

**Serum triglycerides in Alzheimer's disease: Relation to neuroimaging and CSF biomarkers**

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**Abstract** (234/250 words)

**Objective:** To investigate the association of triglyceride (TG) principal component scores with Alzheimer's disease (AD) and the "A/T/N/V" (Amyloid, Tau, Neurodegeneration, and Cerebrovascular disease) biomarkers for AD.

**Methods:** Serum levels of 84 TG species were measured using untargeted lipid profiling of 689 participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI) cohort including 190 cognitively normal older adults (CN) and 339 mild cognitive impairment (MCI) and 160 AD. Principal component analysis with factor rotation was used for dimension reduction of TG species. Differences in principal components between diagnostic groups and associations between principal components and AD biomarkers (including CSF, MRI and [18F]FDG-PET) were assessed using a generalized linear model (GLM) approach. In both cases, the Bonferroni method of adjustment was employed to correct for multiple comparisons.

**Results:** The 84 TGs yielded 9 principal components, two of which consisting of long-chain, polyunsaturated fatty acid-containing TGs (PUTGs), were significantly associated with MCI and AD. Lower levels of PUTGs were observed in MCI and AD compared to CN. PUTG principal component scores were also significantly associated with hippocampal volume and entorhinal cortical thickness. In participants carrying *APOE*  $\epsilon$ 4 allele, these principal components were significantly associated with CSF amyloid- $\beta_{1-42}$  values and entorhinal cortical thickness.

**Conclusions:** This study shows PUTG component scores significantly associated with diagnostic group and AD biomarkers, a finding that was more pronounced in *APOE*  $\epsilon$ 4 carriers. Replication in independent larger studies and longitudinal follow-up are warranted.

**Introduction:** 240/250

Triglycerides (TGs) may represent a risk factor for Alzheimer's disease (AD), yet this relationship is not well understood. TGs are lipids that are comprised of three fatty acids (FA). Blood total TG levels are measured in routine clinical check-ups. Depending on the FAs that contribute to the TG, many combinations of carbon chains and double bonds result, leading to more than 6,000 species encompassed by the term "triglyceride". Conflicting reports exist in the literature regarding TG homeostasis in AD. No relationship between AD and total TGs has been reported,<sup>1</sup> while others suggest that elevated TGs early in life represented a risk factor for increased amyloidosis 20 years later.<sup>2,3</sup> Finally, others found that individuals with probable AD had significantly decreased serum TG levels.<sup>4,5</sup>

Apolipoprotein E (*APOE*)  $\epsilon$ 4 carrier status may serve as a risk factor for altered TG levels in AD. TGs are transported by lipoproteins, due to their lipophilic nature. *APOE* regulates TG homeostasis by acting as a ligand for the TG-rich lipoproteins. The *APOE*  $\epsilon$ 4 allele is a major genetic risk factor for sporadic AD and is associated with a significant decrease in blood TG levels in AD.<sup>5</sup> Here, we examined the association between TGs and AD biomarkers, with and without medication adjustment. We also investigated the effect of *APOE*  $\epsilon$ 4 by stratifying on *APOE*  $\epsilon$ 4 carrier status. This study shows that serum-based TG principal components differ as a function of diagnostic group and are associated with AD biomarkers.

**Methods**

**Study sample:** All individuals used in this study were participants from the Alzheimer's Disease Neuroimaging Initiative Phase 1 (ADNI-1), which is a longitudinal study aimed to explore clinical, genetic, imaging, and biological biomarkers for early AD progression. ADNI-1 is a multicenter consortium across 59 sites in the U.S. and Canada, composed of approximately 200

cognitively normal (CN) older adults, 400 adults diagnosed with mild cognitive impairment (MCI) and 200 adults diagnosed with probable Alzheimer's disease (AD), ranging in age from 47 to 91 years old.<sup>6,7</sup> In partnership with ADNI, the Alzheimer's Disease Metabolomics Consortium (ADMC) provided serum metabolic data for this group of participants. Demographic information, *APOE*, clinical information, neuroimaging and CSF biomarker data were downloaded from the ADNI data repository (<http://adni.loni.usc.edu>).

**Lipid analysis:** Lipid analyses were performed as previously described.<sup>8</sup> In summary, an untargeted lipidomics dataset was generated by the NIH West Coast Metabolomics Center (<http://metabolomics.ucdavis.edu>) using an ultra-high performance liquid chromatography-quadrupole time-of-flight (UHPLC-QTOF) mass spectrometer (Agilent, Santa Clara, California) for 807 baseline serum samples from ADNI-1 participants of the ADMC initiative. Lipid species were annotated by matching accurate mass, isotope abundance, retention times and MS/MS fragmentation spectra in LipidBlast<sup>9</sup> in-silico mass spectral library and measured by signal intensities on the precursor mass level. After data processing, the quality of data was assessed and low-quality lipid species were removed. These quality control (QC) analyses resulted 349 annotated lipids, including 84 triglyceride species. The TG values obtained from the QC step were unadjusted and adjusted<sup>10</sup> for the effect of medication use at baseline.

**Neuroimaging analysis:** MRI scans were processed prior to download as previously described.<sup>11,12</sup> These scans were further processed locally using FreeSurfer version 5.1.<sup>13-15</sup> Regions of interest were extracted, including the bilateral hippocampal volumes, entorhinal cortical thickness, and total intracranial volume (ICV). [<sup>18</sup>F]fluorodeoxyglucose (FDG) PET scans were pre-processed prior to download.<sup>13,16</sup> Standardized uptake value ratio (SUVR) images were created by intensity-normalization using a pons reference region. Mean SUVR values were

extracted for each participant from an overall cortical ROI representing regions where CN>AD from the full ADNI-1 cohort. White matter hyperintensity volumes (WMHI) were assessed using previously described methods.<sup>17,18</sup>

**CSF biomarker analysis:** The CSF biomarker data were downloaded from the ADNI data repository. As previously described,<sup>19</sup> CSF measurements for amyloid  $\beta$  1-42 peptide ( $A\beta_{1-42}$ ), total tau (t-tau), and tau phosphorylated at threonine 181 (p-tau<sub>181P</sub>) were obtained by the validated and highly automated Roche Elecsys electrochemiluminescence immunoassays.

**Cognitive Assessment:** We used the modified Alzheimer's Disease Assessment Scale-cognition sub-scale (ADAS-Cog 13)<sup>20</sup> and memory (ADNI-MEM) as indices of general cognitive performance.<sup>21,22</sup> ADAS-Cog includes eleven items, assessing memory, language, praxis, and orientation. ADAS-Cog 13 includes all items from ADAS-Cog, in addition to delayed recall and cancellation tasks. ADNI-MEM is a memory composite score calculated from the items in several memory tasks including the Rey Auditory Verbal Learning Test (RAVLT), ADAS-Cog, Logical Memory (passage recall), and the Mini-Mental Status Exam (MMSE). Alternate forms were accounted for where applicable.<sup>21</sup>

**A/T/N/V biomarkers:** We used CSF  $A\beta_{1-42}$  levels as a biomarker of amyloid- $\beta$  ("A"), CSF p-tau levels as a biomarker of tau ("T"), structural atrophy on MRI, FDG PET metabolism, and CSF t-tau levels as biomarkers of neurodegeneration ("N"), and white matter hyperintensity volume (WMHI) as a biomarker for microvascular disease burden ("V"), as described in the National Institute of Aging- Alzheimer's Association (NIA-AA) Research Framework.<sup>23</sup> Some ADNI participants had missing data for specific A/T/N/V biomarkers. The specific N available for each biomarker is presented in Table 1.

**Medication adjustment:** Triglyceride data were corrected for 41 major medication classes, including psychiatric, cardiovascular, antidiabetic, anti-hyperlipidemia and dietary supplements. Medication intake was coded as present or absent and a model-based evaluation determined the association of each drug on TG levels, which resulted in drug-corrected serum TG levels. Dimension reduction was then performed on the 84 medication-adjusted triglycerides using Principal Component Analysis (PCA) as implemented in SPSS (v 24). A complete list of medications and procedures were previously reported.<sup>10</sup>

**Education:** Education was measured in years and used as a covariate for MRI endophenotypes and memory tests.

**Statistical analysis:** Dimension reduction was performed on the original 84 triglycerides using Principal Component Analysis (PCA). Initial principal component (PC) extraction was followed by orthogonal rotation to yield principal component-based factor scores. The number of principal components extracted was pre-specified using the standard eigenvalues greater than 1 criterion. “Top contributors” of each rotated principal component were defined as those with a factor loading  $> 0.8$ . All other contributors for each rotated principal component (with a factor loading  $< 0.8$ ) were excluded from further consideration. A series of six separate generalized linear models (GLMs) were performed to assess diagnostic group (CN, MCI, AD) differences across all principal components. Bonferroni adjustment was used to correct for significance of diagnostic group differences across the 9 principal components of triglycerides. In reality, only 6 of the 9 TG PCs were brought forward for further consideration based on our pre-specified preprocessing criteria. Therefore, correcting for 9 PCs of analytes was considered conservative. A series of 14 separate post hoc linear regressions were performed to assess the association of seven A/T/N/V biomarkers with “PC3” and “PC5”. Bonferroni adjustment was used to correct

for the 14 association analyses between “PC3”, “PC5”, and seven A/T/N/V endophenotypes. A series of 14 separate post hoc linear regressions were used to assess the association of seven A/T/N/V biomarkers with “PC3” and “PC5” in *APOE*  $\epsilon$ 4 carriers and non-carriers, separately. Bonferroni adjustment was used to correct for the 14 association analyses between “PC3”, “PC5”, and seven A/T/N/V endophenotypes in *APOE*  $\epsilon$ 4 carriers and non-carriers, separately. Covariates of interest in analyses of the PCA results included age, sex, body mass index (BMI), total triglycerides, and *APOE*  $\epsilon$ 4 carrier status for linear regression. Additional covariates included: years of education for cognitive performance, and years of education and intracranial volume (ICV) for MRI biomarkers. For example, statistical models for “PC5” and diagnosis (a), CSF A $\beta$ 1-42 (b), and hippocampal volume (c), respectively, appeared as follows:

- a) Dependent variable (DV)= “PC5”; Independent variable (IV)=Diagnosis (CN, MCI, AD); Covariates=age, sex, BMI, total triglycerides, *APOE*  $\epsilon$ 4 carrier status;
- b) DV= “PC5”; IV= CSF A $\beta$ 1-42; Covariates= age, sex, BMI, total triglycerides, *APOE*  $\epsilon$ 4 carrier status;
- c) DV= “PC5”; IV= Hippocampal volume; Covariates= age, sex, BMI, total triglycerides, *APOE*  $\epsilon$ 4 carrier status, intracranial volume (ICV), years of education

We did not use total serum cholesterol levels as a covariate because total serum cholesterol levels had no effect on all 6 principal components. We did not use systolic and diastolic blood pressures as covariates because neither variable differed as a function of diagnosis or was associated with A/T/N/V biomarkers in our dataset. Significant associations were defined as  $p < 0.05$  after Bonferroni adjustment to correct for multiple testing in individual analyses.

**Whole brain surface-based analysis:** As previously described, we performed a series of GLM analyses of cortical thickness over the whole brain for “PC3” and “PC5” using SurfStat software

(<http://www.math.mcgill.ca/keith/surfstat/>). We constructed a GLM using age, sex, *APOE*  $\epsilon 4$  status, BMI, total triglycerides, years of education, and ICV as covariates. We corrected for multiple comparisons using the random field theory (RFT) correction method at  $p < 0.05$  significance level.<sup>24</sup>

**Data Availability Statement:** All data used in the analyses reported here are available in the ADNI data repository (<http://adni.loni.usc.edu>).

**Standard Protocol Approvals, Registrations, and Patient Consents:** Written informed consent was obtained at the time of enrollment for imaging and sample collection and protocols of consent forms were approved by each participating sites' Institutional Review Board (IRB).

## Results

In the analysis, we included 689 ADNI participants who had baseline data for the 84 TGs (190 cognitively normal older adults (CN), 339 mild cognitive impairment (MCI) and 160 (AD), after quality control procedures removed participants with non-fasting status ( $n=69$ ). Demographic information is shown in **Table 1**.

**Principal component analysis (PCA) for dimension reduction of TGs:** Dimension reduction with PCA resulted in 9 principal components with eigenvalues  $>1$  (Data available from Dryad (**Table e-1**): <https://doi.org/10.5061/dryad.1dp8r8s>). After selecting for the “top contributors” with a factor loading  $\geq 0.8$  in each component, 6 of 9 components remained for further analysis (Data available from Dryad (**Table e-2**): <https://doi.org/10.5061/dryad.1dp8r8s>).

**Between group differences in TG principal components:** Figure 1 illustrates the profile of group differences between CN and MCI and AD for principal components (“PC3” and “PC5”) after adjusting for multiple testing and covariates. We identified significant group differences in “PC5” between CN and AD ( $p$ -value=  $4.34E-04$ , Cohen’s  $d= 0.386$ ) and between CN and MCI

( $p$ -value= 1.83E-03, Cohen's  $d$ = 0.313; **Figure 1**). MCI and AD did not differ significantly for either "PC3" or "PC5." "PC5" consists of six long-chain, polyunsaturated TGs (PUTGs), with all species containing 8 or more double bonds. Lower levels of PUTGs belonging to "PC5" are seen in MCI and AD compared to CN (Data available from Dryad (**Figure e-1; Table e-3**):

<https://doi.org/10.5061/dryad.1dp8r8s>). In addition, among the six PUTGs, four (TG 60:11, TG 58:9, TG 58:8, and TG 56:8) were significantly lower in MCI and AD compared to CN ( $p$ -value < 0.05) (Data available from Dryad (**Figure e-1**): <https://doi.org/10.5061/dryad.1dp8r8s>). We also identified suggestive group differences in "PC3" between CN and AD ( $p$ -value=0.0533, Cohen's  $d$ = 0.179; **Figure 1**). "PC3" consisted of seven PUTGs, with almost all species containing 2 or more double bonds. Similar to "PC5", AD patients compared to CN showed lower component scores for "PC3" (Data available from Dryad (**Table e-4**):

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**Association of TG principal components with AD biomarkers:** The two principal components that showed significant ("PC5") or suggestive ("PC3") diagnosis group differences were further investigated to assess their associations with continuous A/T/N/V biomarkers for AD. **Figure 2** shows associations of "PC3" and "PC5" with AD biomarkers. Linear regression analysis indicated a significant association between "PC5" and hippocampal volume ("N") ( $p$ -value =0.0243, standardized  $\beta$ =0.135, adjusted  $R^2$  = 0.0683; **Figure 2**) and "PC3" and entorhinal thickness ("N") ( $p$ -value=0.00363, standardized  $\beta$ =0.132, adjusted  $R^2$  = 0.188; **Figure 2**). Lower "PC5" scores were associated with greater brain atrophy. As "PC5" is associated with hippocampal volume, we investigated the association of "PC5" with cognitive performance using ADAS-Cog13 scores and ADNI-MEM. The analysis revealed associations between ADAS-Cog13 ( $p$ -value =0.012, standardized  $\beta$  = -0.099, adjusted  $R^2$  = 0.0579) and ADNI-MEM ( $p$ -

value = 0.00443, standardized  $\beta = 0.115$ , adjusted  $R^2 = 0.0613$ ) with “PC5.” We then performed a detailed whole-brain surface-based analysis of cortical thickness to investigate the effects of “PC3” and “PC5” on cortical atrophy in a spatially unbiased manner. Lower component scores of “PC3” were significantly associated with reduced cortical thickness in bilateral frontal and parietal lobes and right temporal lobe including the entorhinal cortex ( $p$ -value < 0.05; **Figure 3(A)**). Also, lower component scores of “PC5” were significantly associated with reduced cortical thickness in right temporal lobe including the entorhinal cortex ( $p$ -value < 0.05; **Figure 3(B)**).

**Effect of *APOE*  $\epsilon 4$  on TGs:** In order to investigate the effect of *APOE*  $\epsilon 4$  on TGs, we first investigated the presence of an interaction between *APOE*  $\epsilon 4$  status and diagnosis and A/T/N/V biomarkers for AD with principal components. We did not find evidence of significant interactions for any principal components with *APOE*  $\epsilon 4$  carrier status. We then performed an association analysis of principal components with diagnosis and A/T/N/V biomarkers for AD after stratifying on *APOE*  $\epsilon 4$  carrier status. In both the *APOE*  $\epsilon 4$  carrier group and *APOE*  $\epsilon 4$  non-carrier group, we did not find any significant associations of principal components with diagnosis (Data available from Dryad (**Figure e-2**): <https://doi.org/10.5061/dryad.1dp8r8s>). However, in the *APOE*  $\epsilon 4$  carrier group, “PC5” was significantly associated with CSF  $A\beta_{1-42}$  levels ( $p$ -value = 0.0359, standardized  $\beta = 0.228$ , adjusted  $R^2 = 0.101$ ; **Figure 4**) and marginally associated with entorhinal cortical thickness ( $p$ -value = 0.0537, standardized  $\beta = 0.156$ , adjusted  $R^2 = 0.073$ ; **Figure 4**). “PC3” was also significantly associated with entorhinal cortical thickness ( $p$ -value = 9.66E-04, standardized  $\beta = 0.192$ , adjusted  $R^2 = 0.267$ ) (**Figure 4**). In the *APOE*  $\epsilon 4$  non-carrier group, we did not identify any significant associations of principal components with A/T/N/V biomarkers for AD.

**Effect of medication use on TGs:** Using TG values adjusted for the effect of medication use at baseline as a potential confounder, we repeated all analyses. All key findings remained significant after adjustment for medication use (Data available from Dryad (**Figures e-3 and e-4**): <https://doi.org/10.5061/dryad.1dp8r8s> see).

## Discussion

In this study, we found that long-chain, polyunsaturated FA-containing triglycerides (PUTGs) showed significant differences between diagnostic groups (CN, MCI, AD; Data available from Dryad (**Table e-3 and e-4**): <https://doi.org/10.5061/dryad.1dp8r8s>). Lower PUTG component scores in MCI suggest that the changes occur during prodromal stages of disease, though longitudinal studies will be required to confirm this cross-sectional analysis. PUTG component scores were significantly associated with early-AD biomarkers, including hippocampal volume and entorhinal cortical thickness measured from MRI scans. In addition, we observed a significant *APOE*  $\epsilon 4$  effect on PUTG components. In *APOE*  $\epsilon 4$  carriers, we found significant, positive associations between component scores of PUTGs and entorhinal cortical thickness and CSF  $A\beta_{1-42}$  levels, but no significant associations in *APOE*  $\epsilon 4$  non-carriers. The pattern of observed principal component scores suggest that reduction of PUTGs are associated with early stage changes in AD.

The association of PUTGs with atrophy in the entorhinal cortex and hippocampus is noteworthy as these regions are affected in early stages of AD pathophysiology.  $A\beta$  accumulation is an important early change in AD and is associated with *APOE*  $\epsilon 4$  carrier status. Triglycerides (TGs) have been shown to be particularly associated with  $A\beta$ . For example, mouse studies showed that serum TG levels were elevated prior to amyloid deposition,<sup>25</sup> while human studies showed increased serum TGs were associated with increased amyloidosis in cognitively normal

individuals.<sup>3</sup> Longitudinal studies showed that increased midlife TGs predicted amyloidosis and tau pathology 20 years later,<sup>2</sup> providing additional evidence for the early influence of TGs in AD. Thus, it is particularly interesting that we found a selective association of PUTGs with CSF A $\beta$  only in *APOE*  $\epsilon$ 4 carriers. The specific mechanistic role underlying the association of decreased PUTGs and early processes involved in AD pathogenesis remains to be determined, but our results suggest a relationship between a decrease in component scores of PUTGs and early-stage biomarkers in AD. Results remained significant after adjusting for medication use. Thus, exposure to medication by AD and some MCI patients does not appear to account for these results, although some roles cannot be entirely ruled out.

The relationship between TGs and amyloid- $\beta$  may be explained through role of TGs in the lipoprotein peripheral transport of A $\beta$ . Apolipoprotein E (ApoE) is a plasma protein that is involved in lipid transport and metabolism in lipoproteins, including TG-rich lipoproteins.<sup>26</sup> Studies suggest that TG-rich lipoproteins may participate in peripheral A $\beta$  transport and delivery, as evidenced by a study that showed A $\beta$  accumulated in TG-rich lipoproteins.<sup>27</sup> As discussed above, ApoE exists in three major isoforms, ApoE  $\epsilon$ 4, ApoE  $\epsilon$ 3, and ApoE  $\epsilon$ 2. ApoE  $\epsilon$ 4 has been shown unable to complex with A $\beta$  and, peripherally, to associate with triglyceride-rich, very low-density lipoproteins (VLDLs) in contrast to high-density lipoproteins (HDL).<sup>28,29</sup> These studies suggest an important relationship between A $\beta$  and peripheral TG lipoprotein carriers that may provide further insight into aberrant serum PUTG levels in AD.

Omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), have anti-inflammatory and neuroprotective roles, as evidenced by their associations with cognitive improvement in the elderly.<sup>30</sup> In blood, polyunsaturated fatty acids travel in different states: esterified, bound to complex lipids (such as TGs) or lipoproteins, or free-

floating. Triglycerides are created by incorporating three fatty acids onto a carbon backbone, which can then serve to transport and store fatty acids. A previous study, using the same TG dataset, identified EPA and DHA as the primary polyunsaturated fatty acids contributing to PUTG fatty acids of “PC5”.<sup>31</sup> Recently published studies have shown that serum DHA and EPA levels were decreased in AD,<sup>32</sup> and that polyunsaturated fatty acid intake acted to reduced risk for AD.<sup>33</sup> We found that lower component scores of PUTGs were associated with poorer cognitive performance. Due to the importance of these polyunsaturated fatty acids in cognition and neuroinflammation, we believe our findings need further study to determine the overlapping mechanism that may underlie PUTG and polyunsaturated fatty acid aberrations. PUTGs and, by extension, polyunsaturated fatty acids represent links in the gut-liver-brain axis. Triglycerides are synthesized in the liver or absorbed from the gut and have recently been found to cross the blood-brain barrier and accumulate in the brain.<sup>34</sup> PUFAs are rapidly and preferentially incorporated into TGs following postprandial ingestion, suggesting an important role for TGs in PUFA supplementation.<sup>35</sup> The gut-liver-brain communicate through neuronal, neuroimmune, and neuroendocrine pathways. These pathways overlap polyunsaturated fatty acids and AD and may serve an interesting direction to study PUTGs.

A Lipid Hypothesis has been proposed, suggesting that lipid oxidation is the initiating factor for late-onset AD.<sup>36</sup> Lipid oxidation is a key early event in AD that precedes amyloid and neurofibrillary tangle deposition. When lipids are exposed to free radicals, they progress through autooxidation, where reactive oxidative species are released. Previous studies have shown that A $\beta$  aggregation occurred more readily in membranes composed of oxidized lipids,<sup>37</sup> and that polyunsaturated lipids were most vulnerable to oxidative stress.<sup>38</sup> Early-stage AD is characterized by an accumulation of A $\beta$ , potentially resulting from impaired clearance of

pathogenic species by microglia. Early microglial activity is neuroprotective, but progressive cytokine production from microglia, due to aging and disease progression, can reduce A $\beta$  clearance and promote its accumulation. Peripheral inflammatory cytokines have been shown to interact with or pass through endothelial cells on the blood-brain barrier to activate microglia. It is well established that polyunsaturated fatty acid intake is associated with an anti-inflammatory effect. Polyunsaturated fatty acids have been directly linked to the regulation of cytokines by decreasing the expression of proinflammatory pathways. Moreover, polyunsaturated fatty acid intake has been shown to decrease microglial inflammatory activation of A $\beta$ , supporting a neuroprotective role.<sup>39</sup> Decreases in PUTGs could presumably result in decreased availability for polyunsaturated fatty acids and loss of neuroprotective metabolites, thereby suggesting the need for further study into this relationship.

Neural connections between the brain and the gut exist through the vagus nerve and enteric system. The vagus nerve originates in the brain stem and complexes onto the enteric plexus in the gut. These neural connections are important for gastric motility and have been implicated in relaying inflammatory, microbial and nutrient information from the gut to the brain.<sup>40</sup>

Interestingly, dietary fat has shown to activate the vagus nerve and neuroimmunologic pathways.<sup>41</sup> Recent work in Parkinson's disease found that severance of the vagal trunk was protective against PD, suggesting that central nervous system (CNS) invasion may occur from the gut, through the vagus nerve.<sup>42</sup> This study provides an interesting avenue of study for AD due to many links between PD and AD.

The gastrointestinal tract is home to more than 10 times the number of bacteria than cells in the human body, functioning in a mutualistic relationship with their human host.<sup>43</sup> Gut microbiota are known to function in immune responses, nutrient absorption, and regulate gut motility.<sup>44,45</sup>

When gut microbiota is no longer homeostatic (gut dysbiosis), the CNS receives signals to activate inflammatory processes. Alterations in gut microbiota have been linked to neurological conditions, including AD, where gut dysbiosis was associated with memory dysfunction and decreased hippocampal neurogenesis.<sup>46</sup> Polyunsaturated fatty acid intake has been associated with changes in gut microbiota and intestinal excretion of mucosal defense factors that exhibit anti-inflammatory effects. The data suggests an interesting relationship between polyunsaturated fatty acids, gut microbiota and AD and an interesting direction to study PUTGs.

We did not see a significant association between saturated TGs and AD, although we did find a significant difference in PUTG component scores between diagnostic groups. A recent meta-analysis revealed that, over time, saturated fat intake was associated with an increase in AD risk.<sup>47</sup> It is possible that blood TG levels do not accurately represent long-term saturated TG intake and, rather, BMI may better represent the effects of highly saturated diets over time.<sup>48</sup> Further studies are required to investigate these findings.

We hypothesized that APOE  $\epsilon$ 4 carrier status may influence how TGs associate with AD endophenotypes given evidence that APOE  $\epsilon$ 4 polymorphisms influence fasting serum triglyceride levels and play a significant role in AD.<sup>49</sup> We did see a significant association of PUTGs with AD endophenotypes in analyses stratified for APOE  $\epsilon$ 4 carrier status, but there was not an overall modifying effect of APOE  $\epsilon$ 4 carrier status on the association of PUTGs with AD endophenotypes. Although the absence of an overall main effect of APOE  $\epsilon$ 4 carrier status limits generalizability, the association of serum triglyceride subspecies with AD endophenotypes in APOE  $\epsilon$ 4 carriers is of heuristic value and warrants further investigation in a larger, independent study.

There are some limitations to our study. The ADNI observational cohort was designed to be typical of participants who enroll in clinical trials, but is not necessarily representative of the broader community as would be found in epidemiologically derived samples. As ADNI is a largely white sample with high mean education, the present results should not be generalized to community-based populations without further investigation. It will be important to repeat these analyses in more socioeconomically, educationally and racially diverse samples.

The present study of cross-sectional associations is unable to support causal inferences regarding directionality. For example, it is plausible that dietary habits, nutritional intake as well as neurodegeneration influence serum levels of these lipids. Longitudinal assays and clinical follow-up of the present cohort and replication in independent samples will be important to establish potential causal relationships between PUTGs and AD endophenotypes. In addition, functional experiments with lipidomic readouts will ultimately help to clarify underlying mechanisms. Model system studies are required to determine the role and mechanisms of specific classes of TGs in AD initiation and progression. Mechanistic investigations into the cause of decreased PUTG component scores using mouse models of AD may provide insight relevant to early detection and treatment for AD. Although we report modest effect sizes, these effects may indicate important biological relationships that warrant future pursuit with alternate or complementary study design. In multifactorial diseases (such as Alzheimer's disease), high levels of discrimination and confidence are achieved through the combination of multiple "weak" individual markers into a single "strong" multivariate model, a well-known approach in drug and biomarker discovery.<sup>50</sup> Future studies are warranted to investigate the potential role of PUTGs as early detection biomarkers and as targets for therapeutic development. Replication

studies, longitudinal analyses, and functional experimental evaluation in model systems are all future directions for the PUTGs in this study.

In summary, our study investigated the relationship between TG species, AD risk and biomarkers for AD. To our knowledge, this is the first study to show decreased levels of highly unsaturated, long-chain triglycerides in MCI and AD compared to cognitively normal older adults. We also observed an association between decreased PUTG component scores with increased brain atrophy, decreased CSF amyloid- $\beta$  concentration, and the effect of *APOE*  $\epsilon$ 4 carrier status on PUTG components in AD. Our study aimed to increase biological understanding of Alzheimer's disease (AD) pathophysiology and mechanisms through association studies of triglyceride profiles with AD endophenotypes. Our findings identify a specific subcategory of TGs, namely the PUTGs, which appear mechanistically relevant and provide the foundation for future work in therapeutic development. We report evidence that PUTGs are associated with an early prodromal stage of cognitive impairment (i.e., MCI) and early stage biomarkers for AD. Replication in independent samples and longitudinal analysis are needed to elucidate any causal relationships.

#### Appendix: authors

Name	Location	Role	Contribution
Megan Bernath, BS	Indiana University, Indianapolis	Author	Study design, statistical analysis, drafted and revised manuscript
Sudeepa Bhattacharyya, PhD	University of Arkansas for Medical Sciences	Author	Initial analysis of data and edited manuscript

Kwangsik Nho, PhD	Indiana University, Indianapolis	Author	Design and conceptualization of study, edited manuscript
Shannon L Risacher, PhD	Indiana University, Indianapolis	Author	MRI and PET image processing
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## Appendix 2: Co-Investigators

Name and Degree	Institution	Role in ADMC	Contributions
Alexandra Kueider- Paisley, Ph.D.	Duke University Medical Center	Scientific management	Coordinated communication
Colette Blach, M.S.	Duke University Medical Center	Database management	Data storage and organization

Arthur Moseley, Ph.D.	Duke University Medical Center	Duke Proteomics and Metabolomics Core PI	Led and coordinated Proteomics and Metabolomics Core
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Siamak Mahmoudiandehkhordi, Ph.D.	Duke University Medical Center	Bioinformatics	Performed biostatistical review of data
Kathleen Welsh-Balmer, Ph.D.	Duke University Medical Center	AD Clinician	Data Collection
Brenda Plassman, Ph.D.	Duke University Medical Center	AD Clinician	Data Collection
Gabi Kastenmüller, Ph.D.	Helmholtz Zentrum München	bioinformatics	Intellectual contribution to ADMC
Xianlin Han, Ph.D.	University of Texas Health Science Center San Antonio	Lipid expert/metabolomics	Intellectual contribution to ADMC

Peter Meikle, Ph.D.	Bake Heart and Diabetes Institute	Lipids expert, metabolomics	Intellectual contribution to ADMC; data processing
Sarkis Mazmanian, Ph.D.	California Institute of Technology	Gut microbiome expert	Intellectual contribution to ADMC
Mitchel Kling, M.D.	University of Pennsylvania	AD clinician	Data collection
Jon Toledo, Ph.D.	University of Pennsylvania	bioinformatics	Intellectual contribution to ADMC
Cornelia van Duijin, Ph.D.	Oxford University	AD expert	Intellectual contribution to ADMC
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David Wishart, PH.D.	The Metabolomics Innovation Centre Canada (TMIC)	metabolomics	Intellectual contribution to ADMC

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John Newman, Ph.D.	USDA ARS	Metabolomics, bioinformatics	Intellectual contribution to ADMC
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Herman Moreno, M.D.	SUNY Downstate	Neurology, electrophysiology expert	Intellectual contribution to ADMC

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**Table 1.** Demographics of ADNI participants included in the analysis by diagnosis.

	N	CN	N	MCI	N	AD
Age	190	75.29 (4.93)	339	74.25 (7.54)	160	74.43 (7.32)
Sex (Male/Female)	190	94/96	339	218/121	160	79/81
Education	190	15.96 (2.97)	339	15.63 (3.027)	160	14.56 (3.093)
APOE $\epsilon$ 4 status (presence/absence)	190	52/138	339	177/162	160	108/52
BMI	190	26.72 (4.55)	339	26.15 (3.99)	160	25.57 (4.00)
Total triglycerides	190	140.27 (79.77)	339	154.41 (142.53)	160	156.83 (99.091)
Hippocampal volume	189	3483.79 (445.30)	339	3086.87 (528.88)	159	2758.67 (502.71)
Entorhinal Thickness	189	3.41 (0.29)	339	3.11 (0.44)	159	2.76 (0.45)
FDG Global Cortex	77	1.44 (4.095)	171	1.38 (0.15)	77	1.25 (0.13)
WMHI	186	-0.68 (0.70)	338	-0.62 (0.73)	159	-0.44 (0.67)
CSF A $\beta$	88	3.036 (0.23)	168	2.88 (0.22)	84	2.77 (0.20)
CSF t-tau	86	2.37 (0.15)	168	2.46 (0.17)	83	2.52 (0.16)
CSF p-tau	86	1.33 (0.16)	168	1.44 (0.20)	83	1.51 (0.19)
ADAS-13	190	9.16 (4.10)	336	18.56 (6.20)	156	28.72 (7.96)
ADNI-MEM	190	1.014 (0.54)	339	-0.099 (0.60)	160	-0.84 (0.54)

Data are reported as mean (standard deviation) unless otherwise indicated.

*Abbreviations:* CN: cognitively normal older adult controls; MCI: mild cognitive impairment;

AD: Alzheimer's disease; BMI: body mass index; WMHI: white matter hyperintensity volume;

FDG Global cortex: cortical glucose SUVR measured from [<sup>18</sup>F]FDG PET scans; CSF: cerebrospinal fluid; CSF A $\beta$ : CSF amyloid  $\beta$  1-42 peptide (A $\beta$ <sub>1-42</sub>); CSF p-tau: CSF tau phosphorylated at threonine 181 (CSF p-tau<sub>181P</sub>); CSF t-tau: CSF total tau; ADAS-Cog 13: Alzheimer's Disease Assessment Scale-cognition sub-scale.

**Figure 1: Group differences of principal components of triglycerides with diagnosis groups (CN, MCI, and AD)**

(# = 0.0533, \* < 0.05, \*\* <0.01, \*\*\*<0.001)

A series of six separate generalized linear models (GLMs) were performed to assess diagnostic group (CN, MCI, AD) differences across all principal components. Bonferroni adjustment was used to correct for significance of diagnostic group differences across the 9 principal components of triglycerides. Covariates included: age, sex, body mass index (BMI), total triglycerides, and *APOE*  $\epsilon$ 4 status. mean +/- standard error.

*Abbreviations:* PC: principal component; CN: cognitively normal older adult controls; MCI: mild cognitive impairment; AD: Alzheimer's disease.

**Figure 2: Association of triglyceride “PC3” and “PC5” with A/T/N/V biomarkers for AD**

(\* < 0.05, \*\* <0.01)

A series of 14 separate post hoc linear regressions were performed to assess the association of seven A/T/N/V biomarkers with “PC3” and “PC5”. Bonferroni adjustment was used to correct for the 14 association analyses between “PC3”, “PC5”, and seven A/T/N/V endophenotypes. Covariates included: age, sex, body mass index (BMI), total triglycerides, and *APOE*  $\epsilon$ 4 status for all A/T/N/V phenotypes. For MRI biomarkers, we also included years of education and intracranial volume (ICV) as additional covariates. The y-axis colors represent standardized  $\beta$  values from the linear regression analysis, with shades of red indicating a positive standardized  $\beta$  value and gray scale a negative standardized  $\beta$  value.

*Abbreviations:* PC: principal component; CSF: cerebrospinal fluid; CSF A $\beta$ : CSF amyloid  $\beta$  1-42 peptide (A $\beta$ <sub>1-42</sub>); CSF p.tau: CSF tau phosphorylated at threonine 181 (CSF p-tau<sub>181P</sub>); CSF t.tau: CSF total tau (CSF t-tau); WMHI: white matter hyperintensity total volume, FDG Global cortex:

cortical glucose SUVR measured from [<sup>18</sup>F]FDG PET scans; Hippocampal.volume: hippocampal volume; Entorhinal.thickness: entorhinal cortical thickness, “A”= A $\beta$ <sub>1-42</sub> levels as a biomarker of amyloid- $\beta$ , “T”= CSF p-tau levels as a biomarker of tau, “N”= structural atrophy on MRI, FDG PET metabolism, and CSF t-tau levels as biomarkers of neurodegeneration, and “V”= white matter hyperintensity volume as a biomarker for microvascular disease burden.

**Figure 3: Whole-brain surface-based analysis of cortical thickness for “PC3” and “PC5”.**

A whole-brain analysis of cortical thickness across the brain surface was performed to identify the association of two principal components ((a) “PC3” and (b) “PC5”) with brain structure shown as a T-value map and a P-value map. Statistical maps were thresholded using a random field theory for a multiple testing adjustment to a significance level of 0.05. Positive t values (red, yellow) indicate thicker cortical thickness. The *p*-value for clusters indicates significant *p* values with the lightest blue color. Covariates included: age, sex, body mass index (BMI), total triglycerides, *APOE*  $\epsilon$ 4 status, years of education, and intracranial volume (ICV).

**Figure 4: Association of “PC3” and “PC5” with A/T/N/V biomarkers for AD in the *APOE*  $\epsilon$ 4 carrier group**

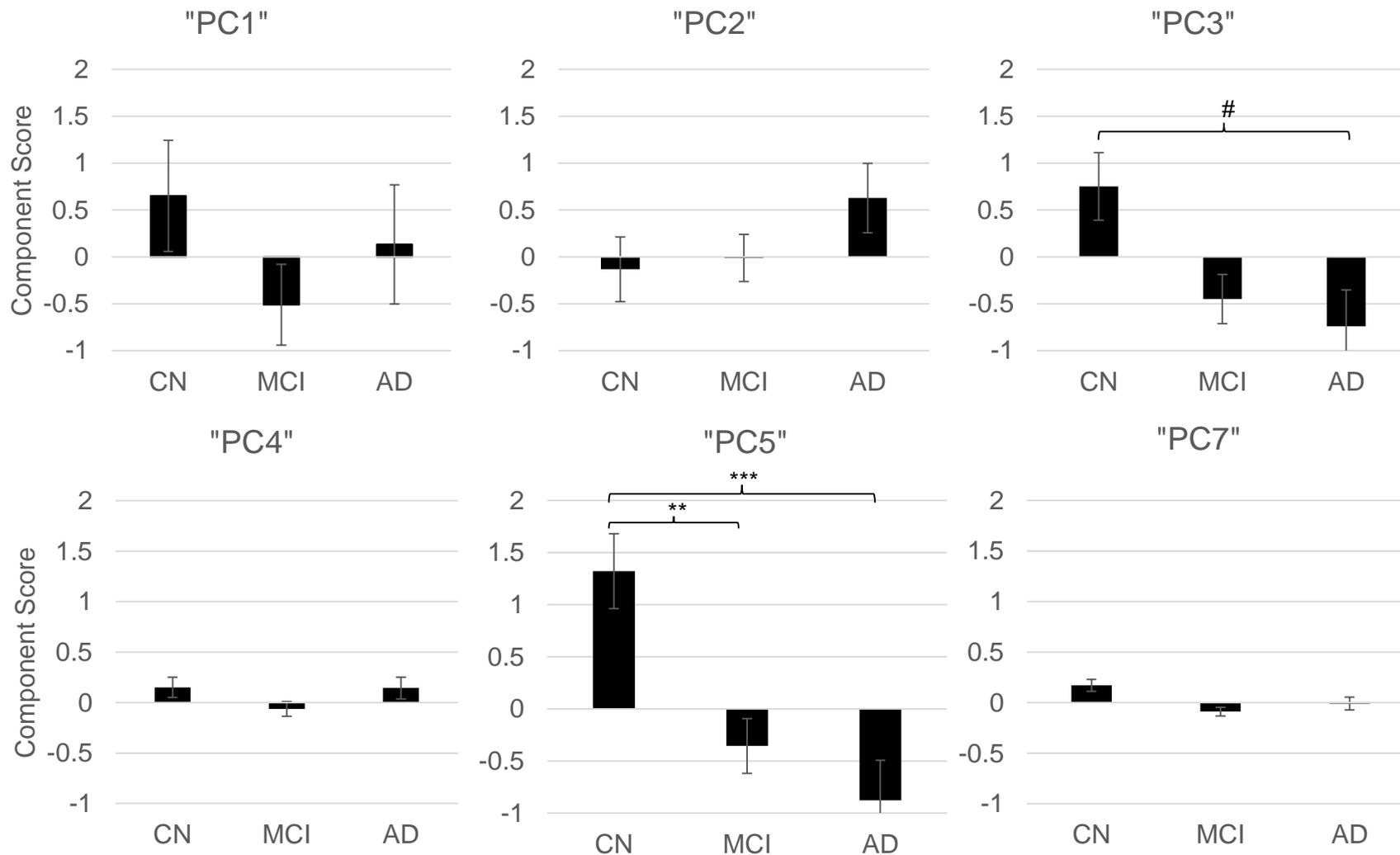
(# = 0.0537, \* < 0.05, \*\* < 0.01, \*\*\* < 0.001)

A series of 14 separate post hoc linear regressions were used to assess the association of seven A/T/N/V biomarkers with “PC3” and “PC5” in *APOE*  $\epsilon$ 4 carriers. Bonferroni adjustment was used to correct for the 14 association analyses between “PC3”, “PC5”, and seven A/T/N/V endophenotypes. Covariates included: age, sex, body mass index (BMI), total triglycerides, and *APOE*  $\epsilon$ 4 carrier status for all A/T/N/V phenotypes. For MRI biomarkers, we also included years of education and intracranial volume (ICV) as additional covariates. The y-axis colors represent

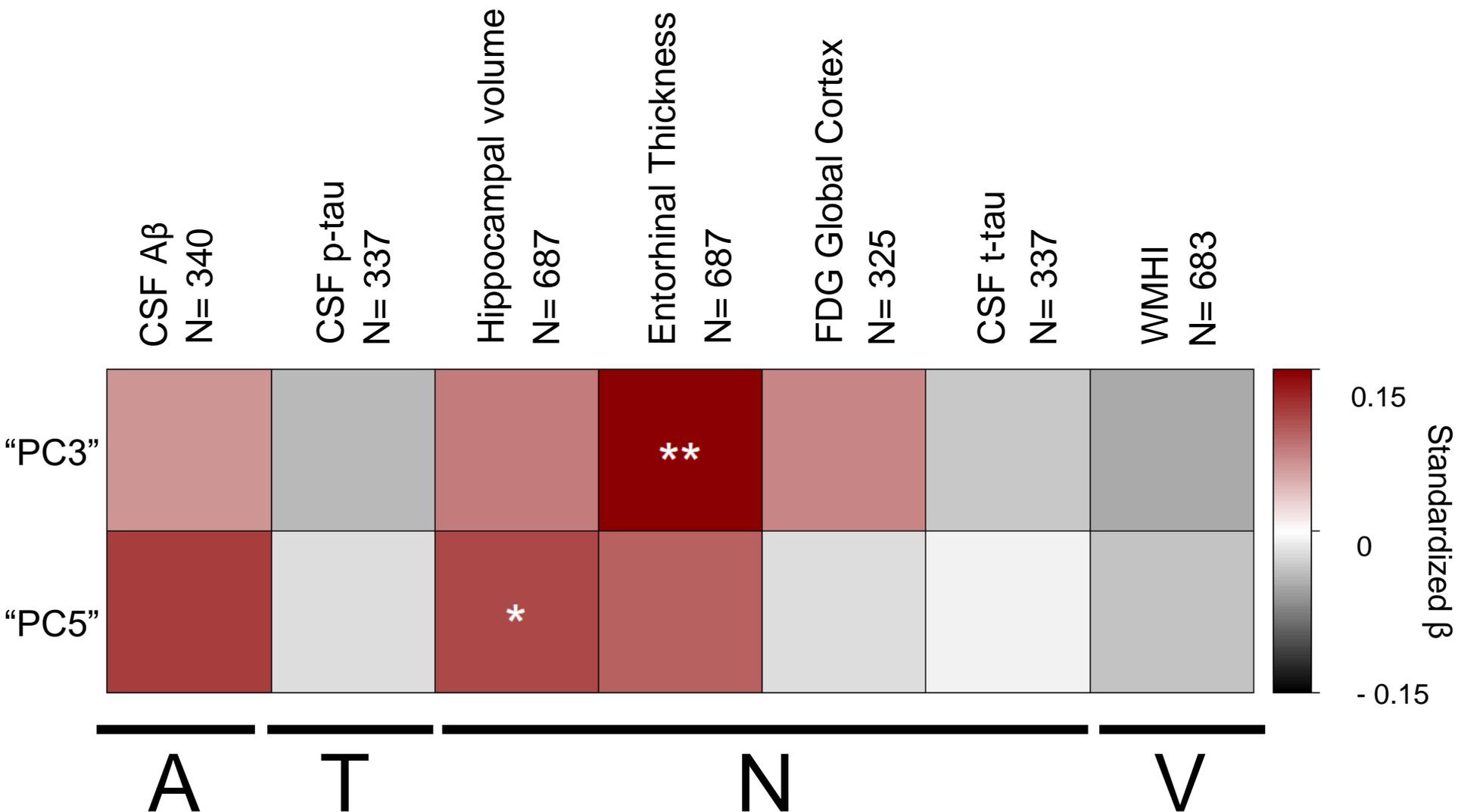
standardized  $\beta$  values from the linear regression analysis, with shades of red indicating a positive standardized  $\beta$  value and gray scale a negative standardized  $\beta$  value.

*Abbreviations:* PC: principal component; CSF: cerebrospinal fluid; CSF A $\beta$ : CSF amyloid  $\beta$  1-42 peptide (A $\beta_{1-42}$ ); CSF p.tau: CSF tau phosphorylated at threonine 181 (CSF p-tau<sub>181P</sub>); CSF t.tau: CSF total tau (CSF t-tau); WMHI: white matter hyperintensity volume, FDG Global cortex: cortical glucose SUVR measured from [<sup>18</sup>F]FDG PET scans; Hippocampal.volume: hippocampal volume; Entorhinal.thickness: entorhinal cortical thickness, “A”= A $\beta_{1-42}$  levels as a biomarker of amyloid- $\beta$ , “T”= CSF p-tau levels as a biomarker of tau, “N”= structural atrophy on MRI, FDG PET metabolism, and CSF t-tau levels as biomarkers of neurodegeneration, and “V”= white matter hyperintensity volume as a biomarker for microvascular disease burden.

**Figure 1: Group differences of principal components of triglycerides with diagnosis groups (CN, MCI, and AD)**

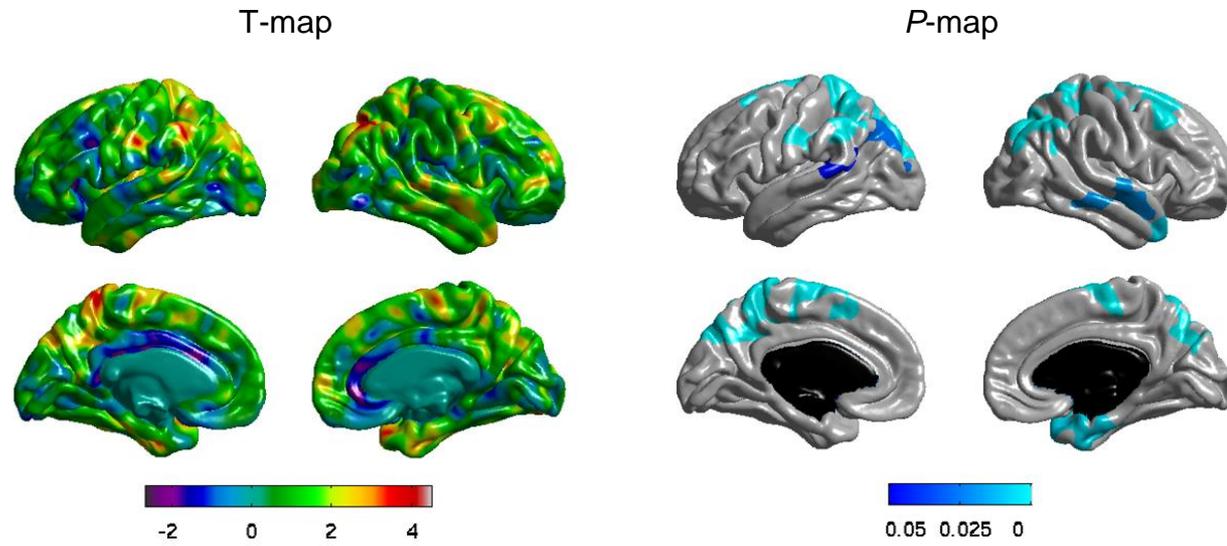


**Figure 2: Association of two principal components (“PC3” and “PC5”) with A/T/N/V biomarkers for AD**

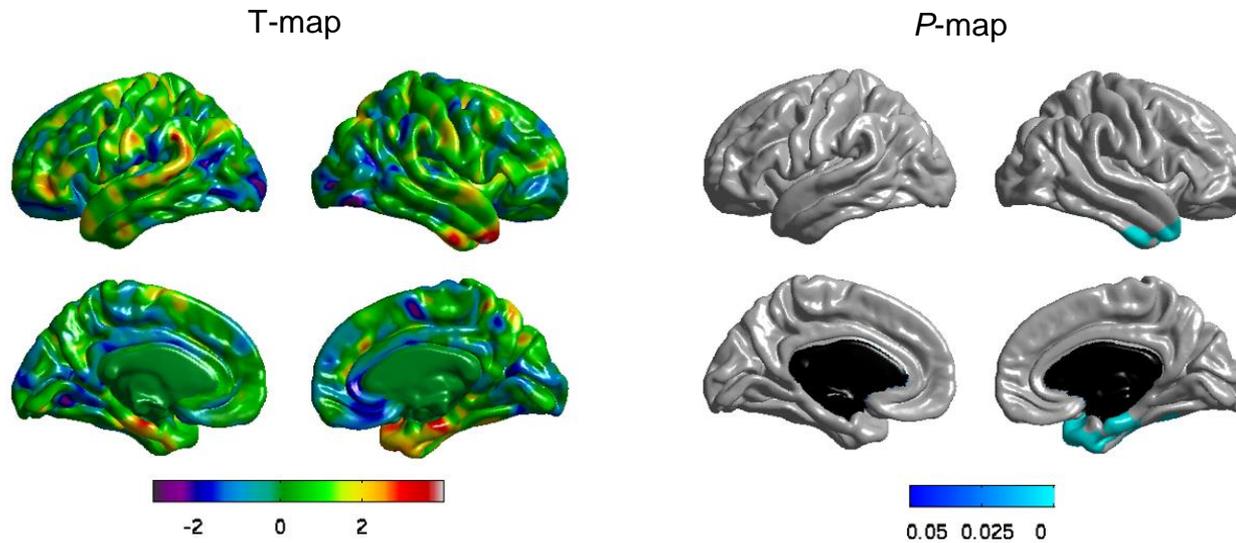


**Figure 3: Whole-brain surface-based analysis of cortical thickness for “PC3” and “PC5”**

A. “PC3”



B. “PC5”



**Figure 4: Association of two principal components (“PC3” and “PC5”) with A/T/N/V biomarkers for AD in the *APOE*  $\epsilon$ 4 carrier group**

