# Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia

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**Abstract Although dementia of the Alzheimer's type (DAT) is the most common form of dementia, the severity of dementia is only weakly correlated with DAT pathology. In contrast, postmortem measurements of cholinergic function and membrane ethanolamine plasmalogen (PlsEtn) content in the cortex and hippocampus correlate with the severity of dementia in DAT. Currently, the largest risk factor for DAT is age. Because the synthesis of PlsEtn occurs via a single nonredundant peroxisomal pathway that has been shown to decrease with age and PlsEtn is decreased in the DAT brain, we investigated potential relationships between serum PlsEtn levels, dementia severity, and DAT pathology. In total, serum PlsEtn levels were measured in five independent population collections comprising >400 clinically demented and >350 nondemented subjects. Circulating PlsEtn levels were observed to be significantly decreased in serum from clinically and pathologically diagnosed DAT subjects at all stages of dementia, and the severity of this decrease correlated with the severity of dementia. Furthermore, a linear regression model predicted that serum PlsEtn levels decrease years before clinical symptoms. The putative roles that PlsEtn biochemistry play in the etiology of cholinergic degeneration, amyloid accumulation, and dementia are discussed.—**Goodenowe, D. B., L. L. Cook, J. Liu, Y. Lu, D. A. Jayasinghe, P. W. K. Ahiahonu, D. Heath, Y. Yamazaki, J. Flax, K. F. Krenitsky, D. L. Sparks, A. Lerner, R. P. Friedland, T. Kudo, K. Kamino, T. Morihara, M. Takeda, and P. L. Wood. **Peripheral ethanolamine plasmalogen deficiency: a logical causative factor in Alzheimer's disease and dementia. *J. Lipid Res.* 2007.** 48: **2485–2498.**

**Supplementary key words** aging • peroxisome • neurodegeneration • amyloid

The most severe consequence of the aging brain is dementia. The number of elderly people is increasing rapidly within our society, and as a consequence, dementia is growing into a major health problem. It has been estimated that 25% of the population older than 65 years has some form of dementia (1) and that the cumulative incidence of dementia in individuals living to the age of 95 years is >80% (2, 3).

The clinical manifestation of dementia can result from neurodegeneration [e.g., dementia of the Alzheimer's type (DAT), dementia with Lewy bodies, and frontotemporal lobe dementia], a vascular event (e.g., multi-infarct dementia) or anoxic event (e.g., cardiac arrest), brain trauma [e.g., dementia pugilistica (boxer's dementia)], or exposure to an infectious agent (e.g., Creutzfeldt-Jakob disease) or a toxic agent (e.g., alcohol-induced dementia) (4). Given that dementia can result from diverse neurological insults, the biochemical mechanism of dementia is likely to be separate and distinct from these precipitating events.

The differential diagnosis of the types and causes of dementia is not straightforward. A prospective study of the prevalence of DAT in people older than 85 years indicated that more than half of the individuals with neuropathological criteria for DAT were either nondemented or incorrectly diagnosed with vascular dementia. As well, 35% of the clinically diagnosed DAT subjects did not exhibit neuropathological features sufficient to support the diagnosis (5). Clearly, dementia can arise from multiple pathological states that are often clinically indistinguishable. Because DAT is the most common type of dementia and

*Manuscript received 20 June 2007 and in revised form 16 July 2007.*

*Published, JLR Papers in Press, August 2, 2007.*
*DOI 10.1194/jlr.P700023-JLR200*

Abbreviations: PtdCho, phosphatidylcholine.

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the percentage of dementias that is DAT increases with increasing age (2), DAT is the obvious model system in which to study the putative underlying biochemical mechanisms of dementia in humans.

Previous studies have shown that ethanolamine plasmalogen (PlsEtn) is depleted in the brains of subjects with DAT (6–9). To determine whether decreased brain levels of PlsEtn in DAT are purely a centrally mediated effect caused by amyloid-β (Aβ) accumulation or whether much broader systemic changes are at play, we investigated the effects of age, dementia severity, and Aβ pathology on serum PlsEtn levels in subjects with various levels of DAT dementia and pathology and a representative healthy general population cohort aged 50 to 95 years.

## METHODS

### Sample extraction

Serum and plasma samples were stored at −80°C until thawed for analysis. All extractions were performed on ice. The phospholipids were extracted from serum/plasma using 1% ammonium hydroxide and ethyl acetate (EtOAc) three times using a serum/plasma: ammonium hydroxide: EtOAc ratio of 1:1:5, followed by two extractions with 0.33 % formic acid and EtOAc using a serum/plasma: formic acid: EtOAc ratio of 1:1:5. Samples were centrifuged between extractions at 4°C for 10 min at 3,500 rpm, and the organic layers were combined. Individual 1.0 ml aliquots of this ethyl acetate extract were then stored at −80°C until analysis.

### LC-MS/MS flow injection analysis

High-throughput screening was performed with a linear ion-trap mass spectrometer (4000 Q TRAP; Applied Biosystems) coupled with the Agilent 1100 LC system. Samples were prepared by adding 15 μl of internal standard (5 μg/ml [24-$^{13}$C]cholic acid [Cambridge Isotope Laboratories, Andover, MA] in methanol) to 120 μl of ethyl acetate fraction of each sample. A 100 μl sample was injected by flow injection analysis and monitored under negative atmospheric pressure chemical ionization mode. The method was based on multiple reaction monitoring (MRM) of one parent/fragment transition for each metabolite and [24-$^{13}$C]cholic acid. Each transition was scanned for 70 ms. Ten percent ethyl acetate in methanol at a flow rate of 360 μl/min was used as the mobile phase. The source parameters were set as follows: curtain gas, 10.0; collision-activated dissociation gas, 8; nebulizing current, −4.0; temperature, 400; ion source gas 1, 30; ion source gas 2, 50; interface heater on. The compound parameters were set as follows: declustering potential, −120.0; entrance potential, −10; nebulizing current, −4.0; collision energy, −40; collision cell exit potential, −15. **Table 1** lists the transitions that were used. A standard curve was generated for all analytes to verify instrument linearity from 100% to 10% of normal serum levels by serial dilution of a healthy normal serum extract with constant concentration of [24-$^{13}$C]cholic acid. All samples were analyzed in a randomized blinded manner and were bracketed by known serum standard dilutions. All standard curves had $r^2$ values of >0.98.

### LC-MS + MS/MS chromatographic conditions

PlsEtn was confirmed to be present and decreased in DAT subjects using an Agilent 1100 HPLC system connected to an Applied Biosystems QSTAR XL mass spectrometer. Normal phase chromatography using an Agilent Zorbax RX-SIL (4.6 × 150 mm, 5 μm) column was used under isocratic conditions [mobile phase (55:40:5 isopropanol-hexane-water) at a flow rate of 1.0 ml/min for a total run time of 15 min]. A column temperature of 35°C and an injection volume of 10 μl were used. Organic solvent extracts (ethyl acetate) of samples were evaporated to dryness under nitrogen gas, and the residue was reconstituted in 100 μl of 55:40:5 isopropanol-hexane-water solution before injection. The QSTAR XL instrument was equipped with an atmospheric pressure chemical ionization (heated nebulizer) source operating in negative mode. Values of the major instrument parameters were as follows: declustering potential, −60; FP, −265; DP2, −15; ion source gas 1, 75; ion source gas 2, 15; curtain gas, 30; nebulizing current, −3; temperature, 400°C; scan range, 50–1,500 amu; accumulation time, 1 s.

### Known standard evaluation

Pure standard of PlsEtn 18:0/20:4 was obtained from Avanti Polar Lipids (Alabaster, AL). The retention time and MS/MS spectra of this standard were compared with those of serum extracts from DAT and cognitively normal subjects (**Fig. 1**). The MS/MS spectra of PlsEtn 18:0/20:4 [1-*O*-1′-(*Z*)-octadecenyl-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine] revealed two principal fragments resulting from the loss of the *sn*-2 acyl group: *m/z* 303, which corresponds to arachidonic acid, and *m/z* 464 as the ketone (**Table 2**). Considering that all PlsEtn have the same basic chemical structure and differ only by different fatty acid side chains and that the principal fragment is the *sn*-2 fatty acid, the theoretical LC-MS/MS parent fragment transitions for eight PlsEtn were determined (Table 1) and confirmed to be present in human serum.

### Quantitative analytical validation of PlsEtn in human serum

Validation of the flow injection methodology was performed using a subset of subjects (12 DAT and 12 cognitive normals comprising six male and six female subjects in each set). Three

TABLE 1. Molecular formulae and MS/MS transitions for the PlsEtn studied

| PlsEtn | Molecular Formula | Isotopic Mass | (M-H-) Mass | Fragment Formula | Fragment Mass | MS/MS Transition |
|---|---|---|---|---|---|---|
| 16:0/18:1 | C39H76N1O7P1 | 701.53591 | 700.5 | C18H33O2 | 281 | 700.5/281.2 |
| 16:0/18:2 | C39H74N1O7P1 | 699.52026 | 698.5 | C18H31O2 | 279 | 698.5/279.2 |
| 16:0/20:4 | C41H74N1O7P1 | 723.52026 | 722.5 | C20H31O2 | 303 | 722.5/303.2 |
| 16:0/22:6 | C43H74N1O7P1 | 747.52026 | 746.5 | C22H31O2 | 327 | 746.5/327.2 |
| 18:0/18:1 | C41H80N1O7P1 | 729.56721 | 728.5 | C18H33O2 | 281 | 728.5/281.2 |
| 18:0/18:2 | C41H78N1O7P1 | 727.55156 | 726.5 | C18H31O2 | 279 | 726.5/279.2 |
| 18:0/20:4 | C43H78N1O7P1 | 751.55156 | 750.5 | C20H31O2 | 303 | 750.5/303.2 |
| 18:0/22:6 | C45H78N1O7P1 | 775.55156 | 774.5 | C22H31O2 | 327 | 774.5/327.2 |
| Free 22:6 | C22H32O2 | 328.24022 | 327.2 | C21H31 | 283 | 327.2/283.2 |

PlsEtn, ethanolamine plasmalogen.

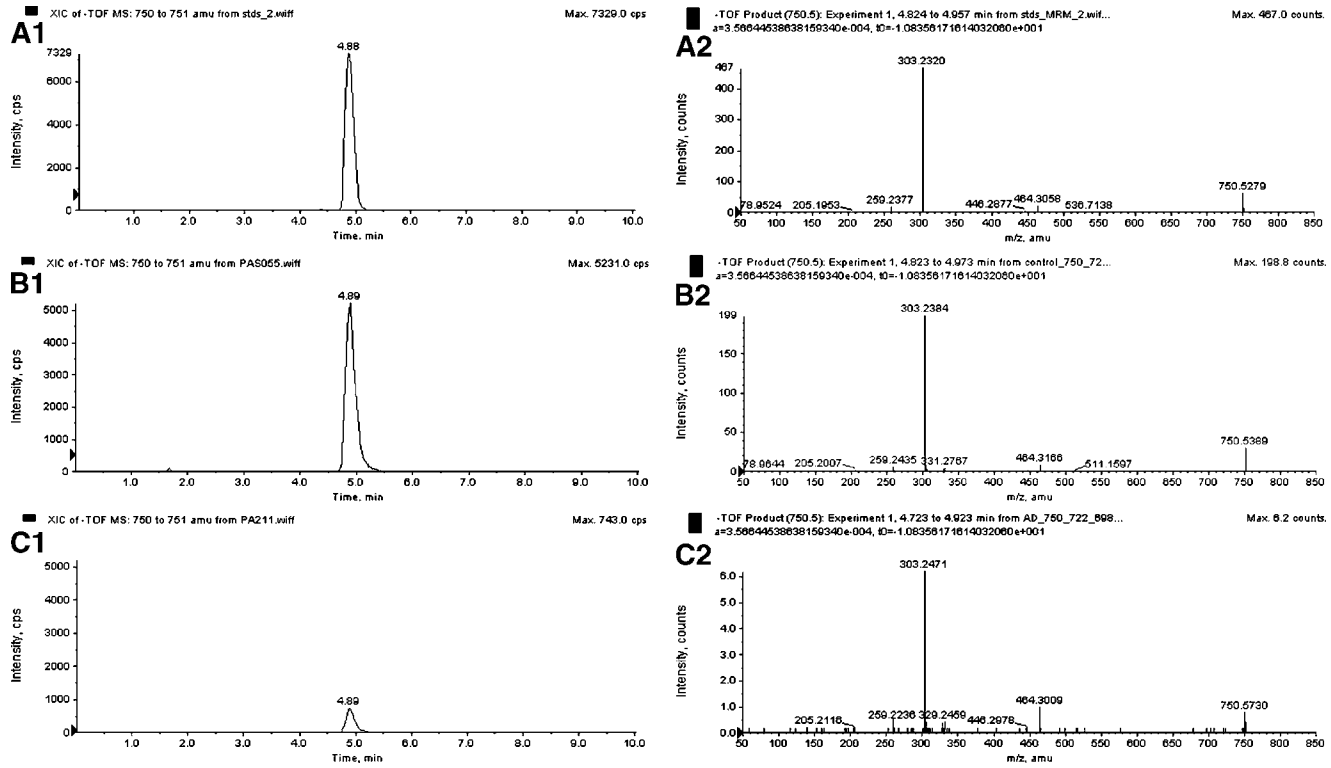



**Fig. 1.** LC-MS and MS/MS analyses of ethanolamine plasmalogen (PlsEtn) 18:0/20:4. A1: Extracted ion chromatogram of mass 750 (M-H-) of a pure standard. A2: MS/MS spectra of parent ion *m/z* 750 at retention time 4.8–5.0 min. B1: Extracted ion chromatogram of 750 from a cognitively normal subject. B2: MS/MS spectra of *m/z* 750 at 4.8–5.0 min. C1: Extracted ion chromatogram of 750 from a dementia of the Alzheimer's type (DAT) subject. C2: MS/MS spectra of *m/z* 750 at 4.8–5.0 min.

analytical methods were compared: full-scan LC time of flight analysis using the above chromatographic conditions (QSTAR XL) and the peak area of the extracted parent ion mass as listed in Table 1; MRM LC-MS/MS analysis (4000 Q TRAP) using the above chromatographic conditions and the peak area of the MRM transitions listed in Table 1; and flow injection analysis (4000 Q TRAP) as described above and using the peak area of the MRM transitions listed in Table 1. The results of this comparison are described in **Table 3**. The robustness and reproducibility of the flow injection analysis method are exemplified by the lower *t*-test *P* values obtained by this method.

## Clinical sample information

For serum collection (PrecisionMed, Bioserve, Case Western, Sun Health), informed consent was obtained from all subjects studied. Serum was collected using standard clinical chemistry practices. No special handling was performed (**Table 4**).

*Cognitive normal subjects (PrecisionMed).* Subjects were confirmed to have no neuropsychiatric disease, no family history of DAT, and a Mini Mental State Examination score ⩾ 28. All subjects were of Caucasian descent.

TABLE 2. MS/MS fragmentation interpretation of PlsEtn 18:0/20:4

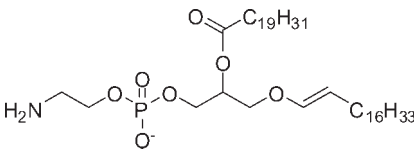
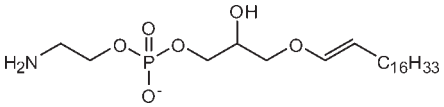
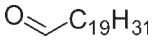
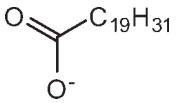
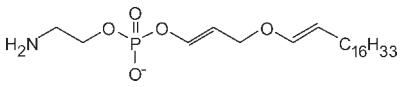
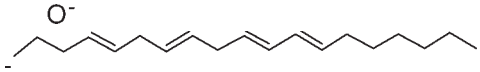
| *m/z* | Formula | Molecular Fragment | Fragment Loss |
|---|---|---|---|
| 750 | $C_{43}H_{77}NO_7P$ |  | $H^+$ |
| 464 | $C_{23}H_{47}NO_6P$ |  |  |
| 303 | $C_{20}H_{31}O_2$ |  |  |
| 259 | $C_{19}H_{31}$ |  | 303 - $CO_2$ |

TABLE 3. Cross-validation of analytical methodology

| Method | PlsEtn 16:0/18:1 | | PlsEtn 16:0/18:2 | | PlsEtn 16:0/20:4 | | PlsEtn 16:0/22:6 | |
|---|---|---|---|---|---|---|---|---|
| | Ratio | *t*-Test | Ratio | *t*-Test | Ratio | *t*-Test | Ratio | *t*-Test |
| QTRAP-FIA | 0.51 | 7.5E-08 | 0.43 | 4.1E-07 | 0.33 | 8.1E-09 | 0.23 | 1.3E-05 |
| QTRAP-LC | 0.32 | 1.5E-04 | 0.33 | 4.0E-05 | 0.26 | 2.1E-04 | 0.22 | 2.6E-04 |
| QSTAR-LC | 0.25 | 1.1E-04 | 0.40 | 4.9E-04 | 0.27 | 1.8E-04 | 0.19 | 1.1E-04 |
| | PlsEtn 18:0/18:1 | | PlsEtn 18:0/18:2 | | PlsEtn 18:0/20:4 | | PlsEtn 18:0/22:6 | |
| Method | Ratio | *t*-Test | Ratio | *t*-Test | Ratio | *t*-Test | Ratio | *t*-Test |
| QTRAP-FIA | 0.41 | 2.4E-07 | 0.40 | 3.0E-07 | 0.30 | 4.5E-08 | 0.21 | 3.2E-05 |
| QTRAP-LC | 0.31 | 1.7E-05 | 0.34 | 8.3E-06 | 0.26 | 9.0E-06 | 0.22 | 2.7E-05 |
| QSTAR-LC | 0.36 | 2.7E-04 | 0.40 | 3.2E-04 | 0.27 | 4.3E-05 | 0.22 | 2.1E-05 |

Comparison of three analytical methods as described in the text. Ratio = mean dementia of the Alzheimer's type (DAT)/mean cognitively normal. FIA, flow injection analysis.

*Probable DAT subjects (PrecisionMed).* Subjects had been diagnosed with probable Alzheimer's disease according to the criteria from the National Institute of Neurological and Communicative Diseases and Stroke/Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA). Brain imaging (computed tomography or MRI) showed cerebral atrophy and no evidence of significant ischemic stroke, brain tumor, or hydrocephalus. No evidence of bipolar disorder, Parkinson's disease, multi-infarct dementia, drug intoxication, thyroid disease, pernicious anemia, luetic brain disease, chronic infections of the nervous system, occult hydrocephalus, Huntington's disease, Creutzfeldt-Jakob disease, or brain tumors was present in any of the subjects studied. All subjects were of Caucasian descent.

*Healthy population normal subjects (Bioserve).* Serum samples were collected from healthy subjects not currently diagnosed with a neurological disease or cancer. No further selection criteria were used.

*Premortem DAT subjects (Case Western).* Serum samples were collected between 1995 and 2001. Fifty subjects were used in this analysis. At the time the serum samples were collected, 13 subjects were diagnosed as having possible DAT, 36 as having probable DAT, and 1 as "other." The time interval from sample collection to death ranged from 0.6 to 12.1 years, with a mean of 4.3 years. The pathological diagnosis was definite for Alzheimer's in all subjects. All subjects were of Caucasian descent.

*Postmortem sample information (Sun Health).* Samples of postmortem serum were obtained from individuals well characterized clinically for cognitive function as part of the brain bank program at the Sun Health Research Institute. The postmortem interval was <4 h in all cases. All control subjects had a Consortium to Establish a Registry for Alzheimer's Disease (CERAD) score of 0. All Alzheimer's disease subjects had CERAD scores of C, definite Alzheimer's disease.

*Japanese DAT subjects (Osaka).* Plasma was collected from 80 subjects diagnosed with probable DAT using NINCDS-ADRDA criteria and 80 healthy age-matched normal subjects who were confirmed to be free of cognitive impairment. All subjects were of Japanese origin and were living in Japan at the time of clinical evaluation and sample collection.

## RESULTS

### Effect of dementia severity on serum plasmalogen levels

The effect of dementia severity was determined using 324 subjects (176 female, 148 male) aged 56 to 95 years comprising 68 cognitively confirmed nondemented subjects and 256 subjects currently diagnosed as having probable

TABLE 4. Summary of clinical data

| Cohort | Age | | Gender | | Number | Mini Mental State Examination | | ADAS-cog | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean | SEM | Male | Female | | Mean | SEM | Mean | SEM |
| Cognitively normal | 77.2 | 0.8 | 32 | 36 | 68 | 29.4 | 0.1 | | |
| DAT (All) | 79.9 | 0.5 | 116 | 140 | 256 | | | | |
| ADAS 5–19 (low) | 79.3 | 0.8 | 40 | 38 | 78 | 17.4 | 0.5 | 15.2 | 0.4 |
| ADAS 20–39 (moderate) | 79.1 | 0.7 | 58 | 54 | 112 | 16.7 | 0.4 | 27.3 | 0.5 |
| ADAS 40–70 (severe) | 82.1 | 1.0 | 18 | 48 | 66 | 4.7 | 0.6 | 56.2 | 1.2 |
| Postmortem control (CERAD 0) | 81.0 | 1.3 | 10 | 9 | 19 | | | | |
| Postmortem DAT (CERAD C) | 78.9 | 1.0 | 10 | 10 | 20 | | | | |
| Population normals (50–59) | 54.1 | 0.3 | 43 | 35 | 78 | | | | |
| Population normals (60–69) | 64.1 | 0.3 | 33 | 37 | 70 | | | | |
| Population normals (70–95) | 77.4 | 0.7 | 34 | 27 | 61 | | | | |
| Osaka normals | 70.3 | 0.8 | 50 | 30 | 80 | | | | |
| Osaka DAT | 72.2 | 0.9 | 51 | 29 | 80 | | | | |
| Case Western CDR 1 | 72.6 | 2.1 | 12 | 1 | 13 | | | | |
| Case Western CDR 2 | 73.7 | 1.8 | 6 | 8 | 14 | | | | |
| Case Western CDR 3 | 76.9 | 2.1 | 11 | 12 | 23 | | | | |
| Case Western CDR 1–3 | 74.9 | 1.2 | 29 | 21 | 50 | | | | |

ADAS-cog, Alzheimer's Disease Assessment Scale – Cognitive Subscale.

DAT. Subjects were grouped into one of four dementia severity cohorts: cognitively normal (CN; Mini Mental State Examination score $\geqslant$ 28) or low [Alzheimer's Disease Assessment Scale – Cognitive Subscale (ADAS-cog score 5–19)], moderate (ADAS-cog 20–39), or severe (ADAS-cog 40–70) cognitive impairment (Table 4). Mean serum levels of eight PlsEtn and free docosahexaenoic acid (DHA; 22:6) were determined for each group (**Fig. 2**). All eight PlsEtn in all dementia subgroups were observed to be significantly reduced relative to cognitive controls (24 pair-wise comparisons, *t*-test *P* values of $2.6 \times 10^{-2}$ to $2.0 \times 10^{-10}$; median = $3.0 \times 10^{-5}$). Free DHA was significantly decreased only in moderately and severely demented subjects ($P < 0.05$).

To investigate whether the observed decrease in serum PlsEtn was attributable to reduced peroxisomal function, increased oxidative stress, or a generalized reduction in phosphatidylglycerylethanolamine (PtdEtn) synthesis, we measured and compared the serum levels of PtdEtn 16:0/18:0, PlsEtn 16:0/22:6, and plasmanylglycerylethanolamine (PakEtn) 16:0/22:6, the immediate metabolic precursor of PlsEtn 16:0/22:6. Because the *sn*-1 position of PakEtn 16:0/22:6 is a simple ether bond and not a vinyl ether bond, as is present in PlsEtn 16:0/22:6, it is not susceptible to oxidative stress. It was observed that the peroxisome-derived PlsEtn 16:0/22:6 and PakEtn 16:0/22:6 were both significantly and equally reduced in all stages of dementia. Nonperoxisomal PtdEtn 16:0/18:0 was not affected, even in severe dementia (Fig. 2C). These data strongly suggested that the observed decrease in serum PlsEtn is likely attributable to either a decreased peroxisomal synthesis capacity or an upregulation of plasmalogen-selective phospholipase $A_2$ (PlsEtn-$PLA_2$) (10, 11) and is less likely to be the result of central nervous system (CNS)-mediated oxidative degradation resulting from Aβ accumulation.

### Linear extrapolation of disease progression and serum plasmalogen depletion

The data in Fig. 2 further indicated that decreased serum PlsEtn correlated with advancing dementia. To investigate this concept in detail, we performed a linear regression analysis using the mean serum PlsEtn 16:0/22:6 level (normalized to CN) of each of the dementia cohorts and the average ADAS-cog score for each of these three cohorts (**Fig. 3**). A very high correlation was observed between the mean serum PlsEtn 16:0/22:6 level and the mean ADAS-cog scores of the three dementia cohorts ($r^2 = 0.99$). However, this linear decrease did not extrapolate back to the CN group (Fig. 3, X vs. CN). Assuming a clinical DAT progression of 7.5 ADAS-cog units per year, this extrapolation predicted that PlsEtn 16:0/22:6 levels began to decline at least 7 years before clinical cognitive impairment (ADAS-cog 15) was evident. These data are consistent with the recent findings of Amieva et al. (12), in which a 9 year prodromal phase of DAT was observed.

### The effect of chronological age on serum DHA-plasmalogen levels

To investigate whether the linear regression model prediction could be verified experimentally, we measured the serum PlsEtn 16:0/22:6 levels in 209 healthy subjects aged 50–95 years of unknown cognitive status but not currently diagnosed with dementia. These subjects were divided into three groups according to age: 50–59, 60–69, and 70–95 years, and compared with the clinically diagnosed DAT and CN cohorts. The results of this analysis revealed

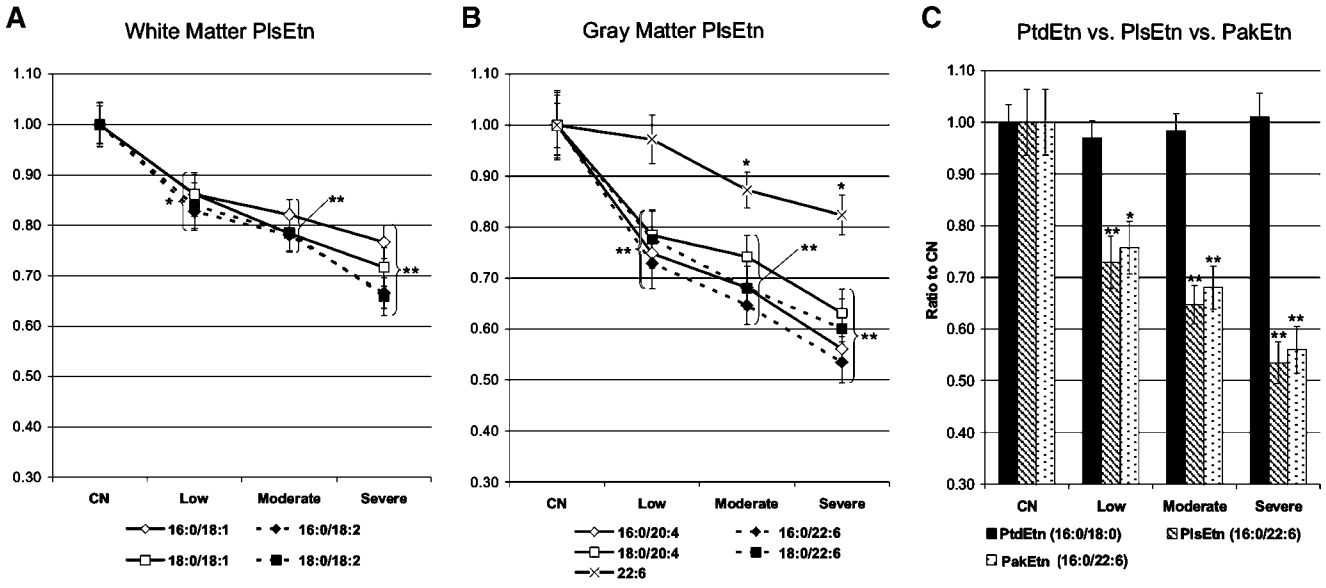



**Fig. 2.** Effects of dementia severity on serum PlsEtn levels. A: Monounsaturated and diunsaturated PlsEtn. B: Polyunsaturated PlsEtn and free docosahexaenoic acid (DHA) (22:6). C: Effects of dementia severity on serum levels of phosphatidylglycerylethanolamine (PtdEtn), PlsEtn, and plasmanylglycerylethanolamine (PakEtn). PlsEtn abbreviations read as follows: fatty acid carbons:double bonds, not including the vinyl ether double bond; position on the glycerol backbone as shown as *sn*-1/*sn*-2. 22:6 represents free DHA. Values are normalized to cognitively normal (CN) levels and expressed as means ± SEM (n = 66–112). * $P < 0.01$, ** $P < 0.001$.

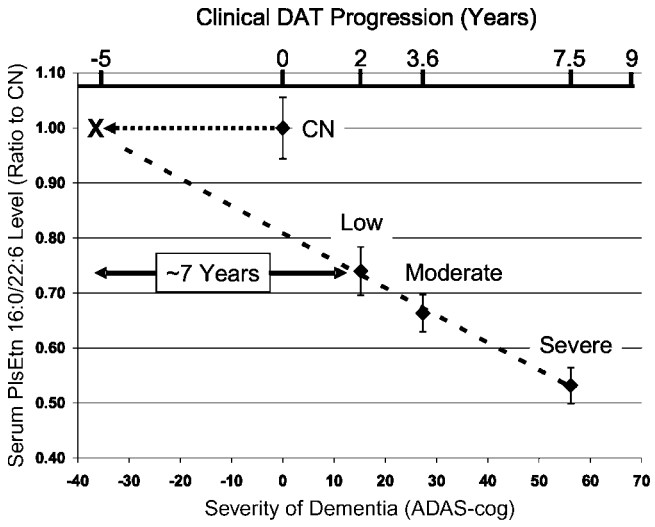



**Fig. 3.** Linear regression analysis of disease severity [Alzheimer's Disease Assessment Scale – Cognitive Subscale (ADAS-cog)] and serum PlsEtn 16:0/22:6 levels in 256 DAT subjects. X = predicted initiation of PlsEtn depletion. Values are expressed as means ± SEM (n = 66–112). Clinical progression assumes 7.5 ADAS-cog points per year.

that the serum levels of PlsEtn 16:0/22:6 in clinically diagnosed DAT subjects were significantly lower than those of all three nondemented age groups. Although there was no statistical difference between the PlsEtn 16:0/22:6 levels between the three age cohorts, the 60–69 year age group had significantly lower levels than the CN group (**Fig. 4A**). It was also observed that the percentage of subjects with very low serum PlsEtn 16:0/22:6 (defined as <50% of CN levels) in the 60–69 year cohort was more than double that observed in either the age 50–59 year cohort or the 70–95 year cohort (Fig. 4B). Considering that the incidence of dementia in the general population begins to increase dramatically after the age of 70 years (1), these data supported the linear extrapolation prediction that serum PlsEtn begins to decrease before the onset of dementia. These data further indicated that a decline in serum PlsEtn 16:0/22:6 was not a general aging phenomenon but that a significant subpopulation exhibited a dramatic decline between the ages of 50 and 69 years, whereas a second subpopulation showed little decline with age.

To investigate the plausibility of this hypothesis, we first assumed that subjects with low plasmalogen levels had a significantly shorter life expectancy than subjects with normal levels. This assumption is supported by the high mortality rate both in DAT (13) and in peroxisomal disorders, in which plasmalogens are abnormally low (14). Therefore, a mortality rate of 75% in 10 years was used. Our second assumption was that the low PlsEtn subpopulation is derived from the normal PlsEtn population. We then applied these assumptions to a hypothetical starting population of 1,000 subjects aged 50–59 years (Fig. 4C). An unanticipated result from this analysis was that for the age 70–95 year cohort to become enriched with normal PlsEtn

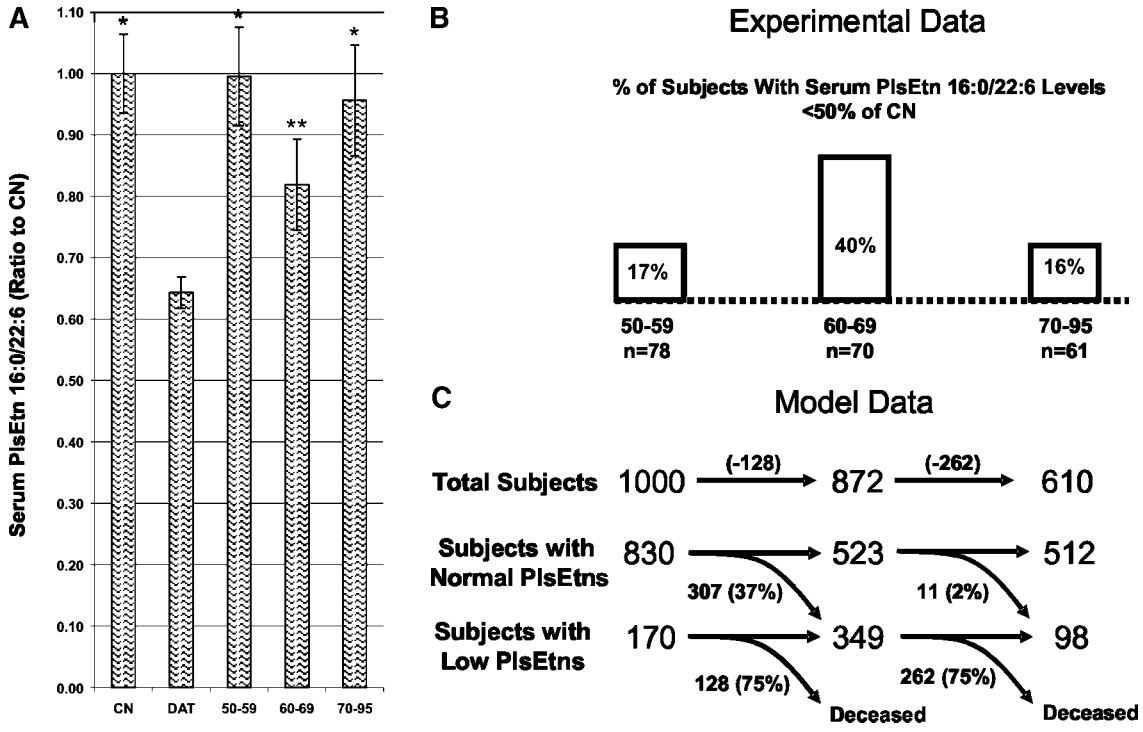



**Fig. 4.** Proposed model of serum PlsEtn levels as a function of age. A: Serum PlsEtn 16:0/22:6 levels in DAT, CN, and general population subjects (by age decade). B: Experimental data indicating what percentage of average risk nondemented subjects have low serum PlsEtn levels by age. C: Hypothetical model showing how an age-related transition from normal to low PlsEtn in combination with a reduced survival benefit of low PlsEtn could explain the observed experimental data. Values are expressed as means ± SEM (n = 61–256). * $P < 0.0001$, ** $P < 0.005$ versus DAT.

relative to the 60–69 year population, the rate of transition from normal to low PlsEtn status must peak in the 55–65 year range and then decrease dramatically and almost stop after age 70 years. This is particularly interesting in that advancing age at death has been shown to be negatively correlated with plaque density in DAT (15).

### Postmortem Alzheimer's disease pathology and serum DHA-plasmalogen levels

To determine the direct effect of Alzheimer's disease pathology status on serum plasmalogen levels, postmortem serum was collected from subjects who were pathologically confirmed to have either Alzheimer's disease pathology (n = 20) or little to no Alzheimer's disease pathology (n = 19) (Table 4). The average postmortem time interval was 2.8 h. Serum levels of PlsEtn 16:0/22:6 were observed to be significantly reduced in the postmortem Alzheimer's disease subjects (55% of control levels; $P = 4.7 \times 10^{-3}$) (**Fig. 5**).

### Serum DHA-plasmalogen levels in clinically diagnosed DAT subjects who were later confirmed to have DAT by postmortem examination

Serum from 50 clinically diagnosed DAT subjects [Clinical Dementia Rating (CDR) 1–3], who were later confirmed to have DAT upon postmortem examination, was analyzed to determine whether serum plasmalogens were decreased in these subjects at the time of their diagnosis. Subjects were grouped into three cohorts according to their CDR rating (Table 4). The time to death was significantly shorter in the CDR 3.0 subjects versus the CDR 1.0 subjects (3.1 vs. 5.2 years; $P = 4.7 \times 10^{-3}$) (**Fig. 6A**). Each of three CDR groups had significantly decreased serum levels of PlsEtn 16:0/22:6 relative to controls (Fig. 6B). Overall, the serum levels of PlsEtn 16:0/22:6 in confirmed DAT subjects were 47% of those of the normal subjects ($P = 3.1 \times 10^{-5}$). Serum levels of PlsEtn 16:0/22:6 were significantly lower in CDR 3.0 subjects than in CDR 1.0 subjects ($P = 7.5 \times 10^{-3}$) (Fig. 6B).

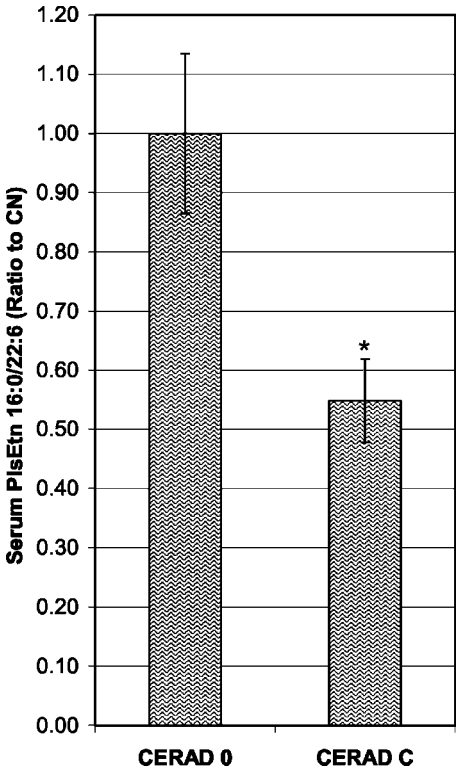



**Fig. 5.** Serum levels of PlsEtn 16:0/22:6 at time of death in subjects with confirmed amyloid deposits (CERAD C) versus age-matched controls confirmed to have little or no amyloid pathology (CERAD 0). Values are expressed as means ± SEM (n = 19–20). * $P < 0.005$.

### Effect of ethnic or environmental differences on serum DHA-plasmalogen levels in DAT

To determine whether geographical location, dietary habits, or ethnicity affected serum plasmalogen levels in DAT, plasma was collected from 80 probable Japanese DAT subjects (NINCDS-ADRDA criteria) and 80 nondemented Japanese subjects living in Japan. Serum PlsEtn 16:0/22:6 levels were significantly reduced in the DAT subjects relative to the controls (**Fig. 7**).

## DISCUSSION

It has been recognized that aging, Alzheimer's disease, and dementia are intricately linked; however, direct causal relationships have yet to be established between them. The prevalence of dementia and Alzheimer's disease increases with advancing age, but all elderly people do not exhibit dementia or develop Alzheimer's disease. Alzheimer's disease neuropathologies do not develop in every elderly person. For those who do develop Alzheimer's disease pathology, this does not guarantee the onset of dementia. Dementia can arise from numerous neurological conditions, but signs of dementia do not automatically develop with advancing age. At this time, the only known causal relationship in dementia is that decreased cognitive function is the result of decreased postsynaptic cholinergic function. With this background in place, the following sections each addresses a fundamental component of late-onset dementia in relation to the data reported here and the relevant literature.

### Plasmalogens and CNS function

PlsEtn play a number of roles in human health and disease [see Farooqui and Horrocks (16) and Nagan and Zoeller (17) for reviews]. In the CNS, their primary function is structural. PlsEtn constitute >80 mol% of the ethanolamine phospholipid pool in nonneuronal brain membranes and >60 mol% in neurons and synaptosomes (7). PlsEtn found in white matter contain predominantly 18:1, 20:1, and 22:4 fatty acids at the *sn*-2 position, whereas in gray matter, 22:6, 20:4, and 22:4 are found in the highest concentrations (18). These differences result in dramatically different membrane structures. A high percentage of monounsaturates at *sn*-2 results in very compact and stable membrane conformations (19, 20), consistent with the

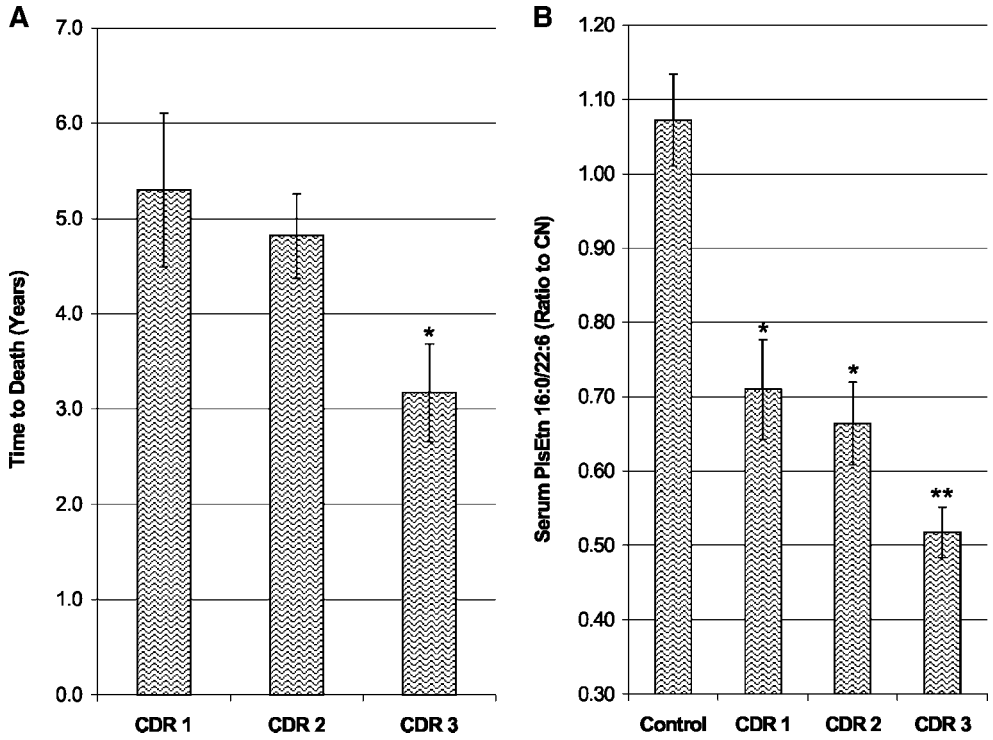



**Fig. 6.** Time to death and serum levels of PlsEtn 16:0/22:6 in pathologically confirmed DAT subjects at 0.6–12.1 years premortem. A: Time interval between time of sampling and time of death in DAT subjects subsequently confirmed pathologically to have DAT as a function of dementia severity at time of sampling. * $P < 0.005$ versus CDR 1.0. B: Serum levels of PlsEtn 16:0/22:6 in samples taken from DAT subjects at 0.6–12.1 years before postmortem confirmation of DAT as a function of dementia severity at time of sampling. Values are normalized to CN levels and expressed as means ± SEM (n = 13–23). * $P < 0.0001$ versus control, ** $P < 0.0001$ versus control, $P < 0.01$ versus CDR 1.0.

function of the myelin sheath. A high percentage of polyunsaturates results in more fluid membrane structures that are required for membrane fusion (21–24), which is consistent with the functions performed by neurons. Plasmalogen-deficient cells exhibit decreased transmembrane protein function (25) and membrane-related intracellular (26) and extracellular (27) cholesterol transport. Proper peroxisomal function also appears to be critical for neuronal migration (25).

### Plasmalogens, peroxisomal dysfunction, plasmalogen-selective phospholipase $A_2$, and aging

Epidemiologically, age is the largest risk factor for the development of DAT. Any hypothesis regarding a causal factor in DAT must show an age association. Our analysis of nondemented controls indicates that the prevalence of subjects with low plasmalogens increases dramatically between 59 and 69 years (Fig. 4B). We have hypothesized that this decrease is not a general aging phenomenon but that a specific subpopulation exhibits this decrease. Fundamentally, the observed decrease in serum plasmalogens can only occur via one of two mechanisms: an increased rate of degradation or a decreased rate of synthesis.

The biosynthesis of plasmalogens was recently reviewed in detail by Nagan and Zoeller (17). The key point is that alkyl-dihydroxy acetone phosphate-acetyltransferase (DHAP-AT) and DHAP synthase are required for the creation of the 1-*O*-alkyl bond and that the formation of this bond occurs exclusively in peroxisomes. Peroxisomal function, as a whole, is known to decline with age (26, 27). Decreased peroxisomal function leads to a decreased synthesis of PlsEtn and DHA (28, 29). DHA synthesis involves chain elongation and the desaturation of 18:3 n-3 fatty acids to 24:6 in the endoplasmic reticulum. The final step of DHA synthesis, β-oxidation to DHA, occurs in the peroxisome (30). Both DHAP synthase (31) and β-oxidase (32) exhibit decreased function with age, and DHA containing PlsEtn is selectively decreased with age (33). In addition, the activity of catalase, the principal peroxisomal enzyme responsible for detoxifying $H_2O_2$, also decreases in activity with age (32, 34, 35) and is believed to be associated with increased lipid peroxidation with age.

A key enzyme involved in the turnover and degradation of PlsEtn is PlsEtn-$PLA_2$. This enzyme has a 30-fold specificity for PlsEtn versus PtdEtn, and in contrast to the above-described decreased enzyme activities, the activity of PlsEtn-$PLA_2$ increases with age (31). Increased PlsEtn-$PLA_2$ activity has been proposed to be involved directly in DAT (36).

Our finding that a subpopulation of aging humans exhibited decreased serum DHA-plasmalogen (Fig. 4C) is consistent with these observations.

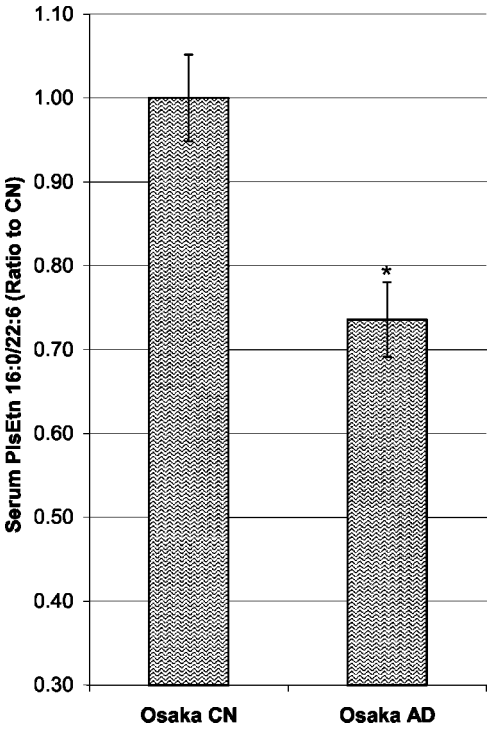



**Fig. 7.** Plasma levels of PlsEtn 16:0/22:6 in Japanese subjects clinically diagnosed as probable for DAT versus age-matched controls. AD, Alzheimer's disease. Values are normalized to CN levels and expressed as means ± SEM (n = 80 per group). * $P < 0.0005$.

### Plasmalogens, membrane cholesterol, and Aβ accumulation

One of the neuropathological hallmarks of DAT is the extracellular accumulation of amyloid in the form of argyrophilic plaques. Our findings clearly indicate an association between low serum PlsEtn and the presence of amyloid plaques in the CNS (Figs. 5, 6B).

In humans, signs of Aβ accumulation start as early as 40 years in nondemented subjects, and the prevalence increases with advancing age (37–40). In mice, genetic conditions that produce 30 times the normal amount of Aβ still fail to result in accumulation until after 8 months of age, which is comparable to 40–50 years of age in humans. Thereafter, Aβ begins to accumulate at an exponential rate and preferentially in cortex and hippocampus versus cerebellum (41). The timing of Aβ accumulation (41) closely matches the timing of decreased peroxisomal activity in mice (42), and our data suggest that the timing of decreased serum PlsEtn closely matches the timing of Aβ accumulation in humans. Other animal models of Aβ accumulation show similar age-dependent profiles (43). In the CNS, membrane PlsEtn decreases correlated with both the temporal and anatomical characteristics of Aβ accumulation (6, 7, 9).

Biological support for the hypothesis that decreased serum PlsEtn may play a causative role in the accumulation of Aβ in DAT involves the role of plasmalogens in cholesterol homeostasis. One plausible theory that explains the sporadic accumulation of Aβ peptides in DAT is a disruption in amyloid precursor protein (APP) processing as a result of increased membrane cholesterol levels (44). This theory is supported by evidence that membrane cholesterol increases with age in both rats and humans (45), that a high-cholesterol diet can increase deposition of Aβ (46), and that Aβ accumulation is closely related to the processing of the cholesterol transport protein apolipoprotein E (41, 46). More than 95% of APP is processed via the nonpathological α-secretase pathway. The pathological process, which leads to Aβ accumulation, occurs via the β-secretase pathway. α-Secretase is located in a phospholipid-rich membrane domain, whereas β-secretase is located in cholesterol-rich lipid rafts. Both of these enzymes are sensitive to changes in membrane cholesterol. When membrane cholesterol is increased, α-secretase activity is decreased (47) and β-secretase activity is increased (48) [see reviews by Puglielli, Tanzi, and Kovacs (44), and Chauhan (49)]. Thus, disturbances in cholesterol processing, such that there is an increase in membrane cholesterol, are believed to play a causal role in Aβ accumulation.

The most specific (and reversible) effect of decreased plasmalogen levels on cell biochemistry is the inhibition of free cholesterol esterification in the plasma membrane (26). This affects both intracellular cholesterol transport and signaling (26) and extracellular reverse cholesterol transport (27). This reduction in LDL-mediated feedback from the plasma membrane to the endoplasmic reticulum results in increased levels of free cholesterol in the plasma membrane. These results likely explain why plasmalogen-deficient cells have been reported to have reduced rates of APP secretion from their membranes (25). There appears to be a tight relationship between PlsEtn and membrane cholesterol levels, because membrane lipid analyses of postmortem DAT subjects have shown that DAT severity is positively correlated with membrane cholesterol (50) and negatively correlated with membrane plasmalogens (7) and that oxidative stress conditions that decrease membrane plasmalogens also increase membrane cholesterol (51).

### Plasmalogens, vesicular fusion, and dementia

Our data clearly indicated that the serum levels of PlsEtn, especially arachidonic acid or DHA containing PlsEtn, are correlated with the severity of cognitive impairment (Figs. 2B, 6B). These results are consistent with the findings of Han, Holtzman, and McKeel (7), who found that PlsEtn levels in frontal cortex gray matter decreased with increasing dementia, and also with those of Catalan et al. (52), who showed that rats fed a DHA-deficient diet exhibited decreased PlsEtn levels in the frontal cortex and decreased cognitive function.

DAT neuropathology in the cortex and hippocampus is poorly correlated with dementia. This pathology is often found in the brains of older persons without dementia or mild cognitive impairment (39, 53–55). The most consistent neurochemical observation in DAT is decreased choline acetyltransferase (ChAT) activity in the neocortex and hippocampus (55–59). Reductions in cortical ChAT activ-

ity, monitored by biopsy or in autopsy samples, correlate with the extent of intellectual impairment in DAT patients (56–58). In addition, these cortical cholinergic deficits have been found in patients examined within 1 year of the onset of symptoms, and cholinesterase inhibitors, which potentiate residual cholinergic transmission, slow the decline in executive memory functions in DAT patients (59). Furthermore, the inhibition of postsynaptic acetylcholine (ACh) activity can directly induce cognitive dysfunction in healthy humans (60).

PlsEtn are unique among neuronal lipids in that they have a high propensity to form an inverse hexagonal phase, which is the essential transitory phase for successful membrane fusion events (18, 19). Optimal vesicular fusion is very sensitive to the amount and type of PlsEtn content. Relatively small reductions in either the vinyl ether content and/or the polyunsaturated fatty acid content of vesicles dramatically reduce the number of successful membrane fusion events (20, 21). Therefore, this mechanism alone is sufficient to explain the correlation between decreased membrane PlsEtn and the severity of cognitive dysfunction in DAT.

### Plasmalogens, vesicular fusion, and the autocannibalism theory of selective cholinergic degeneration

The nontransient, progressive decline of cognitive function in DAT indicates that continual deterioration of the integrity of cholinergic neurons occurs in DAT. Cholinergic neuron integrity is traditionally measured using a proxy measurement of cholinergic neuron-specific biochemistry, specifically, measurement of the activities of acetylcholinesterase and ChAT, the key enzymes for the deactivation and synthesis of ACh, respectively. The relative activity of these enzymes in brain homogenates is presumed to correlate with the density of functional cholinergic axon terminals. Decreased ChAT activity, and thus cholinergic axon density, in cortical regions containing Alzheimer's disease pathology is widely accepted (61). However, to date, there is no widely accepted mechanism to explain this degeneration. Neither the oxidative stress nor the Aβ hypothesis, either alone or in combination, explains the selective degeneration of cholinergic neurons.

The 'Achilles' heel of cholinergic neurons is the choline high-affinity transporter (CHT). When a cholinergic nerve terminal releases ACh into the synaptic cleft during a depolarization event, the released ACh is ultimately degraded to choline and acetate by acetylcholinesterase. This extracellular choline in the synaptic cleft is then rapidly reabsorbed into the presynaptic terminal by the CHT. The reabsorbed choline is preferentially used by ChAT to resynthesize ACh, which is then transported into vesicles by an ACh transporter protein and stored for future depolarizing events. Brain slice studies have shown that as long at the CHT has normal function, the cholinergic terminal can maintain ACh release for extended periods of time using membrane stores of phosphatidylcholine (PtdCho) and PtdEtn (62) and by the extraction of choline from surrounding cells (63). This occurs even in the absence of exogenous choline. However, in the presence of the CHT inhibitor hemicholinium-3, the ability to sustain the release of ACh is reduced dramatically, even in the presence of exogenous supplied choline (64). These data indicate that the proper functioning of the CHT is essential for the sustained release of ACh from cholinergic neurons.

The autocannibalism theory of selective cholinergic denervation, proposed by Wurtman in the mid 1980s, was based upon these observations (65, 66). According to the autocannibalism hypothesis, when there is a depletion in the amount of free choline in the cholinergic nerve terminal, the membrane phospholipids, particularly PtdCho, PtdEtn, and PlsEtn, are broken down to provide more free choline. Specifically, PtdCho is used to directly generate choline and PtdEtn and PlsEtn are used to indirectly generate choline through sequential methylation with *S*-adenosylmethionine and phosphatidylethanolamine methyl transferase (67–69). A prolonged deficiency of choline ultimately leads to cell death.

The only problem with the autocannibalism theory was that a plausible biochemical mechanism by which free choline levels are depleted in DAT was not obvious. Recently, Ferguson et al. (70, 71) made a landmark finding when they showed that the CHT is localized on presynaptic vesicles, not constituently expressed on the presynaptic membrane. This finding indicates that the dynamic regulation of choline uptake via the CHT occurs by an increased density of CHTs at the synapse after a nerve impulse and subsequent deactivation by vesicular endocytosis. This finding revealed a simple mechanism by which free choline levels in the nerve terminal can be compromised: impaired vesicular fusion. Impaired vesicular fusion as a result of a PlsEtn deficiency would be expected to have a similar effect on choline uptake as the presence of hemicholinium-3, albeit via a different mechanism.

### Plasmalogens and DAT neurodegeneration

Neurodegeneration in DAT has been studied extensively. Initial studies of the basal forebrain in DAT suggested that decrements in cortical ChAT were the result of frank loss of nucleus basalis cholinergic magnocellular neurons (72, 73). However, more detailed analyses revealed that cholinergic neurons were generally shrunken and dysfunctional, but not dead, except in late-stage DAT (74–79). These neuronal phenotypic changes without frank neuronal degeneration also occur early in cognitive decline (80). The persistence of shrunken basal forebrain cholinergic neurons in DAT is similar to that seen in experimental studies of retrograde cellular degeneration in the nucleus basalis after axotomy (75).

Studies of ChAT levels in the nucleus basalis and cortex in the same autopsy samples have shown that in 50% of DAT patients, there is a marked loss of cortical ChAT with no reduction in nucleus basalis ChAT (76), suggesting abnormal axonal transport in DAT and that the neurodegeneration originated at the axon terminal, not in the cell body. In this regard, white matter volume (the key component of axon sheaths) is significantly reduced in frontal

(11.9%) and temporal (29.4%) cortex in DAT autopsy samples compared with normal control samples (81). Atrophy of the corpus callosum is also correlated with frontal executive dysfunction in Alzheimer's disease patients (82). These observations have led to suggestions that white matter degeneration is an intrinsic component of DAT (83, 84). Moreover, white matter losses in preclinical DAT in which cortical atrophy is not evident (85) indicate that axonal dysfunction precedes the cortical atrophy observed in clinically manifested DAT. In fact, white matter lesions are prevalent in normal aging, in mild cognitive impairment, and in early-stage DAT before the development of dementia (86, 87). Because mild cognitive impairment is thought to represent a prodromal stage of Alzheimer's disease (79, 80, 88, 89), these observations suggest that white matter lesions occur early in the disease process and may contribute to the subsequent cholinergic dysfunction. These findings are of particular relevance to plasmalogen biochemistry. First, the highest concentration of plasmalogens is in white matter. Second, within white matter, plasmalogen content is significantly reduced in DAT. Specifically, subjects with confirmed DAT exhibited significant decreases in white matter PlsEtn content in all regions, including the cerebellum, independent of dementia status and independent of regional Aβ load (7). Such neuropathological data strongly suggest that a PlsEtn deficiency precedes the clinical course of DAT.

With regard to Aβ accumulation having a causal role in the depletion of gray matter PlsEtn in the later stages of DAT, studies have shown that the direct incubation of oligodendrocytes with Aβ peptides selectively decreased PlsEtn content (90). CNS PlsEtn decreases correlated with both the temporal and anatomical characteristics of Aβ accumulation in animal models (6, 7, 9). Aβ accumulation is also known to directly induce oxidative stress (91–93), which has been shown to directly disrupt vesicular fusion, acetylcholine release, and synaptosomal PtdEtn and PlsEtn content (51). Because oxidative stress preferentially oxidizes PlsEtn over PtlEtn (94, 95) and PlsEtn content is critical for vesicular fusion (21), it is reasonable to hypothesize that in later stages of DAT, Aβ accumulation in the CNS affects neurotransmitter release by reducing membrane PlsEtn content by an oxidative stress mechanism. Peroxisomal proliferation has been shown to inhibit Aβ-induced neurodegeneration (96) and to preserve cognition in early DAT (97); however, the exact mechanism by which this is achieved has not been elucidated.

Overall, these data suggest some early or pre-DAT pathology-associated disease process that affects white matter integrity before the emergence of DAT symptoms and a later DAT pathology-associated process that affects gray matter functioning, which ultimately results in dementia.

### Summary and future directions

The observed decreases of PlsEtn levels early in the DAT disease process may be responsible for the subsequent cholinergic dysfunction that underlies the deterioration of intellectual function in DAT. Because we have come to understand that the majority of cholinergic neurons in the basal forebrain have become smaller and dysfunctional but have not degenerated, correcting the PlsEtn deficit may slow or correct the cholinergic deficit in DAT patients. Of particular note, although the brain contains all of the peroxisomal machinery to synthesize both DHA and PlsEtn, evidence suggests that the liver is the major source of these molecules in the adult. Radiolabeled tracer studies have shown that the dietary precursor of DHA, 18:3, is readily absorbed, stored in triacylglycerols, and then converted to DHA and incorporated into phospholipids in the liver. DHA is then transported to the brain in this phospholipid form via the bloodstream (98). Therefore, a peripheral correction of these phospholipids would be expected to have a CNS effect. Further research to test the above hypotheses is required to better understand the relationships between peroxisomal and/or PlsEtn-$PLA_2$ function, serum and CNS PlsEtn levels, Aβ accumulation, cholinergic neuron dysfunction, and dementia. Regardless, the data presented describe a peripheral metabolic deficiency of PlsEtn in all stages of DAT and predict that this deficiency precedes the clinical manifestation of DAT by many years. These findings are consistent with the known epidemiological, neurochemical, and neuroanatomical course of DAT. As such, clinical trials involving PlsEtn restoration should be undertaken to determine its efficacy in the treatment and/or prevention of DAT.

The authors would like to thank Kirsten A. Taylor for assisting in the preparation, critical reading and review of the manuscript.

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