



## Targeted lipidomics distinguishes patient subgroups in mild cognitive impairment (MCI) and late onset Alzheimer's disease (LOAD)



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### ABSTRACT

**Background:** Diverse research approaches support the concept that a clinical diagnosis of Late-Onset Alzheimer's Disease (LOAD) does not distinguish between subpopulations with differing neuropathologies, including dementia patients with amyloid deposition and dementia patients without amyloid deposition but with cortical thinning. Mild cognitive impairment (MCI) is generally considered the prodromal phase for LOAD, however, while a number of studies have attempted to define plasma biomarkers for the conversion of MCI to LOAD, these studies have not taken into account the heterogeneity of patient cohorts within a clinical phenotype.

**Methods:** Studies of MCI and LOAD in several laboratories have demonstrated decrements in ethanolamine plasmalogen levels in plasma and brain and increased levels of diacylglycerols in plasma and brain. To further extend these studies and to address the issue of heterogeneity in MCI and LOAD patient groups we investigated the levels of diacylglycerols and ethanolamine plasmalogens in larger cohorts of patients utilizing, high-resolution (0.2 to 2 ppm mass error) mass spectrometry.

**Results:** For the first time, our lipidomics data clearly stratify both MCI and LOAD subjects into 3 different patient cohorts within each clinical diagnosis. These include i) patients with lower circulating ethanolamine plasmalogen levels; ii) patients with augmented plasma diacylglycerol levels; and iii) patients with neither of these lipid alterations.

**Conclusions:** These represent the first serum biochemical data to stratify MCI and LOAD patients, advancing efforts to biochemically define patient heterogeneity in cognitive disorders.

**General significance:** Lipidomics offers a new approach for identifying biomarkers and biological targets in cognitive disorders.

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### 1. Introduction

Since cognitive testing lacks the sensitivity required to detect the pre-symptomatic stages of LOAD, researchers continue to search for biomarkers of pre-MCI, MCI, and LOAD [1]. However, there currently are no validated biomarkers for the early detection of MCI or LOAD [2]. In the search for biomarkers of cognitive dysfunction it is essential for researchers to address the complex issue of patient heterogeneity in clinically defined MCI and LOAD patient populations [3–5]. Lipidomics is a powerful analytical platform that is being utilized to address this

complexity [6–17]. While a number of lipid alterations have been reported in LOAD, significant discrepancies between laboratories need to be resolved and a number of reported lipid changes require further validation. This literature has been reviewed a number of times but is restricted to LOAD with minimal available lipidomics data for MCI subjects [1,6,14–15,18–19]. However, previous studies of LOAD have consistently demonstrated decrements in ethanolamine plasmalogen (PIEs) levels in plasma [7–11] and brain [11–16] and increased levels of diacylglycerols (DAGs) in plasma [10–11,17] and brain [11–15]. Pilot studies of MCI plasma and brain also have detected elevated levels of DAGs [10–11], suggesting a role early in the disease process. To extend these observations we performed a targeted high-resolution mass spectrometric analysis of plasma DAGs and PIEs in larger cohorts of MCI and LOAD subjects.

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## 2. Materials and methods

### 2.1. Study participants

This study conforms to The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was conducted accordingly to the guidelines for Good Clinical Practice (European Medicines Agency). Signed informed consent which was written in compliance with local and national ethical guidelines was obtained from each patient or from relatives or a legal guardian prior to patient inclusion in the study [20]. Cognitive function was evaluated with the Mini-Mental State Exam (MMSE) and the Babcock Story Recall Test (BSRT) [20–23]. Serum was collected and stored at -80 °C for biochemical analyses.

### 2.2. Study participants: elderly controls (Table 1)

Elderly controls with no evidence of cognitive decline were included in the study.

### 2.3. Study participants: mild cognitive impairment (MCI, Table 1)

The diagnosis of MCI was made by trained geriatricians based on the presence of short/long-term memory impairment, with/without impairment in other single or multiple cognitive domains, in an individual who didn't meet the standardized criteria for dementia [22]. MMSE along with a battery of neuropsychological tests was used to evaluate the degree of cognitive impairment [23]. Patients with MCI due to known causes (e.g. depression, extensive white matter pathology, vitamin B12 deficiency) were excluded. Subjects affected by severe congestive heart failure (New York Heart Association class III–IV), severe liver or kidney disease, severe chronic obstructive pulmonary disease, and cancer, and those taking NSAIDs, antibiotics or steroids at the time of recruitment were also excluded. Only patients still independent in the activities of daily living (ADLs) were included in the study [20].

### 2.4. Study participants: late onset Alzheimer's Disease (LOAD, Table 1)

A diagnosis of LOAD was made by trained geriatricians according to the NINCDS-ADRDA criteria [22]. All patients (controls, MCI, and LOAD) underwent a brain Computer Tomography (CT) scan at baseline performed with a third-generation scanner at 10 mm thickness (SOMATOM HQ, Siemens Healthcare, Milan, Italy). The CT information was used to support the clinical diagnosis and to diagnose possible brain pathologies associated with secondary cognitive impairment.

### 2.5. Lipid extraction and analysis

Lipids were extracted from 100 µL of serum with methy-tert-butyl ether and methanol containing [<sup>2</sup>H<sub>8</sub>]arachidonic acid, [<sup>13</sup>C<sub>18</sub>]stearic acid, [<sup>13</sup>C<sub>3</sub>]DAG 36:2, [<sup>2</sup>H<sub>31</sub>]phosphatidylethanolamine 34:1, and bromocriptine as internal standards [10–11,22]. Extracts were dried by centrifugal vacuum evaporation and dissolved in isopropanol:methanol:chloroform (4:2:1) containing 15 mM ammonium acetate. Constant infusion lipidomics (5 µL per min) were performed utilizing high-resolution

(140,000 at 200 amu; 0.2–2 ppm mass error) data acquisition [24–25] on an orbitrap mass spectrometer (Thermo Q Exactive). The spray voltage was 3.3 kV, the sheath gas was 10, the capillary temperature was 320 °C, and the S lens RF was 50. Washes (500 µL) with methanol followed by hexane/ethyl acetate (3:2), between samples, were used to minimize ghost effects. In positive ion electrospray ionization (ESI), the ammonium adducts of diacylglycerols (DAG) were quantitated. The cation of bromocriptine was used to monitor for potential mass axis drift. Relative DAG levels were obtained by determining the ratio of the endogenous DAG peak area to the peak area of 1 nanomole of the internal standard [<sup>13</sup>C<sub>3</sub>]DAG 36:2. In negative ion ESI, the anions of phosphatidylethanolamines (PtdE) were quantitated. The anion of bromocriptine was used to monitor for potential mass axis drift. Relative PtdE levels were obtained by determining the ratio of the endogenous PtdE peak area to the peak area of 1 nanomole of the internal standard [<sup>2</sup>H<sub>31</sub>]PtdE 34:1.

### 2.6. Statistical analysis

R values (ratio of endogenous lipid peak area to the peak area of an appropriate internal standard) were calculated. Data are presented as mean ± SEM. Data were analyzed with the Kruskal–Wallis test, followed by the Dunn's t test to compare MCI and LOAD groups to the controls [10–11].

## 3. Results and discussion

### 3.1. Diacylglycerols (DAGs)

For the current targeted lipidomics study we stratified the MCI and AD patient groups (Table 1) based upon their Mini-Mental State Exam (MMSE) score as we have previously reported [10]. In addition, the Babcock Story Recall Test (BSRT) [20–21] was utilized to support the MMSE scores with regard to evaluation of decline in cognitive function for individual patients (Table 2). Utilizing this stratified design our targeted lipidomics analyses detected a number of DAGs that were elevated in the serum of MCI and LOAD patients as we [10–11] and others [14] have previously reported. DAG 34:2 and DAG 36:2 were robust biomarkers being elevated in both MCI and LOAD patients with an MMSE score of 10 to 18 while DAG 36:2 also was elevated in MCI and LOAD patients with an MMSE score of 19 to 24 (Table 2). Our previous studies have shown that DAG 34:2 is comprised mainly of DAG 16:0/18:2 while DAG 36:2 is 66% DAG 18:1/18:1 and 33% DAG 18:0/18:2, with no differences in fatty acid composition of these DAGs between aged-matched control, MCI and LOAD subjects [14]. Elevations in these lipid levels, which are normally tightly regulated [26–27], is of significant clinical interest considering the diverse roles of DAGs as precursors of structural glycerophospholipids and neutral lipids, as structural components of nuclear and endoplasmic reticular membranes, as mediators of signal transduction, as mediators of nuclear signal transduction, and in Golgi transport carrier biogenesis [26–27]. Alterations in any or all of these DAG-dependent functions could contribute to the development of neuronal dysfunction and ultimately to cognitive decline in MCI and LOAD patients.

**Table 1**  
Patient demographics for the clinical cohorts.

Parameter	Controls	MCI-1	MCI-2	LOAD-1	LOAD-2
MMSE	25–30	19–24	10–18	19–24	10–18
N	51	64	13	57	33
Age	76 ± 0.8	78.2 ± 0.062	77 ± 1.3	78 ± 0.7	78.2 ± 0.7
Gender (% Male)	35.3	23.4	38.5	22.8	30.3
Glucose (mg/dL)	95.3 ± 2.1	95.8 ± 1.5	93.4 ± 2.5	96.5 ± 2.5	95.3 ± 3.3
Total Cholesterol (mg/dL)	207.4 ± 4.1	212.8 ± 4.4	210.8 ± 15.6	213.8 ± 4.7	218.3 ± 6.6
HDL/LDL Ratio	0.55 ± 4.1	0.52 ± 0.022	0.44 ± 0.057	0.53 ± 0.023	0.51 ± 0.031
Triglycerides (mg/dL)	109.9 ± 7.7	115.7 ± 6.4	150.4 ± 29.1	110.7 ± 6.0	110.5 ± 7.8

**Table 2**

Mini-Mental Status Exam (MMSE) bracketed scores, Babcock Story Recall Test (BSRT) scores for memory, diacylglycerol (DAG) levels, and ethanolamine plasmalogen (PlsE) levels in control, Mild Cognitive Impairment (MCI), and Late Onset Alzheimer's Disease (LOAD) patients. The DAG and PlsE values are presented as the ratio (R) of the peak area of the endogenous lipid to the peak area of a stable isotope internal standard. Data are presented as mean  $\pm$  SEM. \*,  $p < 0.05$  vs. control. \$,  $[M + NH_4]^+$ ; #,  $[M-H]^-$ .

Parameter	Control	MCI-1	MCI-2	LOAD-1	LOAD-2
MMSE (bracketed range)	25–30	19–24	10–18	19–24	10–18
BRST – immediate (score)	5.1 $\pm$ 0.23	3.8 $\pm$ 0.29*	4.3 $\pm$ 0.90	2.9 $\pm$ 0.32*	2.2 $\pm$ 0.40*
BRST – delayed (score)	6.0 $\pm$ 0.23	3.8 $\pm$ 0.34*	2.3 $\pm$ 0.86*	2.0 $\pm$ 0.30*	1.0 $\pm$ 0.35*
DAG 34:2 (R) 610.54105 <sup>\$</sup>	4.43 $\pm$ 0.22	5.04 $\pm$ 0.19	5.63 $\pm$ 0.32*	5.00 $\pm$ 0.20	5.56 $\pm$ 0.42*
DAG 36:2 (R) 638.57235 <sup>\$</sup>	2.77 $\pm$ 0.13	3.45 $\pm$ 0.12*	3.94 $\pm$ 0.19*	3.52 $\pm$ 0.12*	3.84 $\pm$ 0.26*
PlsE 38:6 (R) 746.51301 <sup>#</sup>	2.66 $\pm$ 0.14	2.06 $\pm$ 0.12*	1.97 $\pm$ 0.18	2.04 $\pm$ 0.11*	2.11 $\pm$ 0.19
PlsE 40:6 (R) 774.54431 <sup>#</sup>	2.17 $\pm$ 0.12	1.58 $\pm$ 0.10*	1.41 $\pm$ 0.12*	1.53 $\pm$ 0.09*	1.51 $\pm$ 0.15*

### 3.2. Ethanolamine plasmalogens (PlsEs)

In the case of serum levels of PlsEs, PlsEs with polyunsaturated fatty acid substitutions were most dramatically affected in both MCI and LOAD patients, as we previously reported for LOAD patients [7–8]. For example, PlsE 38:6 (16:0/22:6) was decreased in the MCI and LOAD cohorts with an MMSE in the range of 19 to 24 while PlsE 40:6 (18:0/22:6) were decreased in both MCI and both LOAD cohorts (Table 2). The decrements in circulating plasmalogen levels monitored in the current study are in contrast with our previous negative findings in a smaller MCI patient cohort [10] and demonstrate the dangers of pilot biomarker evaluations in small patient cohorts. These data also support published observations of decreased peroxisomal function in the liver [28] and brain [29] of LOAD subjects and plasmalogen deficits in LOAD brain [10–16], further suggesting that peroxisomal dysfunction may occur early in the disease process in a subset of patients. Decrements in peroxisomal function and the resulting decreases in circulating and brain plasmalogens may represent a critical biochemical alteration that could lead to cognitive deficits. Plasmalogens are major components of membrane lipid rafts and are essential for neurotransmitter vesicular fusion [6,30]. Alterations in these functions could result in both decreased acetylcholine release and altered postsynaptic signal transduction thereby negatively altering the function of cholinergic pathways involved in cognition [6].

### 3.3. Patient stratification

To further investigate potential heterogeneity within our patient cohorts, we calculated the percentage of patients with DAG levels greater than one standard deviation above the control group mean and the percentage of patients with PlsE levels more than one standard deviation below the control group mean (Table 3). These analyses revealed that as would be expected: i) patient cohorts with the greatest cognitive deficit (MMSE of 10 to 18) had a larger number of patients with alterations in one of these lipid biomarkers relative to patients with less cognitive deficit (MMSE of 19 to 24) and ii) that there was a greater degree of heterogeneity in lipid alterations in the MCI cohorts

**Table 3**

Demonstration of heterogeneity in the patient cohorts via analysis of subjects with DAG levels elevated more than one standard deviation above control values and subjects with ethanolamine plasmalogen (PlsE) levels decreased by more than one standard deviation below control values.

Group	MMSE	$\uparrow$ DAG <sup>1</sup>	$\downarrow$ PlsE <sup>2</sup>	$\leftrightarrow$ DAG or PlsE <sup>3</sup>
MCI-1	19–24	25.5%	17.3%	57.2
MCI-2	10–18	30.0%	45.1%	24.9
LOAD-1	19–24	45.5%	29.6%	24.9
LOAD-2	10–18	40.0%	44.8%	15.2

<sup>1</sup> % of group with DAG levels  $> 1$  SD above the control group levels.

<sup>2</sup> % of group with PlsE levels  $> 1$  SD below the control group levels.

<sup>3</sup> % of group with no increase in DAG or decrease in PlsE levels. There was no overlap between the subjects with elevated DAG levels and the subjects with lowered plasmalogen levels.

relative to the LOAD cohorts. More significantly, we noted that when individual patients with elevated DAGs or decreased PlsEs were assessed there were no patients that possessed both lipid alterations (Table 3). These data clearly demonstrate, at the biochemical level, that there are at least 3 potential subsets of patients within each of the clinical groups in our study: i) patients with lower circulating ethanolamine plasmalogen levels; ii) patients with augmented plasma DAG levels; and iii) patients with neither of these lipid alterations. However, these data do not preclude that analyses of an even larger patient cohort might identify patients possessing both elevated DAG and decreased PlsE levels. This caution is of significant clinical relevance since in our pilot study of a small patient cohort we only monitored an increase of DAG levels in MCI patients but failed to detect any patients with plasmalogen deficits [10], indicating that by chance, our patient cohorts failed to include any of the subset of patients with deficits in circulating ethanolamine plasmalogen levels. The potential role of ApoE phenotype in our observed lipid changes remains to be fully explored since the phenotype of patients in this study was not determined. However, previous studies of decreased circulating PlsEs [8] and increased plasma DAGs [10–11] found no correlation with ApoE phenotype.

## 4. Conclusion

These are exciting new observations which constitute initial efforts to define, at the biochemical level, the heterogeneity of disease processes that can ultimately result in cognitive impairment. Our observations may also be of value in the design of future clinical dementia trials. Inclusion of multiple biochemical parameters to define the heterogeneity of patient groups will aid in avoiding errors such as occurred in clinical evaluations of anti-amyloid therapies in dementia patients with no amyloid deposition [5]. Integration of lipidomics with other omics technologies has the potential to define different pathological mechanisms that lead to cognitive impairment and to distinguish patients at risk for dementia from Non-Demented individuals who possess significant Alzheimer's Disease Neuropathology (NDAN subjects) [31–35]. Ultimately lipidomics may offer an avenue to increase our understanding of the biochemical basis for the cognitive reserve or resistance factors in these NDAN subjects.

In summary, our data differentiate subsets of patients within clinically defined MCI and LOAD groups. The next steps will be to expand the investigation of these lipid biomarkers to larger patient cohorts which are monitored longitudinally with regard to both cognitive and lipidomics parameters, and ultimately brain pathology. Evaluation of these biochemical alterations in MCI and LOAD has the potential to define diverse biochemical pathologies that can ultimately lead to clinical cognitive deficits. Such analyses also will significantly contribute to defining new molecular targets for cognitive disorders and in the design of future therapeutic trials in dementia patients.

## Author contributions

PLW, CC, and GZ contributed to the design of the experiment, analysis of the data, and writing the manuscript. VL and PH conducted the

lipidomics studies while AP, GBV, SV, and GV conducted patient evaluations and collection of serum. All authors have reviewed and approved the manuscript.

### Competing Interests

None.

### Transparency document

The Transparency document associated with this article can be found, in online version.

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