Cerebrospinal Fluid Sphingomyelins in Alzheimer's Disease, Neurodegeneration, and Neuroinflammation

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Abstract

Background: Sphingomyelin (SM) levels have been associated with Alzheimer's disease (AD), but the association direction has been inconsistent and research on cerebrospinal fluid (CSF) SMs has been limited by sample size, breadth of SMs examined, and diversity of biomarkers available.

Objective: Here, we seek to build on our understanding of the role of SM metabolites in AD by studying a broad range of CSF SMs and biomarkers of AD, neurodegeneration, and neuroinflammation.

Aβ: amyloid beta, AD: Alzheimer's disease, ADRC: Alzheimer's Disease Research Center, α-synuclein: alpha synuclein, ANOVA: analysis of variance, CSF: cerebrospinal fluid, CU: cognitively unimpaired, DVR: distribution volume ratio, IL6: interleukin-6, LMM: linear mixed effect model, LP: lumbar puncture, MCI: mild cognitive impairment, MRI: magnetic resonance imaging, NfL: neurofilament light, NTK: Neuro Tool Kit, PET: positron emission tomography, PiB: Pittsburgh compound B, p-tau: phosphorylated tau, p-tau/Aβ: p-tau to amyloid beta 42 ratio, QC: quality control, ROI: region of interest, SM: sphingomyelin, sTREM2: soluble triggering receptor found on myeloid cells 2, WRAP: Wisconsin Registry for Alzheimer's Prevention, YKL40: chitinase-3-like protein 1

Methods: Leveraging two longitudinal AD cohorts with metabolome-wide CSF metabolomics data (n = 502), we analyzed the relationship between the levels of 12 CSF SMs, and AD diagnosis and biomarkers of pathology, neurodegeneration, and neuroinflammation using logistic, linear, and linear mixed effects models.

Results: No SMs were significantly associated with AD diagnosis, mild cognitive impairment, or amyloid biomarkers. Phosphorylated tau, neurofilament light, α-synuclein, neurogranin, soluble triggering receptor expressed on myeloid cells 2, and chitinase-3-like-protein 1 were each significantly, positively associated with at least 5 of the SMs.

Conclusion: The associations between SMs and biomarkers of neurodegeneration and neuroinflammation, but not biomarkers of amyloid or diagnosis of AD, point to SMs as potential biomarkers for neurodegeneration and neuroinflammation that may not be AD-specific.

Key words

Metabolomics, Alzheimer's disease, biomarkers, sphingomyelin, sphingolipid, neurodegeneration, neuroinflammation, cerebrospinal fluid

1. Background

In the US, Alzheimer's disease (AD) is the sixth most common cause of death, and it was estimated to affect five million Americans in 2020, costing $305 billion. Contributing to the enormous cost of AD are the major obstacles posed by the disease: its cause is not fully understood, the most accurate clinical diagnosis relies on postmortem examination, and, while some symptoms can be treated, there is no way to halt progression of the disease.[2] In order to discover new therapeutic targets for AD, we must better understand the changes to the body caused by the disease. One approach to better understanding these changes in AD is metabolomics.

Metabolomics, a way of studying the byproducts of the body's metabolic processes, grants a window into the metabolic state of the body.[4] Metabolomics has proven useful in studying a variety of diseases, including identifying a mechanism of insulin resistance for type 2 diabetes, developing precision medicine approaches to treating cancer, and identifying altered metabolites and their mechanism of change in central nervous system-related disorders such as simian immunodeficiency virus (SIV).[5–8] Metabolomics has also been a valuable tool for studying AD, as it has helped to identify new biomarkers and mechanisms for the disease.[9]

One of the findings from AD metabolomics studies has been the sphingolipid metabolic pathway's association with AD.[10] Sphingolipids are a family of membrane lipids that participate in diverse and quite fundamental cellular processes, such as cell division, differentiation, and death (ref: https://www.hindawi.com/journals/jl/2013/178910/). In mammals, sphingomyelins (SMs) are the most abundant molecule of the sphingolipid metabolic pathway (Supplemental Figure 1).[11] Despite the identification of SMs as associated with AD, there is disagreement as to how they are associated with the disease: some studies show that SMs decrease in progression to AD, while others suggest that they increase.[12,13] Previous research has been limited by sample size, breadth of SMs examined, and diversity of biomarkers available for AD, neurodegeneration, and neuroinflammation, which may have led to the lack of clarity in the role of SMs.

Here, we build on our understanding of the role of SM metabolites in AD. Leveraging two large, longitudinal cohorts with metabolome-wide cerebrospinal fluid (CSF) metabolomics, robust cognitive diagnoses, and a diversity of CSF biomarker and brain imaging measures, we analyzed the relationship between SMs, AD, and markers of neurodegeneration and neuroinflammation. The results shed light on the role of SMs in neurodegeneration and the biological information they best capture.

2. Methods

2.1. Study Cohorts

Data were included from the Wisconsin Registry for Alzheimer's Prevention (WRAP) and the Wisconsin Alzheimer's Disease Research Center (ADRC) cohorts.[14,15] These longitudinal studies of preclinical and clinical AD in middle to older aged adults include CSF metabolomics, AD diagnosis, and CSF biomarker data related to AD, neurodegeneration, and neuroinflammation. All participants included in the current research had at least one lumbar puncture (LP); the CSF samples for WRAP and the Wisconsin ADRC were collected and analyzed by the same staff, following the same protocols. Diagnosis of AD, mild cognitive impairment (MCI), or cognitively unimpaired (CU) was determined by consensus of a committee of dementia specialists.[16]

2.2. CSF samples, biomarkers, and metabolomics

The process through which CSF samples were acquired and biomarker concentrations were measured has been previously described.[17] Briefly, CSF samples were collected in the morning after fasting. Within 30 minutes of collection, samples were mixed, centrifuged, aliquoted, and then stored at -80°C. All CSF sample biomarker assays were performed at the Clinical Neurochemistry Laboratory, University of Gothenburg from March 2019 to January 2020. All biomarker data were taken from the Roche Elecsys® Neuro Tool Kit (NTK), as previously described.[17] Data outside of detectable limits were excluded from our analyses.

For metabolomic analyses, CSF samples were shipped overnight to Metabolon, Inc. (Durham, NC), where samples were also kept frozen at -80°C until analysis.[18] The untargeted metabolomics analysis was performed using Ultrahigh Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). Chemical properties, metabolite identifiers, and pathway information were assigned to each metabolite. All metabolite data underwent a quality control (QC) process; of the 412 metabolites in the initial sample, 13 were removed for missing ≥ 50% of the samples. One reason that metabolites may be missing in ≥ 50% of samples is that they are outside of detectable limits. Nine metabolites were

removed for low variance (interquartile range = 0). Of the 1,172 CSF samples, one was removed for missing ≥ 40% of the metabolite values. 220 samples that were from a clinical trial were also removed. All metabolite values were $\log_{10}$ transformed. After these QC steps, data were available on 390 metabolites from 951 CSF samples taken from 609 individuals.

2.3. Neuroimaging

Detailed methods for radiotracer synthesis and positron emission tomography (PET) and magnetic resonance imaging (MRI) data acquisition, processing and quantification have been previously described.[19] Briefly, anatomical MRI (T1-w and T2-w) underwent multispectral unified tissue class segmentation (SPM12, www.fil.ion.ucl.ac.uk/spm). Regions of interest (ROIs) for PET analysis were defined by applying the deformation field defined during segmentation to the MNI152-space Automated Anatomical Labeling atlas and restricting the subject-space ROIs to voxels with gray matter probabilities greater than 0.3.[20] Reconstructed dynamic Pittsburgh compound B (PiB) PET data acquired from 0-70 minutes post nominal 555 MBq [^{11}C] PiB injection on a Siemens EXACT HR+ or Siemens Biograph Horizon PET/CT were isotopically smoothed, interframe realigned, dynamically denoised, and registered to T1-weighted MRI.[19] Amyloid burden was assessed by averaging distribution volume ratio (DVR) estimates across eight bilateral regions (Logan graphical analysis, cerebellum gray matter reference region, k2'=0.149 min^{-1}; ROIs included: angular gyrus, anterior and posterior cingulate, medial orbital-frontal gyrus, precuneus, supramarginal gyrus, and middle and superior temporal gyri).[21]

2.4. Data Integration

When the data were combined for analysis, further data cleaning measures were performed. CSF data were matched to diagnosis data from the nearest clinic visit within two years. To remove potential correlation between related participants, only the oldest individual from each family group was selected (N = 32 individuals removed). Two overlapping data sets were created to accommodate both cross-sectional and longitudinal data. Both data sets contained metabolomics and demographics information.

The diagnosis data were used to identify associations between diagnosis and SMs using logistic regressions and PiB and SM's using linear regressions. SM-PiB analyses were performed using the diagnosis (cross-sectional) data set because there were not enough visits per individual to analyze it using the biomarker (longitudinal) data set. This data set was limited to one visit per individual, resulting in a total of 493 samples, making these data cross-sectional. The biomarker data set was used to identify associations between CSF biomarkers and metabolites. It included all available visits for each individual to maximize sample size. This data set contained a total sample size of 726 visits from 494 individuals (Table 1). Markers for AD included Aβ42/40 ratio, PiB PET, p-tau to Aβ ratio (p-tau/Aβ), and p-tau.[22–24] The neurodegeneration biomarkers that we used were neurogranin, neurofilament light (NfL), and alpha-synuclein (α-synuclein).[25–27] We also included biomarkers for neuroinflammation: interleukin-6 (IL6), chitinase-3-like protein 1 (YKL40), and soluble triggering receptor found on myeloid cells 2 (sTREM2).[28–30] All biomarker data were checked for skewness and $\log_{10}$-transformed if the skewness was ≥ 2.[31] The biomarkers that were $\log_{10}$-transformed were NfL, p-tau, p-tau/Aβ, and IL6.

The data were divided into two main data sets for analysis. The first was the "Diagnosis" data set, which contained cross-sectional data with 493 individuals with just one visit each. The second was the "Biomarker" data set, which was comprised of 494 individuals (many of whom had multiple visits, range 1-4) and a total of 726 unique visits. In both the Diagnosis and Biomarker data sets, most participants were cognitively unimpaired (CU), white, female, and amyloid- and tau-negative. The average ages in the Diagnosis and Biomarker data sets were 64.1 (SD = 8.9) and 63.4 (SD = 8.3) years, respectively (Table 1).

2.5. Data Analysis

All analyses were performed using R (version 4.0.2) and the "Tidyverse" packages (version 1.3.0).[32] Logistic regressions and linear regressions were performed using the glm and lm functions from the

"stats" package (version 3.6.2).[33] The linear mixed effects regressions (LMMs) using the biomarker data set were performed using the lmer function from the "lme4" and "lmerTest" packages.[34,35] All p-values from regression models were subject to a Bonferroni adjusted threshold of $p \leq 3.47 \times 10^{-4}$ to determine significance ($\alpha = 0.05 / 12$ metabolites / 12 total primary outcomes). To assess the extent to which the SMs represented the same underlying signal, pairwise correlations were calculated between all 72 pairs of SMs.

2.5.1. SM association with AD and MCI diagnoses

Diagnoses of AD and MCI (both relative to CU) were regressed on each metabolite individually, controlling for sex and age as covariates. Pseudo R-squared values to describe model fit were calculated for each regression using the R2 function from the "semEff" package (version 0.4.0).[36]

2.5.2. SM association with biomarkers of AD, neurodegeneration, or neuroinflammation

Linear regressions and LMMs were used to determine the association between SMs and markers of AD, neurodegeneration and neuroinflammation, adjusting for age and sex. For the biomarkers with multiple visits per individual (Aβ42/40, p-tau/Aβ, p-tau, neurogranin, NfL, α-synuclein, IL6, YKL40, and sTREM2), a random intercept for the participant ID was also included. Marginal R-squared values for these models were calculated using the r.squaredGLMM function from the "MuMIn" package (version 1.43.17) to assess model fit.[37]

For amyloid PiB, which was only available for one visit per individual, associations with SMs were analyzed using a linear regression model with sex and age as covariates. Adjusted R-squared values were calculated for these models, to assess model fit.

2.5.3. Independent signals

To determine whether the models for the top SM were significantly improved by adding any of the other SMs, we repeated each of the main analyses that included stearoyl SM while controlling each of the other metabolites. We used an analysis of variance (ANOVA) test to assess whether the model with just one of the SMs was significantly different from the model where that SM and stearoyl SM were both included. We used the anova function from the "stats" package (version 4.0.2) to do this analysis.[33] To ensure that sample sizes were the same between the two groups, samples missing any of the values necessary for either regression were dropped. We compared the R^2 values calculated for each of the regressions before and after removing the necessary samples to perform this analysis to ensure that they were not drastically altered by these lost samples.

2.5.4. Sensitivity Analyses

APOE ε2/ε3/ε4 genotype was determined using competitive allele-specific PCR based KASP genotyping for rs429358 and rs7412.[18] Because *APOE* ε4 count is strongly associated with AD and thus may feasibly influence our results, we performed a sensitivity analysis in which we controlled for the number of C alleles (0, 1, or 2) at the rs429358 SNP, which effectively quantifies the number of ε4 alleles.[38] Only participants whose self-reported ancestry was "White" were retained for this analysis because of previously reported heterogeneity in effect of the *APOE* ε4 allele by race.[39] We added *APOE* ε4 count as a covariate for these analyses.

Some studies of SMs in plasma have suggested that the direction of association of SMs and AD differ based on sex.[40–42] To determine whether there are sex specific differences in associations with SMs in CSF, the SM-AD and SM-biomarker regression analyses were repeated with stratification by sex (Supplemental Table 1).

To understand whether the association between SMs and biomarkers of AD, neurodegeneration, and neuroinflammation change early in AD, we performed the SM-AD and SM-biomarker regressions stratified by amyloid and tau status, in which the main SM-biomarker regressions were performed for

individuals who were amyloid-positive and tau-positive (A+T+), amyloid-positive and tau-negative (A+T-), and amyloid-negative and tau-negative (A-T-) separately (Supplemental Table 2).[43] Amyloid-positive individuals were defined as those with Aβ42/40 values below 0.046 pg/mL, and tau-positive individuals were defined as those with p-tau values above 0.038 pg/mL.[17]

Each of the SM-AD and SM-biomarker regressions were repeated as in the main set of regressions with the noted change for each sensitivity analysis. The regression results were subject to the same Bonferroni-corrected significance threshold of $p = 3.47\times10^{-4}$ used in the main analyses and the p-values and β effect sizes of regressions with significant results were compared to the results of the main set of analyses.

3. Results

3.1. Pairwise correlations

The pairwise correlations between the metabolites showed that the metabolites were all closely correlated with one another (Figure 1). The correlation coefficients were all between 0.46 and 0.89; behenoyl SM (d18:1/22:0, d16:1/24:1) and stearoyl SM (d18:1/18:0) were the least correlated of all of the metabolite pairs (Supplemental Table 3).

3.2. SM associations with AD

Of the SMs analyzed, none showed a significant association with either AD or MCI diagnosis relative to cognitively healthy controls (Supplemental Table 4). Though statistically insignificant, there was a trend towards higher SM levels in AD versus CU (Figure 2A).

3.3. SM associations with biomarkers of AD, neurodegeneration, and neuroinflammation

The associations between the SMs and PiB and the classic CSF biomarkers of AD (Aβ42/40, p-tau/Aβ, and p-tau) were substantially different between measures of amyloid and tau. None of the 12 metabolites were statistically significantly associated with any of the amyloid-related biomarkers (Aβ42/40, PET PiB amyloid burden, and p-tau/Aβ) after multiple testing correction (Table 2). The lack of clear association could be seen as well in the scatterplots for these outcomes plotted against the metabolite levels, where there were large clusters of data points with no clear pattern (Figure 2B). However, the case with the SM-p-tau associations was different; 12 SMs were nominally significantly associated with CSF p-tau, and 6 of those associations remained significant after Bonferroni correction. All of these associations showed a positive direction of association. The metabolite most strongly associated with p-tau was stearoyl sphingomyelin (d18:1/18:0), where $P = 1.83 \times 10^{-23}$. Likewise, the scatterplots of stearoyl SM and p-tau reflected this positive association (Figure 2B).

SMs were clearly associated with all of the biomarkers of neurodegeneration. For all three biomarkers (neurogranin, NfL, α-synuclein), at least 11 of the 12 SMs were nominally significantly associated with each biomarker with a positive direction of effect (Table 3). This positive association is visible in the scatterplots of each outcome plotted against stearoyl SM (d18:1/18:0), the most significant metabolite, where there is a clear association and a consistent, positive trend (see Figure 2C showing the results for stearoyl SM as an example).

The associations between the 12 SMs and biomarkers for neuroinflammation (YKL40, sTREM2, IL6) showed less consistent results than the associations with the biomarkers of neurodegeneration. Of the SM-IL6 associations, only three were nominally significant (Table 4 and Figure 2D). The association between stearoyl sphingomyelin (d18:1/18:0) and IL6 was the most significant ($P = 0.0057$). Also, unlike all of the other associations, most of the SMs were negatively associated with IL6. In contrast, 8 of the 12 SMs were nominally positively associated with YKL40; 5 of these associations remained significant after Bonferroni correction. The most significant of the associations involving YKL40 was that with

stearoyl sphingomyelin (d18:1/18:0) ($P = 1.41 \times 10^{-18}$). Finally, all 12 SMs were nominally significantly associated with sTREM2; 9 remained significant after Bonferroni correction. As with YKL40, the association was in the positive direction, with stearoyl sphingomyelin (d18:1/18:0) having the strongest association ($P = 3.15 \times 10^{-28}$).

Behenoyl SM (d18:1/22:0) yielded no significant associations after Bonferroni correction with any of our biomarkers, while stearoyl SM (d18:1/18:0) had the strongest significant association with all biomarkers. Palmitoyl SM (d18:1/16:0), SM (d18:1/14:0, d16:1/16:0), SM (d18:1/18:1, d18:2/18:0), SM (d18:2/16:0, d18:1/16:1), and stearoyl SM (d18:1/18:0) were significantly associated with the same seven of the ten biomarkers (p-tau, NfL, α-synuclein, neurogranin, sTREM2, IL6, and YKL40) after Bonferroni correction.

3.4. Independent signals

ANOVA tests were performed to determine whether the models were significantly improved by adding an additional SM metabolite to the model with the most significant metabolite, stearoyl SM (Supplemental Table 5). Six models with p-tau as the outcome were significantly improved by the addition of another metabolite predictor. Only one metabolite significantly improved the model for NfL, while there were six metabolites that significantly improved the model for α-synuclein and seven that significantly improved it for neurogranin. Three metabolites significantly improved the models for sTREM2, one significantly improved the models for IL6, and five metabolites significantly improved the model for YKL40.

3.5. Sensitivity Analyses

To ensure that the results of these analyses were not driven by the *APOE* genotype, we repeated each of the SM-AD and SM-biomarker regressions, adding *APOE* ε4 count as a covariate. The results of the sensitivity analysis with the *APOE* ε4 covariate were compared with the main analysis results and were

not substantially different in either the significance of associations or direction of effect (Supplemental Table 6.

To determine whether there were sex-specific differences in the association between SMs and the outcomes, we stratified the data sets by sex and repeated each of the SM-AD and SM-biomarker regressions. The results of this sensitivity analysis were not meaningfully different from the main set of analyses (Supplemental Table 7). The SM-diagnosis and SM-amyloid associations remained insignificant after Bonferroni correction in both groups. The effect size was generally larger in females than in males for P-tau, neurogranin, and YKL40, while it was larger in males for NfL, α-synuclein, and sTREM2.

In the amyloid and tau (AT)-stratified regressions there were no significant SM-diagnosis associations (Supplemental Table 8). There were notably more significant associations and larger effect sizes identified in the A+T+ group than the A+T- group despite a larger sample size in the latter.

4. Discussion

Our study first explored whether CSF SMs were associated with the diagnosis of AD or MCI in our cohorts. Previous studies on this topic have found significant associations but differing directionality.[12,13] We found that none of the SMs were significantly associated with diagnosis of AD or MCI. This result may be because SMs lack specificity to AD and MCI diagnosis. The contrast between our findings and those of previous studies may be explained by population differences or differing diagnostic criteria.

We found that amyloid biomarkers (Aβ42/40, PiB, and p-tau/Aβ) were not significantly associated with any of our SMs. This finding is consistent with previous research in the WRAP cohort that found no association between any of the CSF metabolites and measures of Aβ.[18] It is, however, contradictory to findings of a previous study that examined the association of total SMs with Aβ in CSF, but this may be because of the preclinical nature of the cohort used in our study; a majority of participants were A-T- (see Table 1).[44] However, some SMs were significantly associated with p-tau and several of the

biomarkers for neurodegeneration and neuroinflammation. Five SMs (stearoyl SM (d18:1/18:0), SM (d18:2/16:0, d18:1/16:1), SM (d18:1/18:1, d18:2/18:0), SM (d18:1/14:0, d16:1/16:0), and palmitoyl SM (d18:1/16:0)) were significantly positively associated with p-tau and each of the biomarkers for neurodegeneration that we examined: NfL, p-tau, α-synuclein, and neurogranin. The same five SMs were significantly, positively associated with YKL40 and sTREM2, but not with IL6. The associations of SMs with all of the outcomes we analyzed remained almost completely the same when we controlled for *APOE* ε4 genotype, indicating these results were not likely to be driven by *APOE* genotype. This information seems to indicate that, as neurodegeneration and neuroinflammation are increasing, so too are our SMs, and these five SMs are potential biomarkers for both of those conditions. The association with markers of neurodegeneration and neuroinflammation may explain why studies seem to find associations with SMs in later stages of AD, while we did not observe associations between SMs and amyloid biomarkers.[12,45,46] SMs may only begin changing as neurodegeneration or neuroinflammation occur and neurons begin to die, but not earlier, when amyloid is first beginning to accumulate.

Of the twelve metabolites we analyzed, stearoyl SM (d18:1/18:0) had the strongest associations with most of our biomarkers. This particular metabolite has been previously identified as having a significant, positive association with AD pathology along with SM (18:1/18:1).[46] Stearoyl SM (d18:1/18:0), along with palmitoyl SM (d18:1/16:0), was elevated in plasma of preeclamptic mothers in a previous study, thought to be a result of lipid rafts' (microdomains of the cell membrane) exposure to low oxygen levels.[47] This finding may grant insight into potential mechanisms for the association of CSF SMs with neurodegeneration: as neurons die, by hypoxia or some other mechanism, lipid rafts release their contents, including SMs, into the CSF. [11,13]

The sex-stratified analyses were not meaningfully different from the unstratified analyses. While there were more significant SM-outcome associations in the female than male group, these differences may simply reflect differences in power between the groups as a result of differing sample sizes by

stratification. Similarly, the AT-stratified analyses did not yield results that were meaningfully different from the unstratified analyses; this may be a reflection of the small sample sizes in each of the AT-stratified groups, especially the A-T+ and A+T+. More research with larger sample sizes will be necessary to establish the associations between SMs and various stages of AD.

Our pairwise correlations indicate that all of the CSF SMs we analyzed were correlated, with potential subgroups among the SMs based on chain length. The nested linear models with our most strongly associated metabolite, stearoyl SM (d18:1/18:0), support the correlated nature of the SMs.

This study has certain limitations that must be taken into consideration when interpreting its results. While we were able to produce one of the largest sample sizes of AD and MCI individuals used to study CSF SMs, our samples of individuals with AD and MCI are still relatively small (N = 89) compared to our cognitively normal controls (Table 1), which may have limited our ability to assess diagnosis-related outcomes. We also lack diversity in samples; all individuals included in this study were self-reported white, limiting the generalizability of this study to other populations. As sample sizes for these and other cohorts grow and diversify, we will be better able to investigate the role of SMs across a greater range of populations. We also were limited in the types and quantity of sphingolipids that we examined. Ceramides, particularly, are a type of sphingolipid that has been implicated in AD among other diseases, but we were not able to assess their role here.[48] Nine of the twelve SMs we analyzed were designated as tier 2 compounds by Metabolon. These compounds have a structure that has been confirmed by literature review, but they are not necessarily confirmed by the reference standard.[49] There are also other species of SMs that we were unable to examine because they were not present in our data, though from this study, it did seem that the SMs were highly correlated with each other. Finally, there are likely additional potential confounding variables that we did not control for, which could influence the results of the association analyses.

5. Conclusion

In this study, we examined the relationship between 12 SMs, AD diagnoses and ten different markers of AD, neurodegeneration, and neuroinflammation, providing a comprehensive investigation of the role of SMs and AD pathology. While we found no association between SMs that we analyzed and AD or MCI diagnoses, we did find strong positive associations between the SMs and p-tau, NfL, sTREM2, neurogranin, α-synuclein, and YKL40. We hypothesized that SMs are biomarkers of neurodegeneration and neuroinflammation. There is still much to be learned about SMs in neurodegeneration: testing and proving a mechanism for their role in neurodegeneration, understanding how SM levels differ across neurodegenerative diseases, and characterizing them in diverse cohorts with larger numbers of AD individuals will grant greater insights into the role of these metabolites in AD.

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Conflicts of Interest

HZ, author, has served at scientific advisory boards and/or as a consultant for Alector, Eisai, Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics, Nervgen, AZTherapies, CogRx and Red Abbey Labs, has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). SCJ, author, was a consultant to Roche Diagnostics in 2018.

Table 1. Baseline characteristics of individuals.

Characteristic	Diagnosis data set (Cross-sectional) (n = 493 individuals)	Biomarker data set individuals (n = 494 individuals)	Biomarker data set vis (Longitudinal) (n = 726 visits)
Diagnosis (n, %)			
AD	44 (8.9%)	43 (8.7%)	44 (6.1%)
MCI	40 (8.1%)	42 (8.5%)	45 (6.2%)

CU	409 (82.9%)	409 (82.8%)	637 (87.7%)
Primary Race (n, %)			
White	472 (95.7%)	473 (95.7%)	693 (95.5%)
Black or African American	16 (3.3%)	16 (3.2%)	22 (3.03%)
American Indian or Alaska Native	3 (0.6%)	3 (0.6%)	4 (0.6%)
Asian	1 (0.2%)	1 (0.2%)	3 (0.4%)
Other	1 (0.2%)	1 (0.2%)	4 (0.6%)
Sex (n, %)			
Male	191 (38.7%)	191 (38.7%)	273 (39.4%)
Female	302 (61.3%)	303 (61.3%)	453 (65.4%)
Amyloid and tau status (n, %)			
A-T-	319 (64.7%)	343 (69.4%)	500 (68.9%)
A-T+	61 (12.4%)	64 (13.0%)	85 (11.7%)
A+T+	85 (17.2%)	85 (17.2%)	102 (14.0%)
Age in years (mean, SD)	64.1 (8.9)	63.1 (8.9)	63.4 (8.3)
PET PiB, n = 179 (mean, SD)	172 (9.4)	N/A	N/A

For demographics information in the sex- and amyloid-stratified groups, see Supplemental Tables 1 and 2. All individuals with PET PiB measurements were present in the diagnosis data set.

Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; CU, cognitively unimpaired; CSF, cerebrospinal fluid; PiB, Pittsburgh Compound B; SD, standard deviation.

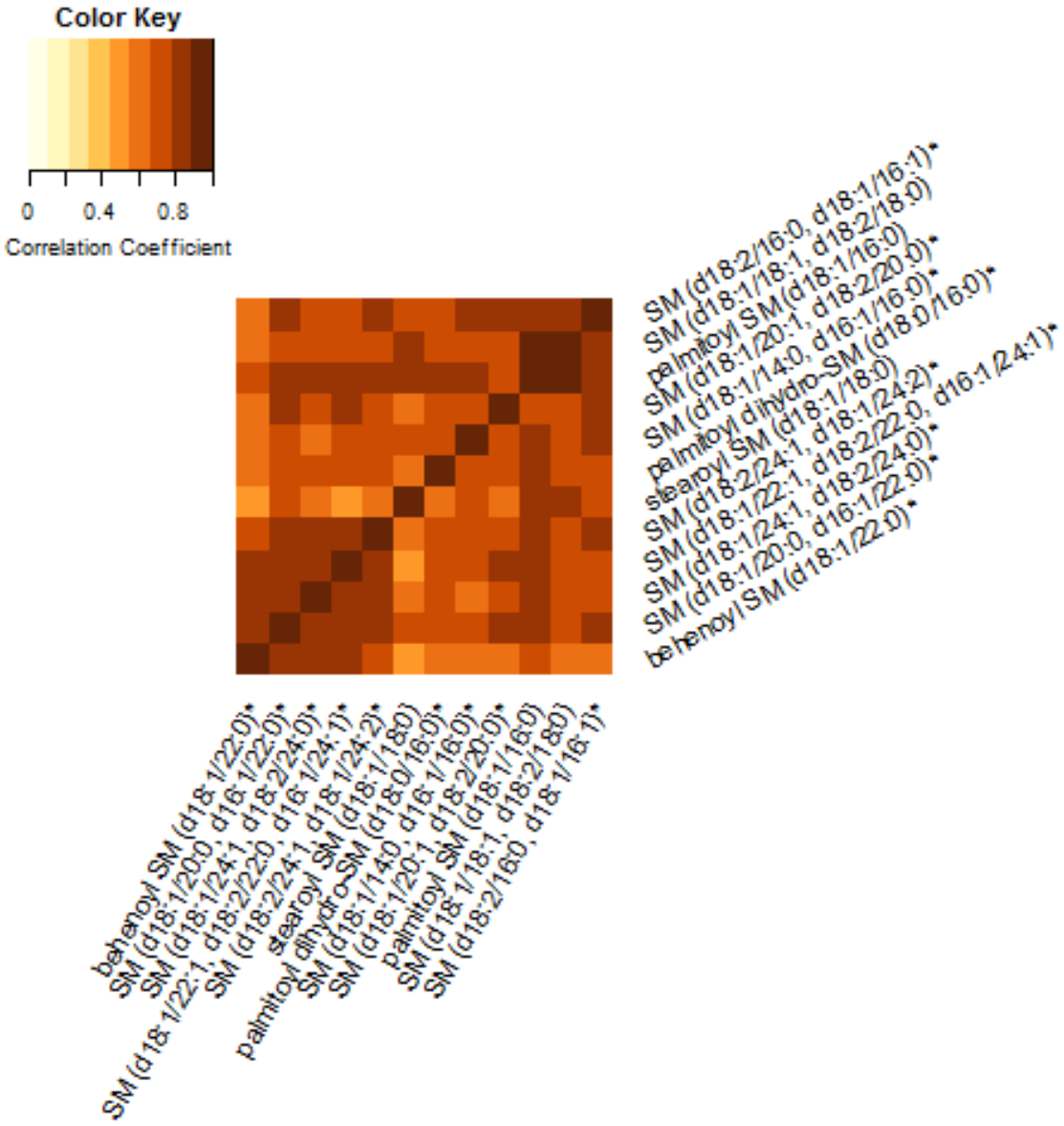

Figure 1. Pairwise correlations of SMs. A heatplot showing the correlation between each metabolite pair. Darker colors indicate stronger correlations (closer to one), while lighter colors indicate weaker correlations (closer to 0). The lowest correlation coefficient was 0.47 and the highest was 0.89.

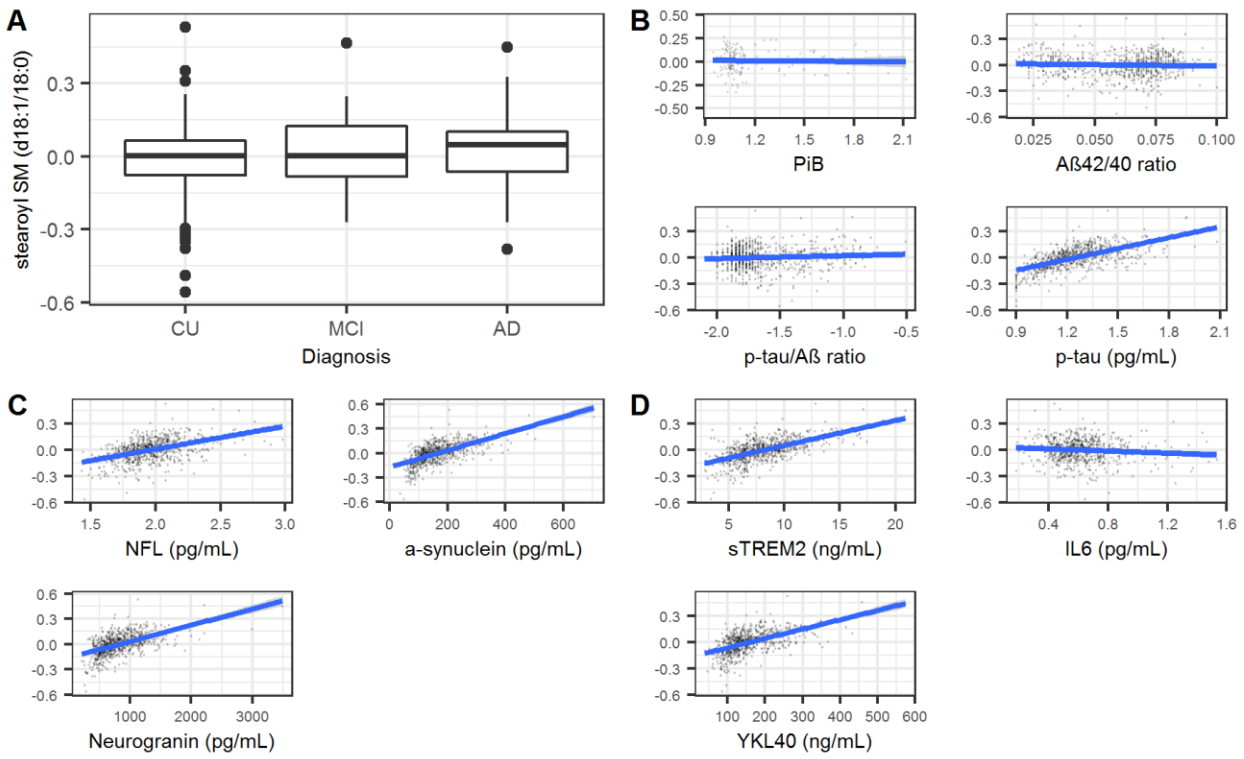

Figure 2. **Plots of the associations between biomarkers and diagnosis data and stearoyl SM (d18:1/18:0).**

Only the results from stearoyl SM (d18:1/18:0) are displayed because it was consistently the most significantly associated SM and all other SMs are correlated with stearoyl SM (correlation coefficient of 0.46-1.0). **A)** Boxplots showing stearoyl SM (d18:1/18:0) levels for individuals with CU, MCI, and AD diagnoses. A small but statistically insignificant increase in metabolite level by AD progression can be seen moving from the CU to AD group. None of the SM-Diagnosis regressions had statistically significant results. B) Scatterplots with each of the four AD specific outcomes on the x-axis of its respective plot and stearoyl SM (d18:1/18:0) on the y-axis of each of the plots. Stearoyl SM was not significantly associated with the measures of amyloid, but was significantly, positively associated with p-tau after Bonferroni correction. **C)** Scatterplots of each of the three neurodegeneration biomarkers (x-axis) plotted against stearoyl SM (d18:1/18:0) (y-axis). Stearoyl SM was significantly, positively associated

with NfL, neurogranin, and α-synuclein after Bonferroni correction. **D)** Scatterplots of each of the three neuroinflammation biomarkers (x-axis) plotted against stearoyl SM (d18:1/18:0) (y-axis). Stearoyl SM was significantly, positively associated with YKL40 and sTREM2 and significantly, negatively associated with IL6 after Bonferroni correction. **A-D:** The units of stearoyl SM (d18:1/18:0) are standardized by log_{10}-transformation as laid out in the methods section of this paper. **B-D:** Best fit lines constructed using linear regression models with 95% confidence intervals are drawn onto each of the scatterplots.

Table 2. Associations between SMs and AD biomarkers.

	PiB			Aβ42/40			p-tau/A	
Metabolite	**β**	**P**	**R^2**	**β**	**P**	**R^2**	**β**	P
behenoyl SM (d18:1/22:0)	0.02	0.85	0.09	-3.54E-04	0.86	0.13	0.02	
palmitoyl dihydro-SM (d18:0/16:0)	0.07	0.60	0.09	-3.00E-04	0.93	0.13	0.01	
palmitoyl SM (d18:1/16:0)	-0.19	0.23	0.10	-1.87E-03	0.68	0.14	0.03	
SM (d18:1/14:0, d16:1/16:0)	-0.05	0.70	0.09	-1.41E-03	0.68	0.14	0.03	
SM (d18:1/18:1, d18:2/18:0)	-0.15	0.38	0.10	-3.22E-03	0.50	0.14	0.07	
SM (d18:1/20:0, d16:1/22:0)	0.01	0.93	0.09	-2.60E-03	0.38	0.14	0.05	
SM (d18:1/20:1, d18:2/20:0)	0.08	0.56	0.09	-2.97E-03	0.35	0.13	0.06	
SM (d18:1/22:1, d18:2/22:0, d16:1/24:1)	0.15	0.19	0.10	-3.62E-03	0.13	0.14	0.05	
SM (d18:1/24:1, d18:2/24:0)	0.05	0.69	0.09	-6.82E-04	0.79	0.14	0.01	
SM (d18:2/16:0, d18:1/16:1)	-0.08	0.57	0.10	-4.76E-03	0.17	0.14	0.09	
SM (d18:2/24:1, d18:1/24:2)	0.06	0.59	0.09	-4.02E-03	0.13	0.14	0.05	
stearoyl SM (d18:1/18:0)	-0.22	0.18	0.10	-1.04E-03	0.84	0.14	0.05	

SM: sphingomyelin; PiB: positron emission tomography Pittsburgh compound B; Aβ42/40: amyloid-beta 42/40 ratio; p-tau/Aβ: phosphorylated-tau_{181} to amyloid-beta 42 ratio; p-tau: phosphorylated-tau; β:

change in the outcome with a one unit increase of the standardized metabolite level; P: P-value of the corresponding regression; R^2: adjusted R^2 for PiB, marginal R^2 for others; bolded values indicate significant results at threshold $p<3.47\times10^{-4}$.

Table 3. Associations between SMs and neurodegeneration biomarkers.

	NfL (pg/mL)			α-synuclein (pg/mL)			Neurog	
Metabolite	**β**	**P**	**R^2**	**β**	**P**	**R^2**	**β**	**P**
behenoyl SM (d18:1/22:0)	0.06	1.23E-03	0.46	31.79	8.96E-04	0.09	46.86	
palmitoyl dihydro-SM (d18:0/16:0)	**0.15**	**2.14E-06**	**0.46**	**87.32**	**1.16E-07**	**0.11**	148.66	
palmitoyl SM (d18:1/16:0)	**0.37**	**1.57E-18**	**0.52**	**254.75**	**1.72E-35**	**0.26**	**608.66**	
SM (d18:1/14:0, d16:1/16:0)	**0.16**	**8.83E-08**	**0.47**	**142.70**	**4.19E-18**	**0.16**	**336.80**	
SM (d18:1/18:1, d18:2/18:0)	**0.37**	**3.99E-17**	**0.51**	**247.38**	**3.30E-32**	**0.25**	**626.89**	
SM (d18:1/20:0, d16:1/22:0)	**0.17**	**2.34E-09**	**0.48**	**103.61**	**1.02E-12**	**0.12**	**207.71**	
SM (d18:1/20:1, d18:2/20:0)	**0.12**	**6.43E-05**	**0.45**	**64.18**	**3.29E-05**	**0.10**	179.55	
SM (d18:1/22:1, d18:2/22:0, d16:1/24:1)	**0.10**	**5.20E-06**	**0.46**	**50.48**	**1.34E-05**	**0.09**	110.05	
SM (d18:1/24:1, d18:2/24:0)	**0.09**	**1.30E-04**	**0.47**	**49.82**	**3.79E-05**	**0.09**	81.38	
SM (d18:2/16:0, d18:1/16:1)	**0.22**	**5.16E-12**	**0.49**	**146.47**	**1.36E-19**	**0.17**	**337.93**	
SM (d18:2/24:1, d18:1/24:2)	**0.14**	**1.02E-08**	**0.48**	**75.29**	**2.36E-09**	**0.10**	111.55	
stearoyl SM (d18:1/18:0)	**0.45**	**1.94E-21**	**0.53**	**325.23**	**1.25E-51**	**0.36**	**994.55**	

SM: sphingomyelin; NfL: neurofilament light; β: change in the outcome with a one unit increase of the standardized metabolite level; P: P-value of the corresponding regression; R^2: marginal R^2 for the corresponding regression; bolded values indicate significant results at threshold $p < 3.47\times10^{-4}$.

Table 4. Associations between SMs and neuroinflammation biomarkers.

	sTREM2 (ng/mL)			IL6 (pg/mL)			YKL4	
Metabolite	**β**	**P**	**R^2**	**β**	**P**	**R^2**	**β**	**P**

behenoyl SM (d18:1/22:0)	0.47	0.03	0.11	-0.02	0.48	0.03	6.87	
palmitoyl dihydro-SM (d18:0/16:0)	**1.64**	**5.15E-05**	**0.12**	0.02	0.75	0.03	19.80	
palmitoyl SM (d18:1/16:0)	**4.55**	**1.06E-16**	**0.17**	-0.10	0.12	0.03	**63.27**	
SM (d18:1/14:0, d16:1/16:0)	**1.91**	**6.67E-07**	**0.12**	-0.07	0.18	0.03	**27.02**	
SM (d18:1/18:1, d18:2/18:0)	**4.30**	**2.70E-14**	**0.17**	-0.17	7.56E-03	0.04	**59.56**	
SM (d18:1/20:0, d16:1/22:0)	**1.48**	**7.69E-06**	**0.12**	-0.06	0.22	0.03	15.09	
SM (d18:1/20:1, d18:2/20:0)	0.88	0.01	0.12	-0.01	0.78	0.02	13.51	
SM (d18:1/22:1, d18:2/22:0, d16:1/24:1)	**1.03**	**9.70E-05**	**0.11**	-0.03	0.40	0.03	9.82	
SM (d18:1/24:1, d18:2/24:0)	0.92	7.96E-04	0.12	-0.03	0.44	0.03	5.99	
SM (d18:2/16:0, d18:1/16:1)	**2.26**	**1.12E-08**	**0.13**	-0.14	8.31E-03	0.04	**31.62**	
SM (d18:2/24:1, d18:1/24:2)	**1.28**	**1.27E-05**	**0.12**	-0.05	0.23	0.03	11.71	
stearoyl SM (d18:1/18:0)	**6.99**	**3.15E-28**	**0.24**	-0.18	5.77E-03	0.04	**105.64**	

sTREM2: soluble triggering receptor found on myeloid cells 2; IL6: interleukin-6; YKL40: chitinase-3-like protein 1; *β: change in the outcome with a one unit increase of the standardized metabolite level; P: P-value of the corresponding regression; R^2: marginal R^2 for the corresponding regression; bolded values indicate significant results at threshold $p<3.47\times10^{-4}$*.

References

1. Facts and Figures. Alzheimer's Disease and Dementia. Accessed August 3, 2020. https://www.alz.org/alzheimers-dementia/facts-figures

2. Treatments. Alzheimer's Disease and Dementia. Accessed August 3, 2020. https://alz.org/alzheimers-dementia/treatments

3. Ozben T, Ozben S. Neuro-inflammation and anti-inflammatory treatment options for Alzheimer's disease. *Clin Biochem*. 2019;72:87-89. doi:10.1016/j.clinbiochem.2019.04.001

4. Wishart DS. Emerging applications of metabolomics in drug discovery and precision medicine. *Nat Rev Drug Discov*. 2016;15(7):473-484. doi:10.1038/nrd.2016.32

5. Bain JR, Stevens RD, Wenner BR, Ilkayeva O, Muoio DM, Newgard CB. Metabolomics applied to diabetes research: moving from information to knowledge. *Diabetes*. 2009;58(11):2429-2443. doi:10.2337/db09-0580

6. L P-C, A P-L. Metabolomics Applications in Precision Medicine: An Oncological Perspective. *Curr Top Med Chem*. 2017;17(24):2740-2751. doi:10.2174/1568026617666170707120034

7. Metabolomic analysis of the cerebrospinal fluid reveals changes in phospholipase expression in the CNS of SIV-infected macaques. - Abstract - Europe PMC. Accessed August 3, 2020. https://europepmc.org/article/pmc/pmc2398736

8. The Role of Metabolomics in Brain Metabolism Research. - Abstract - Europe PMC. Accessed August 3, 2020. https://europepmc.org/article/med/26201839

9. Wilkins JM, Trushina E. Application of Metabolomics in Alzheimer's Disease. *Front Neurol*. 2018;8. doi:10.3389/fneur.2017.00719

10. Han X, Rozen S, Boyle SH, et al. Metabolomics in early Alzheimer's disease: identification of altered plasma sphingolipidome using shotgun lipidomics. *PloS One*. 2011;6(7):e21643. doi:10.1371/journal.pone.0021643

11. Pralhada Rao R, Vaidyanathan N, Rengasamy M, Mammen Oommen A, Somaiya N, Jagannath MR. Sphingolipid Metabolic Pathway: An Overview of Major Roles Played in Human Diseases. Journal of Lipids. doi:https://doi.org/10.1155/2013/178910

12. Mielke MM, Lyketsos CG. Alterations of the sphingolipid pathway in Alzheimer's disease: new biomarkers and treatment targets? *Neuromolecular Med*. 2010;12(4):331-340. doi:10.1007/s12017-010-8121-y

13. Crivelli SM, Giovagnoni C, Visseren L, et al. Sphingolipids in Alzheimer's disease, how can we target them? *Adv Drug Deliv Rev*. Published online January 3, 2020. doi:10.1016/j.addr.2019.12.003

14. Johnson SC, Koscik RL, Jonaitis EM, et al. The Wisconsin Registry for Alzheimer's Prevention: A review of findings and current directions. *Alzheimers Dement Diagn Assess Dis Monit*. 2017;10:130-142. doi:10.1016/j.dadm.2017.11.007

15. Melah KE, Lu SY-F, Hoscheidt SM, et al. CSF markers of Alzheimer's pathology and microglial activation are associated with altered white matter microstructure in asymptomatic adults at risk for Alzheimer's disease. *J Alzheimers Dis JAD*. 2016;50(3):873-886. doi:10.3233/JAD-150897

16. Johnson SC, Koscik RL, Jonaitis EM, et al. The Wisconsin Registry for Alzheimer's Prevention: A review of findings and current directions. *Alzheimers Dement Amst Neth*. 2018;10:130-142. doi:10.1016/j.dadm.2017.11.007

17. Van Hulle. An examination of a novel multipanel of CSF biomarkers in the Alzheimer's disease clinical and pathological continuum, Alzheimer's & Dementia.

18. Darst BF, Lu Q, Johnson SC, Engelman CD. Integrated analysis of genomics, longitudinal metabolomics, and Alzheimer's risk factors among 1,111 cohort participants. *Genet Epidemiol*. 2019;43(6):657-674. doi:10.1002/gepi.22211

19. Johnson SC, Christian BT, Okonkwo OC, et al. Amyloid burden and neural function in people at risk for Alzheimer's Disease. *Neurobiol Aging*. 2014;35(3):576-584. doi:10.1016/j.neurobiolaging.2013.09.028

20. Tzourio-Mazoyer N, Landeau B, Papathanassiou D, et al. Automated anatomical labeling of activations in SPM using a macroscopic anatomical parcellation of the MNI MRI single-subject brain. *NeuroImage*. 2002;15(1):273-289. doi:10.1006/nimg.2001.0978

21. Lopresti BJ, Klunk WE, Mathis CA, et al. Simplified Quantification of Pittsburgh Compound B Amyloid Imaging PET Studies: A Comparative Analysis. *J Nucl Med*. 2005;46(12):1959-1972.

22. Janelidze S, Zetterberg H, Mattsson N, et al. CSF Aβ42/Aβ40 and Aβ42/Aβ38 ratios: better diagnostic markers of Alzheimer disease. *Ann Clin Transl Neurol*. 2016;3(3):154-165. doi:10.1002/acn3.274

23. Fagan AM, Roe CM, Xiong C, Mintun MA, Morris JC, Holtzman DM. Cerebrospinal fluid tau/beta-amyloid(42) ratio as a prediction of cognitive decline in nondemented older adults. *Arch Neurol*. 2007;64(3):343-349. doi:10.1001/archneur.64.3.noc60123

24. Buerger K, Ewers M, Pirttilä T, et al. CSF phosphorylated tau protein correlates with neocortical neurofibrillary pathology in Alzheimer's disease. *Brain J Neurol*. 2006;129(Pt 11):3035-3041. doi:10.1093/brain/awl269

25. Portelius E, Zetterberg H, Skillbäck T, et al. Cerebrospinal fluid neurogranin: relation to cognition and neurodegeneration in Alzheimer's disease. *Brain*. 2015;138(11):3373-3385. doi:10.1093/brain/awv267

26. Dhiman K, Gupta VB, Villemagne VL, et al. Cerebrospinal fluid neurofilament light concentration predicts brain atrophy and cognition in Alzheimer's disease. *Alzheimers Dement Diagn Assess Dis Monit*. 2020;12(1):e12005. doi:10.1002/dad2.12005

27. Majbour NK, Chiasserini D, Vaikath NN, et al. Increased levels of CSF total but not oligomeric or phosphorylated forms of alpha-synuclein in patients diagnosed with probable Alzheimer's disease. *Sci Rep*. 2017;7(1):40263. doi:10.1038/srep40263

28. Kim YS, Lee KJ, Kim H. Serum tumour necrosis factor-α and interleukin-6 levels in Alzheimer's disease and mild cognitive impairment. *Psychogeriatr Off J Jpn Psychogeriatr Soc*. 2017;17(4):224-230. doi:10.1111/psyg.12218

29. Nordengen K, Kirsebom B-E, Henjum K, et al. Glial activation and inflammation along the Alzheimer's disease continuum. *J Neuroinflammation*. 2019;16(1):46. doi:10.1186/s12974-019-1399-2

30. Suárez-Calvet M, Morenas-Rodríguez E, Kleinberger G, et al. Early increase of CSF sTREM2 in Alzheimer's disease is associated with tau related-neurodegeneration but not with amyloid-β pathology. *Mol Neurodegener*. 2019;14(1):1. doi:10.1186/s13024-018-0301-5

31. Joanes DN, Gill CA. Comparing measures of sample skewness and kurtosis. *J R Stat Soc Ser Stat*. 1998;47(1):183-189. doi:https://doi.org/10.1111/1467-9884.00122

32. Tidyverse. Accessed August 31, 2020. https://www.tidyverse.org/

33. stats package | R Documentation. Accessed September 20, 2020. https://www.rdocumentation.org/packages/stats/versions/3.6.2

34. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. *J Stat Softw*. 2015;67(1):1-48. doi:10.18637/jss.v067.i01

35. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest Package: Tests in Linear Mixed Effects Models. *J Stat Softw*. 2017;82(1):1-26. doi:10.18637/jss.v082.i13

36. Murphy M. *SemEff: Automatic Calculation of Effects for Piecewise Structural Equation Models*.; 2020. Accessed October 30, 2020. https://CRAN.R-project.org/package=semEff

37. r.squaredGLMM function | R Documentation. Accessed October 31, 2020. https://www.rdocumentation.org/packages/MuMIn/versions/1.40.4/topics/r.squaredGLMM

38. Liu C-C, Kanekiyo T, Xu H, Bu G. Apolipoprotein E and Alzheimer disease: risk, mechanisms, and therapy. *Nat Rev Neurol*. 2013;9(2):106-118. doi:10.1038/nrneurol.2012.263

39. Kulminski AM, Shu L, Loika Y, et al. APOE region molecular signatures of Alzheimer's disease across races/ethnicities. *Neurobiol Aging*. 2020;87:141.e1-141.e8. doi:10.1016/j.neurobiolaging.2019.11.007

40. Mielke MM, Haughey NJ, Han D, et al. The Association Between Plasma Ceramides and Sphingomyelins and Risk of Alzheimer's Disease Differs by Sex and APOE in the Baltimore Longitudinal Study of Aging. *J Alzheimers Dis JAD*. 2017;60(3):819-828. doi:10.3233/JAD-160925

41. Pujol-Lereis LM. Alteration of Sphingolipids in Biofluids: Implications for Neurodegenerative Diseases. *Int J Mol Sci*. 2019;20(14):3564. doi:10.3390/ijms20143564

42. Darst BF, Koscik RL, Hogan KJ, Johnson SC, Engelman CD. Longitudinal plasma metabolomics of aging and sex. *Aging*. 2019;11(4):1262-1282. doi:10.18632/aging.101837

43. NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease - Jack - 2018 - Alzheimer's & Dementia - Wiley Online Library. Accessed September 14, 2020. https://alz-journals.onlinelibrary.wiley.com/doi/full/10.1016/j.jalz.2018.02.018

44. Mielke MM, Haughey NJ, Bandaru VVR, et al. CSF sphingolipids, β-amyloid, and tau in adults at risk for Alzheimer's disease. *Neurobiol Aging*. 2014;35(11):2486-2494. doi:10.1016/j.neurobiolaging.2014.05.019

45. Savica R, Murray ME, Persson XM, et al. Plasma sphingolipid changes with autopsy-confirmed Lewy body or Alzheimer's pathology. *Alzheimers Dement Diagn Assess Dis Monit*. 2016;3:43-50. doi:10.1016/j.dadm.2016.02.005

46. Koal T, Klavins K, Seppi D, Kemmler G, Humpel C. Sphingomyelin SM(d18:1/18:0) is significantly enhanced in cerebrospinal fluid samples dichotomized by pathological amyloid-β42, tau, and phospho-tau-181 levels. *J Alzheimers Dis JAD*. 2015;44(4):1193-1201. doi:10.3233/JAD-142319

47. Ermini L, Ausman J, Melland-Smith M, et al. A Single Sphingomyelin Species Promotes Exosomal Release of Endoglin into the Maternal Circulation in Preeclampsia. *Sci Rep*. 2017;7(1):12172. doi:10.1038/s41598-017-12491-4

48. Czubowicz K, Jęśko H, Wencel P, Lukiw WJ, Strosznajder RP. The Role of Ceramide and Sphingosine-1-Phosphate in Alzheimer's Disease and Other Neurodegenerative Disorders. *Mol Neurobiol*. 2019;56(8):5436-5455. doi:10.1007/s12035-018-1448-3

49. Tier 1 Metabolite Identifications: A Compass to Rich Research Insights. Metabolon. Published December 11, 2019. Accessed December 22, 2020. https://metabolon.com/tier-1-metabolite-identifications-a-compass-to-rich-research-insights/