added to the cultures at the indicated time points after addition of LPS. Nitrite accumulation in the supernatants at 24 h of culture was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. The error bars are too small to be distinguishable in the figure (numeric data from the left bar: 3.75 ± 0.18, 5.07 ± 0.22, 4.22 ± 0.07, 5.69 ± 0.12, 10.38 ± 0.06, 15.00 ± 0.05, and 25.20 ± 0.28). *P < 0.001 vs. LPS-stimulated cells without PDTC. Data shown are representative of two–three separate experiments.

Fig. 4. LPS stimulation down-regulates α-arrestin 2 expression. RAW264, RAWar, and RAWarr2 cells were stimulated with LPS, and the protein levels of α-arrestin 2 and GAPDH (upper panel) and mRNA expressions of α-arrestin 2 and 18S rRNA (middle panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the band from three separate experiments (mean ± SEM) (lower panel). *P < 0.01 vs. 0 h.

Fig. 5. Forced α-arrestin 2 expression suppresses NO production and NOS II expression.
(A) Cells were stimulated with LPS for 24 h, and nitrite accumulation in the supernatants was measured using the Griess reagent. The results are expressed as means ± SEM from three-well cultures. *P < 0.001 vs. LPS-stimulated RAW264 cells. (B) The protein levels of NOS II and GAPDH (left panel) and mRNA expressions of NOS II and 18S rRNA (light upper panel) were analyzed as in Fig. 1A. Bar graphs show the relative intensity of the PCR bands from three separate experiments (mean ± SEM) (right lower panel). *P < 0.01 vs. corresponding RAW264 cells. Data shown are representative of three–four separate experiments.

Fig. 6. α-arrestin 2 interacts with cytosolic IαBα. Before and after stimulation with LPS for 6 h, cells were lyzed and immunoprecipitated with anti-α-arrestin 2 Abs. Western blotting analysis was performed using anti-IαBα Abs (upper panel). The protein levels of GAPDH in equal amounts of lyzates were used for control (lower panel).

Fig. 7. Cross-talk between α_2AR and TLRs signaling pathways.
(A) α_2AR agonists suppress NF-αB activation by increasing cytoplasmic α-arrestin 2, which stabilizes the NF-αB/IαBα complexes in cytoplasm (a) or by activating CREB which competes CBP with NF-αB in the nucleus (b). (B) TLR4-dependent signals lead to the following steps both in the presence or absence of α_2AR agonists: ① TLR4-dependent down-regulation of α_2AR expression, ② down-regulation of α-arrestin 2, ③ release of NF-αB/IαBα complexes in the cytoplasm, ④ degradation of IαBα, and ⑤ translocation of NF-αB to the nucleus and transcription of its target genes.

Figure 1

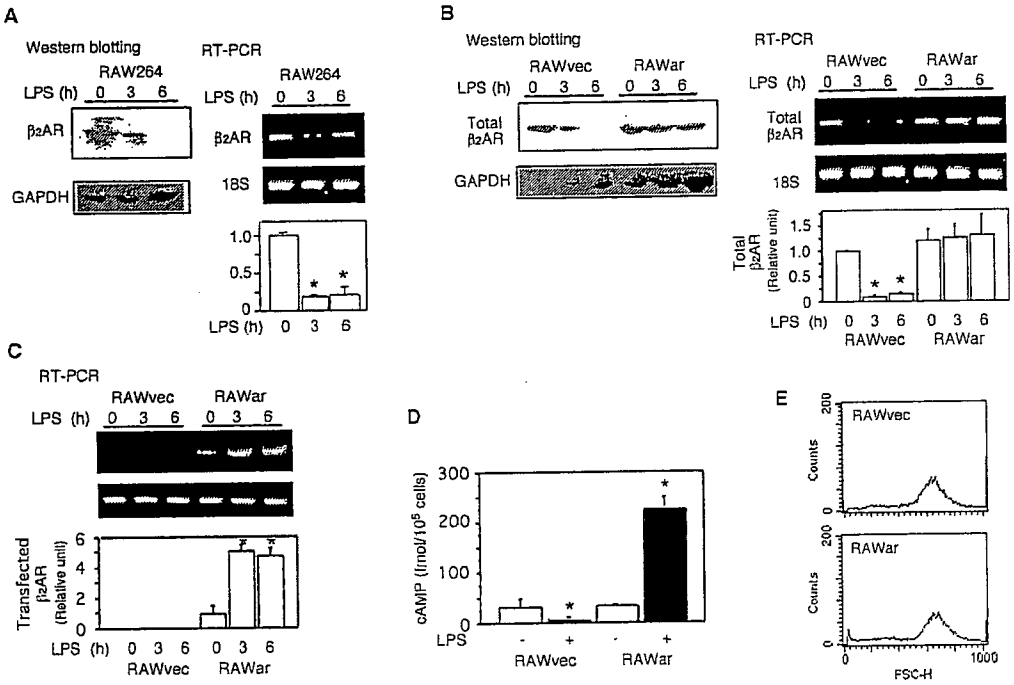

A
Western blotting
RAW264
LPS (h) 0 3 6
β2AR
GAPDH
RT-PCR
RAW264
LPS (h) 0 3 6
β2AR
18S
1.0
0.5
0
LPS (h) 0 3 6
B
Western blotting
RAWvec RAWar
LPS (h) 0 3 6 0 3 6
Total β2AR
GAPDH
RT-PCR
RAWvec RAWar
LPS (h) 0 3 6 0 3 6
Total β2AR
18S
Total β2AR (Relative unit)
1.5
1.0
0.5
0
LPS (h) 0 3 6 0 3 6
RAWvec RAWar
C
RT-PCR
RAWvec RAWar
LPS (h) 0 3 6 0 3 6
Transfected β2AR (Relative unit)
6
4
2
0
LPS (h) 0 3 6 0 3 6
RAWvec RAWar
D
cAMP (fmol/10^5 cells)
300
200
100
0
LPS
- + - +
RAWvec RAWar
E
RAWvec
Counts
200
0
RAWar
Counts
200
0
0 1000
FSC-H

Figure 2

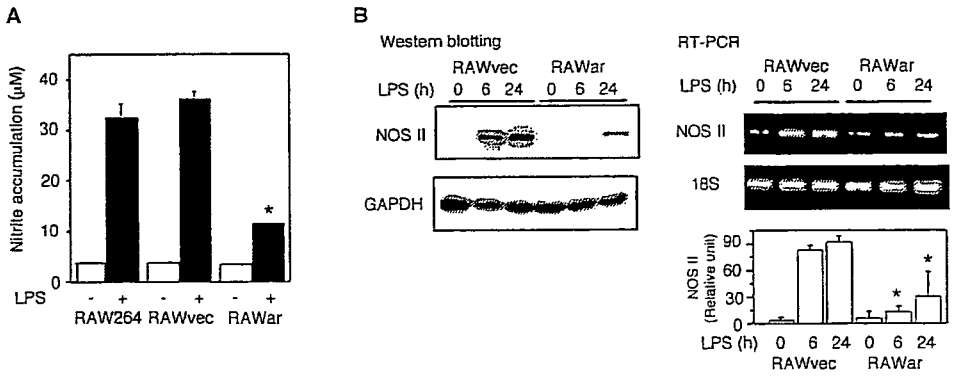

A
Nitrite accumulation (μM)
40
30
20
10
0
LPS
- + - + - +
RAW264 RAWvec RAWar
*
B
Western blotting
RAWvec RAWar
LPS (h) 0 6 24 0 6 24
NOS II
GAPDH
RT-PCR
RAWvec RAWar
LPS (h) 0 6 24 0 6 24
NOS II
18S
NOS II (Relative unit)
90
60
30
0
LPS (h) 0 6 24 0 6 24
RAWvec RAWar
*
*

Figure 3

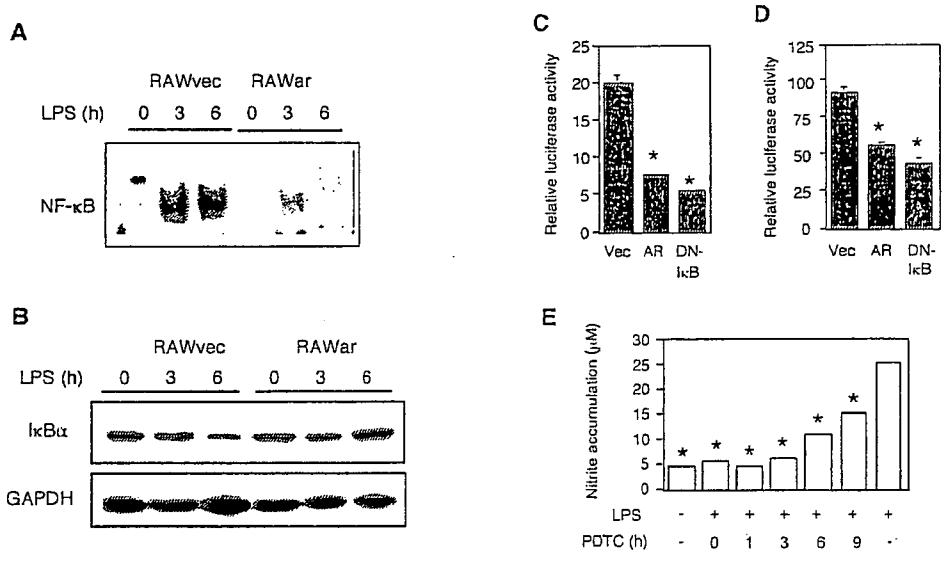

A
RAWvec
RAWar
LPS (h)
0 3 6 0 3 6
NF-κB
B
RAWvec
RAWar
LPS (h)
0 3 6 0 3 6
IκBα
GAPDH
C
Relative luciferase activity
25
20
15
10
5
0
Vec AR DN-IκB
D
Relative luciferase activity
125
100
75
50
25
0
Vec AR DN-IκB
E
Nitrite accumulation (μM)
30
25
20
15
10
5
0
LPS - + + + + + +
PDTC (h) - 0 1 3 6 9 -

Figure 4

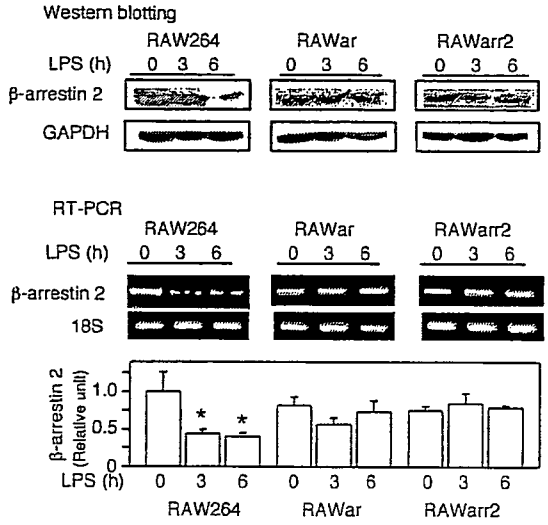

Western blotting
RAW264
RAWar
RAWarr2
LPS (h)
0 3 6
β-arrestin 2
GAPDH
RT-PCR
18S
β-arrestin 2 (Relative unit)
1.0
0.5
0
*

Figure 5

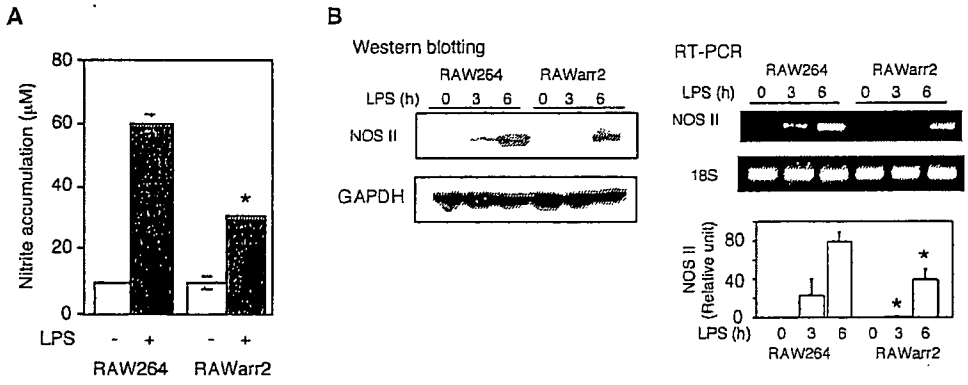

A
Nitrite accumulation (μM)
80
60
40
20
0
LPS - + - +
RAW264 RAWarr2
B
Western blotting
RAW264 RAWarr2
LPS (h) 0 3 6 0 3 6
NOS II
GAPDH
RT-PCR
RAW264 RAWarr2
LPS (h) 0 3 6 0 3 6
NOS II
18S
NOS II (Relative unit)
80
40
0
LPS (h) 0 3 6 0 3 6
RAW264 RAWarr2

Figure 6

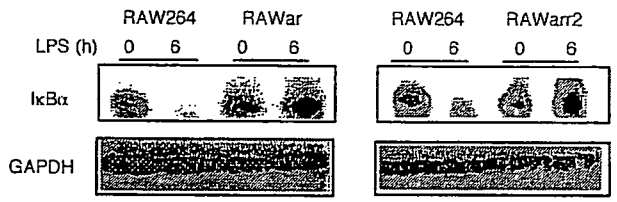
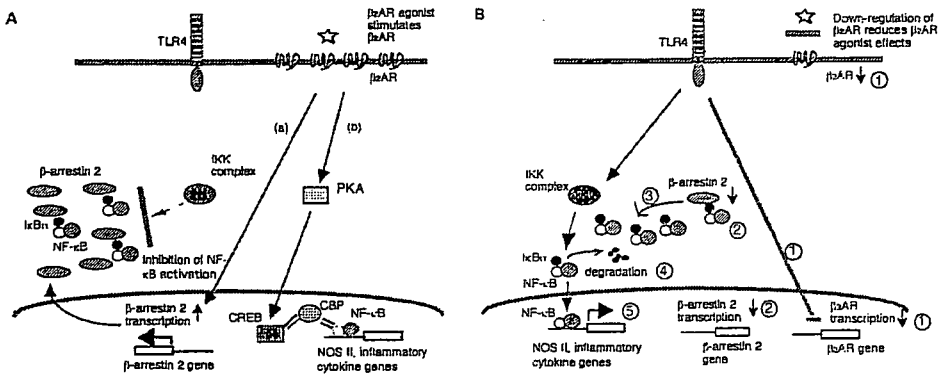


Figure 7


A
TLR4
βzAR agonist stimulates βzAR
βzAR
(a)
(b)
IKK complex
PKA
β-arrestin 2
IκBα
NF-κB
Inhibition of NF-κB activation
β-arrestin 2 transcription
CREB
CBP
NF-κB
β-arrestin 2 gene
NOS II, inflammatory cytokine genes
B
TLR4
Down-regulation of βzAR reduces βzAR agonist effects
βzAR
IKK complex
β-arrestin 2
IκBα
degradation
NF-κB
NF-κB
NOS II, inflammatory cytokine genes
β-arrestin 2 transcription
β-arrestin 2 gene
βzAR transcription
βzAR gene

厚生労働科学研究費補助金（長寿科学総合研究事業）

分担研究報告書

認知症の総合的な予防・治療・介護の確立に関する研究
－身体合併症発症時の一般病院での認知症対応システムの確立－

（ H19-長寿 - 一般 - 023 ）

分担研究者　鷲見　幸彦　国立長寿医療センター外来診療部部長

研究要旨： 認知症患者は高齢者が多く経過中に身体合併症を生じ、一般の急性期病院へ受診を余儀なくされることがあるが、入院直後のせん妄、回復期での離院や転倒といった医療安全の観点からは望ましくない事象が発生することがあり、入院の継続に難渋することが珍しくない。このような状況下での急性期病院の現状を把握するために、愛知県下の臨床研修指定病院 55 施設における認知症への対応の実態調査をおこなうためのアンケート用紙を作成した。

A. 研究目的

認知症患者の増加は著しく、今後も増加していくことが予想されるが、認知症患者は高齢者が多く、その経過中に骨折、肺炎、脳血管障害といった様々な身体合併症を併発する危険性がある。さらに入院直後はせん妄が起こりやすく、回復期には離院や転倒といった医療安全の観点からは望ましくない事象が発生することがあり、入院の継続に難渋することが珍しくない。このような状況下で一般病院（ことに急性期病院）おいていかに認知症患者に対応していくかその指針づくりは急務である。

B. 研究方法

平成19年度は愛知県下の臨床研修指定病院55施設における認知症への対応の実態調査をおこなうためのアンケート用紙を作成した。

C. 研究結果

アンケートは３部にわかれており、第１部は病院代表者または診療部門の責任者が、第２部は内科系、外科系病棟の担当者の方がそれぞれ回答する形式とした。病院代表者をあえて質問対象にしたのは、現時点では認知症患者を積極的に診療することが経営上のメリットには直接関与していないことが指摘されており、病院の経営方針として認知症診療をどの程度考慮しているかを質問のなかに加えた。アンケート内容としては主として１）現在どのくらいの認知症患者を入院で診療しているか２）入院認知症患者の診療において困難を感じる点はなにか３）現状としてどのような対応をしているかについて質問した。現在アンケートを発送、回答待ちの状態である。

D. 考察

平成19年度はアンケートという形式で現状調査をおこなった。今後はアンケート結果から集約できる現状の問題点を把握。アンケートのみでは十分な回答が得られない可能性もあり、いくつかの病院とミニシンポジウムを企画問題点の把握につとめる。その後、介入のモデル病院として可能な病院を 2-3 病院指定し現段階で可能な介入法の検討を行い、当センターを中心にモデル病院で介入を試みる。最終的に対応指針の作成ができればと考えている。

E. 結論

愛知県下の臨床研修指定病院における認知症への対応の実態調査をおこなうためのアンケート用紙を作成した。平成 20 年 3 月には配布

し4月中に回収予定である。

F. 健康危険情報

特になし

G. 研究発表

1) 論文発表

なし

2) 学会発表

なし

H. 知的財産権の出願・登録状況

特になし

デイサービス利用者に対する非薬物療法の無作為化介入研究

大阪大学精神医学教室　武田雅俊

研究趣旨
認知症予防に関し２種類（音読と計算を中心とするプログラムとレクレーション群）の非薬物療法の無作為化介入研究を行っている。対象はデイサービス利用者で週２回、６ヶ月のプログラムに参加する。次の６ヶ月は他方のプログラムに参加する（クロスオーバー法)。評価者は各プログラム実施にかかわらない各施設外のスタッフが行うことによりブラインド化されている。現在約１００名が参加している。
バイオマーカーの開発については血清中plasmalogenがアルツハイマー病で減少していることを見出している。

A　研究目的
①デイサービス利用者に対する非薬物療法の無作為化介入研究。
認知症予防に関しより有効性の高い非薬物的介入プログラムの確立を目指す。
②アルツハイマー病への介入効果を評価するための診断マーカーを開発する。

B　研究方法
①デイサービス利用者を音読と計算を中心とする活動群とレクレーション群にMMSE、性、年齢、教育歴をマッチングさせながら無作為に割り付ける。参加基準は週２回以上デイサービスを利用し介入プログラムに参加できる、介入プログラム参加が困難となるような心身の支障がない、MMSE１５点以上である。プログラム開始前に採血、活動や趣味への好み、服薬、同居状況、合併・既往疾患を調べる。２種類のプログラムは６ヶ月後に交代するクロスオーバー法である。６ヶ月の前後で MMSE, ADAS, FAB、MOSES、FIM、GDS、Zarit を採取する。評価者をブラインド化するため、前の３スケールはプログラム施行とは別で通常のデイサービスにも従事しない外部の専従スタッフが行う。残りについては、施設職員および家族が行う。施設職員についても、どの参加者がどのプログラムに参加しているか知らさないようにし、プログラムもデイサービス通常職員の目に触れない別室で行う。週２回行われるプログラムは毎回 TORS、満足度（VAS）で参加状況をモニターする。音読と計算を中心とする活動群は参加者の能力に合わせた複数の教材を用意している。買い物、旅行などをシュミレーションし、かかったお金の計算を促す。マス計算、参加者の世代が若かったころに使用されていた教科書の音読を行う。レクレーション群は塗り絵、ちぎり絵などを行う。両プログラムとも１回３０分で週２回。またコミュニケーションの量も両プログラムとも同量にするため参加者１~３名に

指導者が１名と、両プログラムとも同じ割合でつく。
研究計画はUMIN Clinical trialに登録した(受付番号R000000878)。
(倫理面への配慮)
施設利用者および家族に対する説明会を3回開催し、研究参加の任意性、内容を説明。研究に関与する現場スタッフに対しても勉強会を頻会に開催し、倫理面の理解を深めるようにしている。
研究計画は大阪大学医学部倫理委員会で承認された。

C　研究結果
①２００６年までに小規模パイロット研究、スタッフのトレーニング、勉強会、説明会をした。介入プログラムの期間はデイサービス参加者の過去のサービス継続実績などを調査し、脱落率が１０－２０％以下になると予想される６ヶ月間を１クールとした。
２００７年はスタッフ教育を続けながら、６施設でデイサービス利用者と家族向けの説明会を開催。リクルートをはじめ、１００名から文書による参加同意を得た。順次介入プログラムを開始。現在中間結果についてまとめているところである。また、活動を円滑に行うためにあたり藤井寺市医師会所属医師対象の説明会を２回、藤井寺市民会館で地域住民対象の認知症の講演会(約1200人参加)もおこなった。
②患者血清を用い、共同研究によりメタボロミクス解析でアルツハイマー病バイオマーカーplasmalogenを同定している。ブラインドで行った測定でもアルツハイマー病と健常高齢者の間で有意な差が確認された（論文参照)。

D　考察　E結論　(今後の方針)
①非薬物療法は認知症予防として期待されている。また介護保険導入後、それぞれのデイサービス施設で様々な活動サービスが工夫されながら提供されている。しかしながら、どのような方法が本当に有効なのかに確かなエビデンスが乏しいまま行われているというのも事実である。本研究はこの重要な疑問に迫り、本当に有効な、非薬物療法やデイサービスでの活動の確立を目指すものである。
順調に行けば、本研究の結果がそろうのは２００８年末、解析は２００９年度にわたる予定である。これまでのところいくらかの遅延はあるもののおおむね順調に進んでおり、中間解析が始まろうとしている。参加者の満足度も良好という印象を得ている。
（多くの協力者のおかげで研究が進んでいるが、現場は手一杯の状況である。また、データーの解析に当たっては、広く柳澤班の先生方をはじめ各分野の専門家の意見を伺うことでよりよいものになればと希望している。）

F　健康危険情報
なし。

G　研究発表
1　論文発表
Peripheral ethanolamine plasmalogen deficiency: A logical causative factor in Alzheimer's disease and dementia.

Goodenowe DB, Cook LL, Liu J, Lu Y, Jayasinghe DA, W K Ahiahonu P, Heath D, Yamazaki Y, Flax J, Krenitsky KF, Sparks DL, Lerner A, Friedland RP, Kudo T, Kamino K, Morihara T, Takeda M, Wood PL.
J Lipid Res. 2007 48(11):2485-98

2　学会発表

Design report: Randomized prospective learning therapy study on elderly Japanese in Osaka
Takashi morihara, Hiroaki Kazui, Ayumi kono, Takahiro Higashi, Masuhiro okuda, Yaeko hata, Hiromi yoshida, Kousuke masuda, Masatoshi Takeda
IPA2007 Osaka Oct 2007

JOURNAL OF LIPID RESEARCH

patient-oriented and epidemiological research

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

Dayan B. Goodenowe,[1,*] Lisa L. Cook,[*] Jun Liu,[*] Yingshen Lu,[*] Dushmanthi A. Jayasinghe,[*] Pearson W. K. Ahiahonu,[*] Doug Heath,[*] Yasuyo Yamazaki,[*] John Flax,[†] Kevin F. Krenitsky,[§] D. L. Sparks,[**] Alan Lerner,[††] Robert P. Friedland,[††] Takashi Kudo,[§§] Kouzin Kamino,[§§,*] Takashi Morihara,[§§] Masatoshi Takeda,[§§] and Paul L. Wood[†††]**

Phenomenome Discoveries, Inc.,[*] Saskatoon, Saskatchewan, Canada; PrecisionMed, Inc.,[†] San Diego, CA; Bioserve, Inc.,[§] Boston, MA; Sun Health Research Institute,[**] Sun City, AZ; Case Western Reserve University,[††] Cleveland, OH; Department of Psychiatry,[§§] Osaka University Graduate School of Medicine, Osaka, Japan; Shoraiso National Hospital,[***] Nara, Japan; and Falk Center for Molecular Therapeutics,[†††] Northwestern University, Chicago, IL

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

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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.

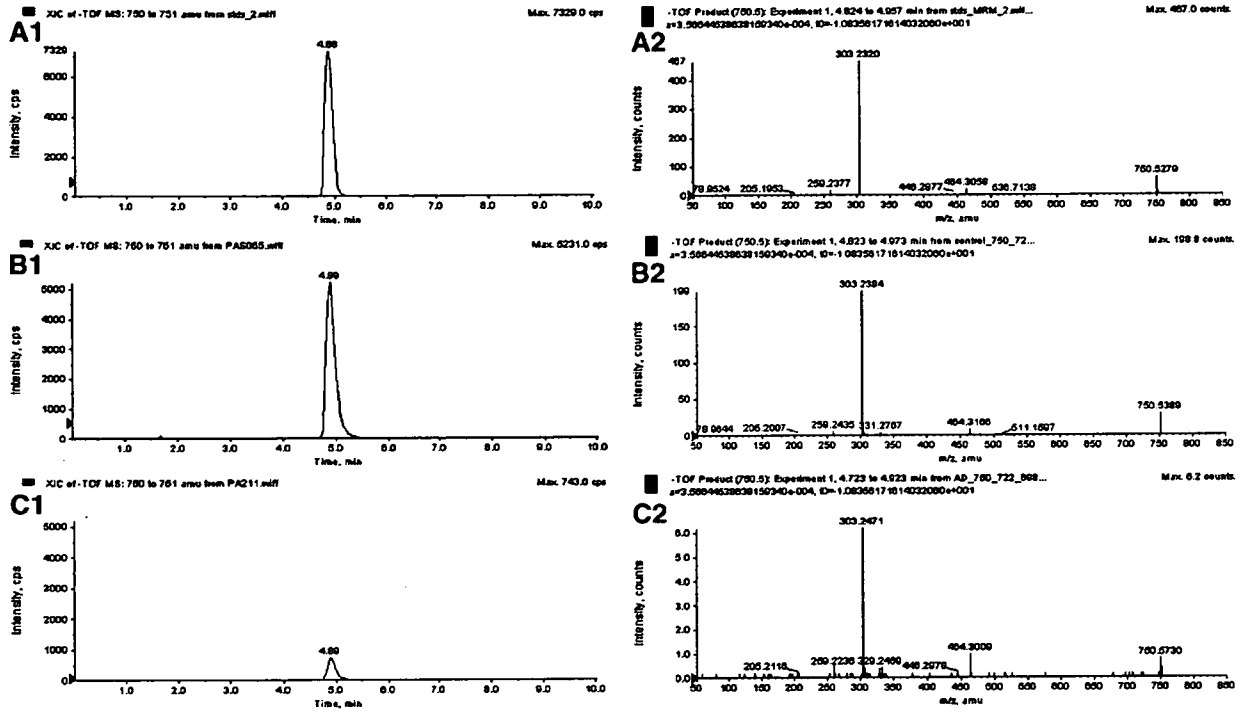


Fig. 1. LC-MS and MS/MS analyses of ethanolamine plasmalogen (PlsEtn) 18:0/20:4. A1: Extracted ion chromatogram of mass 750 (M-H-) of a pure standard. A2: MS/MS spectra of parent ion *m/z* 750 at retention time 4.8–5.0 min. B1: Extracted ion chromatogram of 750 from a cognitively normal subject. B2: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

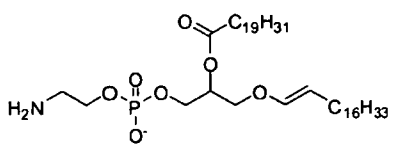
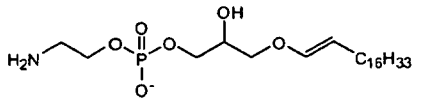
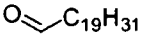
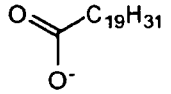
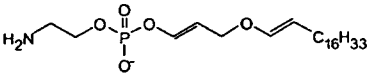
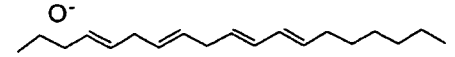
m/z	Formula	Molecular Fragment	Fragment Loss
750	$C_{43}H_{77}NO_7P$		H^+
464	$C_{25}H_{47}NO_6P$		
303	$C_{20}H_{31}O_2$		
259	$C_{19}H_{31}$		303 - CO_2

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 ≥ 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 2.6×10^{-2} to 2.0×10^{-10}; median = 3.0×10^{-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

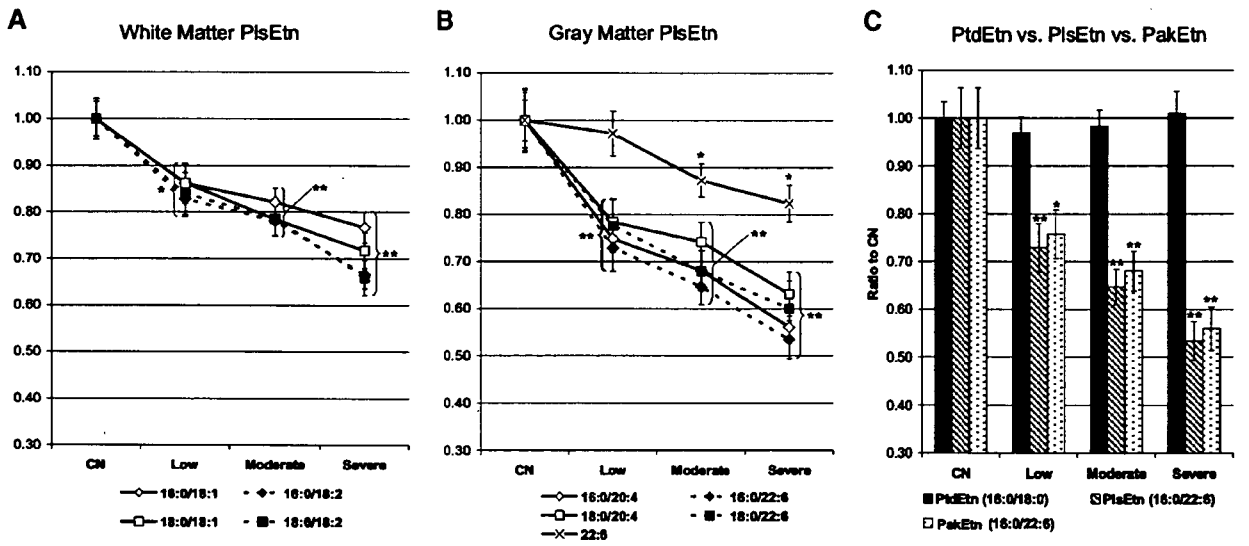


Fig. 2. Effects of dementia severity on serum PlsEtn levels. A: Monounsaturated and diunsaturated PlsEtn. B: Polyunsaturated PlsEtn and free docosahexaenoic acid (DHA) (22:6). C: Effects of dementia severity on serum levels of phosphatidylglycerylethanolamine (PtdEtn), PlsEtn, and plasmanylglycerylethanolamine (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). * $P < 0.01$, ** $P < 0.001$.

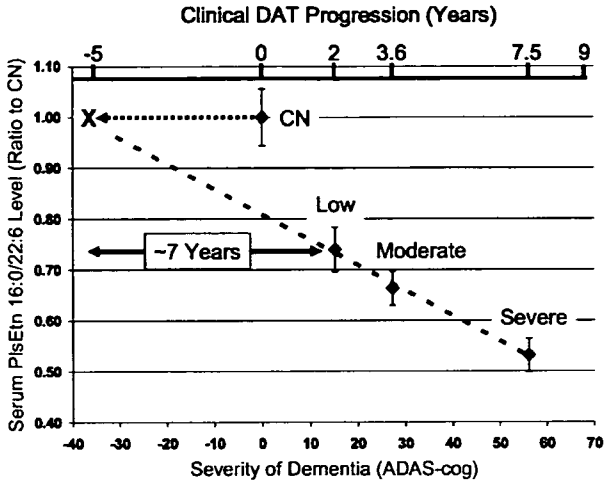


Fig. 3. Linear regression analysis of disease severity [Alzheimer's Disease Assessment Scale – Cognitive Subscale (ADAS-cog)] and serum PlsEtn 16:0/22:6 levels in 256 DAT subjects. X = predicted initiation of PlsEtn depletion. Values are expressed as means ± SEM (n = 66–112). Clinical progression assumes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

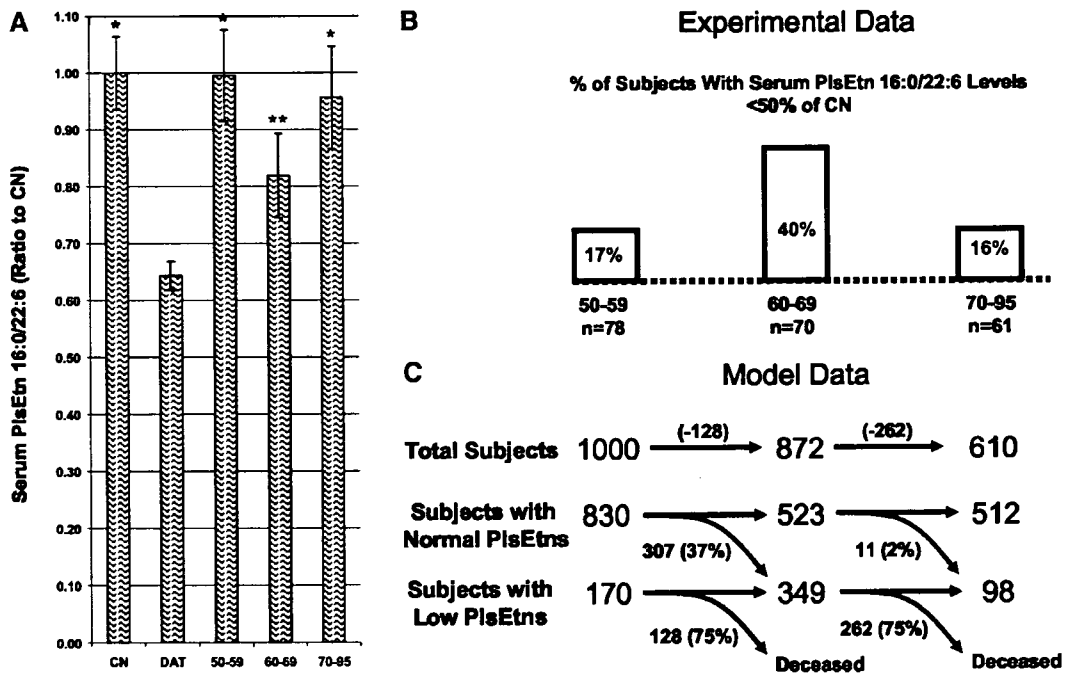


Fig. 4. Proposed model of serum PlsEtn levels as a function of age. A: Serum PlsEtn 16:0/22:6 levels in DAT, CN, and general population subjects (by age decade). B: Experimental data indicating what percentage of average risk nondemented subjects have low serum PlsEtn levels by age. C: Hypothetical model 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).

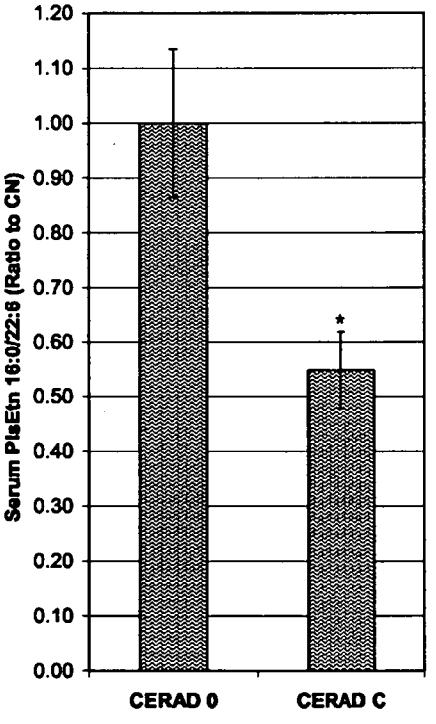


Fig. 5. Serum levels of PlsEtn 16:0/22:6 at time of death in subjects with confirmed amyloid deposits (CERAD C) versus age-matched controls confirmed to have little or no amyloid pathology (CERAD 0). Values are expressed as means ± SEM (n = 19–20). * $P < 0.005$.

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