The Alzheimer's Disease Metabolome: Effects of Sex and *APOE* ε4 genotype

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Significance statement

Research provides substantial evidence that late-onset Alzheimer's disease (AD), the major cause of dementia in the elderly, is a metabolic disease. Besides age, female sex and APOEε4 genotype represent strong risk factors for AD, and at the same time, give rise to large metabolic differences. Our systematic investigation of sex and APOE ε4 genotype differences in the link between metabolism and measures of pre-symptomatic AD using stratified analysis revealed several group-specific metabolic alterations that were not observed without sex and genotype stratification of the same cohort. Pathways linked to the observed metabolic alterations suggest females are more affected by impairment of mitochondrial energy production in AD than males, highlighting the importance of tailored treatment approaches towards a precision medicine approach.

Abstract

Recent studies have provided evidence that late-onset Alzheimer's disease (AD), the major cause of dementia in the elderly, can, at least in part, be considered a metabolic disease. Besides age, female sex and *APOE* ε4 genotype represent strong risk factors for AD. At the same time, they both give rise to large metabolic differences, suggesting that metabolic aspects of AD pathogenesis may differ between males and females and *APOE* ε4 carriers and non-carriers, respectively. Here, we systematically investigated group-specific metabolic alterations by conducting stratified association analyses of 140 metabolites measured in serum samples of 1,517 individuals from the AD neuroimaging initiative with AD biomarkers for Aβ and tau pathology, as well as neurodegeneration. We observed substantial sex differences in effects of 15 metabolites on AD biomarkers with partially overlapping differences for *APOE* ε4 status groups. These metabolites highlighted several group-specific alterations that were not observed in unstratified analyses using sex and *APOE* ε4 as covariates. Combined stratification by both variables uncovered further subgroup-specific metabolic effects limited to the group with presumably highest AD risk, i.e. *APOE* ε4+ females. Pathways linked to the observed metabolic alterations suggest that females experience more expressed impairment of mitochondrial energy production in AD than males. These findings indicate that dissecting metabolic heterogeneity in AD pathogenesis may allow for grading the biomedical relevance of specific pathways for specific subgroups. Extending our approach beyond simple one or two-fold stratification may thus guide the way to personalized medicine.

1. Introduction

Female sex has long been regarded a major risk factor for Alzheimer's disease (AD). It is assumed that out of 5.3 million people in the United States who were diagnosed with AD at age 65 or older, more than 60% are women. Also, estimates indicate that the lifetime risk of developing AD at age 45 may be almost double in females than in males (1, 2). However, the exact role and magnitude of sexual dimorphism in predisposition and progression to AD are controversial (3-6). While age is the strongest risk factor for late-onset AD (LOAD), the higher life expectancy of women only partially explains the observed sex difference in frequency and lifetime risk (7). Complexity is added by several genetic studies showing a significant sex difference in effects of the *APOE* ε4 genotype, the strongest common genetic risk factor for LOAD. These studies report risk estimates for ε4 carriers being higher in females than in males, a finding that seems to be additionally dependent on age (8-13). *APOE* ε4 has also been described to be associated with AD biomarkers in a sex-dependent way with again larger risk estimates for women than for men (9, 14-17), although these findings have not been fully consistent across studies (16, 18). Additionally, studies have suggested that sex differences in AD may change during the trajectory of disease (19), with overall risk for mild cognitive impairment (MCI), the prodromal stage of AD, being higher in males (20, 21), while progression to AD occurs at a faster rate in females, at least partly in *APOE* ε4-dependent ways (3, 8, 10, 19, 22, 23). The mechanisms underlying this sex-linked and partly intertwined *APOE* ε4- and age-dependent heterogeneity in AD susceptibility and severity are only beginning to unravel, calling for novel approaches to further elucidate molecular sex differences in AD risk and biomarker profiles.

Interestingly, all three of the aforementioned major AD risk factors, i.e. age, *APOE* ε4 genotype, and sex, have a profound impact on metabolism (24-30), supporting the view of AD as a metabolic disease (31-33). In recent years, availability of high-throughput metabolomics techniques, which can measure hundreds of small biochemical molecules (metabolites) simultaneously, allows for the study of metabolic imprints of age, genetic variation, and sex very broadly, covering the entire metabolism: (i) Age-dependent differences were observed in levels of phosphatidylcholines (PCs), sphingomyelins (SMs), acylcarnitines, ceramides, and amino acids (29, 34). A panel of 22 independent metabolites explained 59% of the total variance in chronological age in a large twin population cohort. In addition, one of these metabolites, C-glycosyltryptophan, was associated with age-related traits including bone mineral density, lung (30) and kidney function (35). (ii) As expected from APOE's known role in cholesterol and lipid metabolism (36, 37), common genetic variants in this gene were associated with blood cholesterol levels in genome- and metabolome-wide association studies (37, 38). In addition, associations with levels of various SMs were identified (39, 40). (iii) Analogous to age, sex also affects blood levels of many metabolites from a broad range of biochemical pathways. In a healthy elderly population with mostly post-menopausal women, females showed higher levels of most lipids except lyso-PCs, while the levels of most amino acids including branched chain amino acids (BCAAs) were higher in males with the exception of glycine and serine, which were higher in women (24, 25). In addition to studies investigating the impact of age and sex on metabolism separately, Gonzalez-Covarrubias et al. recently reported sex-specific lipid signatures associated with longevity in the Leiden Longevity Study (29). In women, higher levels of ether-PC and SM species were associated with longevity; no significant differences were observed in men. Thus, based on results from large-scale metabolomics studies, aging may influence a wider range of metabolites in women than men, highlighting the need for sex-stratified analyses.

Many of the metabolites affected by female sex, age, and *APOE* genotype such as BCAAs, glutamate, and various lipids appear to be altered in AD independent of these risk factors (39, 41, 42). In patients with MCI, alterations in lipid metabolism, lysine metabolism, and the tricarboxylic acid cycle have been observed (43, 44). In one of the largest blood-based metabolomics studies of AD, we identified metabolic alterations in various stages across the trajectory of the disease. For instance, higher levels of SMs and PCs were observed in early stages of AD as defined by abnormal CSF $A\beta_{1-42}$ levels, whereas intermediate changes, measured by CSF total tau, were correlated with increased levels of SMs and long-chain acylcarnitines (45). Changes in brain volume and cognition, usually noted in later stages, were correlated with a shift in energy substrate utilization from fatty acids to amino acids, especially BCAAs. Other metabolomics studies have reported metabolic alterations in AD which support these findings, including alterations in PCs in AD (44, 46-48) and sphingolipid transport and fatty acid metabolism in MCI/AD compared to cognitively normal (CN) subjects (49). Higher blood concentrations of sphingolipid species were associated with disease progression and pathological severity at autopsy (50). Metabolomics analysis of brain and blood tissue further revealed that bile acids, important regulators of lipid metabolism and products of human-gut microbiome co-metabolism, were altered in AD (51, 52) and associated with brain glucose metabolism and atrophy as well as CSF $A\beta_{1-42}$ and p-tau (53). In most of these studies, sex as well as *APOE* ε4 genotype, were used as covariates. Thus, sex-specific associations between AD and metabolite levels or associations that are modified by sex with opposite effect directions for the two sexes might have been missed in these analyses. Similarly, sex-by-*APOE* genotype interactions would have been masked.

Here, we examine the role of sex in the relationship between metabolic alterations and AD, in order to elucidate possible metabolic underpinnings for the observed sexual dimorphism in AD susceptibility and severity. Using metabolomics data from 1,517 subjects of the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohorts, we specifically investigate how sex modifies the associations of representative A-T-N biomarkers (54, 55) (A: CSF $A\beta_{1-42}$ pathology; T: CSF p-tau; N: region of interest (ROI)-based glucose uptake measured by FDG-PET) with 140 blood metabolites by stratified analyses and systematic comparison of effects between men and women. In downstream analyses, we then inspect sex-differences in metabolic effects on AD biomarkers for dependencies on *APOE* genotype, both by interaction analysis and sub-stratification.

2. Methods

2.1. Study subjects

Data used in the preparation of this article were obtained from the ADNI database (http://adni.loni.usc.edu/). The ADNI was launched in 2003 as a public-private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). For up-to-date information, see www.adni-info.org. Information on data availability and accessibility is available in the **Supplementary Text 1.**

In the current study, we included 1,517 baseline serum samples of fasting participants pooled from ADNI phases 1, GO, and 2. Demographics, diagnostic groups, and numbers and distributions of key risk factors are provided in **Table 1**. AD dementia diagnosis was established based on the NINDS-ADRDA criteria for probable AD. MCI participants did not meet these AD criteria and had largely intact functional performance, meeting predetermined criteria for amnestic MCI (56). Of the 1,517 subjects, 689 were female and 828 male, with 708 *APOE* ε4 carriers and 809 non-carriers. In the combined stratification by sex and *APOE* ε4 status (*APOE* ε4- = 0 copies of ε4, *APOE* ε4+ = 1 or 2 copies of ε4), the *APOE* ε4 non-carriers were separated into 374 females and 435 males, while of *APOE* ε4 carriers 315 were female and 393 male.

2.2. Metabolomics data acquisition

Metabolites were measured with the targeted AbsoluteIDQ-p180 metabolomics kit (BIOCRATES Life Science AG, Innsbruck, Austria), with an ultra-performance liquid chromatography (UPLC)/MS/MS system [Acquity UPLC (Waters), TQ-S triple quadrupole MS/MS (Waters)] which provides measurements of up to 186 endogenous metabolites. Sample extraction, metabolite measurement, identification, quantification, and primary quality control (QC) followed standard procedures as described before (45, 57).

2.3. Metabolomics data processing

Metabolomics data processing followed the processing protocol previously described (45, 57) with a few adjustments. In brief, raw metabolomics data for 182 metabolites was available for 1,681 serum study samples and, for each plate, 2-3 NIST Standard Reference samples were available. Furthermore, we had blinded duplicated measurements for 19 samples (ADNI-1) and blinded triplicated measurements for 17 samples (ADNI-GO and -2) distributed across plates. We first excluded 22 metabolites with large numbers of missing values (> 40%). Then, we removed plate batch effects using cross-plate mean normalization using NIST metabolite concentrations. Duplicated and triplicated study samples were then used to calculate the coefficients of variation (exclusion criterion >20%) and intra-class correlation (exclusion criterion <0.65) for each metabolite. We removed 20 metabolites that violated these thresholds. Next, we excluded non-fasting samples (n=108), imputed missing metabolite data using half the value of the lower limit of detection per metabolite and plate, log2-transformed metabolite concentrations, centered and scaled distributions to a mean of zero and unit variance and winsorized single outlying values to 3 standard deviations. We then used the Mahalanobis distance for detection of multivariate subject outliers, applying the critical Chi-square value for $P < 0.01$ and removing 42 subjects. Finally, metabolites were adjusted for significant medication effects using stepwise backwards selection (for details see (57)). The final QC-ed metabolomics dataset was further restricted to individuals having data on all significant covariates (see Section 2.4. Phenotype data and covariate selection), resulting in the study dataset of 140 metabolites and 1,517 individuals.

2.4. Phenotype data and covariate selection

We limited association analyses of metabolites with AD to early detectable endophenotypes, more specifically to the pathological threshold for CSF $A\beta_{1-42}$, levels of phosphorylated tau protein in the CSF (p-tau), and brain glucose metabolism measured by [^{18}F] fluorodeoxyglucose (FDG)-positron emission tomography (PET). Baseline data on these biomarkers for ADNI-1, -GO, and -2 participants was

downloaded from the LONI online portal at https://ida.loni.usc.edu/. For CSF biomarker data, we used the dataset generated using the validated and highly automated Roche Elecsys electrochemiluminescence immunoassays (58, 59). For FDG-PET, we used a ROI-based measure of average glucose uptake across the left and right angular, left and right temporal and bilateral posterior cingulate regions derived from preprocessed scans (co-registered, averaged, standardized image and voxel size, uniform resolution) and intensity-normalized using a pons ROI to obtain standard uptake value ratio (SUVR) means (60, 61). The pathological CSF $A\beta_{1-42}$ cut-point (1,073 pg/ml) as reported by the ADNI biomarker core for diagnosis-independent mixture modeling (see http://adni.loni.usc.edu/methods/, accessed Oct 2017) was used for categorization since CSF $A\beta_{1-42}$ concentrations were not normally distributed. Processed FDG-PET values were scaled and centered to zero mean and unit variance prior to association analysis, p-tau levels were additionally log2-transformed. Furthermore, we extracted covariates including age, sex, body-mass-index (BMI; calculated using baseline weight and body height), number of copies of the *APOE* ε4 genotype, and years of education. Covariates were separated into forced-in (age, sex, ADNI study phase, and number of copies of *APOE* ε4) and covariates (BMI, education) selectable by backwards selection. ADNI study phase was included to adjust for remaining metabolic differences between batches (ADNI-1 and ADNI-GO/-2 were processed in separate runs), as well as differences in PET imaging technologies.

2.5. Association analyses

Association analyses of the three AD biomarkers with metabolite levels were conducted using standard linear (p-tau, FDG-PET) and logistic (pathological $A\beta_{1-42}$) regression. For pathological CSF $A\beta_{1-42}$, only BMI was additionally selected, while for p-tau and FDG-PET the full set of covariates was used. The stratification variables sex and copies of *APOE* ε4 were excluded as covariates in the respective group-specific association analyses (i.e. sex in sex-stratified and copies of *APOE* ε4 in *APOE* ε4+/- status-stratified analyses, respectively). For identifying metabolic sex-differences, we used linear regression with metabolite levels as the dependent variable and age, sex, BMI, ADNI study phase, and diagnostic group as explanatory variables and retrieved statistics for sex. To adjust for multiple testing, we accounted for the significant correlation structure across the 140 metabolites and determined the number of independent metabolic features (i.e. tests) using the method of Li and Ji (62) to be 55, leading to a threshold of Bonferroni significance of 9.09×10^{-4}. To assess significance of heterogeneity between strata, we followed the methodology of (25, 63) that is similar to the determination of study heterogeneity in inverse-weighted meta-analysis. We further provide a scaled index of percent heterogeneity that is similar to the I^2 statistic (64).

3. Results

In this study, we used CSF biomarkers, FDG-PET imaging, and metabolomics data on 140 metabolites to investigate metabolic effects in relation to sex and AD and their interaction. Out of 1,517 ADNI participants, 1,082 had CSF $A\beta_{1-42}$ and p-tau levels and 1,143 had FDG-PET data available (**Table 1**). We included all individuals with respective data regardless of their diagnostic classification, as we were interested in these three representatives of the A-T-N AD biomarker schema (54, 55) as our main readouts. In this data set, there was no significant difference in the number of *APOE* ε4+/- subjects between females and males ($P > 0.3$). Of the three AD biomarkers, only p-tau levels were significantly different between sexes (corrected $P = 0.01$) with slightly higher levels observed in females.

Previous studies consistently showed widespread metabolic sex-differences, metabolic imprint of genetic variance in the *APOE* locus, as well as significant associations between blood metabolites and AD biomarkers that are independent of (i.e. adjusted for) sex. In the current study, we add the specific examination of the following central questions (**Supplementary Figure 1**): (i) Are metabolic sex-differences changed due to presence of (probable) AD?, (ii) Are metabolite associations with A-T-N biomarkers modified by sex?, and (iii) Is there evidence for *APOE* ε4 status influencing metabolite associations with A-T-N biomarkers that show differences between sexes?

3.1. Metabolic sex-differences are unaffected by MCI and probable AD status

In a first step, we tested whether sex-associated differences in blood metabolite levels differ between patients with probable AD, subjects with late MCI, and CN subjects in the ADNI cohorts. In the complete cohort (n = 1,517), we found 108 of 140 metabolites to be significantly associated with sex after multiple testing correction while adjusting for age, BMI, ADNI study phase, and diagnostic group. 70 of these associations replicate previous findings in a healthy population using a prior version of the same metabolomics platform (25) that provides measurements on 92 out of the 108 metabolites identified in ADNI. All SMs and the majority of PCs were more abundant in women. The majority of biogenic amines, amino acids and acylcarnitines were more abundant in men.

Stratifying subjects by diagnostic group revealed that 53 of the 108 metabolites showing significant sex-differences were also significant in each of the three groups (AD, MCI, CN) alone, while 14 metabolites showed no significant difference in any of the groups, probably due to lower statistical power after stratification (**Supplementary Table 1** and **Supplementary Figure 2**). Significant sex-differences limited to one diagnostic group were found for 8 metabolites (PC aa C34:1, PC ae C34:3, PC ae C36:3, PC ae C36:4, PC ae C38:5, PC ae C40:5, Histidine, C6/C4:1-DC) in patients with probable AD, for 7 metabolites (C0, C3, C9, C18:2, SDMA, Spermidine, t4-OH-Pro) in the MCI group, and for 6 metabolites (PC aa C42:0, PC ae C32:1, PC ae C42:3, SM(OH) C24:1, Sarcosine, Aspartate) in the CN group, although no significant sex-differences were found that were not also significant in the full cohort. Comparisons of beta estimates for sex between AD and CN groups showed no significant effect heterogeneity, indicating reduced power as source for these observed differences. Only PC aa C34:1 showed significant (P = 0.029) heterogeneity between AD patients compared to CN subjects. Interestingly, in the larger healthy cohort used as reference, sex did not significantly affect the blood level of this metabolite when adjusting for the same covariates as in this study (i.e., age and BMI) (25). In summary, we found that sex differences of blood metabolite levels are consistent (if we neglect the reduced power due to stratification) across diagnostic groups and, thus, do not seem to be directly affected by presence of MCI or AD status.

3.2. Sex-stratified analyses reveal substantial differences in the association of AD biomarkers and blood metabolite concentrations between men and women

To investigate whether sex modifies the association between AD endophenotypes and metabolite concentrations, we tested for associations of the three representative A-T-N biomarkers, CSF $A\beta_{1-42}$ pathology, CSF p-tau levels, and brain glucose uptake measured via FDG-PET imaging, with concentrations of 140 blood metabolites. We did this in the full data set, as well as in women and men separately using multivariable linear and logistic regression, followed by analysis of heterogeneity of effects between

sexes. **Table 2** lists the results of these analyses for all metabolite-phenotype combinations, as well as analyses of sex-by-metabolite interaction effects on A-T-N biomarkers, that fulfilled at least one of the following criteria: (i) associations that were significant (at a Bonferroni threshold of $P < 9.09 \times 10^{-4}$) in the full cohort; (ii) associations that were Bonferroni-significant in one of the two sexes; (iii) associations that showed suggestive significance ($P < 0.05$) in one sex coupled with significance for effect heterogeneity between female and male effect estimates. Results for all metabolites, phenotypes and statistical models are provided in **Supplementary Table 2**. Systematic comparison of estimated effects in men and women for all metabolites is shown in **Figure 1**. Based on this comparison, we classified metabolite – A-T-N biomarker associations into homogenous effects if metabolites showed very similar effects in their association to the biomarker for both sexes and heterogeneous effects if effects showed major differences between the sexes with opposite effect directions of the same metabolite for men and women or substantially larger effects in one of the sexes. If an effect was heterogeneous and significant in males but not females or vice versa, we considered it sex-specific.

3.2.1. Homogeneous effects

We refer to **homogenous effects** where similar alterations in metabolite levels are associated with AD biomarkers in men and women. Metabolites with homogenous effects lie on or close to the diagonal going through the first and third quadrant when plotting the effect estimates in women against those in men in **Figure 1**. We identified eight significant homogenous metabolite-phenotype associations with A-T-N biomarkers: CSF $A\beta_{1-42}$ pathology was significantly associated with levels of three related ether-containing PCs (PC ae C44:4, PC ae C44:5, PC ae C44:6). Two of those (PC ae C44:4 and PC ae C44:5) were also significantly associated with brain glucose uptake (FDG-PET) in addition to three other PCs (PC aa C32:1, PC aa C32:0 and PC ae C42:4). For p-tau, we did not identify any homogeneous, overall significant associations. Notably, none of the associations categorized as homogenous showed any indication of effect heterogeneity between sexes, and only one association reached significance in the sex-stratified analyses: higher blood levels of the diacyl-PC PC aa C32:1 were associated with lower glucose uptake in brain in the male stratum alone despite lower power.

3.2.2. Heterogeneous effects

We refer to **heterogeneous effects** where a metabolite shows opposite effect directions for the same phenotype in men and women, or substantially larger effects in one sex leading to significant heterogeneity and/or sex-metabolite interaction. Metabolites showing these types of effects fall mainly into the second or fourth quadrant (with the exception of sex-specific effects) when contrasting the effect estimates for men and women in the plots for the three A-T-N phenotypes in **Figure 1**. In our study, we identified a total of 15 associations in this category (including three sex-specific effects). For CSF $A\beta_{1-42}$, we identified two heterogeneous effects with threonine showing a sex-specific effect (see paragraph below) with greater effect size in males and valine with a larger effect in females: while valine was not significantly associated ($P = 0.78$) with CSF $A\beta_{1-42}$ pathology in males, in females, it showed a nominally significant negative association with an estimated heterogeneity of $I^2 = 49.3\%$. CSF p-tau was the biomarker with the largest number of heterogeneous associations: acylcarnitines C5-DC (C6-OH), C8, C10 (sex-specific), and C2, as well as the amino acid histidine showed stronger associations in females, while the related ether-containing PCs PC ae C36:1 and PC ae C36:2, the amino acids asparagine and glycine,

and one hydroxy-SM (SM (OH) C16:1) yielded stronger associations in males (all I^2 > 50%); associations with FDG-PET revealed three heterogeneous effects, with ether-containing PC ae C40:2, and the acylcarnitine C16:1 (sex-specific) showing a larger effect in males (I^2 = 55.3%), and proline having a larger effect in females (I^2 = 64.8%). Notable, 9 of the 15 reported heterogeneous associations showed opposite effect directions between sexes, and in 7 cases, the interaction term (sex * metabolite) was also significantly (at $P < 0.05$) associated with the respective biomarker.

3.2.3. Sex-specific effects

We refer to **sex-specific effects** where metabolite associations are only significant in one sex with either significant effect heterogeneity between males and females or significant sex-metabolite interaction. In **Figure 1**, metabolites with this effect category fall into the area close to the x- (male-specific) or y- (female-specific) axes of the three effect plots for the different A-T-N phenotypes. In total, we found three instances of this effect type. Male-specific effects were seen for threonine with pathological CSF $A\beta_{1-42}$ (positive association) and C16:1 with FDG-PET (negative association). We also identified a single female-specific effect, where higher levels of the medium-chain acylcarnitine C10 were associated with higher CSF p-tau. This association was simultaneously the strongest seen for p-tau in the analysis of the full cohort, yet seems to be driven by female effects only.

3.3. Stratified analyses by *APOE* ε4 status suggest intertwined modulation of metabolite effects by both sex and *APOE* genotype

Previous reports suggested that the *APOE* ε4 genotype may exert AD risk predisposition in a sex-dependent way (8-13). In order to investigate potential relationships between sex and *APOE* ε4 status on the metabolomic level, we selected the 21 metabolites identified in the previous analyses (**Table 2**) and performed association analyses with the three selected A-T-N biomarkers, now stratified by *APOE* ε4 status and adjusted for sex. Using the same effect categories (homogeneous, heterogeneous, and group-specific) as for the sex-stratified analyses revealed that metabolite effects in *APOE* ε4 carriers vs. non-carriers also show effects from all three categories (**Table 3**): **homogeneous effects** were noted for the overall significant associations of PC aa C32:1, PC ae C44:4, PC ae C44:5, PC aa C32:0, and PC ae C42:4 with FDG-PET. **Heterogeneous effects** again formed the largest group (n = 11), with proline and glycine showing opposite effect directions on CSF $A\beta_{1-42}$ pathology and C8, valine, glycine, and proline having opposite effect directions on FDG-PET for ε4 carriers vs. non-carriers, respectively. 5 metabolites with heterogeneous effects even showed ***APOE* ε4 status-specific effects**: (i) the associations of PC ae C44:6, PC ae C44:4, PC ae C44:5, and PC ae C42:4 with pathological CSF $A\beta_{1-42}$ in *APOE* ε4 carriers. In case of PC ae C44:6, PC ae C44:5, and PC ae C44:4, the group-specific effects were strong enough to drive the signal to overall significance in the full sample. (ii) the association of acylcarnitine C10 with FDG-PET in *APOE* ε4 non-carriers.

3.4. Combined stratification by sex and *APOE* ε4 status identifies metabolic effects specific to female carriers of the ε4 allele

When we stratified separately by sex and *APOE* ε4 status, we observed several metabolites (C8, C10, valine, glycine, and proline) that showed heterogeneous effects on AD biomarkers in both stratifications. To investigate potential additional subgroup-specific effects, we combined the two stratifications and

investigated the selected metabolite set for sex-by-*APOE* ε4 status effect modulations. Although the group of *APOE* ε4-carrying women was the smallest among the four strata, all Bonferroni-significant associations were found in this subgroup (**Table 4**): higher levels of three ether-containing PCs (PC ae C42:4, PC ae C44:5, and PC ae C44:6) were associated with pathological CSF $A\beta_{1-42}$, higher acylcarnitine C10 was associated with increased CSF p-tau, and higher proline levels were associated with decreased FDG-PET values (**Figure 2**). The latter association was not observed in any other of the performed analyses. Of note, except for the association of C10 with p-tau, we found significant ($P < 0.05$) interaction effects between the metabolites and *APOE* ε4 status on their associated endophenotypes in females only, while the effects in males were not significantly ($P > 0.1$) modulated by *APOE* ε4 status.

4. Discussion

In this study, we investigated the influence of sex and *APOE* ε4 status on metabolic alterations related to representative A-T-N biomarkers (CSF $A\beta_{1-42}$ pathology (A), CSF p-tau (T), FDG-PET (N)). By stratified analyses and systematic comparison of the effects estimated for the two sexes, we revealed substantial differences between men and women in their associations of blood metabolite levels with these AD biomarkers, although known sexual dimorphisms of metabolite levels themselves were unaffected by the disease.

Differences between the sexes were largest for associations of metabolites and CSF p-tau levels. Notably, this biomarker was not significantly associated with any metabolite when including all subjects and adjusting for both sex and copies of *APOE* ε4, yet association analysis stratified by sex (but still adjusted for copies of *APOE* ε4) revealed a significant, female-specific metabolite/CSF p-tau association despite the smaller sample size. In contrast, for CSF $A\beta_{1-42}$ and FDG-PET, in addition to heterogeneous, sex-specific effects, we also found homogenous effects, where metabolite concentrations showed the same trends of metabolite levels correlating with CSF $A\beta_{1-42}$ pathology and/or lower brain glucose uptake in both sexes.

For many of the metabolites with different effects for the sexes, we additionally observed significant effect heterogeneity between carriers and non-carriers of the *APOE* ε4 allele, suggesting intertwined modulation of metabolic effects by sex and *APOE* genotype. Indeed, two-fold stratification revealed metabolite associations that were either driven by or even specific to the group with presumably highest risk, *APOE* ε4 carrying females. Our results, thus, demonstrate the importance of stratified analyses for getting insights into metabolic underpinnings of AD that are seemingly restricted to a specific group of patients.

4.1 Metabolic effect heterogeneity suggests sex-specific differences in energy homeostasis, alternative energy sources, and stress response in AD

The metabolites showing effect heterogeneity across AD biomarkers in this study highlight sex-specific dysregulations of **energy metabolism** (acylcarnitines C2, C5-DC/C6-OH, C8, C10 and C16:1 for lipid-based energy metabolism (65); amino acids valine, glycine, and proline as markers for glucogenic and ketogenic energy metabolism (66-68)), **energy homeostasis** (asparagine, glycine, proline, and histidine (67-71)), and (metabolic/nutrient) **stress response** (threonine, proline, histidine (68, 70, 72)). While these pathways

have been linked to AD before, our work presents first evidence and molecular readouts for sex-related metabolic differences in AD.

For instance, in our previous report, we discussed the implication of failing lipid energy metabolism in the context of AD biomarker profiles, starting at the stage of pathological changes in CSF tau levels (45). The current study now provides further insights in this topic, marking this finding to be predominant in females. More specifically, we observed a significant female-specific association of higher levels of acylcarnitine C10 with increased levels of CSF p-tau, with two other metabolites of this pathway (C8 and C5-DC/C6-OH) narrowly falling short of meeting the Bonferroni threshold. This indicates a sex-specific buildup of medium-chain fatty acids in females, suggesting increased energy demands coupled with impaired energy production via mitochondrial beta-oxidation (65).

Interestingly, the significant heterogeneity of association results between sexes for CSF p-tau and glycine, with higher levels of glycine being linked to higher levels of CSF p-tau in men, indicates that energy demands are equally upregulated in males as in females. In contrast to women, men, however, appear to compensate this demand by upregulation of glucose energy metabolism as glycine is a positive marker of active glucose metabolism and insulin sensitivity (67). Findings for acylcarnitines in females are further contrasted by the observed male-specific association of higher levels of the long-chain acylcarnitine C16:1 with decreased brain glucose uptake, which might indicate that in males there is a switch to provision of fatty acids as alternative fuel when glucose-based energy metabolism is less effective. As we did not observe the buildup of medium- and short-chain acylcarnitines as seen in females, we assume that, in males, energy production via mitochondrial beta-oxidation may be sustained, at least in early disease.

Evidence corroborating sex-specific processes in energy homeostasis linked to changes in CSF p-tau levels is provided by the significant heterogeneity estimates for histidine with lower levels of histidine being linked to higher levels of CSF p-tau in women. Depletion of histidine has been shown to be associated with insulin resistance, inflammatory processes, as well as oxidative stress, especially in women with metabolic dysregulation (69, 70).

We further identified a heterogeneous association of valine with lower levels in females ($P < 0.05$), but not in males, with $A\beta_{1\text{-}42}$ pathology. Valine, a BCAA and important energy carrying molecule, has been reported to be associated with cognitive decline and brain atrophy in AD, as well as with risk for incident dementia (42, 45). The lower levels observed in AD are in contrast to other complex phenotypes such as type 2 diabetes, insulin resistance, or obesity (66, 73), where higher levels of BCAAs are found, and may indicate a switch to increased energy consumption via degradation of amino acids in AD. A recent study highlighted decreasing levels of valine as being significantly associated with all-cause mortality (74). Besides implications for energy metabolism, results from our study may thus characterize lower levels of valine also as a marker for increased female vulnerability to pathogenic processes in general and to β-amyloidosis in AD in particular.

4.2 Complex interplay between sex, *APOE* ε4 status, and metabolism

The higher effect size of genetic risk for AD exerted by the *APOE* ε4 allele in females compared to males still awaits molecular elucidation. Here, we tried to elaborate on potential interrelated risk predispositions

from a metabolomic point of view. We therefore investigated if *APOE* ε4 status may also modulate metabolic readouts of AD-linked A-T-N biomarker profiles identified in sex-centered analyses. We found that indeed the majority (68.8%) of observed associations between metabolites and AD biomarkers shows significant heterogeneity between *APOE* ε4 status groups.

Notably, the full set of metabolites yielding significant effect heterogeneity when comparing *APOE* ε4-carriers vs. non-carriers (C8, C10, glycine, proline, and valine) also showed significant heterogeneity estimates in the sex-stratified analyses. We therefore applied two-fold stratification by sex and *APOE* ε4 status to identify potential interactions between both variables (**Supplementary Figure 3**). This analysis revealed several associations that showed Bonferroni significance in the group with presumably the highest AD risk, namely *APOE* ε4+ females. One of those, the significant association of higher proline levels with reduced brain glucose uptake, was not observed in any of the three other strata, in the one-fold stratifications, or in the full sample, emphasizing the value of more fine-granular stratified analyses as proposed here.

4.3 Homogeneous effects seem to represent generic metabolic hallmarks in AD

The heterogeneity of metabolite effects identified in our study might, in part, explain inconsistencies (e.g., (75) vs. (76)) in associations of metabolites and AD reported in different studies (e.g., if sex and *APOE* genotype are distributed differently and sample sizes are small). Besides the heterogeneous, sex-specific effects observed for metabolite associations with CSF $A\beta_{1-42}$ and FDG-PET biomarkers, we also found associations of these biomarkers with metabolites that showed the same effects in women and men. In particular, phosphatidylcholines that presumably contain two long-chain fatty acids with, in total, 4 or 5 double bonds (PC ae C44:4, PC ae C44:5) were significant for both AD biomarkers. Such homogeneous metabolite associations would be expected to replicate well across studies.

To test this assumption in an independent sample, we performed a targeted analysis using the three PCs associated with CSF $A\beta_{1-42}$ pathology in 86 serum samples of subjects in the ROS/MAP cohorts (**Supplementary Text 2**): all three associations were Bonferroni significant (PC ae C44:4 – $P = 3.73 \times 10^{-3}$; PC ae C44:5 – $P = 1.15 \times 10^{-2}$; PC ae C44:6 – $P = 3.28 \times 10^{-3}$) in ROS/MAP with consistent effect directions. Of note, in ROS/MAP, we used a different measure of amyloid pathology (total amyloid load in the brain), which is known to be inversely correlated with CSF $A\beta_{1-42}$ levels (77). This inverse relationship was mirrored by metabolite effect estimates. These results provide evidence for homogeneous associations to be relevant across cohorts.

4.4 Limitations

Our study has several limitations. First, the reported findings are observational and do not allow for any direct causal conclusions. Second, the reported heterogeneity estimates still await replication in an independent cohort with sample sizes appropriate for stratification as well as metabolomics and endophenotypic data available (ROS/MAP sample sizes available to us were too small to be sufficiently powered). Third, it is to be noted that stratified analyses in combination with heterogeneity estimates may identify spurious associations, primarily due to the limited power resulting from group separation. However, we were able to show that for the majority of the non-homogeneous findings reported (60%), the interaction term between metabolite levels and sex were also significant in the pooled analysis. When

stratifying by *APOE* ε4 status, this was true for an even higher fraction of cases (72.7%). This provides an additional line of support for the conclusions drawn in this work. Finally, we only looked at two risk factors, but there may be others (e.g., type 2 diabetes, cardiovascular disease, high blood pressure) that also have metabolic aspects and may reveal even greater molecular heterogeneity.

4.5 Conclusion

Effect heterogeneity between subgroups linked to energy metabolism as reported in this study has several important implications for AD research. First, this heterogeneity could explain inconsistencies of metabolomics findings between studies as observed for AD if participants showed different distributions of variables such as sex and *APOE* ε4 genotype. Second, pooled analysis with model adjustment for such variables as typically applied for sex can mask substantial effects that are relevant for only a subgroup of people. This is also true for combinations of stratifying variables as we demonstrated for the association of proline with brain glucose uptake in female *APOE* ε4 carriers. Consequently, drug trials may be more successful if acknowledging between-group differences and targeting the subgroup with the presumably largest benefit in their inclusion criteria. For energy metabolism in particular, group-specific dietary interventions precisely targeting the respective dysfunctional pathways may pose a promising alternative to *de novo* drug development. Extending our approach by selection of additional variables to further improve stratification may eventually guide the way to personalized medicine.

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Figure captions

Figure 1: Scatter plots showing Z-scores of effect estimates of metabolite associations with A-T-N biomarkers for males (x-axis) versus those for females (y-axis). Homogeneous effects (i.e. those with same effect direction and comparable effect size) are located close to the diagonal, heterogeneous effects are located close to the anti-diagonal, and sex-specific effects are located close to one axis, i.e. x-axis for male-specific and y-axis for female-specific effects, respectively. Homogeneous, overall significant results are depicted as diamonds, effects with significant heterogeneity are drawn as rectangles, and effects significant in only one sex are displayed as triangles. Metabolites additionally marked by an asterisk are significant in one sex only and simultaneously show significant heterogeneity. Sex-specificity is further illustrated by a color scale (blue: females; green: males). On the upper right panel, example boxplots of metabolite residuals (obtained by regressing out included covariates) for each effect type are shown separately for females and males with (in dark red) and without (in light red) CSF $A\beta_{1-42}$ pathology, respectively.

Figure 2: Boxplots showing residuals of proline levels (derived by regressing out covariate effects) for **A:** the full sample; **B:** 1-fold stratifications by sex; **C:** 1-fold stratification by *APOE* ε4 status; and **D:** 2-fold stratification by both sex and *APOE* ε4 status; separately for high (light blue) and low (darker blue; derived by mean-split) FDG-PET values. The only subgroup showing a significant difference in proline levels are *APOE* ε4+ females with substantially higher levels in subjects with lower brain glucose uptake.

Tables

Table 1: Characteristics of the 1,517 ADNI samples included in this study. CN: cognitively normal. SMC: subjective memory complaints; EMCI: early mild cognitive impairment; MCI: mild cognitive impairment; AD: probably Alzheimer's disease; BMI: body-mass-index; *APOE* ε4-/+: non-carriers and carriers of the *APOE* ε4 allele, Path.Abeta-/+: participants who have normal and pathological CSF Abeta levels; respectively.

	global dataset	**CN**	**SMC**	**EMCI**	**MCI**	**AD**
$N_{subjects}$	1517	362	93	270	490	302
Sex (m/f)	828/689	177/185	39/54	149/121	298/192	165/137
Age	73.72 (+-7.25)	74.61 (+-5.77)	72.34 (+-5.70)	71.26 (+-7.63)	74.03 (+-7.63)	74.79 (+-7.77)
BMI	26.86(+- 4.82)	26.99 (+-4.53)	28.46 (+-6.23)	27.96 (+-5.36)	26.45 (+-4.27)	25.88 (+-4.69)
Education	15.88 (+-2.87)	16.24 (+-2.79)	16.78 (+-2.55)	15.95 (+-2.67)	15.84 (+-2.91)	15.16 (+-3.01)
***APOE* ε4-/+**	809/708 *	261/101	64/29	155/115	224/266	105/197
CSF available	1082 *	236	84	245	308	209
Path.Abeta-/+	407/675	134/102	57/27	122/123	75/233	19/190
CSF Abeta	1052.73 (+-601.70)	1324.60 (+-652.13)	1395.01 (+-618.19)	1172.73 (+-569.12)	896.35 (+-501.80)	697.95 (+-431.49)
CSF p-Tau	27.79 (+-14.56)	22.01 (+-9.19)	21.66 (+-9.14)	24.34 (+-14.03)	30.81 (+-14.94)	36.38 (+-16.07)
FDG-PET available	1143 *	247	93	268	318	217
FDG-PET	6.17 (+-0.77)	6.53 (+-0.58)	6.60 (+-0.58)	6.44 (+-0.60)	6.08 (+-0.68)	5.36 (+-0.73)

*** Numbers for combined stratification:**

	***APOE* ε4- females**	***APOE* ε4- males**	***APOE* ε4+ females**	***APOE* ε4+ males**
total	374	435	315	393
CSF available	267	315	222	278
FDG-PET available	278	337	230	298

Table 2: Metabolite associations with A-T-N biomarkers that are either Bonferroni significant in the full sample, one sex, or show nominal significance both in one sex and for effect heterogeneity. Given are regression results for the full sample and both sexes, as well as heterogeneity estimates and the p-value for sex * metabolite interactions.

						males			females			Sex difference			interaction
biomarker	**metabolite**	**effect**	**se**	**p-value**	**effect type**	**effect**	**se**	**p-value**	**effect**	**se**	**p-value**	**t**	**p-value**	**I^2**	**p-value**
Pathological CSF $A\beta_{1-42}$	PC ae C44:6	0.283	0.078	**2.58E-04**	homogeneous	0.282	0.102	5.96E-03	0.299	0.121	1.33E-02	-0.1068	9.15E-01	0.000	8.09E-01
	PC ae C44:4	0.265	0.076	**4.57E-04**	homogeneous	0.274	0.100	6.29E-03	0.255	0.118	3.07E-02	0.11904	9.05E-01	0.000	7.83E-01
	PC ae C44:5	0.260	0.075	**5.23E-04**	homogeneous	0.294	0.100	3.26E-03	0.214	0.116	6.38E-02	0.52233	6.01E-01	0.000	4.34E-01
	Threonine	0.207	0.076	6.72E-03	male-specific	0.372	0.112	**8.83E-04**	0.070	0.108	5.17E-01	1.943	5.20E-02	48.545	**4.03E-02**
	Valine	-0.134	0.083	1.05E-01	heterogeneous	0.032	0.114	7.80E-01	-0.299	0.123	1.50E-02	1.973	**4.85E-02**	49.322	7.65E-02
CSF p-tau	C10	0.084	0.030	4.58E-03	female-specific	0.014	0.042	7.34E-01	0.144	0.042	**6.07E-04**	-2.203	**2.76E-02**	54.613	**2.55E-02**
	C5−DC (C6−OH)	0.103	0.045	2.35E-02	heterogeneous	0.012	0.062	8.52E-01	0.205	0.067	2.27E-03	-2.116	**3.44E-02**	52.740	3.38E-01
	C8	0.064	0.030	3.42E-02	heterogeneous	0.003	0.041	9.39E-01	0.127	0.045	5.11E-03	-2.028	**4.26E-02**	50.692	5.63E-02
	PC ae C36:2	0.056	0.032	8.65E-02	heterogeneous	0.129	0.046	4.80E-03	-0.023	0.046	6.18E-01	2.355	**1.85E-02**	57.535	**2.16E-02**
	Histidine	-0.034	0.031	2.72E-01	heterogeneous	0.033	0.042	4.39E-01	-0.105	0.045	1.97E-02	2.237	**2.53E-02**	55.290	**2.42E-02**
	Asparagine	0.034	0.031	2.84E-01	heterogeneous	0.107	0.045	1.66E-02	-0.052	0.044	2.32E-01	2.550	**1.08E-02**	60.788	**2.16E-02**
	SM (OH) C16:1	0.032	0.031	3.10E-01	heterogeneous	0.091	0.043	3.36E-02	-0.039	0.046	3.99E-01	2.066	**3.89E-02**	51.592	**3.75E-02**
	Glycine	0.030	0.032	3.50E-01	heterogeneous	0.104	0.051	3.94E-02	-0.026	0.040	5.23E-01	2.014	**4.40E-02**	50.346	6.88E-02
	PC ae C36:1	0.028	0.031	3.68E-01	heterogeneous	0.088	0.043	4.17E-02	-0.041	0.044	3.51E-01	2.094	**3.62E-02**	52.251	**3.76E-02**
	C2	0.015	0.028	5.85E-01	heterogeneous	-0.054	0.039	1.67E-01	0.089	0.041	3.02E-02	-2.527	**1.15E-02**	60.430	**1.39E-02**
FDG-PET	PC aa C32:1	-0.127	0.030	**2.32E-05**	homogeneous	-0.140	0.041	**6.31E-04**	-0.110	0.045	1.50E-02	-0.499	6.18E-01	0.000	5.53E-01
	PC ae C44:4	-0.111	0.030	**2.27E-04**	homogeneous	-0.097	0.041	1.80E-02	-0.141	0.045	1.84E-03	0.71633	4.74E-01	0.000	2.21E-01
	PC ae C44:5	-0.105	0.030	**4.07E-04**	homogeneous	-0.112	0.040	5.80E-03	-0.111	0.044	1.30E-02	-0.0207	9.83E-01	0.000	6.02E-01
	PC aa C32:0	-0.107	0.032	**6.85E-04**	homogeneous	-0.125	0.045	5.67E-03	-0.091	0.045	4.25E-02	-0.547	5.84E-01	0.000	7.44E-01
	PC ae C42:4	-0.103	0.031	**8.56E-04**	homogeneous	-0.103	0.042	1.58E-02	-0.112	0.045	1.33E-02	0.15599	8.76E-01	0.000	4.48E-01
	C16:1	-0.103	0.031	9.09E-04	male-specific	-0.165	0.042	**9.64E-05**	-0.029	0.046	5.38E-01	-2.179	**2.93E-02**	54.107	9.94E-02
	PC ae C40:2	-0.053	0.030	7.82E-02	heterogeneous	-0.119	0.042	4.34E-03	0.016	0.044	7.15E-01	-2.238	**2.52E-02**	55.312	5.78E-02
	Proline	-0.023	0.031	4.51E-01	heterogeneous	0.059	0.044	1.77E-01	-0.118	0.044	8.18E-03	2.841	**4.50E-03**	64.801	**7.74E-03**

Table 3: Associations of metabolites identified in the sex-centric analysis with A-T-N biomarkers that are either Bonferroni significant in the full sample, in *APOE* ε4+ or *APOE* ε4- subjects, or show nominal significance both in one *APOE* ε4 status group and for effect heterogeneity. Given are regression results for the full sample and both *APOE* ε4 status groups, as well as heterogeneity estimates and the p-value for *APOE* ε4 status * metabolite interactions.

						***APOE* ε4+**			***APOE* ε4-**			***APOE* ε4 status difference**			**interaction**
biomarker	**metabolite**	**effect**	**se**	**p-value**	**effect type**	**effect**	**se**	**p-value**	**effect**	**se**	**p-value**	**t**	**p-value**	**I^2**	**p-value**
Pathological CSF $A\beta_{1-42}$	PC ae C44:6	0.283	0.078	**2.58E-04**	specific to ε4+	0.630	0.150	**2.50E-05**	0.158	0.090	7.96E-02	-2.705	**6.83E-03**	63.030	**2.80E-03**
	PC ae C44:4	0.265	0.076	**4.57E-04**	specific to ε4+	0.565	0.148	**1.30E-04**	0.139	0.088	1.13E-01	-2.478	**1.32E-02**	59.645	**5.80E-03**
	PC ae C44:5	0.260	0.075	**5.23E-04**	specific to ε4+	0.609	0.145	**2.64E-05**	0.129	0.087	1.37E-01	-2.837	**4.56E-03**	64.749	**3.07E-03**
	PC ae C42:4	0.242	0.078	1.98E-03	specific to ε4+	0.564	0.148	**1.32E-04**	0.114	0.092	2.15E-01	-2.589	**9.61E-03**	61.382	**5.64E-03**
	Proline	-0.075	0.081	3.52E-01	heterogeneous	0.176	0.142	2.15E-01	-0.202	0.100	4.40E-02	-2.173	**2.98E-02**	53.982	1.58E-01
	Glycine	0.060	0.082	4.60E-01	heterogeneous	0.363	0.154	1.83E-02	-0.102	0.100	3.05E-01	-2.538	**1.11E-02**	60.604	**7.89E-04**
FDG-PET	PC aa C32:1	-0.127	0.030	**2.32E-05**	homogeneous	-0.087	0.045	5.35E-02	-0.162	0.042	**1.34E-04**	-1.210	2.26E-01	17.332	3.58E-01
	PC ae C44:4	-0.111	0.030	**2.27E-04**	homogeneous	-0.115	0.047	1.39E-02	-0.114	0.041	6.34E-03	0.023	9.82E-01	0.000	8.63E-01
	PC ae C44:5	-0.105	0.030	**4.07E-04**	homogeneous	-0.122	0.046	8.34E-03	-0.102	0.041	1.30E-02	0.326	7.44E-01	0.000	6.39E-01
	PC aa C32:0	-0.107	0.032	**6.85E-04**	homogeneous	-0.135	0.047	4.39E-03	-0.082	0.045	6.58E-02	0.818	4.14E-01	0.000	3.69E-01
	PC ae C42:4	-0.103	0.031	**8.56E-04**	homogeneous	-0.131	0.047	5.79E-03	-0.086	0.043	4.51E-02	0.701	4.83E-01	0.000	3.98E-01
	C10	-0.057	0.029	5.14E-02	specific to ε4-	0.037	0.046	4.17E-01	-0.135	0.040	**7.17E-04**	-2.840	**4.51E-03**	64.793	**4.96E-03**
	C8	-0.051	0.031	9.96E-02	heterogeneous	0.038	0.046	4.04E-01	-0.138	0.043	1.58E-03	-2.794	**5.20E-03**	64.215	**6.37E-03**
	Valine	0.036	0.032	2.49E-01	heterogeneous	-0.040	0.048	4.08E-01	0.106	0.044	1.68E-02	2.234	**2.55E-02**	55.233	9.50E-02
	Glycine	-0.032	0.031	3.00E-01	heterogeneous	-0.140	0.047	3.05E-03	0.059	0.044	1.80E-01	3.092	**1.99E-03**	67.653	**3.29E-03**
	Proline	-0.023	0.031	4.51E-01	heterogeneous	-0.100	0.047	3.39E-02	0.048	0.043	2.64E-01	2.324	**2.01E-02**	56.977	6.35E-02

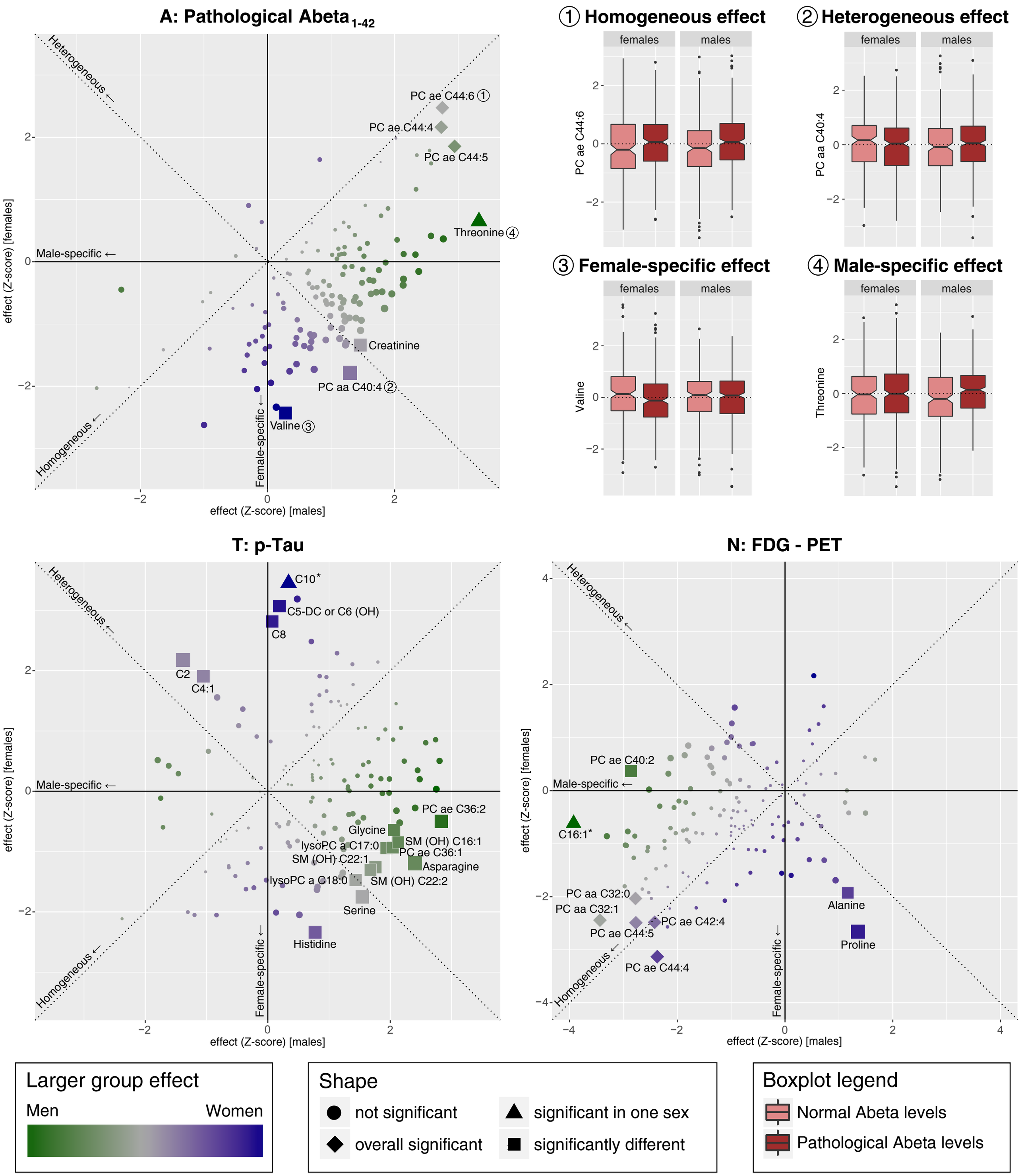
Table 4: Significant metabolite effects in the combined stratification (sex by *APOE* ε4 status) on A-T-N biomarkers are driven by or limited to *APOE* ε4+ females. Given are regression results for the full sample, *APOE* ε4+ males, *APOE* ε4+ females, as well as heterogeneity estimates by sex and *APOE* ε4 status. The only metabolite showing effect heterogeneity for both stratification variables was proline in its association with FDG-PET values.

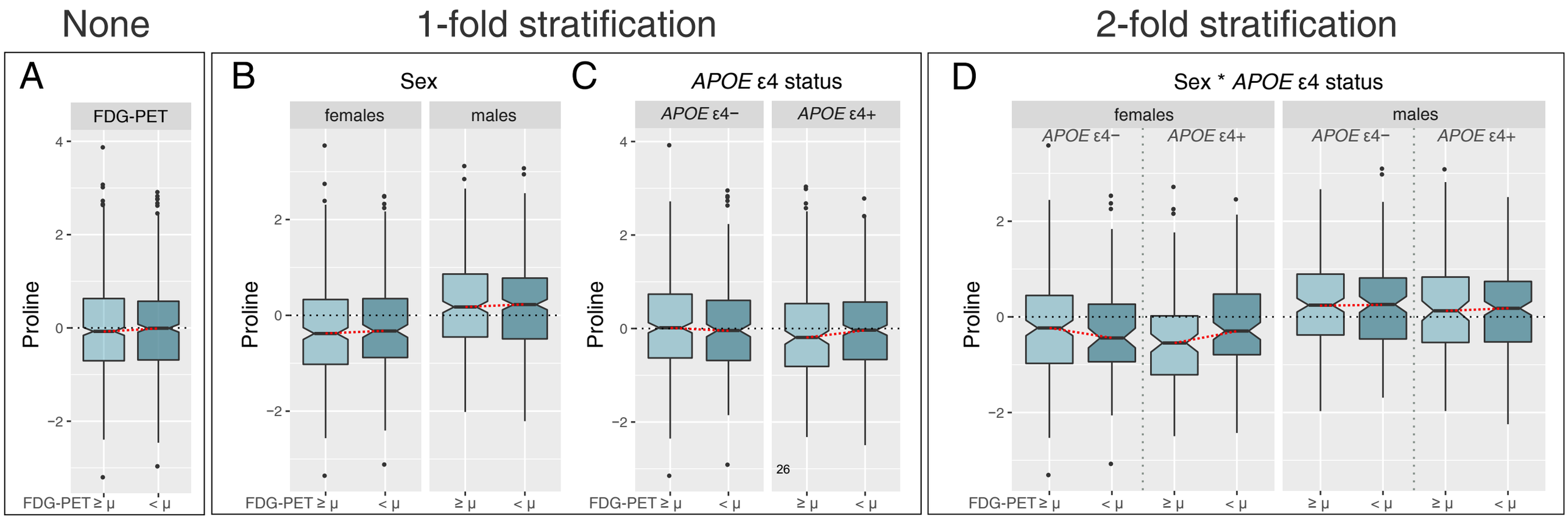
				Sex difference		***APOE* ε4 status difference**		***APOE* ε4+ males**		***APOE* ε4+ females**	
biomarker	**metabolite**	**effect**	**p-value**	**p-value**	**I^2**	**p-value**	**I^2**	**effect**	**p-value**	**effect**	**p-value**
Pathological CSF $A\beta_{1-42}$	PC ae C44:6	0.283	**2.58E-04**	9.15E-01	0.000	**6.83E-03**	63.03	0.463	1.68E-02	0.922	**1.90E-04**
	PC ae C44:5	0.26	**5.23E-04**	6.01E-01	0.000	**4.56E-03**	64.749	0.521	6.17E-03	0.761	**8.29E-04**
	PC ae C42:4	0.242	1.98E-03	7.58E-01	0.000	**9.61E-03**	61.382	0.42	3.15E-02	0.761	**8.65E-04**
CSF p-tau	C10	0.084	4.58E-03	**2.76E-02**	54.613	6.16E-01	0	-0.064	3.24E-01	0.264	**1.21E-04**
FDG-PET	Proline	-0.023	4.51E-01	**4.50E-03**	64.801	**2.01E-02**	56.977	0.046	4.76E-01	-0.272	**8.22E-05**

Figure captions

Figure 1: Scatter plots showing Z-scores of effect estimates of metabolite associations with A-T-N biomarkers for males (x-axis) versus those for females (y-axis). Homogeneous effects (i.e. those with same effect direction and comparable effect size) are located close to the diagonal, heterogeneous effects are located close to the anti-diagonal, and sex-specific effects are located close to one axis, i.e. x-axis for male-specific and y-axis for female-specific effects, respectively. Homogeneous, overall significant results are depicted as diamonds, effects with significant heterogeneity are drawn as rectangles, and effects significant in only one sex are displayed as triangles. Metabolites additionally marked by an asterisk are significant in one sex only and simultaneously show significant heterogeneity. Sex-specificity is further illustrated by a color scale (blue: females; green: males). On the upper right panel, example boxplots of metabolite residuals (obtained by regressing out included covariates) for each effect type are shown separately for females and males with (in dark red) and without (in light red) CSF $A\beta_{1\text{-}42}$ pathology, respectively.

Figure 2: Boxplots showing residuals of proline levels (derived by regressing out covariate effects) for **A:** the full sample; **B:** 1-fold stratifications by sex; **C:** 1-fold stratification by *APOE* ε4 status; and **D:** 2-fold stratification by both sex and *APOE* ε4 status; separately for high (light blue) and low (darker blue; derived by mean-split) FDG-PET values. The only subgroup showing a significant difference in proline levels are *APOE* ε4+ females with substantially higher levels in subjects with lower brain glucose uptake.


A: Pathological Abeta1-42
Heterogeneous ←
Male-specific ←
Homogeneous ←
Female-specific ←
PC ae C44:6 ①
PC ae C44:4
PC ae C44:5
Threonine ④
Creatinine
PC aa C40:4 ②
Valine ③
effect (Z-score) [females]
effect (Z-score) [males]
① Homogeneous effect
females
males
PC ae C44:6
② Heterogeneous effect
PC aa C40:4
③ Female-specific effect
Valine
④ Male-specific effect
Threonine
T: p-Tau
C10*
C5-DC or C6 (OH)
C8
C2
C4:1
PC ae C36:2
Glycine
SM (OH) C16:1
lysoPC a C17:0
PC ae C36:1
SM (OH) C22:1
Asparagine
lysoPC a C18:0
SM (OH) C22:2
Serine
Histidine
N: FDG - PET
PC ae C40:2
C16:1*
PC aa C32:0
PC aa C32:1
PC ae C42:4
PC ae C44:5
PC ae C44:4
Alanine
Proline
Larger group effect
Men
Women
Shape
not significant
significant in one sex
overall significant
significantly different
Boxplot legend
Normal Abeta levels
Pathological Abeta levels


None
1-fold stratification
2-fold stratification
A
FDG-PET
B
Sex
females
males
C
APOE ε4 status
APOE ε4−
APOE ε4+
D
Sex * APOE ε4 status
females
APOE ε4−
APOE ε4+
males
APOE ε4−
APOE ε4+
Proline
4
2
0
−2
FDG-PET ≥ μ
< μ
≥ μ
< μ
26

Supplementary Information for:
The Alzheimer's Disease Metabolome: Effects of Sex and *APOE* ε4 Genotype

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Supplementary Text – Data Availability

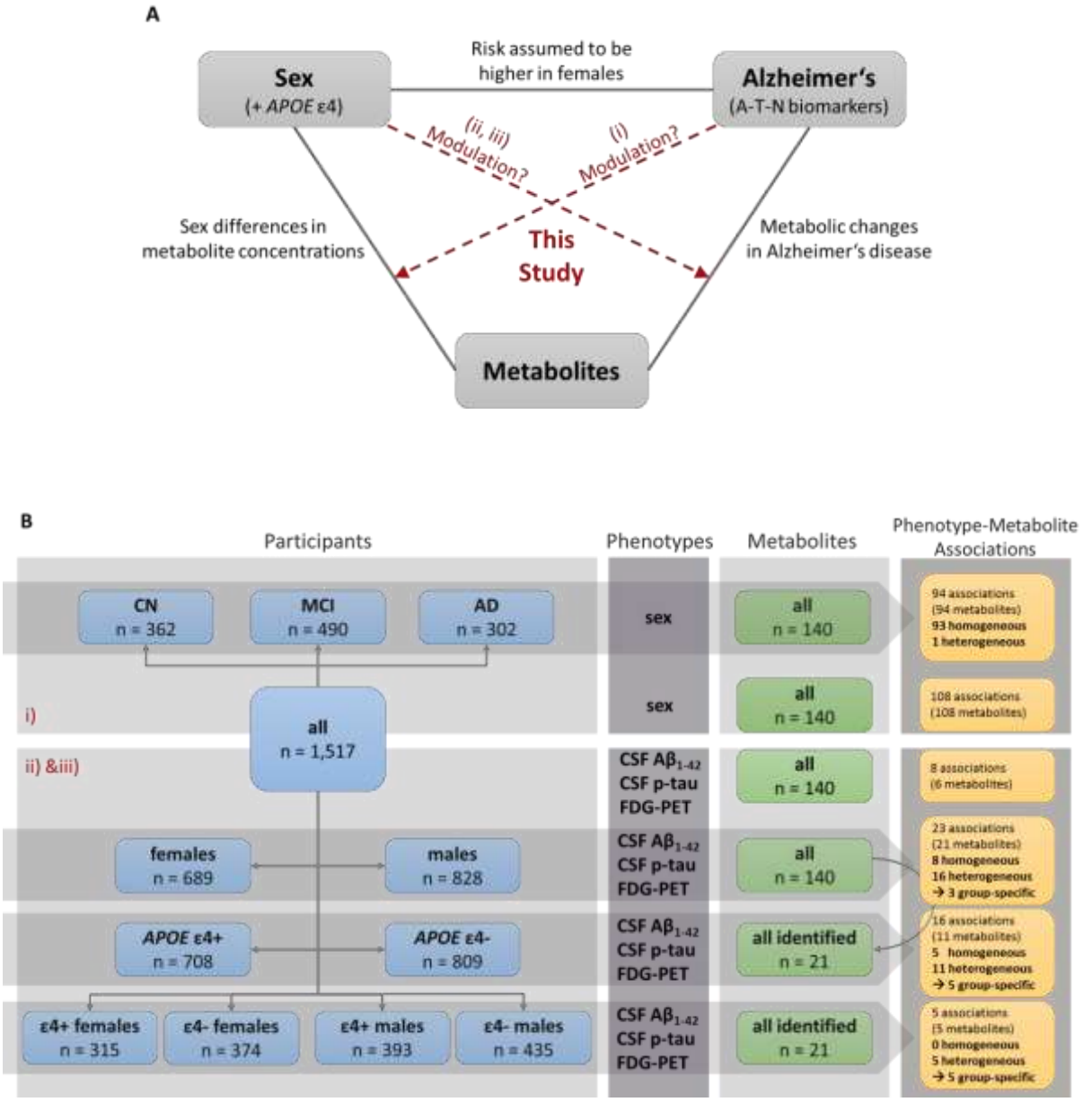
Data use restrictions prohibit the distribution of any ADNI clinical or demographic data outside of LONI. Researchers can apply for access to the ADNI data at http://adni.loni.usc.edu/data-samples/access-data/. Data use restrictions prohibit the distribution of any ROSMAP data. Researchers can apply for access to the ROSMAP data at https://www.radc.rush.edu/ . Data for the ADNI-1 cohort is accessible via http://dx.doi.org/10.7303/syn5592519 . ADNI GO/2 data is accessible via http://dx.doi.org/10.7303/syn9705278 and ROSMAP data is accessible via http://dx.doi.org/10.7303/syn10235592

Supplementary Text - Replication Analysis in ROS/MAP

To replicate a subset of the findings reported in this manuscript in an independent cohort, we used metabolomics data obtained from pre-mortem serum samples of 86 deceased participants of the Religious Orders Study and the Rush Memory and Aging Project (ROS/MAP), who had agreed to post-mortem neuropathological examinations, using the same metabolomics kit (AbsoluteIDQ-p180). Of the 86 total participants, 24 were females / 62 males; 52 CN / 24 MCI / 7 AD; mean age was 87.77 (± 6.01) years. Metabolomics data processing was performed very similar as for the ADNI, except that we used a pool of study samples randomly injected across plates instead of NIST standard plasma, and median- instead of mean-based quotient batch removal. We then did a targeted analysis to replicate associations of PC ae C44:4, PC ae C44:5, and PC ae C44:6 with $A\beta_{1-42}$ pathology using post-mortem, neuropathology-derived measures of total amyloid load in the brain. This phenotype was transformed to square root values to get values closer to a normal distribution. Linear regression models were adjusted for age at blood draw, sex, study cohort (ROS vs. MAP), race, number of copies of APOE ε4, as well as years of education. All three p-values were Bonferroni significant when adjusting for three test (p-value threshold of $P < 1.667$), complete result statistics were:

biomarker	metabolite	effect	se	p-value
total amyloid in the brain	PC ae C44:4	0.30741	0.10277	0.00373
	PC ae C44:5	0.2656	0.10257	0.01149
	PC ae C44:6	0.30992	0.10212	0.00328

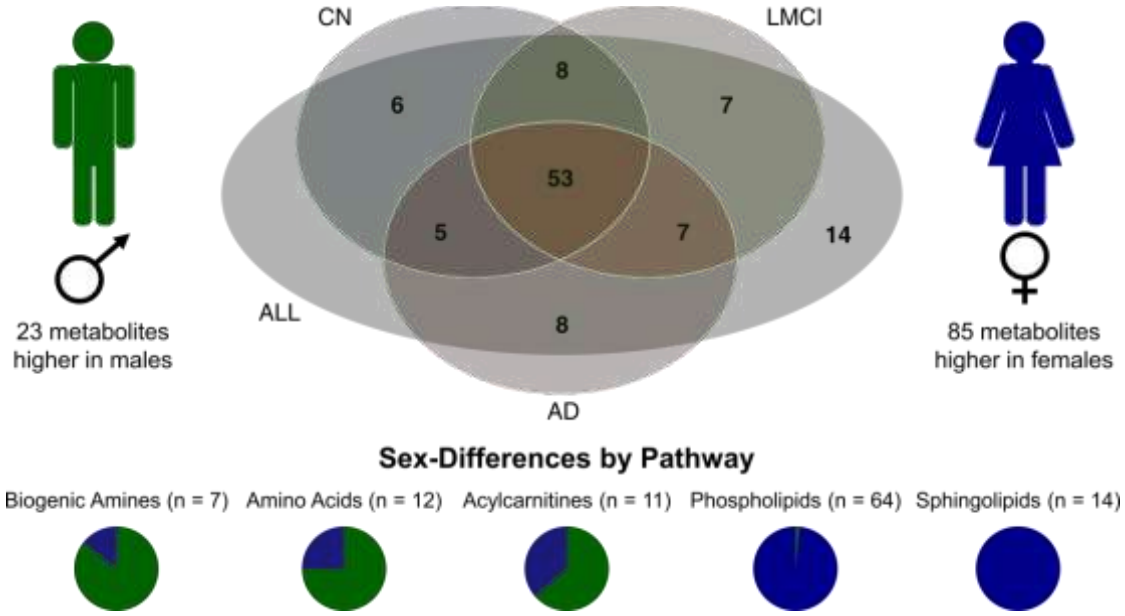
Supplementary Figure 1: Study rationale and workflow



Supplementary Figure 1: Study rationale and workflow. A) This study aims to investigate the relationship between AD, sex, and metabolic readouts in a systematic fashion. The background of this work is: firstly, it has been reported that AD risk may be increased in females; secondly, there are strongly pronounced, highly significant, and often replicated sex differences in metabolite concentrations in the general, healthy population; and, thirdly, we and others have shown that there are significant associations of metabolite levels with AD and its biomarkers. In the current study, we examined: (i) if clinical diagnosis of MCI or AD influences metabolic sex differences as seen in healthy controls, (ii) if sex modulates associations of metabolite levels with three AD biomarkers across the A-T-N spectrum, and, (iii), if effects of metabolites showing sex-based effect heterogeneity in their

associations with AD are also modulated by *APOE* ε4 status. **B)** To address the three research questions of this study, we first performed analyses of sex-metabolite associations for 140 metabolites in the ADNI cohort stratified by diagnostic group (question i). Subsequently, we performed phenotype (A/T/N)-metabolite associations for 140 metabolites in the ADNI cohort stratified by sex (question ii) and stratified by *APOE* ε4 status; additionally, we performed phenotype (A/T/N)-metabolite associations for the 21 significantly associated metabolites after stratification by sex plus *APOE* ε4 status (question iii).

Supplementary Figure 2: Metabolic sex differences in the ADNI cohorts



Supplementary Figure 2: Metabolic sex differences in the ADNI cohorts. We tested whether sex-associated differences in blood metabolite levels differ between patients with probable AD, subjects with MCI, and CN subjects in the ADNI cohorts. We found 108 of 140 metabolites to be significantly associated with sex after multiple testing correction while adjusting for age, BMI, ADNI study phase, and diagnostic group. 70 of these associations replicate previous findings in a healthy population using. All SMs and the majority of PCs were more abundant in women. The majority of biogenic amines, amino acids, and acylcarnitines were more abundant in men. Stratifying subjects by diagnostic group revealed that 53 of the 108 metabolites showing significant sex-differences were also significant in each of the three groups (AD, MCI, CN) alone, while 14 metabolites showed no significant difference in any of the groups, probably due to lower statistical power after stratification. No significant sex-differences were found that were not also significant in the unstratified analysis.

Supplementary Figure 3: Boxplots for all 21 metabolites identified in this study in relation to A-T-N biomarkers in 2-fold stratified analyses

In a separate file, we provide boxplots for all 21 metabolites identified in this study to show their relation to A-T-N biomarkers (A: pathological CSF $A\beta_{1-42}$; T: mean-split CSF p-tau levels; N: mean-split FDG-PET values) for 2-fold stratified analyses by both sex and *APOE* ε4 status. *APOE* ε4 status groups are plotted in separate panels, females and males are distinguished by color (f: blue, m: green), and binarized biomarker groups are emphasized by lighter (lower-risk biomarker profile) and deeper (higher-risk biomarker profile) colors.