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Discovery and Confirmation of Diagnostic Serum Lipid Biomarkers for Alzheimer's Disease Using Direct Infusion Mass Spectrometry

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Abstract. Alzheimer's disease (AD) is a neurodegenerative disorder lacking early biochemical diagnosis and treatment. Lipids have been implicated in neurodegenerative disorders including AD. A shotgun lipidomic approach was undertaken to determine if lipid biomarkers exist that can discriminate AD cases from controls. The discovery study involved sera from 29 different stage AD cases and 32 controls. Lipid extraction was performed using organic solvent and the samples were directly infused into a time-of-flight mass spectrometer. Differences between AD cases and controls were detected with 87 statistically significant lipid candidate markers found. These potential lipid markers were reevaluated in a second confirmatory study involving 27 cases and 30 controls. Of the 87 candidates from the first study, 35 continued to be statistically significant in the second confirmatory set. Tandem MS studies were performed and almost all confirmed markers were characterized and classified. Using a Bayesian lasso probit regression model on the confirmed markers, a multi-marker set with AUC = 0.886 was developed comparing all stages of AD with controls. Additionally, using confirmed biomarkers, multi-marker sets with AUCs >0.90 were developed for each specific AD Clinical Dementia Rating versus controls, including the earliest stage of AD. More conservative and likely more realistic statistical analyses still found multi-marker sets that appeared useful in diagnosing AD. Finally, using ordinal modeling a set of markers was developed that staged AD accurately 70% of the time, $p = 0.0079$. These results suggest that these serum lipidomic biomarkers may help diagnose and perhaps even stage AD.

Keywords: Alzheimer's disease, biomarkers, diagnosis, disease staging, lipidomics, mass spectrometry

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and the most common cause of age-related dementia [1, 2]. As many as 25 million people worldwide may have AD with more than 4 million new cases reported every year [3]. General risk factors for AD include age, female sex, and certain genetic backdrops. However, the actual risk of an individual to develop AD cannot be determined currently. This in turn has limited drug trials.

Several hypotheses exist for AD etiology. The most common theory proposes that increased amyloid-β protein (Aβ) deposits, resulting from the cleavage of amyloid-β protein precursor by β-secretase and γ-secretase, lead to amyloid plaque formation and

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AD [4]. However, recent results from a clinical trial of a drug targeting Aβ accumulation did not reduce AD progression [5]. Another hypothesis proposes the cause to be formation of neurofibrillary tangles of abnormally phosphorylated tau protein leading to loss of synapses and neurons with the resulting AD sequelae [6].

Mild cognitive impairment (MCI) precedes AD but also occurs with other types of dementia. The definitive diagnosis of AD can only be made by post-mortem histopathological analysis. Currently, the clinical diagnosis of AD is still subjective and based on neuropsychological evaluation, including memory tests, and in the research setting neuroimaging. Early diagnosis is challenging, although clinical centers specializing in AD appear better able to identify subjects with MCI that will progress to AD, but more generally accuracies vary from 20–100% [7].

More quantitative methods of AD diagnosis exist but are not routinely used. These include fluoro-deoxyglucose positron emission tomography (FDG-PET) to assess reduced glucose metabolism [8], magnetic resonance imaging (MRI) to determine hippocampal volume, [9], and PET using ligands such as Pittsburgh compound B (^{11}C-PIB) that bind Aβ plaques [10, 11]. Other ligands, e.g., (S)-[^{18}F]-THK5117, target tau aggregates and may be useful [12]. Some of these techniques are quite sensitive but are not routinely used due to their clinical complexity, risk and substantial expense [13].

Studies of Aβ and phosphorylated tau levels in cerebrospinal fluid have found them to be reasonably accurate for mid to late stage AD but of limited use in early stage AD [7]. Blood levels of tau protein are below the detection limit for many assays [14], and the measurement of serum or plasma Aβ peptide has provided poor diagnostic value for AD in a number of studies [15, 16] and do not correlate with the severity of dementia [17]. While there is a clear increase in AD risk for individuals having the apolipoprotein E ε4 (ApoE4) allele or alleles, ApoE genotyping provides unacceptably low predictive values [18].

Currently then, there are as yet no accepted serologic or radiologic tests to diagnose AD, most especially early stage AD. Imaging using radio-ligands is promising but is expensive, time consuming, and invasive. Consequently, there is an unmet need to find serum biomarkers for AD, especially for very early stage disease. Moreover, other assessments such as the future risk of AD, rates of AD progression, and responsiveness to therapies may be possible with as yet undiscovered serum biomarkers.

Lipids are frequently altered by disease but also occasionally act as participants [19]. In AD, neurodegeneration involves the breakdown of cell membranes, resulting in abnormal abundances of membrane lipids [20]. Also, there is a strong evidence of oxidative damage in the brains of AD patients [21, 22]. Sustained oxidative stress can increase oxidized lipid species, e.g., isoprostanes [23] or malondialdehyde [24]. Some studies have demonstrated alterations in ceramides with differing stages of AD [25–27]. Others observed the breakdown of phosphatidylcholines accompanying neurodegeneration [20]. More recently, a mass spectrometric metabolomics approach, that included measurement of some plasma lipids, has shown substantial promise in identifying individuals who will develop cognitive impairment and AD [28, 29].

Therefore, it is reasonable that 'global' or broad characterization of alterations in the serum lipidome between AD cases and controls would reveal biomarkers for the diagnosis of AD, perhaps even for early AD. The research reported here tested this hypothesis by applying an untargeted, label free, global lipidomic approach that employs direct electrospray ionization, time-of-flight mass spectrometry (ESI-TOFMS) to the extracts of the serum of AD patients and controls. Diagnostically useful AD lipid biomarker panels were sought and the results suggest that such lipid biomarkers exist and represent in many cases biologically plausible changes in consequence of AD.

METHODS

Study population

The serum specimens were obtained from the Knight Alzheimer's Disease Research Center (Knight ADRC) at the Washington University School of Medicine, St. Louis, MO. The study was approved by Institutional Review Boards (IRB) at both the Knight ADRC and Brigham Young University (BYU) for specimen analysis.

In an initial discovery study, sera from 29 cases and 32 age- and gender-matched controls were analyzed. AD cases included patients having different stages of AD as indicated by previously determined Clinical Dementia Ratings (CDR). Cases included 10 subjects with MCI, also termed very mild AD (CDR 0.5), 10 with mild AD (CDR 1), and 9 with moderate AD (CDR 2). A second confirmatory study included

27 cases and 30 controls. AD cases included 9 patients with CDR 0.5, 9 with CDR 1, and 9 with CDR 2. Postmortem autopsies to unequivocally confirm AD were not available for these cases. The demographics of the patients whose samples were used for the discovery and confirmatory studies are summarized in Tables 1A and 1B. Cases and controls excluded patients with co-morbidities including thyroid disease, diabetes, etc. Medication records were not available for these subjects. Blood specimens at the time of collection, were allowed to clot at room temperature for 30 min followed by separation of serum by centrifuge with its removal and aliquoting followed by immediate freezing. Serum specimens were maintained frozen at –80°C until used in these studies.

Sample preparation

Lipids were extracted from serum using a published method [30]. This was selected over four other lipid extraction methods because it provided more peaks with better signal to noise [31]. To 200 μL of serum, 1.8 mL of a mixture of hexane and isopropanol (3:2, v:v) and 300 μL of 0.5 M KH_2PO_4 were added to a glass tube followed by 60 s of vortexing. The stoppered tubes were further mixed on a motorized shaker at room temperature for 1 h at 80 rpm. To enhance phase separation, 150 μL of water was added and the samples were then centrifuged at 2000 × g for 12 min. The upper organic phase containing the lipids was collected and dried under nitrogen. Dried lipid samples were re-dissolved in 200 μL of chloroform: methanol (3:1) and stored at –80°C until MS analysis.

Mass spectrometric analysis of the lipid extract

To 20 μL of the sample extract, 23 μL of chloroform, 46 μL of methanol, and 14 μL of 12 μM ammonium acetate were added and mixed. The samples were directly injected into the mass spectrometer (6530 LC/MS ESI-QTOF, Agilent Technologies, Santa Clara, CA) through an electrospray ionization (ESI) source operated in the positive ion mode. A syringe pump injected samples at a flow rate of 10 μL/min for 4 min. The ESI spray needle had an i.d. of 120 μm. The capillary voltage was set at 3500 V. MS data was collected from mass to charge ratios (m/z) of 100–3000 with an acquisition rate of 1 spectrum/s. The drying gas was set to 5 L/min at 325°C with a nebulization gas pressure of 1.03 bar. Mass Hunter-Qualitative software (Agilent) was used for data analysis. Each specimen generated a mass spectrum from m/z 100 to 3000. A peak list having m/z values for each peak with their abundances was generated from the mass spectrum.

To reduce analytical variability all MS peaks were normalized. For normalization, seven abundant peaks representing different classes of lipids but with similar abundances between comparison groups were chosen as a reference set. The sum of the intensities (ion counts) of these seven peaks was used for normalizing all the peaks in each mass spectrum.

For the second confirmatory study, all the samples were processed and directly infused into the MS instrument using the same method. All the candidate markers significant in the initial, discovery set were reanalyzed for their diagnostic performance in the second confirmation set.

Chemical characterization of the replicating lipid biomarkers by tandem MS

Tandem MS studies were performed on all 35 replicating lipid AD biomarkers. Fragmentation was accomplished using collision-induced dissociation on an ESI-quadrupole-TOFMS. Both a QSTAR Pulsar 1 quadrupole (Applied Biosystems /Thermo Fisher, Foster City, CA) and an Agilent 6530 Q-TOF MS were employed using the positive ion mode.

For the QSTAR, the capillary voltage set to 4200 V. Samples were injected directly at a flow rate of 2 μL/min and a specific precursor ion targeted. A spectral acquisition rate of 1 spectrum/s was used. Focusing and de-clustering potentials were set to 290 V and 65 V. Collision-induced dissociation was performed using nitrogen or argon gas. Multiple fragmentation energies were used. MS/MS spectra were collected for 2 min. The multi-channel analyzer function was used to sum all 120 MS/MS spectra improving signal to noise.

For the Agilent 6530, samples were injected at the flow rate of 10 μL/min with the capillary voltage set to 3500 V. The drying gas flow rate and temperature were 5 L/min and 300°C. The targeted MS/MS mode was used to select and fragment the parent ion of interest with MS/MS spectra collected 1 spectrum /s. Multiple collision energies were used to obtain maximum fragmentation coverage. Individual scans were summed to improve the signal to noise.

Exact mass studies were accomplished by reference to a set of internal standards added to specimens. Lipidmaps.org was used to tentatively determine all the possible lipid species for each parent peak mass.

Table 1
1A AD demographics for the first discovery set of samples

	N	Gender	Age (mean+SD)	Apo E4 status
Cases	29	62% F, 38% M	79.7 ± 6.6	Allele E4 positive - 20 Allele E4 negative - 9
Controls	32	56% F, 44% M	78.1 ± 6.3	Allele E4 positive - 7 Allele E4 negative - 25

1B AD demographics for the second confirmatory set of samples

	N	Gender	Age (years)	ApoE4 status
Cases	27	59% F, 41% M	79.6 ± 8.5	Allele E4 positive - 14 Allele E4 negative - 13
Controls	30	40% F, 60% M	77.2 ± 6.2	Allele E4 positive - 12 Allele E4 negative - 18

Furthermore, product ions, neutral fragment losses, fragmentation patterns and exact masses of parent and daughter ions were evaluated and where possible compared with published fragmentation patterns or with in silico predicted fragmentations to clarify the classes and composition of the markers.

Statistical analyses

All lipids whose monoisotopic peaks had an ion count >150 (~2,000) were normalized and subjected to a two-tailed Student's *t*-test. Peaks having a p-value < 0.05 in the first study were further reevaluated in a second, independent, confirmatory study involving different subjects and their specimens. The markers that continued to show p-values < 0.05 in the second set were then considered candidates and used for biomarker panel development. In addition, species with p-values < 0.10 were also included recognizing that they might be more specific to a subgroup and hence be complementary.

Models of both binary classification (i.e. cases versus controls) and ordinal regression (i.e., marker intensity versus AD stage) models were created. A few missing values were estimated from multivariate imputation using fully conditional specification [32]. Analyses used R software and packages (see the Supplementary Material for details).

Binary classification models

Several methods were compared via cross-validation and a Bayesian lasso probit regression model was chosen [33]. A fuller rationale and further details of the model framework are provided in the Supplementary Material.

Four classification models were created: (1) comparing controls to all stages of AD, (2) controls to CDR 0.5, (3) controls to CDR 1, and (4) controls to CRD 2. Model 1 had $n = 57$; 30 controls and 27 cases. Model 2 had $n = 39$; 30 controls and 9 AD CDR 0.5. Model 3 had $n = 39$; 30 controls and 9 AD CDR 1 and model 4 had $n = 39$; 30 controls and 9 AD CDR 2.

Ordinal model

Some markers showed changes that progressed with disease stage. A Bayesian Lasso Probit Ordinal regression model was used. This ordinal model utilizes a normally-distributed latent response variable and the Bayesian lasso formulation of Park and Casella [33]. Because of sparse numbers for CDR 0.5 and 1, they were combined for the ordinal analysis. For details about the model framework, see the Supplementary Material.

RESULTS

We tested the hypothesis that there would be one or more serum lipids useful in the diagnosis of patients with AD. A 'global' or 'shotgun' serum lipidomics approach was used to seek and confirm lipid biomarkers that correctly classified patients clinically diagnosed with AD, including those with early stage AD. The approach was used in two independent patient series and results from both appear to support the hypothesis.

Discovery of candidate serum lipid AD diagnostic biomarkers

The first study revealed 87 lipids of interest ($p < 0.05$). These are summarized in Table 2.

Table 2
Candidate AD lipid biomarkers from first study

m/z	*p* value	Higher in
228.2	0.06	Controls
229.1	0.10	Controls
243.2	0.004	Controls
281.1	2.6×10^{-5}	Controls
282.2	8.8×10^{-5}	Controls
295.1	6.7×10^{-5}	Controls
371.35	0.001	Controls
387.17	0.04	Cases
391.17	0.009	Controls
415.2	2.3×10^{-5}	Controls
430.3	0.03	Cases
432.2	2.6×10^{-5}	Controls
437.1	0.01	Controls
447.3	2.2×10^{-10}	Cases
453.1	0.07	Cases
463.3	0.02	Cases
488.3	0.004	Controls
496.3	1.1×10^{-6}	Cases
514.4	0.01	Cases
520.3	0.0004	Cases
522.3	5.4×10^{-6}	Cases
524.3	2.7×10^{-5}	Cases
577.5	0.008	Cases
579.5	0.001	Cases
601.5	0.04	Cases
602.4	0.02	Cases
603.5	0.01	Cases
610.5	0.05	Cases
611.3	0.1	Cases
614.5	0.07	Controls
616.4	0.08	Controls
620.4	0.004	Controls
627.4	0.07	Cases
630.5	0.03	Cases
639.4	0.03	Cases
640.6	0.0001	Controls
642.6	5.3×10^{-9}	Controls
654.3	7.4×10^{-7}	Controls
664.6	0.1	Controls
666.9	0.01	Controls
669.6	4.4×10^{-6}	Controls
688.6	0.01	Controls
701.5	0.008	Controls
703.5	0.02	Controls
714.6	0.07	Controls
717.5	0.06	Controls
724.5	0.0007	Controls
727.5	2.3×10^{-10}	Cases
729.6	3.6×10^{-8}	Cases
731.6	0.04	Controls
742.6	0.07	Controls
744.6	0.04	Controls
750.5	4×10^{-5}	Controls
752.5	0.001	Controls
754.5	0.03	Controls
759.1	0.007	Controls
760.8	0.09	Controls
760.9	0.08	Controls
761.1	0.08	Controls
766.6	0.02	Controls
768.6	0.0008	Controls
778.5	0.005	Controls
786.6	0.02	Controls
792.6	0.005	Controls
794.6	0.02	Controls
799.6	0.1	Controls
810.8	0.02	Controls
812.6	0.07	Controls
820.7	0.07	Cases
822.7	0.03	Cases
824.6	0.02	Cases
842.6	0.02	Cases
844.7	0.06	Controls
846.7	0.01	Cases
848.7	0.02	Cases
850.7	0.03	Cases
860.7	0.01	Cases
862.7	0.007	Cases
864.6	0.003	Cases
881.7	0.0005	Cases
886.7	0.02	Cases
888.8	0.01	Cases
890.8	0.01	Cases
907.8	0.009	Cases
912.7	0.005	Cases
915.8	2.3×10^{-5}	Cases
930.8	0.01	Cases

(*Continued*)

Confirmation of candidate AD biomarkers from the first study

A second confirmatory study evaluated the performance of the 87 candidates from the discovery set in samples from other subjects. Of the 87 markers, 35 continued to show statistically significant or near significant *p*-values comparing AD cases and controls. These are listed in Table 3.

Chemical characterization of the validated AD diagnostic lipid biomarkers

Tandem MS studies were performed on all 35 confirmed, replicating lipid biomarkers to chemically characterize them. The exact mass, elemental composition, lipid class and molecular components were successfully determined for most (See Table 4). The rationales for these structural chemical assignments are explained hereafter (Table 4).

Tandem MS studies on biomarker m/z 430.4 produced fragments at m/z 165 and m/z 205 consistent with the published fragmentation pattern of vitamin E [34]. Additional peaks in the broader fragmentation MS^2 spectrum and the predicted elemental composi-

Table 3
Candidate markers continued to be statistically significant in the second confirmatory set

Exact mass	*p* value in first discovery set	*p* value in confirmatory set	Higher in
229.14	0.1	0.003	Controls
430.38	0.03	0.001	Cases
488.39	0.004	0.09	Controls
496.33	10^{-5}	0.06	Cases
514.38	0.01	0.01	Cases
522.35	10^{-6}	0.02	Cases
577.51	0.008	0.009	Cases
601.52	0.04	0.02	Cases
603.53	0.02	0.003	Cases
602.44	0.02	0.006	Cases
610.53	0.05	0.001	Cases
620.42	0.004	0.0003	Controls
630.47	0.03	0.007	Cases
703.56	0.02	0.1	Controls
714.62	0.07	0.002	Controls
724.52	0.0007	0.04	Controls
778.54	0.005	0.09	Controls
766.57	0.02	0.07	Controls
799.66	0.1	0.05	Controls
820.73	0.07	0.02	Cases
822.75	0.03	0.01	Cases
824.62	0.02	0.01	Cases
842.61	0.02	0.007	Cases
846.75	0.01	0.002	Cases
848.77	0.02	0.004	Cases
850.78	0.03	0.01	Cases
862.78	0.007	0.007	Cases
860.77	0.01	0.006	Cases
864.61	0.001	0.08	Cases
881.74	0.0005	0.1	Cases
886.78	0.02	0.01	Cases
888.80	0.01	0.008	Cases
890.81	0.005	0.003	Cases
912.76	0.005	0.05	Cases
930.84	0.01	0.01	Cases

tion ($C_{29}H_{50}O_2^+$) from exact mass studies were also consistent with its being vitamin E [34].

Fragmentation spectra of markers, m/z 496.3 and 522.3, displayed a prominent peak at m/z 184.07 corresponding to a phosphocholine moiety. Fragments indicating neutral losses from the parent precursor ion suggested fatty acids as components that were determined by comparing theoretical fragments generated by the Lipid MS predictor feature of Lipid Maps with the experimentally observed fragments. Elemental compositions were predicted by exact mass studies. Both peaks were classified as lysophosphatidylcholines (LPC) with m/z 496.3 and m/z 522.3 likely being LPC-16:0 and LPC-18:1. Our MS data was also consistent with previously published spectra [35].

The elemental composition of candidate marker m/z 577.5 was predicted to be $C_{37}H_{69}O_4^+$. This species is likely a diacylglycerol remnant produced by the neutral loss of a fatty acid from a triacylglycerol during the ionization step [36], but it could potentially arise from the cleavage of the head group from a parent phospholipid (i.e., M+H$^+$ – head group), e.g., a glycerophosphoserine. We also observed product ion peaks at m/z 265 and m/z 239, that have been shown previously to be acylium ions (RCO^+) of 18:1 and 16:0 fatty acids [36].

Elemental compositions of markers m/z 601.5 and m/z 603.5 were likely $C_{39}H_{69}O_4^+$ and $C_{39}H_{71}O_4^+$ and are predicted to be diacylglycerol remnants arising from the neutral loss of a fatty acid from a triacyglycerol (TAG) during ionization or possibly due to the removal of the head group from a glycerophosphoserine, glycerophosphoethanolamine, glycerophosphoglycerol, or glycerophosphatidylcholine. Based on the published literature, the acylium ions we observed in the MS^2 spectrum of marker 601.5 were consistent with

Table 4
Chemical characterization of the replicating lipid biomarkers

m/z	Exact mass	Adduct	Elemental composition	Class	Possible identity
229.1	229.14		$C_{12}H_{21}O_4^+$	Novel lipid	
430.4	430.38	M^+	$C_{29}H_{50}O_2^+$	Sterol	Vitamin E
488.4	488.39	$M+NH_4^+$	$C_{27}H_{50}O_{6+}NH_4^+$	Triacylglycerol	TG-8:0/8:0/8:0
496.3	496.33	$M+H^+$	$C_{24}H_{50}NO_7P+H^+$	Lysophosphocholine	LysoPC 16:0
514.4	514.38				—
522.3	522.35	$M+H^+$	$C_{26}H_{52}NO_7P+H^+$	Lysophosphocholine	LysoPC 18:1
577.5	577.51	M^+	$C_{37}H_{69}O_4^+$	Diacylglycerol remnant	16:0/18:1
601.5	601.52	M^+	$C_{39}H_{69}O_4^+$	Diacylglycerol remnant	18:1/18:2
603.5	603.53	M^+	$C_{39}H_{71}O_4^+$	Diacylglycerol remnant	18:1/18:1
602.4	602.44		$C_{44}H_{58}O^+$	Novel lipid	
610.5	610.53		$C_{41}H_{70}O_3^+$	Novel lipid	
620.4	620.42		$C_{43}H_{56}O_3^+$ or $C_{47}H_{56}$	Novel lipid	
630.4	630.47		$C_{46}H_{62}O^+$	Novel lipid	
703.5	703.56	$M+H^+$	$C_{43}H_{76}N_2O_6+H^+$	Sphingomyelin	SM(d18:1/16:0) or SM(d16:1/18:0)
714.6	714.61	$M+NH_4^+$	$C_{49}H_{76}O_{2+}NH_4^+$	Cholesterol ester	C22:6 cholesterol ester
724.5	724.52	$M+H^+$	$C_{41}H_{74}NO_7P+H^+$	Glycerophosphatidylethanolamine	PE(16:0p/ 20:4)
778.5	778.57	$M+H^+$	$C_{45}H_{80}NO_7P+H$	Glycerophosphatidylethanolamines	PE(18:0p/ 22:5)
766.6	766.57	$M+H^+$	$C_{44}H_{80}NO_7P+H^+$	Glycerophosphocholine	PC(O-16:1/20:4)
799.6	799.66	$M+H^+$	$C_{46}H_{91}N_2O_6P+H^+$	Sphingomyelin	Insufficient fragments
820.7	820.73	$M+NH_4^+$	$C_{51}H_{94}O_{6+}NH_4^+$	Triacylglycerol	TG-16:0/16:1/16:1
822.7	822.75	$M+NH_4^+$	$C_{51}H_{96}O_{6+}NH_4^+$	Triacylglycerol	TG-16:0/16:0/16:1
824.6					
842.6	842.61	$M+H^+$	$C_{46}H_{84}NO_{10}P+H^+$	Glycerophosphocholine	PC(18:0/20:4)+OOH
846.7	846.75	$M+NH_4^+$	$C_{53}H_{96}O_6+NH_4^+$	Triacylglycerol	TG-16:0/16:1/18:2
848.7	848.77	$M+NH_4^+$	$C_{53}H_{98}O_6+NH_4^+$	Triacylglycerol	TG-16:0/16:1/18:1
850.7	850.78	$M+NH_4^+$	$C_{53}H_{100}O_6+NH_4^+$	Triacylglycerol	TG16:0/16:0/18:1
862.7	862.78	$M+NH_4^+$	$C_{54}H_{100}O_6+NH_4^+$	Triacylglycerol	TG16:0/17:0/18:2
860.7	860.77	$M+NH_4^+$	$C_{54}H_{98}O_6+NH_4^+$	Triacylglycerol	TG16:1/17:1/18:1
864.6					
881.7	881.74	$M+H^+$	$C_{56}H_{100}O_6+H^+$	Triacylglycerol	TG-16:0/18:0/20:5
886.7	886.78	$M+NH_4^+$	$C_{56}H_{100}O_6+NH_4^+$	Triacylglycerol	TG-17:0/18:1/18:3
888.8	888.80	$M+NH_4^+$	$C_{56}H_{102}O_{6+}NH_4^+$	Triacylglycerol	TG-17:0/18:1/18:2
890.8	890.81	$M+NH_4^+$	$C_{56}H_{104}O_{6+}NH_4^+$	Triacylglycerol	TG-17:0/18:0/18:2
912.7	912.76	$M+NH_4^+$	$C_{57}H_{98}O_7+NH_4^+$	Oxidized triacylglycerol	18:1/18:2/18:2 epoxide
930.8	930.84	$M+NH_4^+$	$C_{59}H_{108}O_{6+}NH_4^+$	Triacylglycerol	TG-18:1/18:2/20:0

loss of an 18:1 (m/z 265) and 18:2 (m/z 263) fatty acid [36]. The fragmentation spectrum of marker m/z 603.5 was consistent with acylium ions from an 18:1 (m/z 265) fatty acid [36].

The markers with m/z 229.1, 602.4, 610.5, 620.4, and 630.4 while very likely lipids yielded tandem MS fragmentation patterns and fragments not previously described in the literature or in lipid databases. Their predicted elemental compositions, based on exact mass studies, are summarized in Table 4.

A dominant peak at m/z 184.06 was observed in the MS^2 spectra of markers with m/z 703.5 and 799.6, indicating a phosphocholine containing species. Therefore, the fragmentation spectra of the markers m/z 703.5 and m/z 799.5 were most consistent with and predicted to be sphingomyelins (SMs) (for 703.5: SM (d18:1/16:0) or SM (d16:1/18:0)) [37] but for 799.5 while likely a SM, no neutral loss fragments were observed.

Fragmentation studies of marker m/z 714.6 resulted in an ion m/z 369.4. This fragment is known to be highly characteristic of cholesterol esters [38]. The fragmentation results were consistent with and suggested it to be a cholesterol ester of docosahexaenoic acid (22:6) [39].

The markers m/z 724.5 and m/z 778.5 were very likely glycerophosphoethanolamines (GPETn) as indicated by a characteristic neutral loss peak of 141 amu. MS/MS spectra of m/z 724.5 displayed product ions at m/z 364 and m/z 361 consistent with previous studies as being a16:0p fatty acid group at the *sn*-1 position and a 20:4 fatty acid at *sn*-2 position [40]. Its putative identification as (16:0p/20:4) GPETn was also consistent with its predicted elemental composition of $C_{41}H_{74}NO_7P+H^+$. Similarly, fragmentation of the marker m/z 778.5 yielded product ions at m/z 392 and m/z 387, potentially indicating the presence of an 18:0p fatty acyl group at the *sn*-1

position and a 22:5 acyl group at the *sn*-2 position as reported previously [40] and was predicted to be (18:0p/22:5) GPETn by its elemental composition of $C_{45}H_{80}NO_7P + H^+$.

A product ion at m/z 184.06 was also observed for markers m/z 766.6 and 842.6 indicating glycerophosphocholines (PCs). Based on its MS^2 fragmentation pattern, Lipid Maps, and exact mass studies, our results were most consistent with m/z 766.6 being an oxidized PC (O-16:1/20:4). The marker m/z 842.6 displayed fragment ions at m/z 808.6 and m/z 824.6, likely corresponding to loss of hydrogen peroxide (H_2O_2) and water consistent with previous published literature [41]. An additional fragment ion at m/z 524.3 indicated the presence of a C18:0 fatty acid. All this suggests a hydroperoxide group attached to PCs (18:0/20:4).

The markers m/z 824.6 and m/z 864.6 overlapped a peak adjacent to them and could not be fully mass resolved. The fragmentation spectra displayed a product ion at m/z 184.06 indicating a phosphocholine group, but it cannot be conclusively determined as to which precursor peak it belonged.

Those peaks with m/z 488.3, 820.7, 822.7, 846.7, 848.7, 850.7, 860.7, 862.7, 886.7, 888.8, 890.8, 912.7, and 930.8 were highly likely to be ammoniated adducts of TAGs as indicated by a product ion representing the neutral loss of ammonia characteristic of TAGs that readily form ammonium adducts [42]. The species m/z 881.7 was potentially the protonated form ($M+H^+$) of a TAG. While the protonated ion is less common that the ammoniated, it does occur as observed with TG standards. The neutral loss of acylium ions allowed for prediction of fatty acid components but not location (Table 4).

The species with m/z 912.8 displayed fragment ions at m/z 599.5, m/z 601.6, and m/z 615.5 corresponding to diacylglycerol fragments of $(18:2/18:2)^+$, $(18:2/18:1)^+$, and $(18:1/18:2_{epoxide})^+$ consistent with an epoxide of 18:1/18:2/18:2 $(OLL_{ep})+NH_4^+$ as published previously [43].

Modeling of multi-marker sets of the verified diagnostic AD biomarkers to increase their ability to classify AD subjects

Statistical modeling was performed on the 35 validated markers to develop multi-marker sets with higher diagnostic capability. Several panels involving 10 or fewer markers were found to have AUCs >0.80, the best of which provided an AUC of 0.866 comparing all stages of AD to controls with a corresponding sensitivity of 93% at a specificity of 80%. A summary of the selected markers, coefficient estimates, 95% credible intervals, and probabilities for the best four models are found in Supplementary Tables 1–4. Receiver operator characteristic curves (ROC curves) for each analysis are shown in Fig. 1. Panels providing high sensitivities and high specificities were found for combined AD stages and individual AD stages, as grouped by CDR (Table 5).

Internal cross-validation modeling to predict future performance of AD diagnostic biomarker sets

The apparent performance of a model is almost always optimistic, so additionally a Bayesian Lasso Probit Regression analysis with internal validation via n-fold cross-validation was performed to obtain more unbiased estimates for future model performance. The results are provided in Table 6. More complete summaries of the cross-validated performance of the four models are given in the Supplementary Material.

Ordinal modeling to stage AD disease using the validated AD lipid biomarkers

Some markers changed in proportion to disease stage and could be potentially useful in staging AD. To test this, a Bayesian Lasso Probit Ordinal regression model that included n-fold cross validation was used. This optimized set of biomarkers correctly assigned disease stage to 70.2% of subjects in cross validation. A summary of the selected markers is given in Table 7. Additional information about the framework is provided in the Supplementary Material. Figure 2 shows selected markers that increased or decreased with advancing stage of disease.

This ordinal model may also be used for binary classification (i.e., control versus any stage of AD). Binary performance of the cross-validated model provided an AUC of 0.806, a classification rate of 79%, a sensitivity of 78% at a specificity of 80%.

DISCUSSION

The early diagnosis of AD remains challenging but most concede that a method to diagnose early AD would facilitate drug development and evaluation.

Newer ligand imaging methods may allow earlier AD diagnosis; however, they are expensive, invasive, and time intensive and not suitable for routine

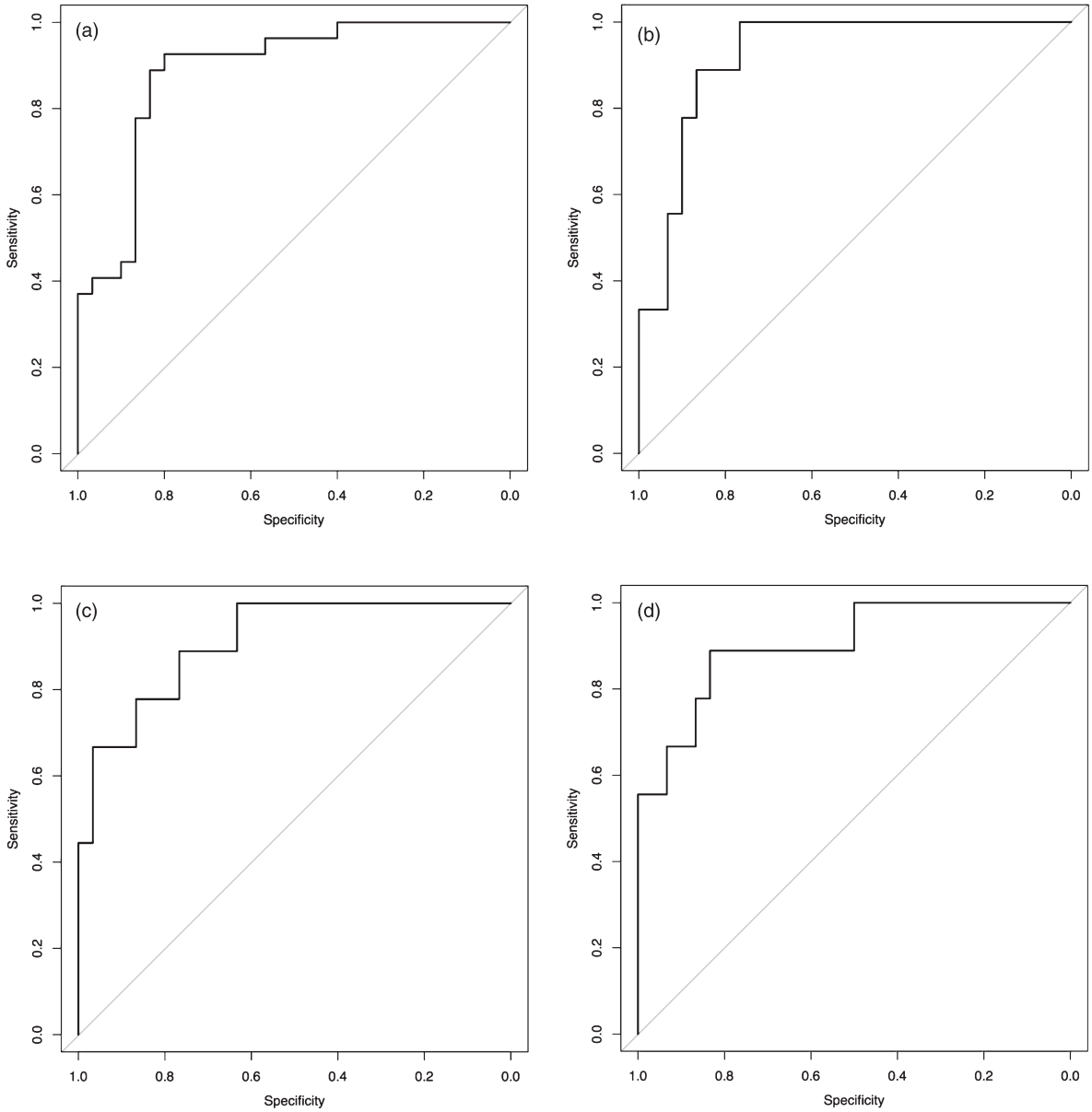

Fig. 1. Optimized combination of diagnostic serum lipid biomarker sets for binary classification of AD subjects versus controls. Receiver operator characteristic curves (ROC) were generated by a Bayesian Lasso Probit Regression model. A) The ROC curve for any stage AD versus control included 9 lipid biomarkers having mass to charge ratios of 229.1, 430.4, 514.5, 602.4, 610.5, 620.4, 630.5, 714.6, and 824.6. The AUC was 0.886 with a sensitivity of 92.6% with a specificity of 80.0%. B) The ROC curve for CDR 0.5 versus controls and included biomarkers having mass to charge ratios of 229.1, 514.4, 602.4, 603.5, 610.5, 620.4, 630.5, 714.6, and 824.6. The AUC was 0.922 with a sensitivity of 100% at a specificity of ∼78%. C) The ROC curve for CDR 1 versus controls and included biomarkers having mass to charge ratios of 430.4, 514.4, 620.4, 630.5, 714.6, and 824.6. The AUC was 0.911 with a sensitivity of 100% at a specificity of ∼78 %. D) The ROC curve for CDR 2 versus controls and included biomarkers having mass to charge ratios of 229.1, 430.4, 514.4, 602.4, 603.5, 620.4, 630.5, 714.6, and 842.7. The AUC was 0.904 with a sensitivity of ∼94% at a specificity of ∼78%.

screening or assessment. Moreover, recent studies suggest that a sensitive measurement of Aβ may not account for the earliest changes in AD [5]. Still, many believe that neurodegeneration in AD begins before the appearance of clinical symptoms. Such changes would necessarily involve molecular changes. Consequently, there is a reasonable presumption that very early AD can be biochemically

Table 5
Optimized verified biomarker combinations for controls versus any stage AD or versus individual AD CDR stage

Model	AUC	Classification Rate	Sensitivity	Specificity	Threshold
CTL versus All AD	0.886	0.86	0.93	0.80	0.52
CTL versus AD CDR 0.5	0.922	0.82	0.88	0.87	0.47
CTL versus AD CDR 1	0.911	0.80	0.78	0.87	0.46
CTL versus AD CDR 2	0.904	0.85	0.89	0.83	0.54

CTL, control subjects; AD, Alzheimer's disease; CDR, Clinical dementia rating. There were 9 markers in the set of CTL versus All AD, 9 markers in the set of CTL versus AD CDR 0.5, 9 markers in the set of CTL versus CDR 1, and 6 markers in the set of CTL versus AD CDR 2. Binary comparisons used Bayesian Lasso Probit Regression Classification Models.

Table 6
Cross validated biomarker performance of the previous verified biomarker sets considered in Table 5

Model	AUC	Classification Rate	Sensitivity	Specificity	Threshold
CTL versus All AD	0.786	0.79	0.73	0.74	0.48
CTL versus AD CDR 0.5	0.696	0.67	0.30	0.79	0.48
CTL versus AD CDR 1	0.685	0.74	0.40	0.79	0.48
CTL versus AD CDR 2	0.815	0.77	0.50	0.89	0.48

CTL, control subjects; AD, Alzheimer's disease; CDR, Clinical dementia rating. There were 9 markers in the set of CTL versus All AD, 9 markers in the set of CTL versus AD CDR 0.5, 9 markers in the set of CTL versus CDR 1 and 6 markers in the set of CTL versus AD CDR 2. Binary comparisons used n-fold cross validated Bayesian Lasso Probit Regression Classification Models.

Table 7
Set of optimized verified biomarkers that showed progression with disease stage and allowed for staging of AD

Marker (m/z)	Coefficient Estimate*	Lower Boundary	Upper Boundary
229.13	–0.126	–0.557	0.161
514.38	0.117	–0.205	0.593
602.40	0.105	–0.207	0.556
620.42	–0.407	–1.037	0.030
630.47	0.092	–0.229	0.519
703.56	0.122	–0.163	0.552
724.52	–0.080	–0.429	0.180
778.54	–0.175	–0.693	0.111
799.66	–0.080	–0.417	0.180
824.60	0.209	–0.096	0.682
842.66	0.088	–0.183	0.443

A 10-fold cross-validated Bayesian Lasso Probit Ordinal Regression Model was used. This model had a classification rate of 0.702, p-value 0.0079. A positive coefficient estimate indicates that the marker increased in abundance with an increase in disease severity whereas a negative coefficient estimate changed inversely with disease stage. This same set of markers could also be used for binary classification of cases and controls. CTL, control subjects; AD, Alzheimer's disease; CDR, Clinical dementia rating. There were 9 markers in the set of CTL versus All AD, 9 markers in the set of CTL versus AD CDR 0.5, 9 markers in the set of CTL versus CDR 1 and 6 markers in the set of CTL versus AD CDR 2.

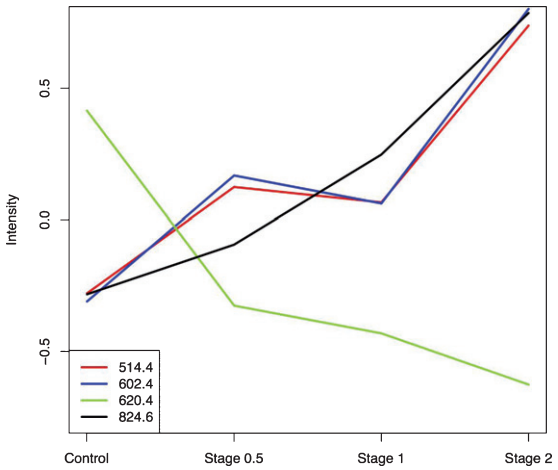

Fig. 2. Serum lipid biomarkers showing progressive changes with disease stage. The y-axis plots the mean peak abundance for a specific lipid marker versus the disease stage as indicated by AD CDR rating on the x-axis. Five biomarkers that demonstrated strong positive or negative correlations with AD stage are plotted (m/z 514.4, 602.4, 620.4, 824.6, listed in inset). The overall Bayesian Lasso Probit Ordinal Regression model had a classification rate of 0.70, $p = 0.0079$.

identified with a simple blood test. Indeed, other recent work suggests that peptides and lipids are altered early in AD [44].

Lipids are an emerging area of biomedical interest [45], even in the pathogenesis of AD [6] and may reflect pathology or even mediators of disease [46].

We and others have employed a global lipidomic approach using direct infusion mass spectrometry to interrogate serum. Direct infusion mass spectrometry using ESI-MS can be quantitative and is faster than other MS analyses [47] and allows for the screening of many diverse lipids. This study sought lipid changes that discriminated AD cases from controls, especially

patients with very early stage AD. It is also recognized that such markers may provide insight into AD pathology.

Our approach discovered and confirmed 35 lipid biomarkers differentiating any stage AD from controls across two independent studies. We observed alterations in sterols, SMs, PCs, glycerophosphoethanolamines (PEs), LPCs, DAGs, and TAGs. Combinations provided panels with ROC curves having AUCs greater than 0.90 for all-stage AD, with a sensitivity of 93% and a specificity of 80%. Other combinations of these 35 markers provided sensitivities approaching or above 90% at specificities near or above 80% for each CDR stage. While the numbers were few, the results nonetheless suggest strongly that serum lipid biomarkers are present that diagnose AD, even very early stage AD.

Limitations

Although we performed a confirmation study with 35 markers replicating, it is recognized that further validation is needed. Moreover, the cases included here did not have autopsies and therefore a definitive AD diagnosis based on neuropathological data is not available. It is possible that marker levels change with disease stage but markers were not measured repeatedly in the same subjects. While equivalent numbers of men and women of comparable age were included as cases and controls, markers specific to gender were not evaluated. Likewise, specimens from other neurologic or neurodegenerative diseases were not available to analyze to determine the specificity of these markers. However, our using multi-marker panels increases the probability of disease specificity. Diet may affect some lipids, e.g., TAGs, but none of the TAG markers figured into the optimized panels. Moreover, dietary differences would increase measurement variability making it harder to find significant, replicating markers. Effects due to medications cannot be ruled out but the subjects had no intercurrent disease. Nevertheless, one cannot rule out drugs contributing to the differences seen. Other AD biomarker studies, including those targeting Aβ and tau, have used similar subject sets and have also not had the numbers to assess the potential contribution of dozens of variables. While our results were consistent and promising, these limitations should be addressed in future studies to fully validate these markers.

Biological plausibility

Direct infusion ESI-MS interrogated ∼2000 serum lipids having diverse biological functions. As such, a second element of this study was to chemically classify these biomarkers and to consider, based on published research, the biologic plausibility of the changes we observed.

Marker m/z 430.4 was likely vitamin E and was found to be increased in AD. Previous results found decreased vitamin E levels in the cerebrospinal fluid of AD patients [48, 49]. It is possible that AD cases may have selectively had increased supplement use, but we do not have data to support or refute this in these subjects.

Markers m/z 496.3 and 522.3 were higher in AD cases and were LPCs. Neurodegeneration, a feature of AD, results in breakdown of cell membranes [20] and the release of membrane lipids. Other studies report the activation of phospholipase A_2, leading to increased production of LPCs [20]. Furthermore, LPCs serve as precursors of platelet activating factor involved in inflammation [50]. There is strong evidence of inflammation in the brains of AD patients [51, 52]. Our results appear consistent with previous findings.

Markers m/z 703.5 and m/z 799.6 were SMs and reduced in AD. This is consistent with a previous study [53]. SMs are metabolized to ceramides which can affect cell differentiation, proliferation, and apoptosis [54] and may be involved in atherosclerosis [55], another feature of AD [56, 57].

Biomarkers m/z 724.5 and m/z 778.5 were decreased in AD cases and were PEs. Ethanolamine plasmalogen, representing ∼90% of all PEs, is enriched in white and grey matter of the brain and helps provide an axon sheath and acts as a source of lipid messengers participating in signal transduction in grey matter. Several studies report reduction in plasmalogen with worsening AD [58, 59], likely contributing to myelin sheath defects and axonal dysfunction. Plasmalogen deficiency could result in synapse loss [60], another feature of AD [61]. This same process, if somewhat more generalized, might explain the reduced PE seen in AD patients in our study.

The marker m/z 842.6 was predicted to be an oxidized PC having a hydroperoxide group. It was increased in AD cases. Oxidative stress is observed in neurodegenerative disorders [62], including AD [63], resulting in increased lipid oxidation. Similarly, marker m/z 912.7 was found to be an ammoniated

adduct of an oxidized TAG and was higher in AD cases. F2-isoprostane, produced from free radical peroxidation of polyunsaturated fatty has been reported to be higher in AD patients [64].

Several markers, those with m/z 820.7, 822.7, 846.7, 848.7, 850.7, 860.7, 862.7, 881.7, 886.7, 888.8, 890.8, and 930.8, were TAGs and higher in AD cases. Elevated TAG levels precede amyloid deposition in an AD mouse model [65], although the connection is unclear.

While our study provided robust, previously undescribed diagnostic biomarkers for AD, it is not the only study to describe serum lipid AD biomarkers. A previously published metabolomics study used a water miscible organic/water mixture to extract polar metabolites and highly polar lipids and analyzed them by LC-MS. While the approach was described as untargeted, it appears to survey a defined set of metabolites and polar lipids that this group had assessed. Their biomarker panel identified normal individuals who would phenoconvert to MCI or AD within 3 years with high accuracy [28]. A follow up study of the same specimens found additional potential markers from these same specimens [29]. Both studies importantly were prospective and found markers predicting phenoconverters. These reports differ from ours in a number of ways: They appear targeted. They measure different molecules that included single amino acids and water soluble metabolites. In their study, only ten subjects phenoconverted and half of these demonstrated conversion to MCI, not always an early stage of AD. Moreover, there was likely no opportunity, given the very small numbers, to have matched cases and controls or to assess biomarker panels for individual stages of AD or the ability to stage AD as was done in our study. There was no overlap in specific biomarkers between our and their studies, but there were similar alterations in PCs for both groups. At a minimum, our study provides complementary biomarkers and affirms that a lipidomics approach can likely diagnose and potentially stage AD.

Conclusion

The results of these studies suggest that useful serum lipid biomarkers can be found that may allow the diagnosis of most patients with AD and based on the two sets of cases and controls studied here, differences were observed for all stages, including very early AD. The majority of these biomarkers had altered expression consistent with known AD changes and abnormalities, some of which potentially contribute to the disease. These are novel, previously undescribed lipid biomarkers for AD. Some appeared to change progressively with disease stage. If verified, they might allow for staging AD. It would also be interesting to know if these particular markers might be altered prior to clinical AD changes, a finding that might aid in the design of drug trials.

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SUPPLEMENTARY MATERIAL

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REFERENCES

[1] Schneider JA, Aggarwal NT, Barnes L, Boyle P, Bennett DA (2009) The neuropathology of older persons with and without dementia from community versus clinic cohorts. *J Alzheimers Dis* **18**, 691.

[2] Schneider JA, Arvanitakis Z, Bang W, Bennett DA (2007) Mixed brain pathologies account for most dementia cases in community-dwelling older persons. *Neurology* **69**, 2197-2204.

[3] Ferri CP, Prince M, Brayne C, Brodaty H, Fratiglioni L, Ganguli M, Hall K, Hasegawa K, Hendrie H, Huang Y, Jorm A, Mathers C, Menezes PR, Rimmer E, Scazufca M (2006) Global prevalence of dementia: A Delphi consensus study. *Lancet* **366**, 2112-2117.

[4] Hardy J (2009) The amyloid hypothesis for Alzheimer's disease: A critical reappraisal. *J Neurochem* **110**, 1129-1134.

[5] Roher AE, Maarouf CL, Kokjohn TA, Belden C, Serrano G, Sabbagh MS, Beach TG (2016) *Am J Neurodegener Dis* **5**, 158-170.

[6] Maccioni RB, Muñoz JP, Barbeito L (2001) The molecular bases of Alzheimer's disease and other neurodegenerative disorders. *Arch Med Res* **32**, 367-381.

[7] Jack C, Petersen RC, Xu YC, O'Brien PC, Smith GE, Ivnik RJ, Boeve BF, Waring SC, Tangalos EG, Kokmen E (1999) Prediction of AD with MRI-based hippocampal volume in mild cognitive impairment. *Neurology* **52**, 1397-1397.

[8] Samuraki M, Matsunari I, Chen W-P, Yajima K, Yanase D, Fujikawa A, Takeda N, Nishimura S, Matsuda H, Yamada M (2007) Partial volume effect-corrected FDG PET and

grey matter volume loss in patients with mild Alzheimer's disease. *Eur J Nucl Med Mol Imaging* **34**, 1658-1669.

[9] Hampel H, Bürger K, Teipel SJ, Bokde AL, Zetterberg H, Blennow K (2008) Core candidate neurochemical and imaging biomarkers of Alzheimer's disease. *Alzheimers Dement* **4**, 38-48.

[10] Jack CR, Lowe VJ, Senjem ML, Weigand SD, Kemp BJ, Shiung MM, Knopman DS, Boeve BF, Klunk WE, Mathis CA, Petersen RC (2008) 11C PiB and structural MRI provide complementary information in imaging of Alzheimer's disease and amnestic mild cognitive impairment. *Brain* **131**, 665-680.

[11] Forsberg A, Engler H, Almkvist O, Blomquist G, Hagman G, Wall A, Ringheim A, Långström B, Nordberg A (2008) PET imaging of amyloid deposition in patients with mild cognitive impairment. *Neurobiol Aging* **29**, 1456-1465.

[12] Jonasson M, Wall A, Chiotis K, Saint-Aubert L, Wilking H, Sprycha M, Borg B, Thibblin A, Eriksson J, Sörensen J, Antoni G, Nordberg A, Lubberink M (2016) Tracer kinetic analysis of (S)-18F-THK5117 as a PET tracer for assessing tau pathology. *J Nucl Med* **57**, 574-581.

[13] Panegyres PK, Rogers JM, McCarthy M, Campbell A, Wu JS (2009) Fluorodeoxyglucose-positron emission tomography in the differential diagnosis of early-onset dementia: A prospective, community-based study. *BMC Neurol* **9**, 41.

[14] Tang BL, Kumar R (2008) Biomarkers of mild cognitive impairment and Alzheimer's disease. *Ann Acad Med Singapore* **37**, 406-405.

[15] Mayeux R, Honig L, Tang M-X, Manly J, Stern Y, Schupf N, Mehta P (2003) Plasma Aβ40 and Aβ42 and Alzheimer's disease Relation to age, mortality, and risk. *Neurology* **61**, 1185-1190.

[16] Fukumoto H, Tennis M, Locascio JJ, Hyman BT, Growdon JH, Irizarry MC (2003) Age but not diagnosis is the main predictor of plasma amyloid β-protein levels. *Arch Neurol* **60**, 958-964.

[17] Irizarry MC (2004) Biomarkers of Alzheimer disease in plasma. *NeuroRx* **1**, 226-234.

[18] Patterson C, Feightner JW, Garcia A, Hsiung G-YR, MacKnight C, Sadovnick AD (2008) Diagnosis and treatment of dementia: 1. Risk assessment and primary prevention of Alzheimer disease. *Can Med Assoc J* **178**, 548-556.

[19] Watson AD (2006) Thematic review series: Systems biology approaches to metabolic and cardiovascular disorders. Lipidomics: A global approach to lipid analysis in biological systems. *J Lipid Res* **47**, 2101-2111.

[20] Klein J (2000) Membrane breakdown in acute and chronic neurodegeneration: Focus on choline-containing phospholipids. *J Neural Transm* **107**, 1027-1063.

[21] Huang X, Moir RD, Tanzi RE, Bush AI, Rogers JT (2004) Redox-active metals, oxidative stress, and Alzheimer's disease pathology. *Ann NY Acad Sci* **1012**, 153-163.

[22] Butterfield DA, Perluigi M, Sultana R (2006) Oxidative stress in Alzheimer's disease brain: New insights from redox proteomics. *Eur J Pharmacol* **545**, 39-50.

[23] Praticò D (2010) The neurobiology of isoprostanes and Alzheimer's disease. *Biochim Biophys Acta* **1801**, 930-933.

[24] Bet L, Calabrese E, Bava L, Magni E, Rapuzzi S, Pezzoli G, Mariani C (1999) Malondialdheyde production features in platelets of Parkinson and Alzheimer patients and in physiological aging. *Neurosci Res Commun* **25**, 33-41.

[25] Svennerholm L, Gottfries CG (1994) Membrane lipids, selectively diminished in Alzheimer brains, suggest synapse loss as a primary event in early-onset form (type I) and demyelination in late-onset form (type II). *J Neurochem* **62**, 1039-1047.

[26] Han X, Holtzman DM, McKeel DW, Kelley J, Morris JC (2002) Substantial sulfatide deficiency and ceramide elevation in very early Alzheimer's disease: Potential role in disease pathogenesis. *J Neurochem* **82**, 809-818.

[27] Cutler RG, Kelly J, Storie K, Pedersen WA, Tammara A, Hatanpaa K, Troncoso JC, Mattson MP (2004) Involvement of oxidative stress-induced abnormalities in ceramide and cholesterol metabolism in brain aging and Alzheimer's disease. *Proc Natl Acad Sci U S A* **101**, 2070-2075.

[28] Mapstone M, Cheema AK, Fiandaca MS, Zhong Z, Mhyre TR, MacArthur LH, Hall WJ, Fisher SG, Peterson DR, Haley JM, Nazar MD, Rich SA, Berlau DJ, Peltz CB, Tan MT, Kawas CH, Federoff HJ (2014) Plasma phospholipids identify antecedent memory impairment in older adults. *Nat Med* **20**, 415-418.

[29] Fiandaca MS, Zhong X, Cheema AK, Orquiza MH, Chidambaram S, Tan MT, Gresenz CR, FitzGerald KT, Nalls MA, Singleton AB, Mapstone M, Federoff HJ (2015) Plasma 24-metabolite panel predicts preclinical transition to clinical stages of Alzheimer's disease. *Front Neurol* **6**, 237.

[30] Hara A, Radin NS (1978) Lipid extraction of tissues with a low-toxicity solvent. *Anal Biochem* **90**, 420-426.

[31] Anand S, Young SA, Esplin MS, Peaden B, Tolley HD, Porter TF, Varner MW, D'Alton ME, Jackson BJ, Graves SW (2016) Detection and confirmation of serum lipid biomarkers for preeclampsia using direct infusion mass spectrometry. *J Lipid Res* **57**, 687-696.

[32] van Buuren S, Groothuis-Oudshoorn K (2011) MICD: Multivariate imputation by chained equations in R. *J Stat Software* **45**, 1-67.

[33] Park T, Casella G (2008) The Bayesian lasso. *J Amer Stat Assoc* **103**, 681-686.

[34] Touboul D, Roy S, Germain DP, Chaminade P, Brunelle A, Laprévote O (2007) MALDI-TOF and cluster-TOF-SIMS imaging of Fabry disease biomarkers. *Intl J Mass Spectrom* **260**, 158-165.

[35] Chughtai K, Jiang L, Greenwood TR, Glunde K, Heeren RM (2013) Mass spectrometry images acylcarnitines, phosphatidylcholines, and sphingomyelin in MDA-MB-231 breast tumor models. *J Lipid Res* **54**, 333-344.

[36] Hsu F-F, Turk J (2009) Electrospray ionization with low-energy collisionally activated dissociation tandem mass spectrometry of glycerophospholipids: Mechanisms of fragmentation and structural characterization. *J Chromatogr B* **877**, 2673-2695.

[37] Enomoto H, Sugiura Y, Setou M, Zaima N (2011) Visualization of phosphatidylcholine, lysophosphatidylcholine and sphingomyelin in mouse tongue body by matrix-assisted laser desorption/ionization imaging mass spectrometry. *Anal Bioanal Chem* **400**, 1913-1921.

[38] Liebisch G, Binder M, Schifferer R, Langmann T, Schulz B, Schmitz G (2006) High throughput quantification of cholesterol and cholesteryl ester by electrospray ionization tandem mass spectrometry (ESI-MS/MS). *Biochim Biophys Acta* **1761**, 121-128.

[39] Yu S, Dong J, Zhou W, Ruiyue Y, Hongxia L, Haijian Z, Tianjiao Z, Hanbang G, Shu W, Chuanbao Z, Wenxiang C (2014) A rapid and precise method for quantification of fatty acids in human serum cholesteryl esters by liquid chromatography and tandem mass spectrometry. *J Chromatogr B* **960**, 222-229.

[40] Berry KAZ, Murphy RC (2004) Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. *J Amer Soc Mass Spectrom* **15**, 1499-1508.

[41] Uchikata T, Matsubara A, Nishiumi S, Yoshida M, Fukusaki E, Bamba T (2012) Development of oxidized phosphatidylcholine isomer profiling method using supercritical fluid chromatography/tandem mass spectrometry. *J Chromatogr A* **1250**, 205-211.

[42] Byrdwell W, Neff WE (2002) Dual parallel electrospray ionization and atmospheric pressure chemical ionization mass spectrometry (MS), MS/MS and MS/MS/MS for the analysis of triacylglycerols and triacylglycerol oxidation products. *Rapid Commun Mass Spectrom* **16**, 300-319.

[43] Zeb A (2012) Triacylglycerols composition, oxidation and oxidation compounds in camellia oil using liquid chromatography–mass spectrometry. *Chem Phys Lipids* **165**, 608-614.

[44] Shah DJ, Rohlfing F, Anand S, Johnson WE, Alvarez MTB, Cobell J, King J, Young SA, Kauwe JSK, Graves SW (2015) Discovery and confirmation of novel serum biomarkers diagnosing Alzheimer's disease. *J Alzheimers Dis* **49**, 317-327.

[45] Wenk MR (2005) The emerging field of lipidomics. *Nat Rev Drug Discov* **4**, 594-610.

[46] Hu C, van der Heijden R, Wang M, van der Greef J, Hankemeier T, Xu G (2009) Analytical strategies in lipidomics and applications in disease biomarker discovery. *J Chromatogr B* **877**, 2836-2846.

[47] Han X, Gross RW (2005) Shotgun lipidomics: Electrospray ionization mass spectrometric analysis and quantitation of cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom Rev* **24**, 367-412.

[48] Mangialasche F, Kivipelto M, Mecocci P, Rizzuto D, Palmer K, Winblad B, Fratiglioni L (2010) High plasma levels of vitamin E forms and reduced Alzheimer's disease risk in advanced age. *J Alzheimers Dis* **20**, 1029.

[49] Kontush A, Schekatolina S (2004) Vitamin E in neurodegenerative disorders: Alzheimer's disease. *Ann NY Acad Sci* **1031**, 249-262.

[50] Bazan NG, de Turco EBR, Allan G (1995) Mediators of injury in neurotrauma: Intracellular signal transduction and gene expression. *J Neurotrauma* **12**, 791-814.

[51] Akiyama H, Barger S, Barnum S, Bradt B, Bauer J, Cole GM, Cooper NR, Eikelenboom P, Emmerling M, Fiebich BL, Finch CE, Frautschy S, Griffen WST, Hampel H, Hull M, Landreth G, Lue L-F, Mrak R, Mackenzie IR, McGeer PL, O'Banion MK, Pachter J, Pasinetti G, Plata-Salaman C, RogersJ, Rydel R, Shen Y, Streit W, Strohmeyer R, Tooyoma I, Van Muiswinkel FL, Veerhuis R, Walker D, Webster S, Wegrzyniak B, Wenk G, Wyss-Coray T (2000) Inflammation and Alzheimer's disease. *Neurobiol Aging* **21**, 383-421.

[52] Heneka MT (2006) Inflammation in Alzheimer's disease. *Clin Neurosci Res* **6**, 247-260.

[53] He X, Huang Y, Li B, Gong C-X, Schuchman EH (2010) Deregulation of sphingolipid metabolism in Alzheimer's disease. *Neurobiol Aging* **31**, 398-408.

[54] Merrill A, Schmelz E, Dillehay D, Spiegel S, Shayman J, Schroeder J, Riley R, Voss K, Wang E (1997) Sphingolipids—the enigmatic lipid class: Biochemistry, physiology, and pathophysiology. *Toxicol Appl Pharmacol* **142**, 208-225.

[55] Ichi I, Nakahara K, Miyashita Y, Hidaka A, Kutsukake S, Inoue K, Maruyama T, Miwa Y, Harada-Shiba M, Tsushima M, Kojo S (2006) Association of ceramides in human plasma with risk factors of atherosclerosis. *Lipids* **41**, 859-863.

[56] Rodríguez JJ, Jones VC, Verkhratsky A (2009) Impaired cell proliferation in the subventricular zone in an Alzheimer's disease model. *Neuroreport* **20**, 907-912.

[57] Casserly I, Topol EJ (2004) Convergence of atherosclerosis and Alzheimer's disease: Inflammation, cholesterol, and misfolded proteins. *Lancet* **363**, 1139-1146.

[58] Han X, Holtzman DM, McKeel DW (2001) Plasmalogen deficiency in early Alzheimer's disease subjects and in animal models: Molecular characterization using electrospray ionization mass spectrometry. *J Neurochem* **77**, 1168-1180.

[59] Ginsberg L, Rafique S, Xuereb JH, Rapoport SI, Gershfeld NL (1995) Disease and anatomic specificity of ethanolamine plasmalogen deficiency in Alzheimer's disease brain. *Brain Res* **698**, 223-226.

[60] Ginsberg L, Xuereb JH, Gershfeld NL (1998) Membrane instability, plasmalogen content, and Alzheimer's disease. *J Neurochem* **70**, 2533-2538.

[61] Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, Hansen LA, Katzman R (1991) Physical basis of cognitive alterations in Alzheimer's disease: Synapse loss is the major correlate of cognitive impairment. *Ann Neurol* **30**, 572-580.

[62] Migliore L, Fontana I, Colognato R, Coppede F, Siciliano G, Murri L (2005) *Neurobiol Aging* **26**, 587-9563.

[63] Sultana R, Mecocci P, Mangialasche F, Cecchetti R, Baglioni M, Butterfield DA (2010) Increased protein and lipid oxidative damage in mitochondria isolated from lymphocytes from patients with Alzheimer's disease: Insights into the role of oxidative stress in Alzheimer's disease and initial investigations into a potential biomarker for this dementing disorder. *J Alzheimers Dis* **24**, 77-84.

[64] Song F, Poljak A, Smythe GA, Sachdev P (2009) Plasma biomarkers for mild cognitive impairment and Alzheimer's disease. *Brain Res Rev* **61**, 69-80.

[65] Burgess BL, McIsaac SA, Naus KE, Chan JY, Tansley GHK, Yang J, Miao F, Ross CJD, van Eck M, Hayden MR, van Nostrand W, St George-Hyslop P, Westaway D, Wellington CL (2006) Elevated plasma triglyceride levels precede amyloid deposition in Alzheimer's disease mouse models with abundant Aβ in plasma. *Neurobiol Dis* **24**, 114-127.