Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology[S]

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Abstract **We performed unbiased analysis of steroid-related compounds to identify novel Alzheimer's disease (AD) plasma biomarkers using liquid chromatography-atmospheric pressure chemical ionization-mass spectroscopy. The analysis revealed that desmosterol was found to be decreased in AD plasma versus controls. To precisely quantify variations in desmosterol, we established an analytical method to measure desmosterol and cholesterol. Using this LC-based method, we discovered that desmosterol and the desmosterol/cholesterol ratio are significantly decreased in AD. Finally, the validation of this assay using 109 clinical samples confirmed the decrease of desmosterol in AD as well as a change in the desmosterol/cholesterol ratio in AD. Interestingly, we could also observe a difference between mild cognitive impairment and control. In addition, the decrease of desmosterol was somewhat more significant in females. Receiver operating characteristic (ROC) analysis between controls and AD, using plasma desmosterol shows a score of 0.80, indicating a good discrimination power for this marker in the two reference populations and confirms the potential usefulness of measuring plasma desmosterol levels for diagnosing AD. Further analysis showed a significant correlation of plasma desmosterol with Mini-Mental State Examination scores. Although larger sample populations will be needed to confirm this diagnostic marker sensitivity, our studies demonstrate a sensitive and accurate method of detecting plasma desmosterol concentration and suggest that plasma desmosterol could become a powerful new specific biomarker for early and easy AD diagnosis.**—Sato, Y., I. Suzuki, T. Nakamura, F. Bernier, K. Aoshima, and Y. Oda. **Identification of a new plasma biomarker of Alzheimer's disease using metabolomics technology.** ***J. Lipid Res.*** **2012.** 53: **567–576.**

Supplementary key words desmosterol • cholesterol • lipidomics • mild cognitive impairment patients • mass spectrometry

Alzheimer's disease (AD) is a neurodegenerative disorder of the central nervous system characterized by a progressive loss of short-term memory accompanied by a gradual loss of cognitive functions (1). AD pathology is characterized by brain atrophy reflecting neuronal and synaptic loss and by the presence of amyloid plaques and neurofibrillary tangles. AD pathogenic mechanisms contributing to neuronal loss and brain dysfunction are still unclear.

Biomarkers are very useful for diagnosing and monitoring disease progression (2) and are important for patient selection, monitoring side-effects, aiding selection of appropriate patient treatment, and helping new drug discovery. For the clinical studies of AD therapeutics, there is an increasing need for diagnostic markers to ensure that therapies are targeted at the right patient population, to initiate early treatment when disease-modifying drugs will be available, and to monitor disease progression (3). Several studies have investigated AD biochemical biomarkers in various tissues including blood (4, 5) and cerebrospinal fluid (CSF) (4). Of those approaches, CSF β x-42 and tau protein phosphorylation are currently considered the most useful biomarkers (6, 7), although they cannot predict conversion from mild cognitive impairment (MCI) to AD accurately and are not useful for guiding drug treatment. In addition, analyzing those markers requires performing delicate CSF collection from patients. Hence, access to less-invasive biomarkers found in easy-to-acquire fluids such as plasma would accelerate and reduce the cost of AD diagnosis and offer windows of opportunity for selecting and treating patients with disease-modifying drugs once they are available.

Metabolomics has particular relevance to drug discovery and development, because metabolites often mirror the end result of genomic and protein perturbations in disease and are most closely associated with phenotypic changes. Current metabolomics research involves the identification and quantification of hundreds to thousands of small-molecular-mass

A Core Research for Evolutional Science and Technology (CREST) grant made this study possible.

Manuscript received 10 November 2011 and in revised form 25 December 2011.

Published, JLR Papers in Press, December 27, 2011
DOI 10.1194/jlr.M022376

Abbreviations: AD, Alzheimer's disease; CSF, cerebrospinal fluid; LC/APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectroscopy; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; NIA, National Institute on Aging; RI, refractive index; ROC, receiver operating characteristic; RSD, relative standard deviation; SIM, selected-ion monitoring; UV, ultraviolet.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of five figures and two tables.

metabolites (<1,500 Daltons) in cells, tissues, or biological fluids. The aims of such studies are typically to understand new diagnosis biomarkers, to understand the mechanism of action of therapeutic compounds, and to uncover the pharmacodynamics and kinetic markers of drugs in patients and in preclinical in vivo and in vitro models (8). We had previously established in our laboratory a metabolomics platform focused on hydrophilic cationic compounds (9) and could identify 55 hydrophilic AD biomarker candidates in human AD CSF using nano-LC/MS.

Lipidomics is one of the metabolomics approaches used to analyze lipid species in biological systems (10–12). Investigating lipid biochemistry using a lipidomics approach will not only provide insights into the specific roles of lipid molecular species in healthy individuals and patients but will also assist in identifying potential biomarkers for establishing preventive or therapeutic approaches for human health (10, 13–15). Lipidomics has recently captured attention, owing to the well-recognized roles of lipids in numerous human diseases such as diabetes, obesity, atherosclerosis, and Alzheimer's disease (13, 16–18). In support of the hypothesis that lipid dysfunction plays an important role in AD pathogenesis, our laboratory also previously established a lipidomics method for comprehensive phospholipids evaluation that identified 31 phospholipids as AD biomarker candidates in human plasma using LC/MS (18). Moreover, additional studies have suggested that AD associates with other lipid metabolism pathways and lipid carrier proteins such as apoE (19–22).

In this study, we applied lipidomics technologies we developed in-house that focus on sterols, to find new AD plasma biomarkers. The reliable quantification method we established allows the detection of selected lipids and validates the change of the candidate in AD. The specificity of candidate was also confirmed using unrelated neurological disorders samples to clarify the specificity of the biomarker candidate.

MATERIALS AND METHODS

Chemicals and reagents

Most of the reagents used in the experiments were of analytical grade and were purchased from Wako Pure Chemicals Co. (Osaka, Japan). All other chemicals and solvents were analytical reagent grade. Zymosterol was purchased from Steraloids, Inc. (Newport, RI). Desmosterol and cholesterol were purchased from Sigma Aldrich (Poole; Dorset, UK). 5α-Cholesta-7,24-dien-3β-ol, cholesta-5,7-dien-3β-ol, and cholesta-5,8-dien-3β-ol were purchased from Sumika Technoservice Corporation. Cholesterol-25,26,26,26,27,27,27-D7 (D7-cholesterol) was purchased from Kanto Chemicals Co., Inc. (Tokyo, Japan). Desmosterol-26,26,26,27,27,27-D6 (D6-desmosterol) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL).

Samples collection

Plasma and CSF samples of elderly controls and subjects diagnosed with AD, MCI, schizophrenia, and Parkinson's disease were purchased from PrecisionMed, Inc. (San Diego, CA) and stored at −80°C until use. For circadian rhythm studies, plasma samples of healthy volunteers were collected at various time points during the day (9:30, 11:00, 13:00, 15:00, and 17:00) and stored at −80°C until use.

Sample preparation for unbiased sterol analysis in plasma

The deep-frozen plasma samples were thawed on ice, and 25 μl of plasma was spiked with 50 μl of 5 μg/ml D7-cholesterol in ethanol as internal standard. One hundred microliters of 50% potassium hydroxide (w/v) was then added to the solution, mixed thoroughly, and incubated at 70°C for 60 min. Following the incubation, 2 ml hexane and 0.5 ml PBS (pH 6.8) were added and mixed well. The solution was centrifuged for 10 min at 2,000 *g*, and the upper organic phase was transferred to a new tube. The lower layer was extracted with an additional 1 ml hexane, which was also added to the organic phase extract. The solvents were evaporated to dryness under a nitrogen gas stream at 40°C, the pellet was reconstituted in 100 μl ethanol, and the solution was subjected to liquid chromatography-atmospheric pressure chemical ionization-mass spectroscopy (LC/APCI-MS) analysis.

Unbiased sterol analysis with LC/MS

The omics study of neutral lipids was performed using a Shimadzu 20 AD system with an SIL-20AC auto-sampler, a CTO-20A column oven, and an LTQ Orbitrap mass spectrometer (ThermoFisher; San Jose, CA) with an APCI probe. For each run, we injected a total of 20 μl sample onto a 3.0 inner diameter (ID) × 100 mm Shim-Pak XR-ODS column (Shimadzu Corporation; Kyoto, Japan) at a flow rate of 0.8 ml/min, with a total run time of 60 min. The gradient used consisted of solvent A (water-methanol, 50:50) and solvent B (methanol) starting at 40% B, ramping to 90% B over 20 min, holding at 90% B for 10 min, ramping to 100% B over 10 min, holding at 100% B for 10 min, back to 40% B in 0.1 min, and then holding to 10 min. The mass spectrometer was operated in the positive-ion mode. The spray voltage was set at 2.5 kV. A cycle of one full fourier transform (FT) scan mass spectrum (200–1,000 *m/z*, resolution of 30,000) followed by three data-dependent MS/MSs acquired in the linear ion trap with normalized collision energy (setting of 35%) was repeated continuously. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcalibur data system.

Data analysis of unbiased sterol analysis and statistical analysis

The mass spectrometric data were acquired using Xcalibur, and the initial metabolomics profiling was performed using in-house-developed Mass ++ data analysis software (http://groups.google.com/group/massplusplus) to obtain a peak list and align retention times and peak area, which is normalized with internal standard (D7-cholesterol). One-way variance analysis (ANOVA) was applied to the data. Finally, some peaks, whose ANOVA values were lower than 0.05 and fold change more than 2 or less than half, were picked up for further identification.

Identification of reliable candidate

Individual primary methanolic stock solutions of desmosterol, zymosterol, 5α-cholesta-7,24-dien-3β-ol, cholesta-5,7-dien-3β-ol, and cholesta-5,8-dien-3β-ol were prepared at concentrations high enough that when combined, a secondary dilution of each in a 10 ml volume of methanol resulted in concentrations of 20–30 μg/ml for each compound. This solution was subjected to LC/MS analysis to compare retention times.

The identification study of unknown peak A was formed using a Shimadzu 20AD system with a SIL-20AC auto-sampler, a CTO-20AC column oven, and an LCMS-2010EV high-performance single-quadrupole mass analyzer (Shimadzu) equipped with an APCI probe. For each run, we injected a total of 20 μl sample onto a 4.6 mm ID × 250 mm YMC-Pack Pro C18 RS column (YMC, Inc.; Wilmington, NC) at a flow rate of 1 ml/min, with a total run time of 70 min for desmosterol analysis or 20 min for cholesterol analysis. Column temperature was

maintained at 50°C. For desmosterol analysis, the gradient used consisted of solvent A (water-methanol, 50:50) and solvent B (methanol) starting at 85% B for 45 min, ramping to 100% B over 0.1 min, holding at 100% B for 10 min, back to 85% B in 0.1 min, and then holding to 15 min. For the cholesterol analysis, the mobile phase B flow rate was 1 ml/min isocratically. The mass spectrometer was operated in the positive-ion mode. MS conditions were as follows: resolution, ±0.15 Da; capillary temperature, 250°C; APCI vaporizer temperature, 400°C; ionization voltage, 4.5 kV; sheath gas flow, 2.5 l/min; and drying gas pressure, 0.02 MPa. To increase sensitivity, selected-ion monitoring (SIM) was used in this study. Programmed SIM was used, in which specific ions were monitored for compound throughout the chromatographic run. Ions monitored for identification and quantification of desmosterol and its isomers were *m/z* 367.3 and 369.3 for cholesterol.

Sample preparation for sterol analysis in CSF

The deep-frozen CSF samples were thawed on ice, and 100 μl of CSF was spiked with 100 μl of 1 μg/ml D7-cholesterol in ethanol and 100 μl of 100 ng/ml of D6-desmosterol in ethanol as internal standard. One hundred microliters of 50% potassium hydroxide (w/v) was then added to the solution, mixed thoroughly, and incubated at 70°C for 60 min. Following the incubation, 2 ml hexane and 0.5 ml PBS (pH 6.8) were added and mixed well. The solution was centrifuged for 10 min at 2,000 *g*, and the upper organic phase was transferred to a new tube. The lower layer was extracted with an additional 1 ml hexane, which was also added to the organic-phase extract. The solvents were evaporated to dryness under a nitrogen gas stream at 40°C, the residue was dissolved in 100 μl of ethanol, 20 μl of this solution was injected into the LC/APCI-MS system in desmosterol analysis, and 2 μl of this solution was injected in cholesterol analysis of CSF.

Sample preparation for desmosterol and cholesterol analysis and method validation with LC/MS

For preparation of standard stock solutions, desmosterol and cholesterol were dissolved in ethanol at a concentration of 0.1 mg/ml. Samples were diluted to concentrations of 10, 30, 100, 300, 1,000, 3,000, 10,000, and 30,000 ng/ml using ethanol for desmosterol and 0.1, 0.2, 0.5, 1, 2, 5, and 10 mg/ml using ethanol for cholesterol for the calibration curve in the plasma analysis. And samples were diluted to concentrations of 1, 2, 5, 10, 20, 50, 100, and 200 ng/ml using ethanol for desmosterol and 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μg/ml using ethanol for cholesterol for the calibration curve in CSF analysis. Internal standard stock solutions containing 500 ng/ml or 100 ng/ml of D6-desmosterol and 200 μg/ml or 1 μg/ml of D7-cholesterol in ethanol were also prepared in ethanol for plasma or CSF analysis, respectively. In the calibration study, a 25 μl or 100 μl aliquot of each standard solution was mixed with 100 μl of each internal standard solution in ethanol for plasma or CSF analysis and evaporated under a nitrogen gas stream at room temperature. The residue was dissolved in 100 μl ethanol, 20 μl of this solution was injected into the LC/APCI-MS system in desmosterol analysis, and 2 μl of this solution was injected in cholesterol analysis of CSF. The residual 10 μl was diluted with 1990 μl ethanol for analysis of cholesterol, and 2 μl of this solution was injected into the LC/APCI-MS system in cholesterol analysis of plasma. The LC/MS is LCMS-2010EV high-performance single quadrupole mass analyzer. SIM was used in this study. Programmed SIM was also used in these studies. Ions monitored for quantification of desmosterol were *m/z* 367.3 and 373.3 (D6-incorporated standard); cholesterol ions were *m/z* 369.3 and 376.3 (D7-incorporated standard).

The calibration curves were constructed by $1/x^2$ weighted least-squares linear regression of the peak area ratio of the analyte to the internal standard, against the concentration of each compound. For the accuracy and precision studies using human plasma, the interassay precision, expressed as relative standard deviation (RSD), was determined by the extraction and analysis of the four different samples. Briefly a 25 μl aliquot of human plasma was added to 25 μl aliquots of 0, 30, 300, or 10,000 ng/ml stock solution of desmosterol, or 25 μl aliquots of 0, 0.2, 1, or 5 mg/ml stock solution of cholesterol were separately spiked into the plasma. Then the concentrations of desmosterol and cholesterol were analyzed with LC/APCI-MS on the same day (n = 3) and on a different day (n = 3). The relative error was calculated as [(observed concentration−theoretical concentration)/spiked concentration] × 100 (%), and the precision was obtained in terms of the RSD.

Sample preparation for quantitative analysis with GC-MS

Samples were aliquoted and frozen at −80°C until assayed. Plasma concentration of desmosterol was measured by a modified method using combined GC-MS as described previously (23). In brief, 1 μg D6-desmosterol (100 μl from a stock solution of D7-desmosterol in ethanol; 10 μg/ml) was added to 100 μl plasma. One hundred microliters of 50% potassium hydroxide was then added to the solution, mixed thoroughly, and incubated at 70°C for 60 min. Following the incubation, 2 ml hexane and 0.5 ml PBS (pH 6.8) were added and mixed well. The solution was centrifuged for 10 min at 2,000 *g*, and the upper organic phase was transferred to a new tube. The lower layer was extracted with an additional 1 ml hexane, which was also added to the organic-phase extract. The organic solvents were evaporated, and the residual sterols were derivatized to TMS ethers by adding 1 ml TMS reagent (pyridine-hexamethylsisilazane-trimethylchlorosilane, 9:3:1[Supelco; Bellefonte, PA]) and incubation for 1 h at 64°C. The derivatization reagent was evaporated under nitrogen, and the silyl sterol ethers from plasma were dissolved in 160 μl *n*-decane. Eighty microliters of the solution was transferred into micro-vials for GC-MS SIM, and an aliquot of 2 μl was injected in a splitless mode at 280°C by an automated sampler and injector.

GC-MS SIM

GC-MS SIM was performed on a GC-2010 (Shimadzu) combined with a GCMS-QP2010 plus (Shimadzu) equipped with a DB-XLB 122-1232 fused silica capillary column (J&W Scientific, Inc.; Folsom, CA) (30 m × 0.25 mm, i.e., ×0.25 μm film thickness) in the splitless mode using helium (1 ml/min) as the carrier gas as described previously (23, 24). The temperature program was as follows: 150°C for 1 min, followed by 20°C/min up to 260°C, and 10°C/min up to 280°C (for 15 min). For the modified GC-MS method, the temperature program was as follows: 150°C for 1 min, followed by 20°C/min up to 250°C (for 55 min). Neutral sterols were monitored as their TMS derivatives in the SIM mode using the following masses: D6-desmosterol (internal standard) at *m/z* 447 [M^+-CH_3] and desmosterol at *m/z* 441 [M^+-CH_3]. Peak integration was performed manually, and sterols were quantified from SIM analysis against internal standard (D7-desmosterol) using standard curves of desmosterol.

Statistical analysis using receiver operating-characteristic curve

Receiver operating-characteristic (ROC) curves were analyzed with sensitivity (*y* axis) versus specificity (*x* axis), and area under the curve (AUC) was calculated by using the commercially available software package GraphPad Prism. An AUC of 1.0 indicates a test with perfect discrimination between elderly control and patients. An AUC of 0.5 indicates a test with no discriminatory power.

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RESULTS

Unbiased sterol analysis of plasma samples

GC with flame ionization detection (25, 26), HPLC with ultraviolet (UV) detection (27, 28), and HPLC with refractive index (RI) detection (29) are among the most frequently used methods for sterol analysis. However, these methods are limited because they cannot quantify and distinguish minor variants of endogenous sterols with sufficient sensitivity and specificity (29). MS is a powerful detection method that is suitable for GC and HPLC systems. These detectors are not only superior in terms of sensitivity but are also highly specific compared with flame ionization and UV and RI detectors. GC-MS has been widely accepted as a reliable analytical method for the determination of sterols in biological samples (30–32). However, in recent years, HPLC-MS or HPLC-MS/MS have become popular because these methods do not always require deconjugation and derivatization steps before analysis (33, 34). Moreover, although HPLC methods do not affect the decomposition of some labile sterols, such as 24S, 25-epoxycholesterol, the high temperatures achieved during GC methods can lead to the degradation of unstable sterols (35, 36). In addition, HPLC is easily connected with ESI and APCI sources, and APCI is particularly applicable to neutral lipids, such as cholesterol, inasmuch as those compounds are less polar and relatively low-molecular-weight compounds (29, 37–39). In this paper, we used the LC/APCI-MS method and applied this method to the discovery of plasma and CSF AD biomarkers.

Using our optimized analytical procedure, many peaks were detected from 25 μl of human plasma. After peak selection and retention time alignment of samples from 10 AD patients and 10 elderly healthy controls, we looked for unknown peak differences, with criteria being a fold change less than 0.5 or more than 2, and *P* value of less than 0.05. One candidate that met these criteria had a fold change and *P* value of 0.36 and 0.009, respectively (see supplementary Fig. IA, C), so we decided to further characterize this metabolite from its molecular mass.

The exact mass of the peak is 367.3349 with 60,000 resolutions, which indicates that the formula of this cationic ion is $C27H43^{+}$ (see supplementary Fig. IB). From this result and chemical properties estimated from the extraction method, we predicted that this compound was either a cholesterol metabolite or one of its precursors. Because cholesterol and its related compounds emerged as a cationic ion, [M-H2O+H]+ in APCI positive mode, we concluded that this candidate should have an OH functional group in its structure. Looking at cholesterol metabolic pathways and reported bypath pathway, we hypothesized that this candidate is a cholesterol-related compound, with a sterol backbone and one more double bond than cholesterol, like desmosterol, zymosterol, 5α-cholesta-7,24-dien-3β-ol, cholesta-5,7-dien-3β-ol, and cholesta-5,8-dien-3β-ol.

To identify the precise structure and establish the analytical method of biomarker candidate, unknown peak A, we purchased all compounds whose formula is $C_{27}H_{45}O$ and whose structure backbone is sterol, such as desmosterol, zymosterol, 5α-cholesta-7,24-dien-3β-ol, cholesta-5,7-dien-3β-ol, and cholesta-5,8-dien-3β-ol. Next, we established the analytical method that allows separation of all compounds and identification of the structure of the biomarker candidate. This step was thought to be necessary because the exact mass of those compounds is completely identical and we could not distinguish those isomers using only exact mass and MS/MS fragmentation data.

In addition to the LC method, the typical separation methods used to determine cholesterol and its metabolites and precursors are based on the application of GC. Because these methods are time and labor intensive, we tried first to establish a method that could separate endogenous isomers of desmosterol using an LC/MS method to identify the biomarker candidate.

From the results of several experiments, it was established that organic modifiers can affect chromatographic behavior of desmosterol isomers. From several experiments, organic modifiers can affect the chromatographic behavior of desmosterol isomers (40). The use of methanol or ethanol in the mobile phase can sharpen sterol peaks, potentially by contributing to the solubility of desmosterol isomers (**Fig. 1B**). Although 7, 24-cholestadien-3β-ol and 8, 24-cholestadien-3β-ol could not be separated using this method, we could separate all other targeted sterols within 45 min using this HPLC system. Using this method, we could identify unknown peak A using the information of elution time of each compound on the ODS column. The result of the chromatogram of human plasma indicated that unknown peak A was well separated and has the same elution profile as desmosterol (Fig. 1A). Unknown peak A is therefore without a doubt desmosterol, as indicated by the exact mass of protonated molecules and elution time of unknown peak A and desmosterol standard.

Development of analytical method and its validation

Next, we tried to develop the method to quantify desmosterol and cholesterol using LC/MS. Our method turned out to provide a good separation from interfering peaks with sufficient linearity, and the analysis can be performed with an excellent correlation (greater than 0.999). The deviations of calibration standards from nominal concentrations were less than 6.6% for all points in the calibration range. The assay validation presented in **Tables 1**and **2** demonstrates that the method simultaneously determines desmosterol in human plasma in a range of 10–30,000 ng/ml and also determines cholesterol in human plasma in a range of 0.1–10 mg/ml. Noticeably, the deviations were less than 5.0% also for a low concentration range of desmosterol. Given that plasma desmosterol concentrations are approximately 200–1,000 ng/ml, our novel analytical method can therefore evaluate tiny changes in desmosterol levels.

Patient studies

There has been some publication referring to the relationship between AD and desmosterol, but there is no clear evidence in the current literature on changes of AD plasma

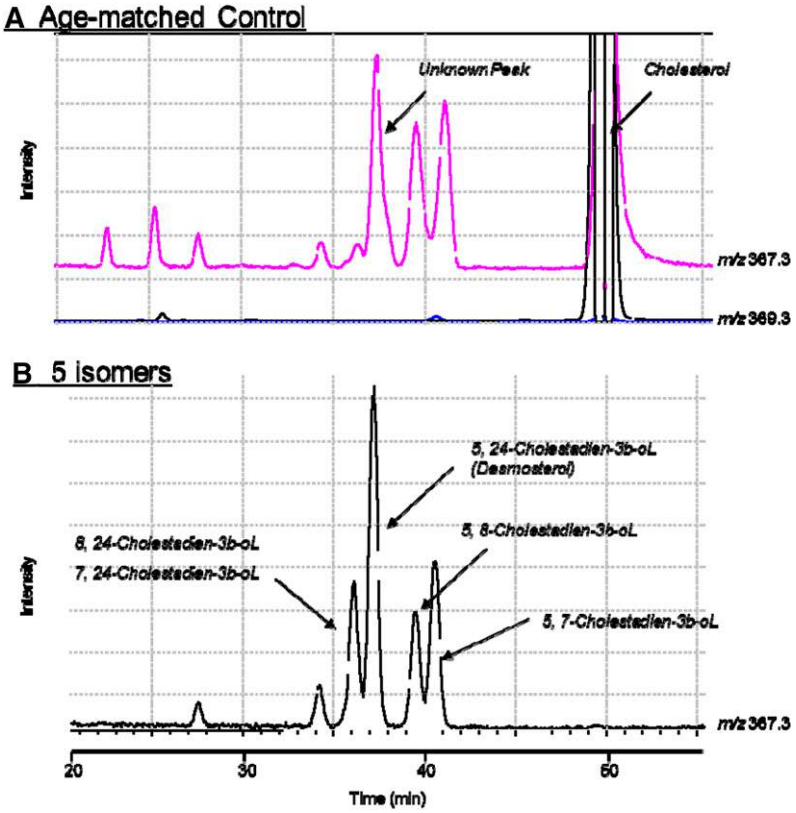

Fig. 1. Extracted ion chromatograms in the positive-ionization mode of *m/z* 367.3 and 369.3 of age-matched control sample (A) and standards of five isomers (B).

desmosterol, although many scientists have tried to detect such changes. First, our method was applied to the determination of desmosterol and cholesterol in human plasma from 10 AD and 10 elderly healthy control plasma samples. The assay results are shown in **Table 3**. Initially, no significant changes were observed in plasma cholesterol; however, the level of desmosterol in plasma was significantly decreased between AD patients (434 ± 130 ng/ml) and the elderly healthy controls (848 ± 199 ng/ml). Interestingly, a decrease in the desmosterol/cholesterol ratio was also observed in AD patients (control, 460 ± 115 and AD, 272 ± 87). The variation in desmosterol levels and desmosterol/cholesterol ratios in plasma was significant with a *P* value of less than 0.001 in AD patients (Table 3).

As we were developing our LC/MS analysis, a report came out looking at desmosterol and cholesterol levels in AD patient plasma and CSF using the GC method. In that report, by Kolsch et al., no changes in plasma desmosterol were observed (23). We were therefore interested in understanding why our initial data could demonstrate a difference of desmosterol in AD patients compared to controls, as opposed to the GC method. To do so, we performed the original GC method (23) as reported using our same samples to clarify whether we could see significant change of desmosterol. As a result, we also could not see the significant change of desmosterol with this GC method as reported by Kolsch et al. Even though we used the same plasma sample set, we could find significant difference in AD and control with our LCMS method (see supplementary Fig. IIA). Using this reported GC-MS method, we could see interference of quantification of plasma desmosterol by overlapped peaks with desmosterol peak, although this method is capable of separating desmosterol from its four isomeric compound peaks (see supplementary Fig. IIA). And we observed higher desmosterol levels with this GC-MS method compare with our LC-MS method (see supplementary Fig. IIIA). Actually, the desmosterol concentration means we detected were less than half those detected by Kolsch et al., (our data, approximately 0.64 μg/ml; their data, approximately 1.8 μg/ml). In addition, low correlation between two concentrations measured by LC-MS and GC-MS was observed ($r^2 = 0.52$; see supplementary Fig. IIIA). Those results strongly suggested that the difference in concentration could be due to the ability of the original method to

TABLE 1. Accuracy and precision of the analysis of desmosterol in human plasma (n = 3)

	Blank			30 ng/ml			300 ng/ml			1,000 ng/ml		
Desmosterol	Observed concentration: (ng/ml)	RSD%	RE%	Observed concentration: (ng/ml)	RSD%	RE%	Observed concentration: (ng/ml)	RSD%	RE.%	Observed concentration: (ng/ml)	RSD%	RE%
intra-day	864	1.7	—	893	1.2	−2.3	1,176	1.3	4.0	11,506	2.4	6.4
	805	1.9	—	834	1.6	−1.7	1,095	2.6	−3.3	11,003	1.9	2.0
	833	0.9	—	866	1.6	10.4	1,150	2.2	5.8	11,573	0.7	7.4
inter-day	834	3.6	—	865	3.4	2.1	1,141	3.6	2.2	11,288	3.6	4.5

TABLE 2. Accuracy and precision of the analysis of cholesterol in human plasma (n = 3)

	Blank			0.2 mg/ml			1 mg/ml			5 mg/ml		
Cholesterol	Observed concentration (mg/ml)	RSD%	RE%	Observed concentration (mg/ml)	RSD%	RE%	Observed concentration (mg/ml)	RSD%	RE%	Observed concentration (mg/ml)	RSD%	RE.%
intra-day	1.47	2.2	—	1.68	2.4	3.8	2.47	2.3	0.2	6.73	2.5	5.3
	1.47	2.3	—	1.64	3.1	−11.7	2.42	0.7	−4.8	6.86	1.2	7.9
	1.46	3.2	—	1.66	3.1	2.0	2.51	1.4	4.8	6.85	1.1	7.9
inter-day	1.46	0.4	—	1.66	1.1	−1.9	2.46	1.8	0.0	6.86	0.1	7.9

correctly separate desmosterol from metabolites having a similar structure, and that desmosterol concentration would fluctuate due to the change of the interfering peak concentration.

To clarify whether this discrepancy is due to overlapping peaks, the reported GC-MS method was modified to improve the separation of desmosterol and interfering peaks and was applied to evaluate desmosterol levels using the same original samples. The results indicate that we could see a significant change of plasma desmosterol after improving the separation of desmosterol from interfering peaks (see supplementary Fig. IIB). Moreover, when we used a modified GC-MS method, whose separation between desmosterol and interfering peaks was improved, the difference of desmosterol concentration measured by LC-MS and GC-MS became more similar (see supplementary Fig. IIIB). This result suggests that our LC-MS method is better able to purify desmosterol compared with previously published GC methods and that our LC method is more suitable for measuring plasma desmosterol accurately.

Taken together, our findings cast doubt on previous reports that failed to demonstrate any changes in desmosterol levels in AD patients compared to elderly controls. Most likely, the inability to observe changes was due to the lack of separation from interfering peaks. Our method is therefore a novel method that allows more-precise desmosterol quantification and separation of desmosterol from its endogenous isomers and other interference peaks compared with previously published methods. On the other hand, we cannot completely rule out the possibility that diet differences between American and German patients (our and Kolsch's sample set, respectively) may contribute to our findings. We have now started to analyze additional experiments in various populations with our detection methodology.

Metabolites, proteins, and peptides present in CSF are thought to better reflect metabolic processes taking place in the brain, as compared with plasma. Previously Kolsch et al. (23) investigated CSF and plasma levels of cholesterol and of its desmosterol in AD patients and nondemented controls. They found CSF levels of cholesterol ($P = 0.011$, fold change = 0.9) and absolute levels of desmosterol ($P < 0.001$, fold change = 0.9) to be lower in AD patients as compared with controls, but no change in the desmosterol/cholesterol ratio was observed ($P = 0.68$, fold change = 1.0) (23). Because our method turned out to be more suitable for desmosterol analysis due to its good separation with interfering peaks compared with the original GC method, we were interested in reevaluating desmosterol and cholesterol levels of AD and elderly healthy controls and determining whether the changes were similar between plasma and CSF. The results showed that the levels of desmosterol and cholesterol in CSF were not significantly different between AD patients and controls, but a significant decrease of the desmosterol/cholesterol ratio was observed in AD patients' CSF (AD: fold change 0.62; P value, 0.001) (Table 3). In addition, the correlation between desmosterol:cholesterol in plasma and CSF is shown in **Fig. 2**. The result shows a good correlation between those parameters in our study ($r^2 = 0.60$). As mentioned before, variation of CSF constituents is likely to better reflect changes in brain associated with AD. Considering the good correlation with CSF and plasma desmosterol:cholesterol, it seems that plasma desmosterol levels reflect sterol metabolism of the brain.

Next, to understand whether the changes in desmosterol or the desmosterol/cholesterol ratio change in plasma and CSF are AD specific, we analyzed those biomarker levels in schizophrenia and Parkinson's disease patients' samples. As shown in Table 3 and supplementary Table I, desmosterol levels are decreased only in AD plasma compared with elderly controls, and no changes in plasma level were

TABLE 3. Plasma and CSF levels of cholesterol, desmosterol, and desmosterol/cholesterol ratio in AD patients (n = 10) and elderly healthy controls (n = 10)

				AD vs Control	
		Control (n = 10)	AD (n = 10)	Fold change	P
Plasma	Cholesterol (mg/ml)	1.88 ± 0.32	1.64 ± 0.43	0.87	n.s.
	Desmosterol (ng/ml)	848 ± 199	434 ± 130	0.51	0.00003
	Desmosterol:cholesterol (ng/mg)	460 ± 115	272 ± 87	0.59	0.0007
CSF	Cholesterol (μg/ml)	3.55 ± 0.83	5.34 ± 2.79	1.51	n.s.
	Desmosterol (ng/ml)	4.39 ± 1.32	4.09 ± 2.36	0.93	n.s.
	Desmosterol:cholesterol (ng/μg)	1.26 ± 0.31	0.780 ± 0.224	0.62	0.001

Desmosterol and cholesterol were measured by LC/APCI-MS as described in the Materials and Methods section. n.s., not significant.

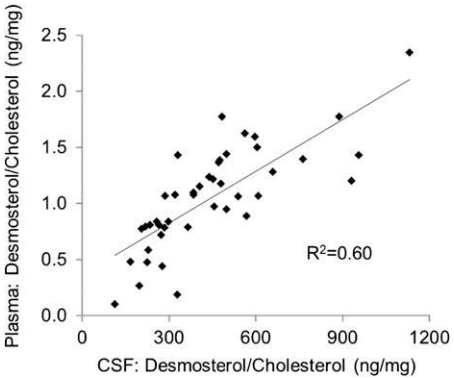

Fig. 2. Correlation between the values of desmosterol in human plasma and the desmosterol/cholesterol ratio in CSF (n = 43).

observed in schizophrenia and Parkinson disease patients, hinting that desmosterol could be considered as a candidate AD biomarker in plasma and CSF.

As is often the case, circadian rhythms can affect diagnostic markers, hence the importance of evaluating whether sampling time affects biomarker levels. As shown in supplementary Fig. IV, no marked circadian rhythm affected desmosterol levels or the plasma desmosterol/cholesterol ratio from healthy volunteers. Those results suggest that sampling time does not affect detected desmosterol levels. Perhaps surprisingly, the result revealed that concentrations of plasma desmosterol in two different healthy volunteers vary significantly (see supplementary Fig. IVA). However, no difference between the two subjects on each sampling time point after normalization with cholesterol (see supplementary Fig. IVB) was observed. This result indicates the importance of normalization of plasma desmosterol with plasma cholesterol when we are comparing the individual values.

To further validate desmosterol and the desmosterol/cholesterol ratio as candidate AD biomarkers, we next analyzed desmosterol levels in plasma using 109 different clinical samples: 42 elderly controls, 26 MCI, and 41 AD patients (see supplementary Table II). To the best of our knowledge, there have been no publications describing the quantification of desmosterol in MCI patients. As shown in **Fig. 3A, D**, we could see a significant change in MCI patients as well as AD patients (Fig. 3A, D). Given that plasma desmosterol starts to change in MCI patients, there is a possibility that plasma desmosterol level could be used as an early-stage dementia diagnostic marker. Interestingly, we observed a sex difference in desmosterol levels (Fig. 3B, C). The change of desmosterol in female patients is more significant compared with that in male patients. This phenomenon was also observed in the plasma desmosterol/cholesterol ratio (Fig. 3E, F). It is not clear what is causing this difference, but this phenomenon has not been previously described and will require further validation in large-scale studies.

Next, we confirmed the feasibility of using plasma desmosterol level and CSF desmosterol/cholesterol ratio as AD diagnostic markers with ROC plot analysis. ROC plots provide a statistical method to assess the diagnostic accuracy of a test (or biomarker) that has a continuous spectrum of test results (41). The ROC curve is a graphical display of the trade-offs of the true-positive rate (sensitivity) and false-positive rate (1-specificity) corresponding to all possible binary tests that can be formed from this continuous biomarker (41). Each classification rule, or cut-off level, generates a point on the graph. The closer the curve follows the left-hand border and then the top border of the ROC space, the more accurate the test. **Figure 4A,** B shows ROC plot analysis using plasma desmosterol and plasma desmosterol/cholesterol

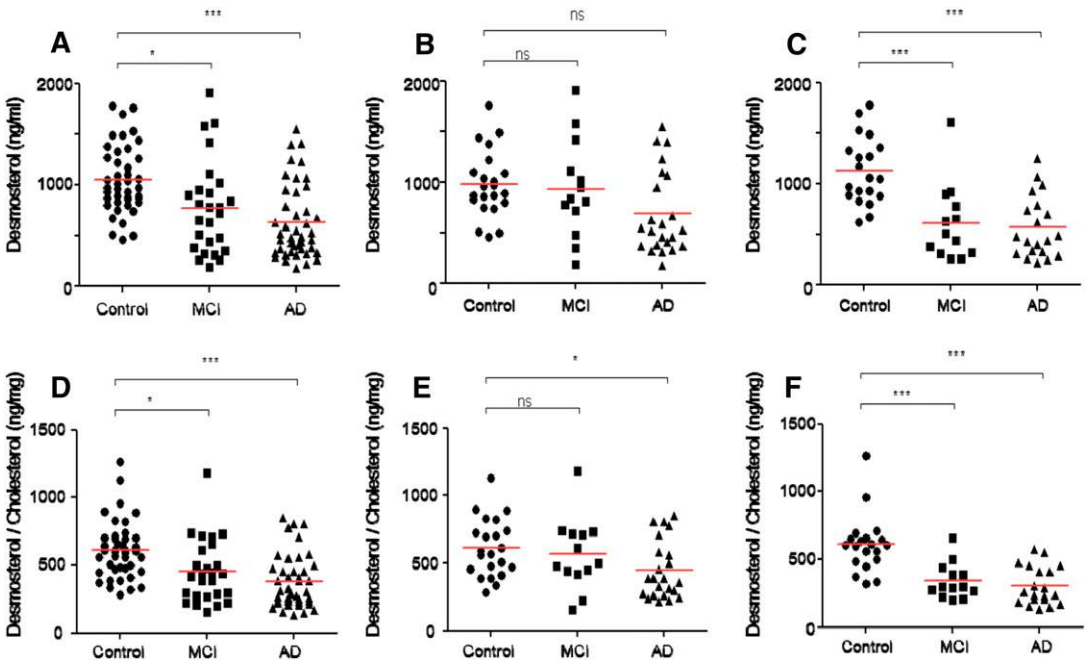

Fig. 3. Comparison of plasma desmosterol level in all patients (A), male (B), and female (C). And comparison of plasma desmosterol/cholesterol ratio in all patients (D), male (E), and female (F). $*P < 0.05$, $***P < 0.001$, n.s., not significant.

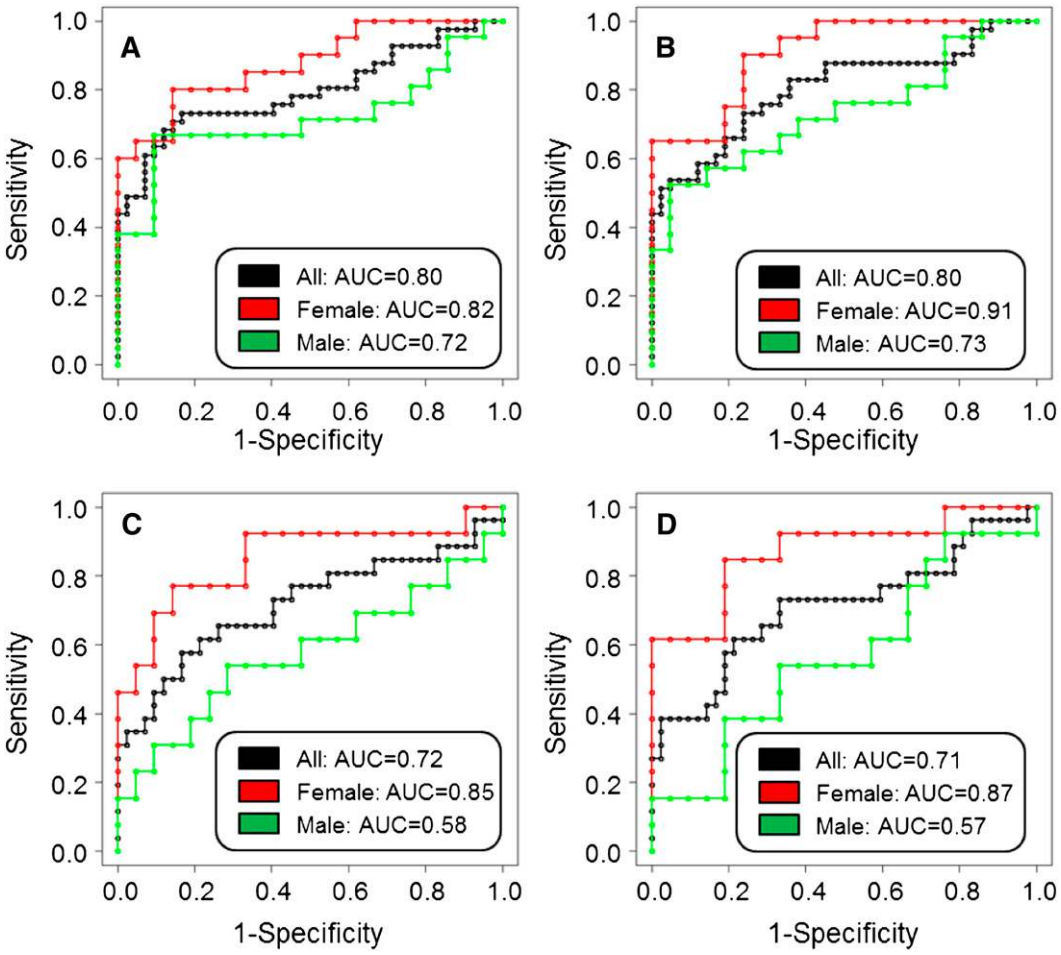

Fig. 4. ROC plot analysis using plasma desmosterol value (A) or plasma desmosterol/cholesterol ratio (B) in AD (n = 41) versus control (n = 42). And ROC plot analysis using plasma desmosterol value (C) or plasma desmosterol/cholesterol ratio (D) in MCI (n = 26) versus control (n = 42).

ratio in elderly controls versus AD patients, respectively. The AUC of ROC curve using plasma desmosterol and desmosterol/cholesterol ratio is 0.80, indicating a rather high discrimination power of those markers in the two reference populations and confirms the validity of those parameter measurements in AD. From our results, it is also evident that normalization by cholesterol is a better diagnostic marker than desmosterol value alone, particularly in females (AUC on AD versus control: desmosterol, 0.82; desmosterol/cholesterol, 0.91).There is also a sex difference in those diagnostic markers: actually, ROC areas of females using plasma desmosterol and the desmosterol/cholesterol ratio are bigger than the area of males (Fig. 4A, B). As indicated in Fig. 4B, it is revealed that setting specificity at 76% in the desmosterol/cholesterol ratio of females gave a sensitivity value of 90%. And for males, setting specificity to 76% gave a sensitivity value of 62%, indicating that there is gender difference, and a good sensitivity of this marker is observed in females.

According to the current consensus criteria proposed by the National Institute on Aging (NIA) for ideal AD biomarker specificity and sensitivity, the biomarker should have a diagnostic sensitivity >85% for detecting AD and a specificity >75% for distinguishing between other dementias (5). Our value of the desmosterol/cholesterol ratio of females meets this diagnostic sensitivity and selectivity, indicating that the desmosterol/cholesterol ratio in female plasma could become a strong AD diagnosis biomarker. We are planning to confirm this finding in other dementia types, including patients with dementia with Lewy body, and frontal temporal dementia in the near future.

Figure 4C, D show ROC plot analysis using plasma desmosterol and the plasma desmosterol/cholesterol ratio in elderly control versus MCI patients, respectively. The AUC of ROC curve using plasma desmosterol and the desmosterol/cholesterol ratio are 0.72 and 0.71, respectively, indicating a moderate discrimination power of those markers in the two reference populations and confirming the validity of those parameter measurements in MCI. As with the AD versus control analysis, we also found a sex difference in those diagnostic markers. As seen in Fig. 4D, it is revealed that setting specificity at 80% in the desmosterol/cholesterol ratio of females gave a sensitivity value of 85%. On the other hand, for males, setting specificity at 80% gave a sensitivity value of 38%. Particularly for females, we can get good sensitivity and specificity as an MCI bio-

marker, and this value also meets the NIA criteria, indicating the potential usefulness of desmosterol as a plasma MCI biomarker.

Next, we also looked at the desmosterol level in relation to age, gender, and Mini-Mental State Examination (MMSE) scores as a measure of disease severity (supplementary Table III). No significant correlation ($P > 0.05$) of desmosterol or the desmosterol/cholesterol ratio in plasma and CSF has been found with the apoE4 allele carrier; however, a significant correlation between plasma desmosterol or the desmosterol/cholesterol ratio has been observed with MMSE score and age ($P < 0.05$). Both desmosterol and the desmosterol/cholesterol ratio in plasma correlate with lower MMSE score and increased age. In addition, we could also observe a sex difference in this analysis. As shown in supplementary Fig. V, the desmosterol/cholesterol ratio in females correlates better with MMSE score than with its ratio in males. As for r values between plasma desmosterol/cholesterol ratio and MMSE score in males and females, values are 0.19 ($P > 0.05$) and 0.54 ($P < 0.05$), respectively.

Those results also indicated that we could use simply 25 μl of plasma for AD and MCI diagnosis, especially in female patients. Although this finding will have to be validated in larger studies, our data suggest the possible use of plasma desmosterol as noninvasive plasma biomarkers to help AD diagnosis in patient populations, especially when combined and normalized with cholesterol levels. We are now investigating whether normalization by other endogenous sterols would further improve the accuracy of this biomarker. In addition, we will report correlations between already-reported AD candidate biomarkers and desmosterol in combination studies using this new AD plasma biomarker in the near future.

CONCLUSION

A sensitive focused metabolomics method of biological samples has been developed and applied to AD biomarker discovery. We were able to find a biomarker candidate that changes in AD compared with plasma from healthy elderly controls. Furthermore, the unknown peak with statistical significance was identified as desmosterol from its exact mass and the comparison of retention time between this peak and standard. Next, we have developed the analytical method of desmosterol with LC/MS, which does not need derivatization and allows a good separation with another endogenous desmosterol isomer. Using this method, we have analyzed the concentration of desmosterol in human AD and elderly controls, and have shown that desmosterol plasma level and the desmosterol/cholesterol ratio in the same patients was significantly decreased in AD. Surprisingly, we found that this difference of plasma desmosterol in AD could not be detected with a previously validated GC-MS method due to the lack of separation of interfering peaks. We believe this to be the reason that no one before us has been able to show desmosterol as an AD plasma diagnostic biomarker candidate. Then we measured desmosterol and cholesterol in the same patient's CSF to clarify the relationship between plasma and CSF value of desmosterol. As a result, it is revealed that the CSF desmosterol/cholesterol ratio correlates well with its plasma ratio, suggesting that plasma desmosterol does reflect brain sterol synthesis and/or sterol metabolism. Additionally, the specificity of this change was confirmed using other neuronal disease patients' plasma and CSF, suggesting the specific association of desmosterol with Alzheimer's disease and/or with cognitive decline.

Finally, large-scale evaluation using one hundred clinical samples was performed to confirm desmosterol potential as a noninvasive plasma biomarker. In this study, we could find a significant desmosterol and desmosterol/cholesterol ratio change in MCI and AD plasma compared with healthy elderly controls. Interestingly, these changes are more prominent in plasma of female AD patients. In support of plasma desmosterol as a potential AD biomarker, we also found a significant correlation with worsening MMSE scores.

This study is the first to report that plasma desmosterol levels are decreased in AD and MCI. And future studies will be able to confirm whether desmosterol could become an attractive plasma AD biomarker that could perhaps also be utilized for diagnosis and as well as for monitoring noninvasively the effect of future AD drugs on disease progression.

The authors thank Dr. Keith M. Wilcoxen, who provided carefully considered feedback and valuable comments. Special thanks also go to Ms. Hiroko Shinjo, whose support and information have helped very much throughout the production of this study.

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