identify distinct metabolic signatures and drivers of LOAD

The analytical pipeline used in the study is presented in Figure 1. Consensus networks provide metabolic signatures defined as subnetworks containing metabolites within three steps upstream of Dx node. Metabolic key drivers are the immediate (1-step) metabolite(s) upstream of Dx node in each network (Figure 2, Table S4). To investigate the common metabolic signature of LOAD, we first built a background network by using 656 AD and CN samples without patient stratification (Figure 2A). This network identified changes in six phosphatidylcholines (PCs) with PC aa C36:6 as an immediate upstream driver regardless of sex or *APOE* genotype (Table S4A). The differential expression (DE) analysis confirmed significant changes in thirteen PCs, three sphingomyelins (SMs), four acylcarnitines, and citrulline (Table S6A).

To reveal sex-specific differences, we built consensus metabolic networks using residuals of 161 AD and 177 CN males and 133 AD and 185 CN females. The male consensus network (Figure 2B) identified changes in amino acids valine, isoleucine, lysine, and tryptophan mediated by alpha-amino adipic acid (alpha-AAA; Table S4B). The DE analysis confirmed increased levels of three acylcarnitines and a decrease in sarcosine, two PCs, and one sphingomyelin (SM; Table S6B). Levels of valine, a metabolite directly connected to alpha-AAA, were decreased by more than 7-fold ($P = 0.13$, Table S6B). The female consensus network (Figure 2C) was dominated by reduced levels of four PCs, one SM and tryptophan, and an increase in creatinine (Table S6C). These data suggest that AD was mainly associated with changes in amino acids in males, and PCs and tryptophan in females.

To define *APOE*-specific metabolic signatures, we built consensus networks using residuals of 193 AD and 101 CN *APOE* ε4+ and 101 AD and 261 CN *APOE* ε4−. The *APOE* ε4+ consensus network (Figure 2D) revealed a homogeneous signature of six PCs mediated by PC aa C34:4 (Table S4D). The DE analysis confirmed significant changes in four PCs (Table S6D). The *APOE* ε4− consensus network (Figure 2E) identified

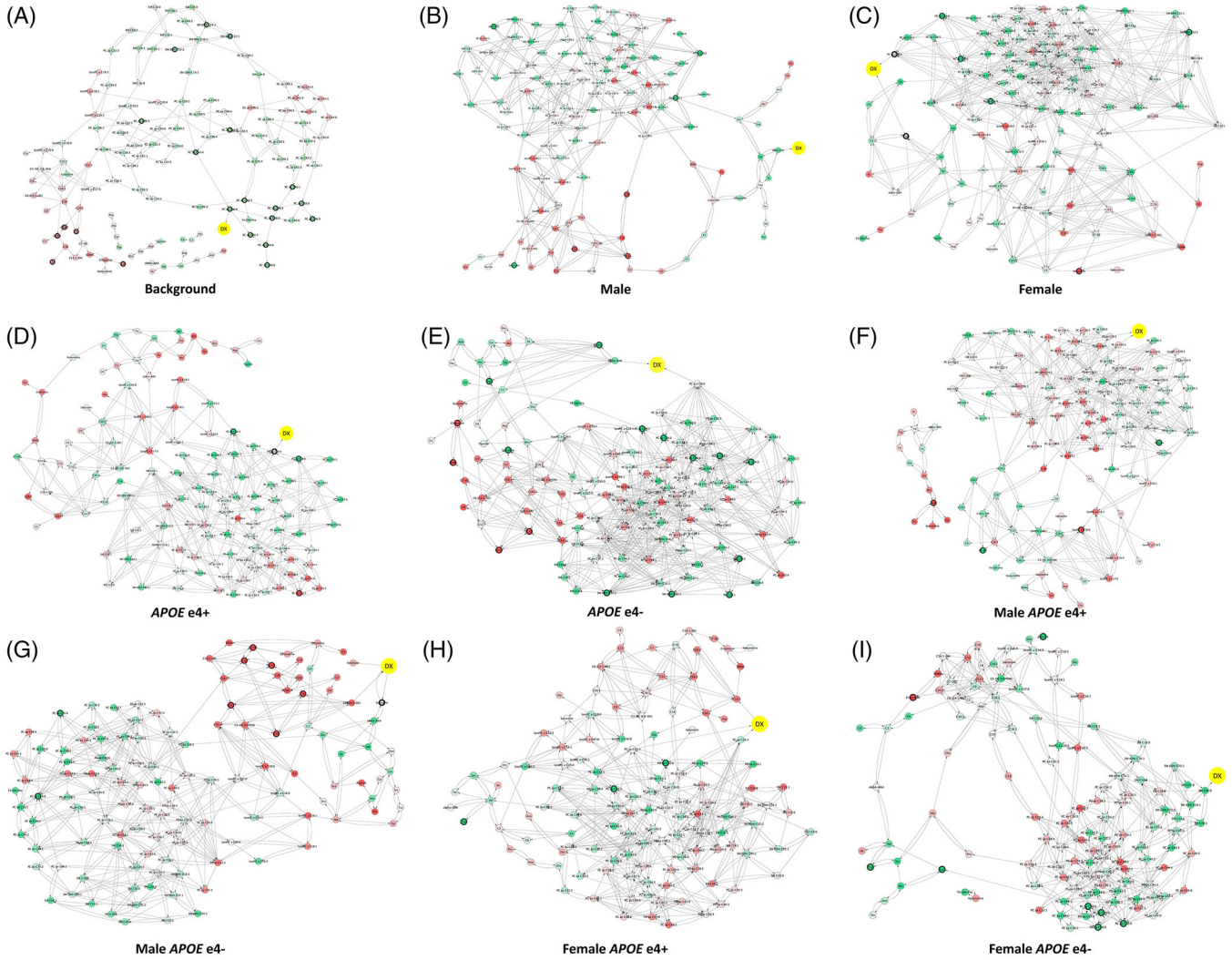

FIGURE 2 Sex- and apolipoprotein E (*APOE*)-specific consensus predictive metabolic network. To build consensus causal predictive metabolic network, we subsampled 100 datasets and constructed 100 metabolic networks per patient group. The 95% confidence interval is calculated per edge. The consensus network models were used to identify the upstream metabolites and pathways associated with Alzheimer's disease (AD) in background with all 656 AD and cognitively normal (CN) samples (A), males (B), females (C), *APOE* ε4+ (D), *APOE* ε4– (E), male *APOE* ε4+ (F), male *APOE* ε4– (G), female *APOE* ε4+ (H), female *APOE* ε4– (I). Red color indicates metabolites metabolite level is increased in AD compared to CN; green color indicates metabolite level is decreased in AD compared to CN. Significant differential expression metabolites are indicated with black circles

a mixed signature of valine, isoleucine, alpha-AAA, tryptophan, creatinine, lysine, proline, two acylcarnitines, and 26 PCs mediated by PC aa C38:0 and alpha-AAA (Table S4E). The DE analysis confirmed a significant decrease in nine PCs, four SMs, sarcosine, lysine, and valine, and an increase in creatinine and citrulline, three acylcarnitines, and a pro-inflammatory agent symmetric dimethylarginine (Table S6E). These results demonstrate that the *APOE* ε4 allele specifically affects the metabolism of PCs.

Next, we built male *APOE* ε4+, male *APOE* ε4–, female *APOE* ε4+, and female *APOE* ε4– consensus networks by using 107/47, 54/130, 86/54, and 47/131 AD/CN residuals, respectively (Figure 2F-2I). The male *APOE* ε4+ network (Figure 2F) identified a homogeneous signature of 22 PCs and 5 SMs (Table S4F). The DE analysis revealed significant decreases in taurine and carnitine C7-DC, and an increase in asparagine and lyso-PC a C18:0. While not statistically significant, levels of alanine and lysine decreased by more than 8- and 3-fold, respectively, whereas levels of glycine, threonine, ornithine, and glutamate increased by more than 20-, 5-, 3-, and 3-fold, respectively. The male *APOE* ε4– consensus network (Figure 2G) identified changes in ten acylcarnitines, five amino acids, and three lyso-PCs (Table S4G). A decrease in sarcosine and two PCs and an increase in six acylcarnitines were significant in LOAD compared to CN. While not significant, levels of branched chain amino acids (BCAA) valine and isoleucine and amino acids lysine, glutamate, isoleucine, and arginine were decreased while levels of citrulline, glycine, creatinine, alanine, and taurine were increased from 2- to 13-fold (Table S4G).

The female *APOE* ε4+ consensus network (Figure 2H) identified a PC-dominant signature (Table S4H). DE analysis revealed significantly decreased PCs and essential amino acid L-tryptophan. Levels of alanine and lysine decreased by more than 18- and 6-fold,

TABLE 1 Characteristics of the 1152 ADNI subjects in this study

Demographics	CN	LMCI	AD
Sample size	362	496	294
Sex (M/F)	177/185	307/189	161/133
Age(yr.)	74.61(+/-5.66)	74.11(+/-7.57)	74.71(+/-7.85)
BMI (kg/m^2)	27.04(+/-4.51)	26.49(+/-4.32)	25.87(+/-4.71)
Education(yr.)	16.20(+/-2.79)	15.86(+/-2.91)	15.19(+/-2.99)
***APOE*ε4 +/-**	101/261	265/231	193/101
Clinic Assessment			
ADAS-Cogtotal score	5.99(+/-3.04)	11.56(+/-4.50)	19.34(+/-6.75)
Memory function (ADNI_MEM)	0.95(+/-0.53)	−0.05(+/-0.57)	−0.73(+/-0.52)
Executive function (ADNI_EF)	0.74(+/-0.70)	0.014(+/-0.78)	−0.82(+/-0.84)
CSF Pathology			
CSF p181-Tau	25.54(+/-14.80)	35.36(+/-17.36)	41.64(+/-19.63)
CSF Aβ1-42	207.67(+/-54.47)	163.48(+/-53.57)	143.64(+/-41.86)

Notes: Metabolomics datasets from the Biocrates p180 platform used in the current analyses for the ADNI-1 and ADNI-GO/2 cohorts are available via the Accelerating Medicines Partnership-Alzheimer's Disease (AMP-AD) Knowledge Portal and can be accessed at https://doi.org/10.7303/syn5592519(ADNI-1) and https://doi.org/10.7303/syn9705278(ADNI GO-2). The full complement of clinical and demographic data for the ADNI cohorts are hosted on the LONI data sharing platform and can be requested at http://adni.loni.usc.edu/data-samples/access-data/.

Abbreviations: AD, Alzheimer's disease; ADAS-Cog, Alzheimer's Disease Assessment Scale–Cognitive Subscale; ADNI, Alzheimer's Disease Neuroimaging Initiative; *APOE* ε4−/+: non-carriers and carriers of the *APOE* ε4 allele; BMI, body mass index; CSF Aβ1-42: Cerebrospinal fluid amyloid beta 1–42 protein. CSF p181-Tau, Cerebrospinal fluid phosphorylated tau protein at threonine 181 (p181tau); CN, cognitively normal; LMCI, late mild cognitive impairment; yr., years.

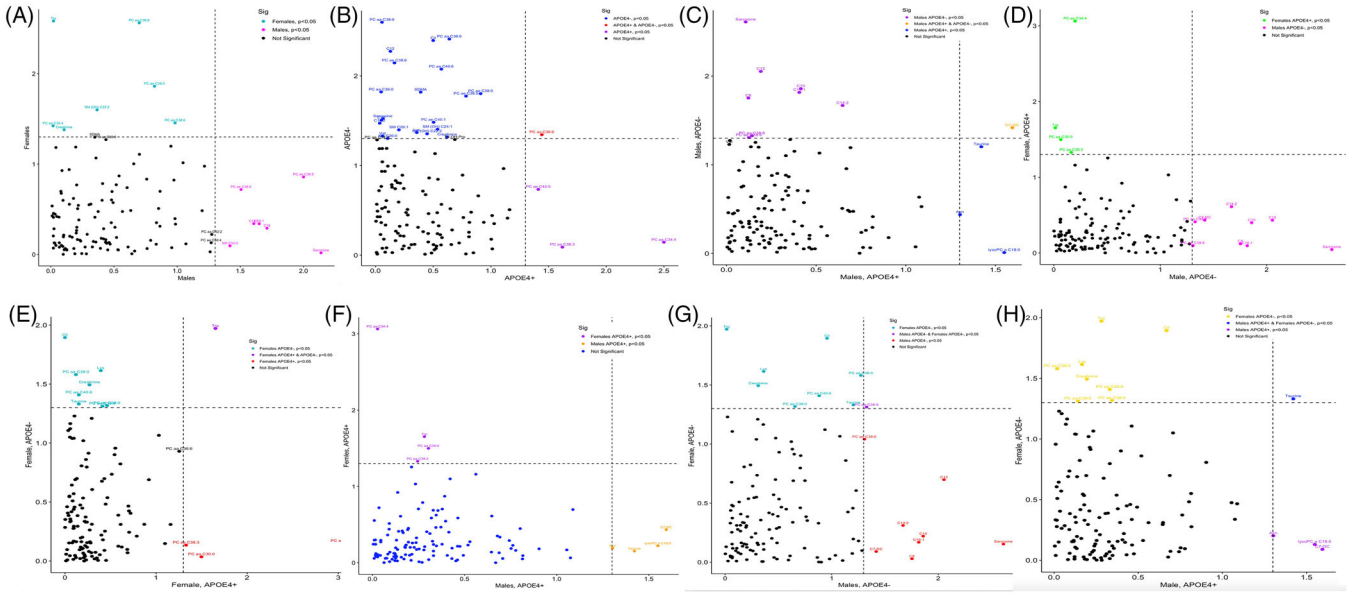

FIGURE 3 Sex- and apolipoprotein E (*APOE*)-specific metabolic differential expression analysis. The significant *P*-value < 0.05 of differentially produced metabolites are compared between patient groups to illustrate the specificity and commonality of Alzheimer's disease (AD)-associated metabolic signatures to sex and *APOE* genotype. (A) male vs. female; (B) *APOE* ε4+ vs. *APOE* ε4−; (C) male *APOE* ε4+ vs. male *APOE* ε4−; (D) male *APOE* ε4− vs. female *APOE* ε4+; (E) female *APOE* ε4+ vs. female *APOE* ε4−; (F) male *APOE* ε4+ *vs* female *APOE* ε4+; (G) male *APOE* ε4− vs. female APOE ε4+; (H) male *APOE* ε4+ vs. female *APOE* ε4−

respectively, whereas glycine, proline, and arginine increased by more than 6-, 4-, and 4-fold, respectively, though not statistically significantly. In female *APOE* ε4− carriers, the consensus network discovered a mixed signature of eight SMs and three PCs (Figure 2I, Table S4I). The DE analysis revealed significantly decreased four PCs and amino acids L-tryptophan, taurine, lysine, as well as significantly increased citrulline and creatine. A summary for each group is presented in Table 2.

3.2 | Heterogeneity in patient group–specific metabolic key drivers

The consensus network analysis revealed distinct patterns of homogeneity/heterogeneity in the metabolic signatures within each patient group. While this network captures the most robust signal relative to all individuals in each patient group, it doesn't address the endogenous metabolic heterogeneity associated with different subpopulations within the same group. To further investigate inherent metabolic heterogeneity, we calculated the significance (FDR adjusted) of robustness and confidence for every key driver in each group (Table S5). Robustness of metabolic drivers was determined based on their connection to Dx with positive 95% CI, significant robustness, and confidence. Despite multiple potential metabolic drivers identified in each subpopulation group, the following metabolites robustly connected to Dx: males: alpha-AAA; females: PC aa C36:6 and tryptophan; *APOE* ε4+: PC aa C34:4; *APOE* ε4−: PC aa C38:0, alpha-AAA, and serotonin; male *APOE* ε4+: PC ae C36:3 and PC aa C40:2; male *APOE* ε4−: C6 and sarcosine; female *APOE* ε4+: PC aa C34:4, PC ae C36:4, and L-tryptophan; and female *APOE* ε4−: SM C26:0 (Figure 4, Table 2, Table S5).

3.3 | Metabolic network cross-validation using sex- and *APOE*-specific biomarker panel

To validate metabolic networks and key drivers, we trained an ensemble of machine learning models to select a subset of metabolites based on the network model and drivers with changes significantly associated with the disease state in each patient group. The prediction performance was evaluated with averaged AUC by cross-validation in the ADNI data (Figure 5). In each patient group, we trained different models and compared AUCs of each model to eight sets of input features: Set 1—all 127 metabolites; Set 2—significant DE metabolites; Set 3—network-derived metabolites; Set 4—combination of all 127 metabolites plus age, education, body mass index (BMI); Set 5—significant DE metabolites plus demographics; Set 6—network-derived metabolites plus age, education length, BMI; Set 7—network-derived metabolites plus significant DE metabolites; Set 8—network-derived metabolites plus significant DE metabolites and age, education, BMI. The network-derived metabolites were extracted from the neighbor (within three-step undirected) subnetwork of the Dx node in respective networks.

We found that the prediction accuracy (AUC) of the network-derived metabolites (Set 3, Figure 5 green line) robustly and significantly outperformed those predicted by using all metabolites in the data (Set 1, Figure 5 black line) and only significant ($P < 0.05$) DE metabolites derived from the linear regression model (Set 2, Figure 5 purple line) across all patient groups. Adding patient demographics to Sets 1 and 3 greatly improved individual prediction accuracy. However, the same pattern was observed in their relative accuracy, that is, the AUC produced by the network-derived metabolite with patient demographics (Set 6, Figure 5 orange line) consistently outperformed the accuracy predicted by adding demographics to either all metabolites in the data (Set 4, Figure 5 pink line) or only significant ($P < 0.05$) DE metabolites (Set 5, Figure 5 blue line) across all patient groups. When significant DE metabolites were added to the combination of network-derived features with patient demographics (Set 8, Figure 5 red line), a marginal improvement in AUC was observed in all *APOE* ε4−, female *APOE* ε4+, and female *APOE* ε4− groups, with a slight decrease in AUC observed in males, male *APOE* ε4+, male *APOE* ε4−, suggesting that significant DE metabolites derived from linear regression added no further power in prediction of Dx given the network structure and patient demographics. Data suggest that the metabolic network-derived features with or without age, BMI, and education significantly improved the prediction accuracy compared to the other feature sets, DE metabolites, and all 127 metabolites in the data, across all patient groups. These data indicate that the predictive network model used in this study is more sensitive than a traditional regression method in detecting weaker relations of metabolic changes with disease state in sex- and *APOE*-specific patient groups. The best performing feature set with or without demographics, respectively, was selected for each group as the biomarker panel (Table S7 in supporting information).

3.4 | Blood-based metabolic biomarker panels associate with cognitive decline

To evaluate the association of selected biomarker panels with clinical cognitive assessment (diagnosis, Alzheimer's Disease Assessment Scale–Cognitive subscale [ADAS-Cog] score, memory, and executive function), we calculated the eigen expression to recapitulate the primary variance component (the first principal component) for each panel and fitted a linear regression model between the eigen expression of the first principal component and cognitive measures. Significance of association is shown in Figure 5I (Table S8). Network-derived biomarker panels for each patient group with or without demographics were all significantly associated with the Dx. Of 16 panels (Table S7, two selected panels per group times eight patient groups), 13 and 15 panels were significantly associated with memory (ADNI_MEM) and the overall cognition (ADAS-Cog Total Score), respectively. Of 16 panels, 10 were significantly associated with the executive function composite score (ADNI_EF). Two biomarker panels approached statistical significance with the executive function composite score: network-derived features with demographics for male *APOE* ε4+ (FDR = 0.0667) and network-derived features

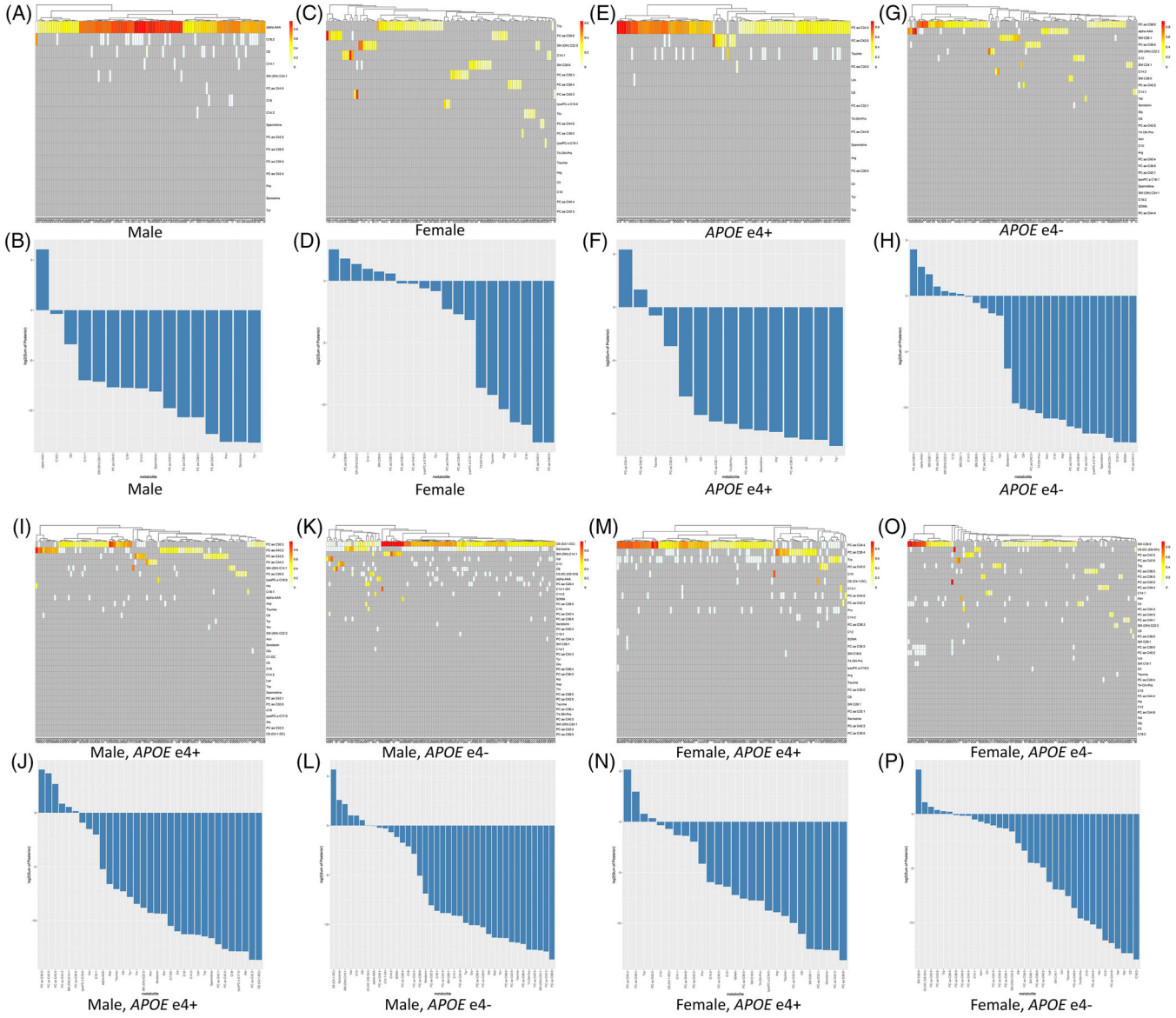

FIGURE 4 Sex- and apolipoprotein E (*APOE*)-specific metabolic heterogeneity. In each group, the confidence and robustness of metabolic drivers were shown in the heatmap where the X-axis represents 100 different networks and Y-axis represents candidate key drivers in 100 networks. Each row in the heatmap represents a vector of 100 posterior probability values of the edge from a key driver to diagnosis derived from 100 networks, and the bar plot is ranked based on the log2 of the sum of the 100 posterior values per key driver in male (A,B), female (C,D), *APOE* ε4+ (E,F), *APOE* ε4− (G,H), male *APOE* ε4+ (I,J), male *APOE* ε4− (K,L), female *APOE* ε4+ (M,N), female *APOE* ε4− (O,P)

without demographic for female *APOE* ε4+ (FDR = 0.0678). Two biomarker panels approached statistical significance with overall cognition score: network-derived features with (FDR = 0.0655) and without (FDR = 0.0667) demographics. These results indicate that patient-specific metabolic networks and network-derived biomarker panels are associated with clinical cognitive assessment.

4 | DISCUSSION

Using an advanced computational method, we demonstrated that LOAD is associated with metabolomic profiles defined by sex and *APOE* genotype. Based on patient group–specific network models, we identified key drivers, differentially produced metabolites, and metabolic signatures of the disease. Unstratified analyses identified changes in lipid homeostasis, with carnitines, PCs, and SMs as most affected metabolites that differentiated AD from CN. Further stratification by sex revealed that metabolic changes in AD males were associated with amino acids while lipids remained predominantly affected in females. Stratification by sex and *APOE* did not affect lipid-dominant metabolic signatures in females while in *APOE* ε4+ males, metabolic drivers and signatures changed from amino acids, especially BCAAs, to lipids comparable to *APOE* ε4+ females. In *APOE* ε4− males and females, metabolic changes were more diverse compared to *APOE* ε4+ and

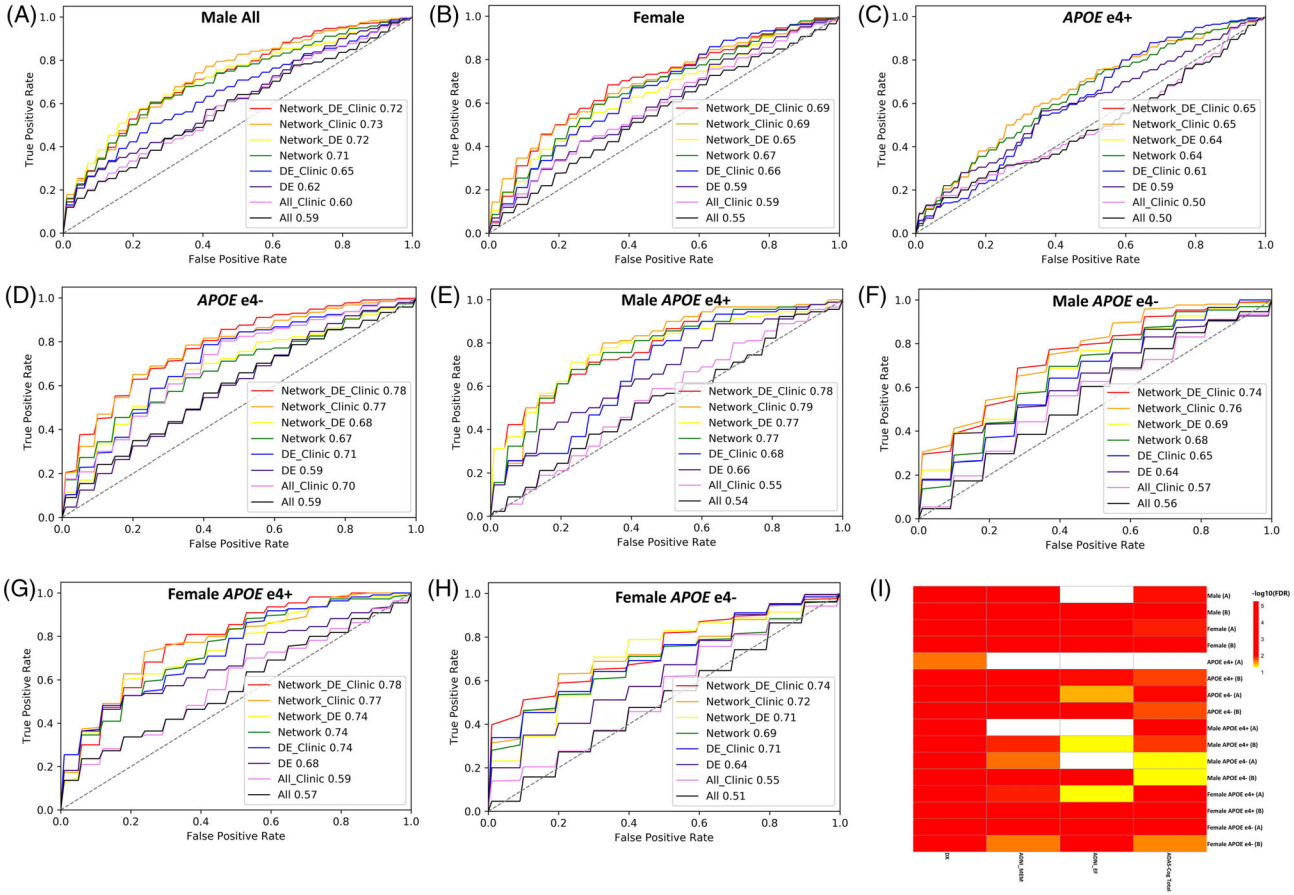

FIGURE 5 Biomarker panel and cross-validation accuracy for Alzheimer's disease (AD) diagnosis. The prediction performance of diagnostic biomarker panels derived from different sets of features are compared in each patient group. The number in the figure represents the averaged cross-validation area under the curve with eight feature sets respectively in male (A), female (B), apolipoprotein E (*APOE*) ε4+ (C), *APOE* ε4− (D), male *APOE* ε4+ (E), male *APOE* ε4− (F), female *APOE* ε4+ (G), male *APOE* ε4− (H); All, all 127 metabolites in the data; All Clinic, all 127 metabolites in the data combines with age, body mass index (BMI) and/or education; differential expression (DE): significant DE metabolites; DE Clinic: significant DE metabolites combined with age, BMI, and/or education; Network: biomarkers derived from metabolic network; Network_DE: biomarkers derived from the combination of significant DE and metabolic network; Network_DE_Clinic: biomarkers derived from combination of significant DE metabolites, metabolic network and age, BMI, and/or education. Network_Clinic: biomarkers derived from metabolic network and age, BMI, and/or education; (I) Two selected optimal biomarker panel association with clinical assessment and cognitive decline: The association of the two selected biomarker panel with and without age, BMI, and education in each patient group (A: biomarker pane derived from metabolic network; B: biomarker pane derived from combination metabolic network plus age, BMI, and/or education) with diagnosis (Dx) and clinical assessments (Alzheimer's Disease Assessement Scale-Cognitive subscale Total Score, memory function [ADNI_MEM] and executive function [ADNI_EF])

included lipids and amino acids. The identified metabolic alterations are consistent with previous reports in which changes in blood levels of PCs, SMs, acylcarnitines, ceramides, and amino acids differentiated mild cognitive impairment (MCI) and AD from CN.[11,14,18,19,27–30]

In addition to replicating sex-specific differences in serum metabolites associated with AD,[14] our analyses also generated multiple novel and important findings by identifying metabolic signatures and key drivers in patient groups stratified by the intersection of sex and *APOE* genotype, which has not been previously reported. We demonstrate the following. (1) Previously observed amino acid–centered metabolic signatures and drivers in AD males are true for males without *APOE* ε4. (2) Metabolic signatures and drivers for *APOE* ε4+ males shifted from amino acids to lipids (PCs and SMs) similar to changes observed in *APOE* ε4+ females. This important finding highlights the ability of *APOE* ε4 genotype to significantly influence metabolic changes overriding sex-specific differences observed in serum metabolites in AD males and females. (3) The metabolic shift is more subtle between *APOE* ε4+ and *APOE* ε4− females, which is confined to different lipid species, that is, the shift from a SM-dominant metabolic signatures and drivers in *APOE* ε4− to a PC-dominant signatures and drivers in *APOE* ε4+. Overall, our novel findings indicate that *APOE* ε4 genotype drives metabolic signature to a PC-focused profile regardless of the patient sex.

The replicative validity and novelty of our findings emphasizes the importance of these metabolic pathways for AD. We identified changes in numerous individual lipids, including PCs and lysoPCs, as key drivers or components of metabolic signatures associated with cognition. PCs and SMs are integral constituents of the plasma membrane. The reduced PC levels observed in AD may reflect abnormal

TABLE 2 Summary of sex- and *APOE*-specific metabolic signature, key drivers, and DE metabolites in each patient group

A. Overall.DE	Overall.KD	Overall signature
C12↑	PC aa C36:6	PC aa C36:6
C18↑		PC ae C38:0
C18:1↑		PC aa C34:4
C18:2↑		PC ae C40:6
Citruline↑		PC aa C30:0
PC aa C34:4↓		PC aa C38:4
PC aa C36:0↓		
PC aa C36:5↓		
PC aa C36:6↓		
PC aa C38:0↓		
PC aa C38:3↓		
PC aa C38:6↓		
PC aa C40:6↓		
PC aa C42:6↓		
PC ae C36:5↓		
PC ae C38:0↓		
PC ae C38:6↓		
PC ae C40:1↓		
SM (OH) C22:1↓		
SM (OH) C22:2↓		
SM (OH) C24:1↓		
B. Male.DE	**Male.KD**	**Male.Signature**
C18↑	alpha-AAA	alpha-AAA
C18:1↑		Val
C18:2↑		Ile
PC ae C36:5↓		Lys
PC ae C38:6↓		Trp
Sarcosine↓		
SM C24:0↓		
C. Female.DE	**Female.KD**	**Female.Signature**
Creatinine↑	PC aa C36:6	PC aa C36:6
PC aa C34:4↓	Trp	Trp
PC aa C36:6↓		PC aa C30:0
PC aa C38:6↓		PC ae C38:0
PC ae C38:0↓		PC ae C40:6
SM (OH) C22:2↓		PC aa C34:4
Trp↓		PC aa C36:5
		PC aa C38:6
		Tyr
		C3
		Val
		PC ae C30:0

(Continues)

TABLE 2 (Continued)

C. Female.DE	Female.KD	Female.Signature
		PC aa C32:1
		PC aa C32:0
		PC aa C38:0
		PC aa C40:6
		PC ae C40:1
		PC ae C42:3
		PC ae C42:2
		PC aa C42:1
		PC ae C40:5
		PC ae C38:6
		PC aa C34:3
		PC aa C40:4
		PC aa C38:4
		PC aa C38:5
		PC ae C40:4
		Ala
		Asn
		C4
		alpha-AAA
		C0
		Ile
		Lys
D. Male.APOE e4+.DE	**Male.APOE e4+.KD**	**Male.APOE e4+.Signature**
Asn↑	PC ae C36:3	PC ae C36:3
C7-DC↓	PC aa C40:2	PC aa C40:2
lysoPC a C18:0↑		PC ae C34:3
Taurine↓		PC ae C34:2
		PC ae C34:1
		SM C16:0
		PC aa C42:2
		PC aa C40:3
		PC ae C32:1
		PC ae C30:0
		PC ae C36:4
		PC ae C36:2
		PC ae C38:3
		PC aa C32:0
		PC ae C34:0
		PC ae C36:1
		PC aa C36:1
		SM C24:1
		SM C24:0

(Continues)

TABLE 2 (Continued)

D. Male.APOE e4+.DE	Male.APOE e4+.KD	Male.APOE e4+.Signature
		PC ae C32:2
		PC ae C40:3
		SM C16:1
		SM (OH) C14:1
		PC aa C42:1
		PC ae C42:3
		PC ae C42:1
		PC ae C42:2
E. Female.APOE e4+.DE	**Female.APOE e4+.KD**	**Female.APOE e4+.Signature**
PC aa C30:0↓	PC aa C34:4	PC aa C34:4
PC aa C34:4↓	PC ae C36:4	PC ae C36:4
PC aa C38:3↓		PC aa C30:0
Trp↓		PC aa C34:3
		PC aa C36:6
		PC aa C40:4
		PC aa C38:5
		PC ae C34:3
		PC ae C36:5
		PC ae C36:3
		PC ae C38:5
		PC ae C38:4
		PC ae C30:0
		PC aa C32:1
		PC aa C32:0
		PC ae C34:0
		PC ae C38:0
		PC aa C40:6
		PC aa C36:5
		PC aa C38:6
		PC aa C38:3
		PC ae C42:1
		PC ae C40:4
		PC aa C40:5
		PC aa C38:4
		PC ae C40:1
		PC aa C42:5
		PC ae C38:6
		PC ae C32:2
		PC ae C32:1
		PC ae C34:2
		SM C16:0
		PC ae C34:1
		PC aa C40:3

(Continues)

TABLE 2 (Continued)

E. Female.APOE e4+.DE	Female.APOE e4+.KD	Female.APOE e4+.Signature
		PC ae C36:2
		PC ae C40:5
		PC ae C44:5
		SM (OH) C16:1
		C18
F. Male.APOE e4-.DE	**Male.APOE e4-.KD**	**Male.APOE e4-.Signature**
C10↑	C6 (C4:1-DC)	C6 (C4:1-DC)
C12↑	Sarcosine	Sarcosine
C14:2↑		alpha-AAA
C16:1↑		C8
C7-DC↑		C10
C8↑		C16:1
PC aa C38:6↓		C10:2
PC ae C38:6↓		C5-DC (C6-OH)
Sarcosine↓		C12
		C14:1
		C18:1
		lysoPC a C16:0
		lysoPC a C18:0
		C9
		lysoPC a C17:0
		Glu
		Val
		Ile
G. Female.APOE e4-.DE	**Female.APOE e4-.KD**	**Female.APOE e4-.Signature**
Citrulline↑	SM C26:0	SM C26:0
Creatinine↑		SM (OH) C22:1
Lysine↓		SM C26:1
PC aa C38:0↓		SM (OH) C24:1
PC aa C38:6↓		SM C24:0
PC aa C40:6↓		SM (OH) C22:2
PC ae C38:0↓		SM C24:1
Taurine↓		PC ae C40:3
Trp↓		PC ae C40:2
		SM (OH) C16:1
		PC ae C44:5

Abbreviations: *APOE*, apolipoprotein E; DE, differential expression.

membrane functions including synaptic transmission and processing of the amyloid precursor protein contributing to Aβ production.[31] Furthermore, alterations in PCs may contribute to increased inflammation, one of the underlying mechanisms of LOAD.[32,33] The panel of PCs and carnitines predicted the conversion from CN to AD/amnestic MCI with sensitivity and specificity of 90%[27,34] yielding improvements to

previous reports for which stratification was not used.[35–37] L-Carnitine and acylcarnitines play an essential role in energy metabolism transporting activated long-chain fatty acids into mitochondria for β-oxidation. They also mediate the metabolism of BCAAs, neuromodulation, antioxidant, and anti-apoptotic functions in the brain.[38,39] Consistent with our findings, changes in multiple carnitines (e.g., C12, C12:1, C14:1, and C8) contributed to discriminating AD from CN.[40–42] A recent study with the same metabolomics data conducted in both *ante mortem* blood and *post mortem* brain samples in two community-based longitudinal aging and dementia cohorts reported that decanoylcarnitine C10, pimelylcarnitine C7-DC, and tetradecadienylcarnitine C14:2 significantly predicted a lower AD risk after a 4.5-year follow-up, independent of age, sex, and education.[34] However, the most important changes in carnitines and amino acids detected in AD patients associate with sex-specific dysregulation of energy metabolism.[2,43]

Altered glucose uptake in the brain detected using FDG-PET occurs decades before onset of AD symptoms, suggesting that metabolic deficits are an upstream event specific to LOAD.[43,44] Thus, changes in carnitines, fatty acids, and amino acids, BCAA in particular, may indicate differential compensatory mechanisms for alternative energy substrates in AD males and females.[45,46] High levels of carnitines may indicate a buildup of fatty acids, suggesting increased energy demands coupled with impaired energy production via mitochondrial β-oxidation.[46] Male-specific metabolic signatures identified herein included alpha-AAA and BCAA valine and isoleucine. BCAAs are important energy carrying molecules associated with cognitive decline and brain atrophy in AD.[47] Changes in their levels could indicate a switch to increased energy consumption via degradation of amino acids. The biogenic amine alpha-AAA is a degradation product of lysine and is involved in mechanisms of neurotransmission.[48,49] Higher levels of serum alpha-AAA are associated with decreased cognitive function.[5] Consistent with previous observations, we detected positive associations of AD cognitive function with multiple amino acids, including tryptophan, citrulline, sarcosine, aspartic acid, and taurine.[5,11,14,18–20]

Our study has several limitations. First, the AbsoluteIDQ-p180 system is a targeted metabolomic platform with limited set of metabolites, including amino acids (21), biogenic amines (21), hexose (1), acylcarnitines (40), lysophosphatidylcholines (14), phosphatidylcholines (76), and sphingolipids (15). Use of this platform enabled a direct comparison of the results reported herein, generated using advanced analytical computational analysis, to previously generated findings using the same platform.[14] Our novel computational systems biology approach enabled findings that strongly support utility of the targeted metabolomic biomarker translational approach for individualized medicine. Future large-scale metabolomics analyses could provide greater detail to support the metabolic pathways reported herein while also identifying additional pathways. From a translational perspective the replication of affected pathways using the targeted metabolomic platform coupled with a novel systems biology computational approach enabled identification of sex-, APOE genotype-, and AD stage–specific phenotypes. These findings provide the foundation for personalized therapeutic interventions and simultaneously a biomarker strategy to determine target engagement and therapeutic efficacy. Second, the metabolic data are inherently susceptible to environmental influences and personal factors. Such variability is further amplified in stratified analyses like ours, where although starting with thousands of patients, stratification reduces group size resulting in smaller detection power. Thus, most of significant DE didn't survive multiple-testing correction. Therefore, potentially important findings could be missed by using conventional analytical methods such as DE and linear regression. We addressed this problem by using a more sensitive network model than conventional methods. Our network model exploited the conditional independence derived from the robust covariance structure to overcome the relatively small effect size in metabolic data due to random noise and small detection power due to reduced number of patients by stratification, which is not well handled by linear regression and correlation-based methods.[14] Herein, we demonstrated that our network approach is more robust and sensitive for detecting true associations over conventional methods. This may explain why previous studies have not discovered that *APOE* ε4 status overrides sex-specific difference in serum AD metabolites though a similar effect was observed in a recent study with humanized APOE mice.[30] Although our network approach to some extent can mathematically alleviate the issue of low detection power, the number of subjects in each group was relatively small and studies in larger longitudinal cohorts are warranted to confirm these results. Our Alzheimer's Disease Metabolomics Consortium (ADMC) is conducting comprehensive metabolic profiling across metabolomic platforms to provide broad biochemical coverage of the metabolome to map metabolic failures across trajectory of disease.

In summary, we provide a compelling systems biology analytical platform for metabolomics data analysis. We identified sex- and *APOE*-specific metabolic signatures associated with clinical diagnosis and cognitive assessment and key metabolic drivers that could be evaluated as therapeutic targets with a potential to shift the trajectory of the disease. The metabolic signatures and key drivers demonstrated clear metabolic differences in sex and *APOE* genotype and highlighted the potential of *APOE* ε4 genotype overriding sex difference in human serum metabolic associated with AD. In addition, we identified serum metabolic panels significantly associated with clinical diagnosis and cognitive assessment in each patient subgroup. This is the first study to establish patient-specific serum metabolic biomarkers predictive of disease diagnosis that significantly associated with clinical cognitive assessment for individual groups of patients stratified by sex and *APOE* genotype (Figure 5I, Table S9). Based on the biomarker panel of network-derived metabolites and demographic features, we identified that education attainment and BMI are two most common biomarkers shared by five out of eight patient groups, followed by tryptophan (four out of eight), a set of PCs (PC aa C42:6, PC ae C36:5, PC ae C40:2, PC ae C42:5, PC ae C36:0), and age (3 out of 8). Interestingly, we identified valine, creatinine, lysine, C16, SM C26:1, lysoPC a C16:1, lysoPC a C18:0, lysoPC a C18:2, lysoPC a C20:3, PC aa C32:1, PC ae C38:5, PC ae C42:3 as unique markers for males; alpha-AAA and sarcosine as specific markers for *APOE* ε4− males; C14:1-OH, PC aa C40:3, PC ae C30:0, SM (OH) C22:1, and taurine as unique markers for *APOE* ε4+ males; PC aa C34:4 as a specific marker for *APOE* ε4− females; and PC aa C38:0 as a specific marker for *APOE* ε4+ females.

Our study provides an initial but critical step toward developing personalized and precision medicine for AD and an operational

strategy to achieve that goal, which integrates clinical cognitive assessment, metabolomic profiling, and a computational network model to identify targeted therapeutic strategies for subsets of patients.

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CONFLICTS OF INTEREST

RC is the founder of INTelico Therapeutics LLC and co-founder of PATH Biotech LLC. RKD in an inventor on a series of patents on use of metabolomics for the diagnosis and treatment of CNS and ther diseases and holds founding equity in Metabolon Inc., Chymia LLC and PsyProtix. This study was not supported by any of above companies. The other authors declare no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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