

Research Report

A Lead Study on Oxidative Stress-Mediated Dehydroepiandrosterone Formation in Serum: The Biochemical Basis for a Diagnosis of Alzheimer's Disease

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Abstract. Alzheimer's disease (AD) is a progressive, yet irreversible, neurodegenerative disease for which there are limited means for its ante-mortem diagnosis. We previously identified a brain- and cell-specific oxidative stress-mediated mechanism for dehydroepiandrosterone (DHEA) biosynthesis present in rat, bovine, and human brain, independent of the cytochrome P450 17 α -hydroxylase/17,20-lyase (CYP17) enzyme activity found in the periphery. This alternative pathway is induced by pro-oxidant agents, such as Fe²⁺ and amyloid- β peptide. Using brain tissue specimens from control and AD patients we subsequently provided evidence that DHEA is formed in the AD brain by the oxidative stress-mediated metabolism of an unidentified precursor, thus depleting the levels of the precursor present in the blood stream. Here, we tested for the presence of this DHEA precursor in human serum using a simple Fe²⁺-based reaction and determined the amounts of DHEA formed. A total of 86 subjects were included in this study: 19 male and 20 female AD patients; 18 male and 22 female age-matched controls; and 4 men and 3 women with mild cognitive impairment. Serum oxidation resulted in a dramatic increase of DHEA level in control patients, whereas only a moderate or no increase was observed in the AD patients. The DHEA variation after oxidation correlated with the patients' cognitive and mental status. These results suggest that the comparison of DHEA levels in patient serum before and after oxidation could provide a useful tool to diagnose AD.

Keywords: Alzheimer's disease, blood, DHEA, diagnosis, gas chromatography, mass spectrometry, mild cognitive impairment, oxidative stress

INTRODUCTION

Intensive research carried out in recent years has shown that some steroids synthesized in the nervous system, called "neurosteroids," display beneficial neu-

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roprotective properties and improve altered cognitive processes such as learning and memory [1]. These neurosteroids may be of particular importance in the treatment of diseases where neurodegeneration is predominant, including age-dependent dementia, stroke, Alzheimer's disease (AD), and Parkinson's disease [2]. AD remains the most common form of dementia in the elderly [3], and there is currently no definitive diagnostic tool other than postmortem histological analysis of the brain tissue. In the human body [4], dehydroepiandrosterone (DHEA) is a major neurosteroid [5] that is present at high levels in the brain [6] and exerts a broad range of biological effects [7]. Its neurotrophic effects were first reported in mouse brain cell cultures [8], and DHEA has also been found to protect rat and human hippocampal neuronal cells against oxidative stress-induced cellular damage [9]. Given the neuroprotective and cognitive-enhancing properties of DHEA, it has been hypothesized that elevated DHEA levels described in AD brain tissue potentially represent an adaptive or compensatory process [10].

In the peripheral steroidogenic tissue, DHEA synthesis from pregnenolone requires the microsomal enzyme cytochrome P450 17 α -hydroxylase/17,20 lyase (CYP17) [11, 12]. DHEA is also synthesized in the brain, although the biology of its synthesis remains to be elucidated. Indeed, neither CYP17 nor its activity have been detected in the adult brain [13]. We recently demonstrated the presence in the brain of an alternative Fe²⁺-dependent pathway mediating DHEA formation [14]. In this particular metabolic pathway, DHEA synthesis in the brain is mediated by oxidative stress from an unidentified precursor [15, 16]. *In vitro* oxidation of brain tissue and serum of nondemented subjects resulted in the increased formation of DHEA, whereas brain tissue and serum from AD patients showed no increase in DHEA formation [16]. These results suggested that a yet to be identified precursor of DHEA in the oxidative pathway was absent from the serum of AD patients and led us to believe that this specific trait could be used for diagnosis of the disease.

To investigate this hypothesis, the sera of 86 subjects were analyzed using a sensitive analytical method based on gas chromatography coupled to quadrupole electronic impact mass spectrometry (GC/Q-EI-MS). Here, we show that a blood test based on *in vitro* oxidative stress-mediated DHEA increases may be a valuable diagnostic tool to identify AD at an early stage, and to differentially diagnose AD from mild cognitive impairment (MCI).

MATERIAL AND METHODS

Chemicals

DHEA, the derivatization reagent bis-(trimethylsilyl)trifluoroacetamide (BSTFA), and (NH₄)₂Fe(SO₄)₂·6 H₂O were purchased from Sigma (Oakville, ON, Canada). Ethyl acetate, toluene, and water were purchased from Merck (Kirkland, QC, Canada).

Serum samples collection

A total of 86 subjects were included in the study. The subjects included 41 men and 45 women, of which 39 were age-matched controls, 23 had mild AD, 3 had moderate AD, 14 had severe AD, and 7 had MCI. All subjects were recruited from the Memory Disorders Program, Georgetown University Medical Center. Each subject was given a thorough physical and neurological examination, laboratory blood tests, and brain scan, and tested using the Mini Mental State Examination (MMSE) to assess cognitive mental status [17]. Peripheral blood samples were taken by venipuncture and collected in heparinized tubes. We avoided using EDTA-containing tubes to prevent any interaction with our biochemical protocol. Tubes containing whole blood were kept at room temperature and laboratory processing was performed within 1 h. Serum was separated by centrifugation at 3000g for 10 min at 4°C. The sera were collected, aliquoted in 1 mL volume, and stored at -80°C. The details of subjects repartition by age class and diagnosis is given Table 1. Protocols for the use of human samples were approved by the Georgetown University Internal Review Board.

Serum sample Fe²⁺ treatment and extraction

A liquid/liquid procedure was used to extract the samples. The extraction was initiated by adding a volume of 4 mL ethyl acetate. After decantation and centrifugation for 5 min at 1000g, the organic phase was dried under nitrogen stream. The extraction process was repeated three times. The dried residue, containing all steroids and sterols, was reconstituted in 1 mL of ethyl acetate. At the same time, 1 mL of each serum sample was incubated with 200 μ L of a 10 mM Fe²⁺ solution at 37°C for 2 h and extracted as described above.

Table 1
Subject repartition by age, diagnosis, and MMSE

		Diagnosis					Total
		Aged-matched control	AD patients			MCI	
			Mild	Moderate	Severe		
Men	Age range (years)	63–106	53–86	n/a	67–86	67–80	53–106
	<i>n</i>	19	14	n/a	4	4	41
Women	Age range (years)	51–108	71–88	55–63	58–108	72–81	51–108
	<i>n</i>	20	9	3	10	3	45
MMSE range		29–30	21–28	13–20	<12	26–29	

n/a = not applicable.

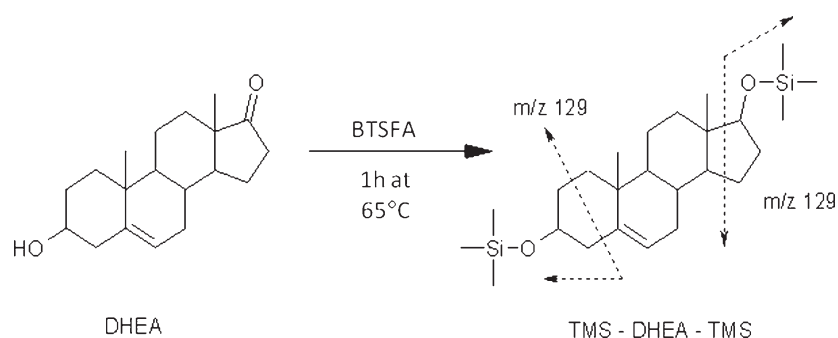


Fig. 1. DHEA derivatization reaction using BSTFA.

Sample derivatization

Five hundred microliters of the organic extract were dried under nitrogen stream and derivatized using 100 μ L of BSTFA. The reaction was done at 65°C over 1 h (Fig. 1). Samples were dried under nitrogen stream and reconstituted in 20 μ L of toluene. One microliter was submitted for gas chromatographic analysis.

Gas chromatography – quadrupole electronic impact mass spectrometry (GC/Q-EI-MS) analysis

The gas chromatography/mass spectrometry system consisted of a GC 17A gas chromatograph equipped with an AOC-20 autosampler and a QP5050A quadrupole electronic impact mass spectrometer (Shimadzu, Columbia, MD, USA). The separation was performed on a fused silica capillary column (30 m \times 0.25 mm i.d.) coated with a 0.25- μ m layer of methylsilicone plus 5% phenyl-methylsilicone. The injection port was equipped with a double gooseneck splitless liner with deactivated siltek coating (Restek Co., Bellefonte, PA, USA). The samples were analyzed using RealTime GCMS software that was programmed as follows: initial oven temperature was maintained at 120°C for 3 min, then increased by 40°C/min to 310°C, and maintained for 5 min. The injection port and the interface

temperature were set at 280°C and 300°C, respectively. Helium was used as a mobile phase at a flow rate of 1.2 mL/min. Acquisition began 8 min after the sample injection. The detector voltage was set at 1.2 kV and the quadrupole electronic impact mass spectrometer was set at the ionization energy of 65 eV. Derivatized DHEA was monitored in a single ion mode (SIM) for the most abundant DHEA ion fragments, m/z 129 and m/z 73.

Calibration curve

The calibration curve was calculated with a derivatized DHEA external standard using six concentrations in toluene: 10^{-5} , 5×10^{-5} , 10^{-4} , 5×10^{-4} , 10^{-3} , and 5×10^{-3} mg/mL.

Statistical analyses

Equal intra-group variances were determined by Brown-Forsythe F-test. In the case of equal variances, one-way analysis of variance (ANOVA) followed by *t*-test and correlation analyses was performed. In the case of unequal variances, Welch ANOVA was used for group comparisons. All statistical analyses were performed using JMP8 (SAS Institute Inc., Cary NC, USA).

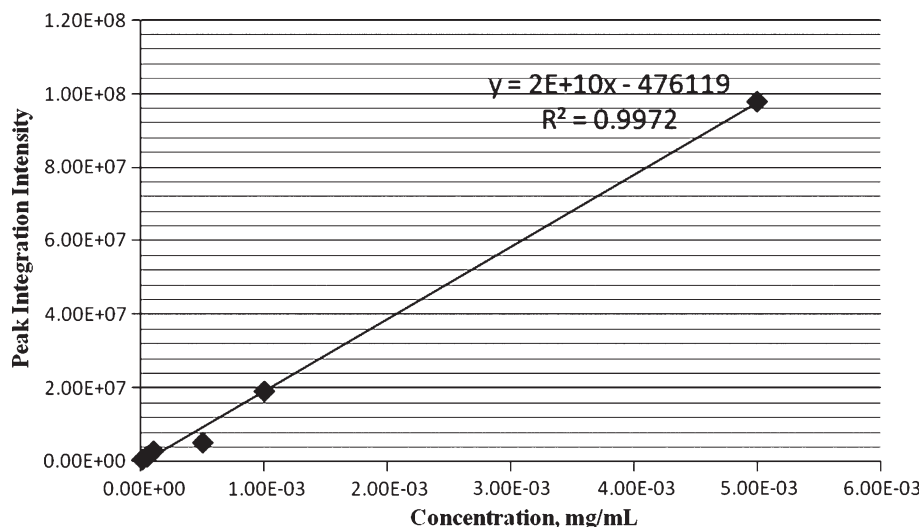


Fig. 2. DHEA-TMS external standard calibration curve.

RESULTS

Calibration curve

A six point calibration curve was obtained using a derivatized DHEA external standard. The DHEA concentration ranged from 10^{-5} to 5×10^{-3} mg/mL. The linear regression analysis yielded the following equation: $y = 10^2x - 476119$ with $r^2 = 0.997$, where y is the peak height, x is the DHEA-TMS concentration, and r^2 is the correlation coefficient (Fig. 2). Sensitivity and repeatability were evaluated by repeating derivatization and injection ($n=5$) at 10^{-8} – 10^{-4} mg/mL concentrations. We also calculated the detection limit ($S/N=3$) of the method, which was 0.3 pg/mL. The retention time of the standard derivatized DHEA was 9.08 min (Fig. 3A). The Q-EI mass spectrum showed a major peak at m/z 129, corresponding to the typical fragmentation of a 3-OH substituted steroid (Fig. 3B).

DHEA quantitation

After evaluation, DHEA was quantified in serum. The sera of the 86 subjects were extracted and derivatized using BSTFA reagent as described in the Methods Section. Figures 4 and 5 show full chromatograms of serum extracts from an AD and an age-matched control patient before (Figs 4A and 5A), and after (Figs 4B and 5B), oxidation. DHEA was identified by monitoring the specific fragment ions m/z 129 and m/z 73 in

mass spectrum at the same retention time found for the external standard (9.08 min) (Fig. 3).

DHEA increase is correlated with cognitive impairment in AD patients

The DHEA serum levels were measured based on the external standard method. For each subject, DHEA measurement was performed before and after oxidation. Brown-Forsythe's F-test showed equal intra-group variances (F ratio=0.846, Prob>F=0.473). The range of individual DHEA concentration values obtained for each group was very broad and did not reflect the severity of the pathology (Table 2, Fig. 6A–D). Ferrous-induced serum oxidation resulted in DHEA formation and an increase in DHEA concentration in the sera of age-matched control subjects (Table 2, Fig. 6A). The increase in individual DHEA values was less consistent, or even nonexistent, in the AD groups (Table 2, Fig. 6B–D). As a percentage of baseline, the DHEA increase was $52.59 \pm 7.71\%$ ($n=39$) in the age-matched control group (Fig. 6E). The mild and severe AD groups displayed significantly lower ferrous-mediated DHEA increases ($14.22 \pm 6.03\%$, $n=23$, $p<0.001$ and $3.52 \pm 7.39\%$, $n=14$, $p<0.001$, respectively) than the control group. The moderate group showed a trend toward a lower DHEA increase as well ($16.80 \pm 18.86\%$, $n=3$, $p=0.137$). This lack of significance may be due to the low number of subjects in this particular group. Bivari-

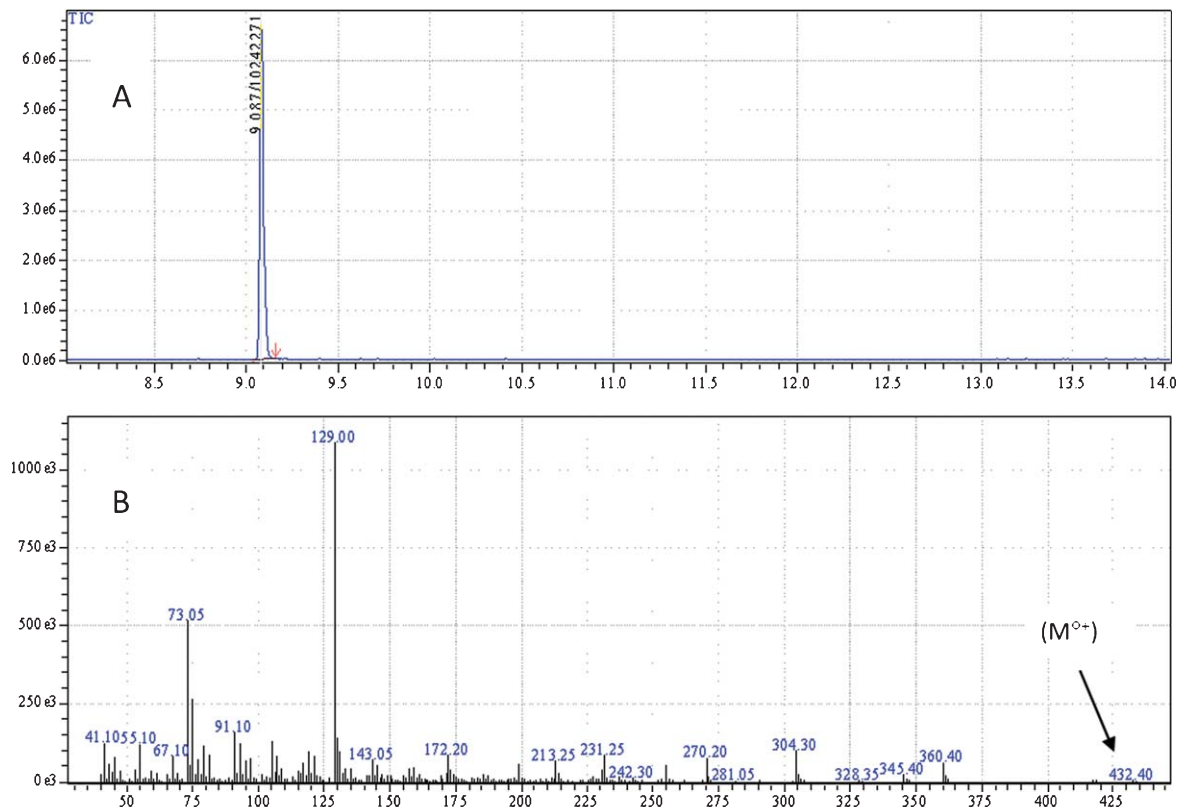


Fig. 3. A) DHEA-TMS chromatogram with the retention time of 9.08 min. B) DHEA-TMS quadrupole electronic impact mass spectrum showing the molecular ion m/z 432 and the most abundant ion fragments m/z 129 and m/z 73. Spectra were determined using standard solutions.

ate analysis showed a positive correlation between the level of cognitive impairment (MMSE) and the %DHEA increase ($r=0.41$, $p=2 \times 10^{-4}$, Fig. 6E). The characteristic elliptic shape of the 50% (green) and 90% (blue) density confirmed the normal repartition of the bivariate and the presence of an effect of the MMSE factor on the %DHEA increase.

To determine whether the correlation between %DHEA increase and cognitive impairment may be a function of the severity of the clinical phenotype, we recalculated the correlation factor as well as the bivariate normal ellipse that define the relationship between %DHEA increase and MMSE by omitting either the moderate/severe group or the mild group. The correlation coefficient ($r=0.389$) of mild AD versus control groups was lower than the moderate/severe and control groups ($r=0.454$) used for the analysis. This was confirmed by a flatter bivariate ellipse in the latter case.

MCI and AD biochemical differential diagnostic

Brown-Forsythe's F-test showed unequal intra-group variances (F ratio=6.572, Prob>F=0.0001).

Welch ANOVA analysis showed inter-group differences in the oxidation-induced DHEA increase (F ratio=5.485, Prob>F=0.0103). In the AD groups, MCI subjects displayed a broad range of basal DHEA concentration values (Table 2, Fig. 7A). Oxidation of sera from patients suffering from MCI showed, for the most part, an increase in DHEA levels (Table 2, Fig. 7A). Overall, the percent increase was significantly higher in MCI subjects than in age-matched controls ($131.23 \pm 76.46\%$, $n=7$, $p=0.0054$) (Fig. 7B), and other AD groups (Fig. 7B).

%DHEA increase/MMSE correlation and gender

When gender was included as a variable, ANOVA revealed that the %DHEA increase was a function of the severity of the symptoms, with age-matched controls displaying the highest increase, regardless of gender (Fig. 8A, 8C). The %DHEA increase/MMSE correlation was higher for women ($r=0.473$, $p=0.0016$) (Fig. 8D) than for men ($r=0.335$, $p=0.0426$) (Fig. 8B).

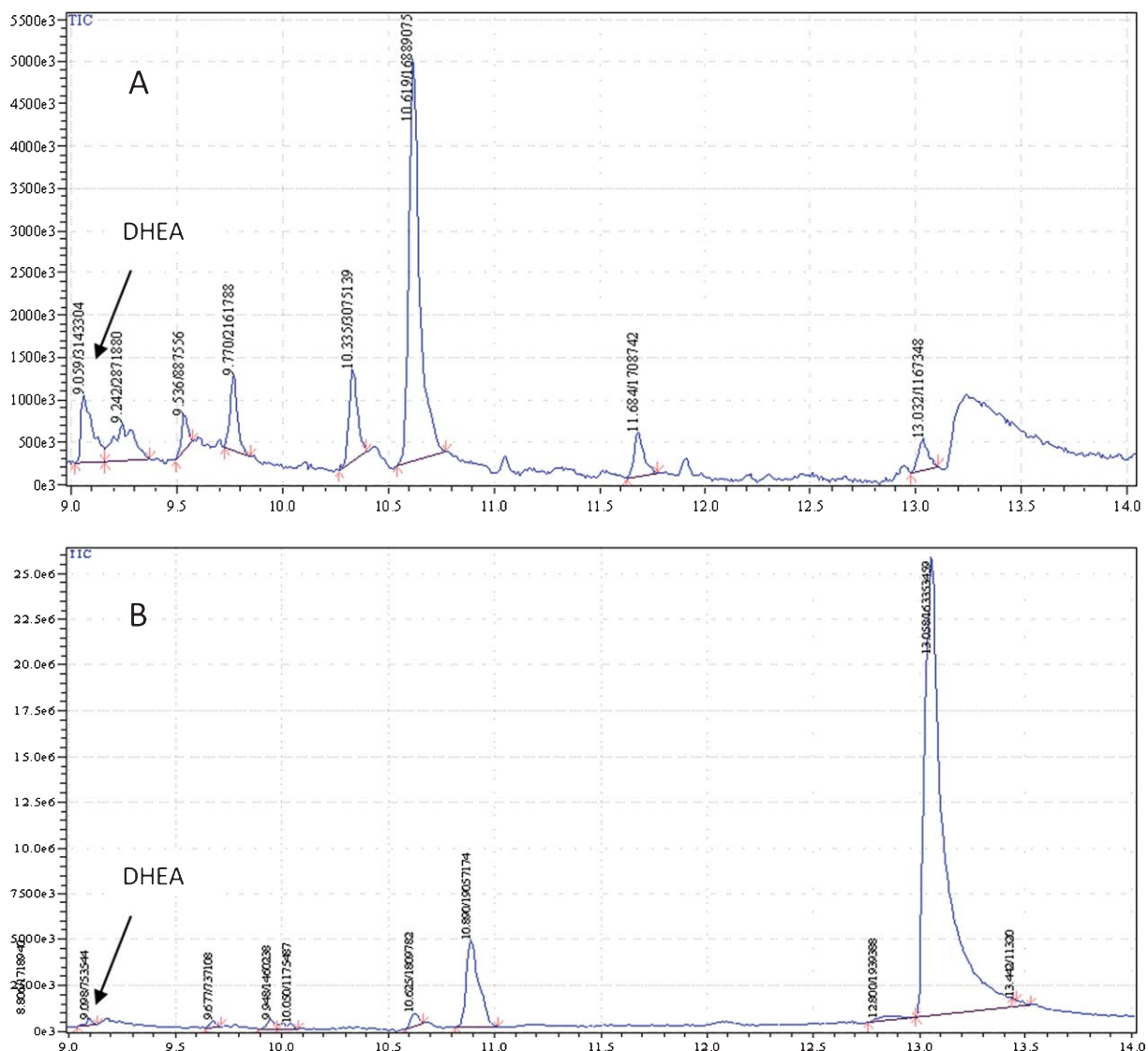


Fig. 4. High-resolution GC-SIM serum extracts chromatogram. A) Age-matched control before Fe^{2+} oxidation; B) age-matched control after Fe^{2+} oxidation.

DISCUSSION

We previously reported the presence of an alternative pathway for DHEA formation from an unknown precursor through oxidative stress induced by Fe^{2+} in rat, mouse, and human cells, as well as in brain samples [11, 14, 15]. Based on these observations we postulated that such a finding might help in the development of a simple non-invasive way to diagnose AD. Thus, we investigated the potential of sera from control and AD patients to form DHEA in response to an exogenous oxidative stress. A number of attempts have been made in the past to link oxidative stress with the pathogenesis

of AD [18]. In addition, high levels of DHEA have been found in brain tissue, independent from any peripheral brain tissue [9, 19]. Here, we report results from a clinical study conducted on 86 subjects showing that a blood test based on *in vitro* oxidative stress-mediated DHEA increases may be successfully used to diagnose AD at an early stage and to differentially diagnose AD from MCI.

Over the past four decades, numerous studies have evaluated circulating levels of neuroactive steroids, especially DHEA, in adult men [20–22]. Some authors used radioimmunoassay (RIA) following HPLC analysis [23], while others have developed new gas

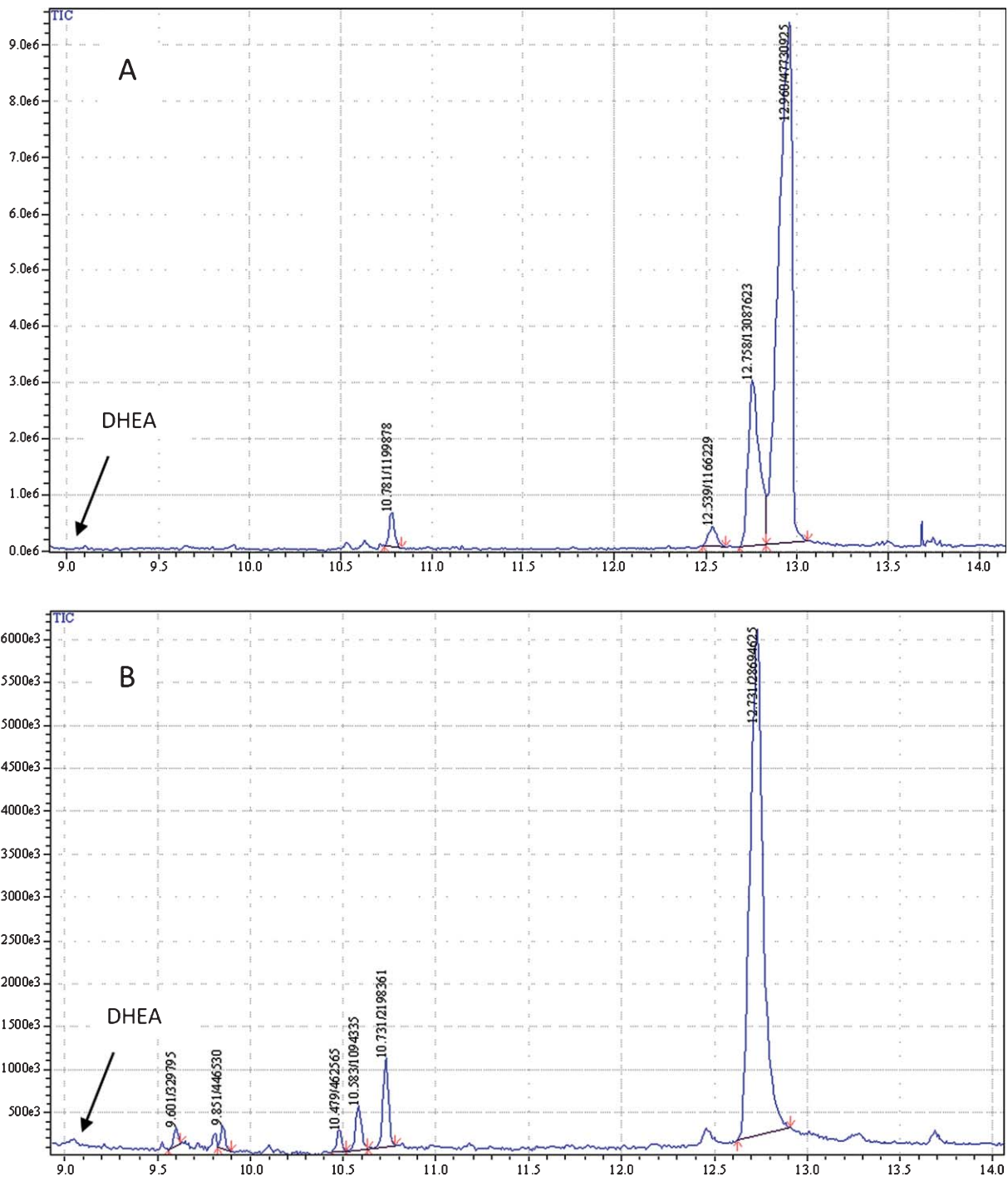


Fig. 5. High-resolution GC-SIM serum extracts chromatogram. A) Alzheimer's disease before Fe^{2+} oxidation; B) Alzheimer's disease after Fe^{2+} oxidation.

chromatography-mass spectrometry (GC-MS) methods [24–26] for profiling [27–29] and measurement of neuroactive steroids in serum, brain, and cerebrospinal fluid [30–32]. However, these analytical methods usu-

ally require relatively large sample volumes and are not sensitive enough to define physiological concentrations of the steroids under investigation [26, 33]. Recently, two studies reported elevated DHEA levels

Table 2
DHEA concentration range per group before and after serum oxidation

	DHEA range, pg/mL	
	Pre-oxidation	Post-oxidation
Control	80–13565	136–14794
Mild	0–6988	0–7591
Moderate	4774–5777	4997–8837
Severe	0–19961	0–16050
MCI	795–10221	2456–12456

in postmortem frontal cortex, hippocampus, hypothalamus, and cerebrospinal fluid of AD patients using RIA preceded by HPLC purification [16, 34]. In this

study, DHEA in the silylated form was detected and quantified by GC-MS in the sera before and after oxidation. Due to thermal instability and low volatility of the steroids, the chemical derivatization of DHEA hydroxyl and ketone functions by trimethylsilyl residues was necessary to make DHEA thermally stable and amenable to the GC-MS analysis [35]. Moreover, previously used gas chromatographic methods [26, 33] showed high detection limits varying between 0.1 and 10 ng/mL. In our method, the calculated detection limit was 0.3 pg/mL using the same S/N = 3. The main advantages of the GC/Q-EI-MS method used in this study are its sensitivity, repeatability, and the small

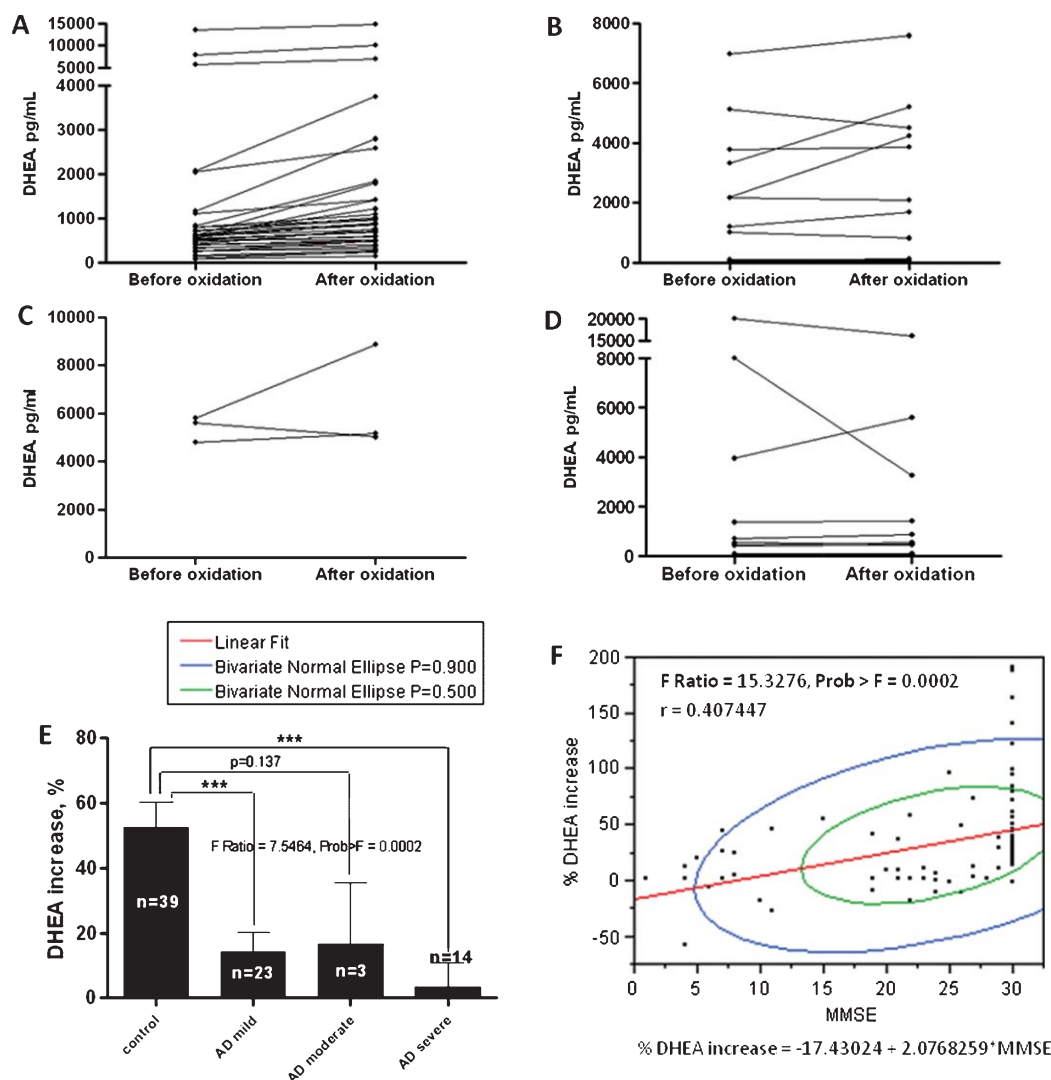


Fig. 6. Variation of the DHEA concentration (individual values and %) before and after Fe^{2+} oxidation of human sera from AD and age-matched control patients. A) Age-matched control; B) mild AD; C) moderate AD; and D) severe AD. E) ANOVA followed by *t*-test, mean \pm SEM, *** $p < 0.001$. F) Correlation between MMSE score and %DHEA variables and bivariate analysis of the normal repartition of the points in the 50th and 90th percentiles.

volume required (1 μ L) for DHEA-TMS detection. The development of this method allowed us to detect and quantitate even low DHEA concentrations in sera.

Recently published studies indicated that DHEA serum levels vary between 300 and 2000 pg/mL [35]. Our results showed that the range of DHEA levels measured in the serum of age-matched control subjects, although having a higher upper limit, was close to the values reported in other published studies, and in the same range depending on age scales and MMSE values [16, 31, 33, 36, 37]. Similar to our previous finding [16], our data confirmed that there was no significant difference in basal serum DHEA levels between AD subjects and age-matched controls (Table 2). Indeed, although the upper limit of the DHEA concentration range was found to be much lower in patients with moderate and mild AD than in age-matched controls, our results showed a complete overlap of the DHEA concentration range among all groups (Table 2). In our previous study [16], sera from age-matched control ($n=4$) and AD ($n=6$) patients were oxidized with Fe^{2+} . A dramatic increase of DHEA level was reported in the sera of healthy subjects, indicating the presence of an alternative DHEA precursor that can be transformed upon oxidation. However, no change in the DHEA levels was observed in the sera from AD patients, indicating the lack of the alternative precursor in these samples. We therefore hypothesized that a still unknown precursor is present in the brain and has the capacity to be effluxed into the blood stream and metabolized to DHEA in the presence of oxidative stress. We further hypothesized that this precursor is absent in AD serum because it is already converted within the brain by the disease-mediated oxidative stress. The present study confirmed our preliminary data.

In vitro oxidation of sera from aged-matched control subjects led to an over 50% increase in DHEA levels compared to their respective baseline. This increase was significantly higher than what we observed in the AD groups, where there were only 14% and 3% increases in the mild and severe AD groups, respectively. Although lower, the post-oxidation DHEA increase observed in the moderate AD group was not significantly different from that measured in the age-matched controls. This apparent discrepancy most likely reflects the low number of subjects in this group. A larger number of subjects with moderate AD would probably have reduced the standard deviation and refined the statistical analysis. Nevertheless, oxidative stress-mediated DHEA increase was inversely correlated with cognitive impairment, indicating a strong

impact of the MMSE factor on %DHEA increase. To determine if the correlation was influenced by the severity of disease, we conducted statistical analyses omitting either the moderate/severe or the mild group. Interestingly, the correlation factor was better when only age-matched control and moderate/severe AD groups were compared than when the mild AD group was included. This suggests that at an early stage of the disease, inter-individual differences due to the multifactor nature of AD may make the biochemical diagnostic less straightforward than at a later stage, when these differences have a tendency to disappear because of worsening pathology.

Interestingly, the correlation between cognitive impairment and percent DHEA increase in the oxidative pathway seemed to be stronger in women than in men, in whom the relationship appeared less linear. In addition, the correlation factor was much stronger for women in the low range of MMSE whereas it was stronger in the higher MMSE group for the men. However, more samples would be needed to be analyzed before reaching any conclusions on gender differences in the evolution and outcomes of AD and their detection using the proposed methodology. MCI is currently proposed as a transition state between normal aging and dementia [38]. Although MCI is a rather elusive entity for which no consensual definition has been provided, it is commonly divided into two groups: amnesic MCI which is thought to be an early stage of AD; and non-amnesic MCI which is associated with cognitive alterations other than memory, and is thought to prefigure frontotemporal, vascular, or Lewy body dementia [38]. Because MCI is considered an early stage of dementia and, early diagnosis can lead to clinically relevant treatment, a diagnostic tool for early stage identification of the disease is required to allow clinicians to discriminate MCI from AD. The present study demonstrates that, unlike the AD subjects, oxidation of sera collected from MCI patients led to a dramatic increase in DHEA concentration, far beyond what we observed with age-matched control subjects. The nature of the biochemical alterations observed in MCI as compared to AD patients suggests that MCI is not simply a symptom of pre-AD but might be more complex and heterogeneous, as shown by the amplitude of the standard deviation. Indeed, this intriguing preliminary set of data could be the sign of a functional overcompensation in MCI as recently described by functional MRI [39]. Nevertheless, this subset of data demonstrate that monitoring oxidative stress-mediated DHEA formation in sera allows one to discriminate MCI from healthy subjects, and more

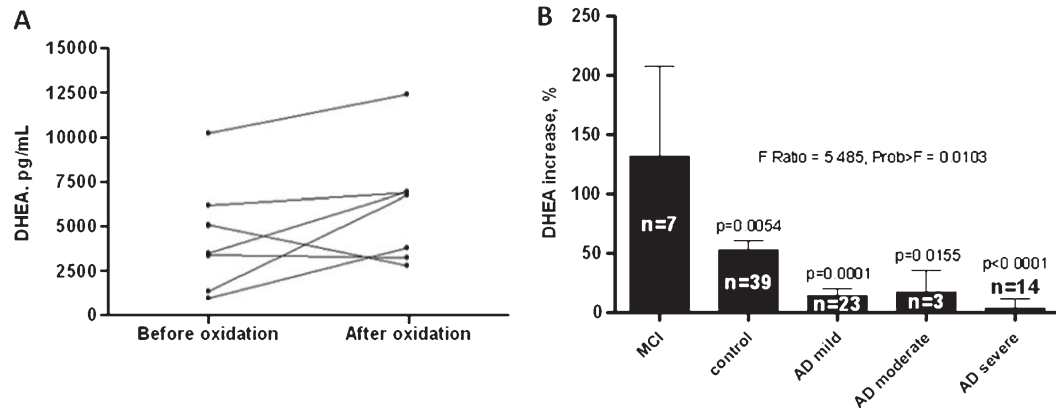


Fig. 7. Variation of the DHEA concentration (individual values and %) before and after Fe^{2+} oxidation of human sera from MCI patients. A) Individual values of MCI patients, B) ANOVA followed by t-test, mean \pm SEM. p values are comparisons between MCI and all other groups.

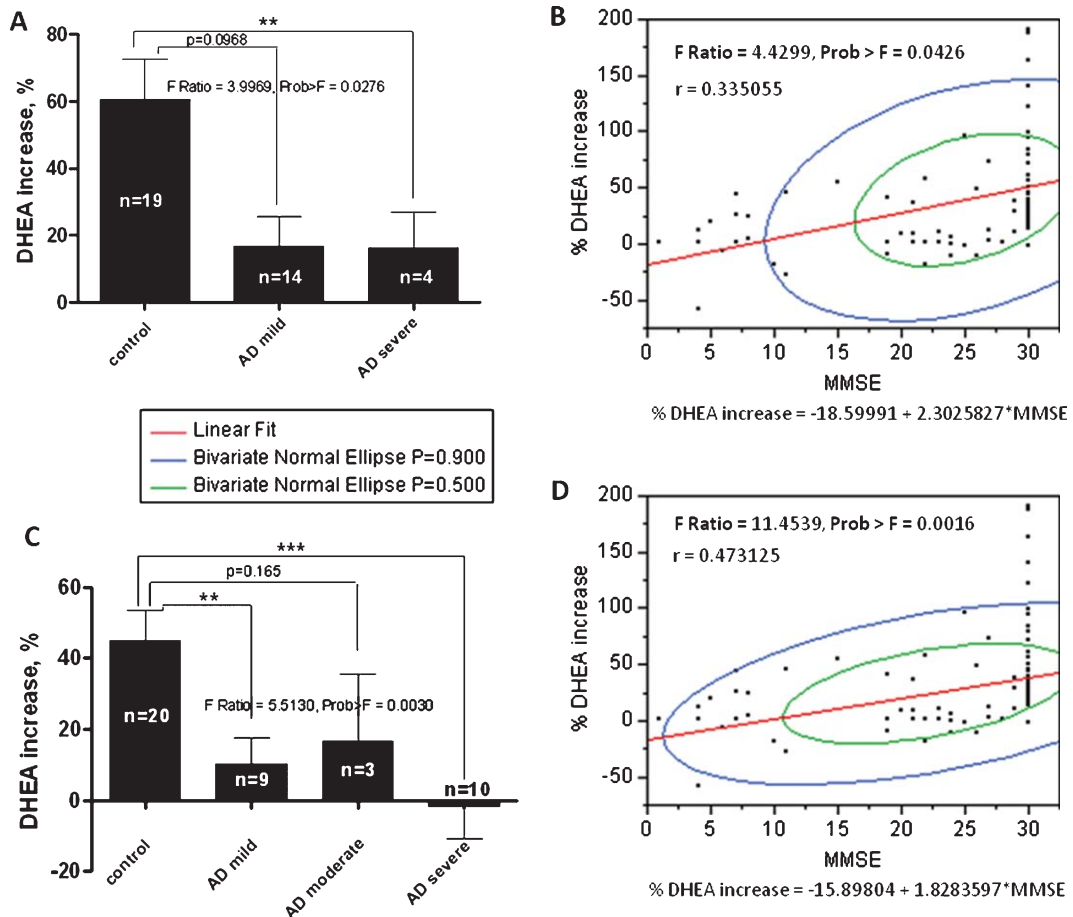


Fig. 8. Gender effect on the variation of the DHEA concentration (%) before and after Fe^{2+} oxidation measured in men (A and B) and women (C and D). A) ANOVA followed by t -test, mean \pm SEM, $**p < 0.01$. B) Correlation between MMSE score and %DHEA variables and bivariate analysis of the normal repartition of the points in the 50th and 90th percentiles. C) ANOVA followed by t -test, mean \pm SEM, $**p < 0.01$, $***p < 0.001$. D) Correlation between MMSE and %DHEA variables and bivariate analysis of the normal repartition of the points in the 50th and 90th percentiles.

so from AD patients, regardless of the severity of the disease status.

Many candidate disease-modifying therapies that target the underlying pathogenic mechanism of AD are currently in clinical trials. However, the clinically relevant implementation of any therapy is dependent on the reliability of the diagnosis. Currently, the diagnosis of AD follows a logical sequence: the family history information, the mental assessment, and the physical examination which, thus far, focuses on neurological signs [40]. An accurate, easy, and specific non-invasive biochemical test that correlates with clinical findings is needed. The results presented herein show that a blood assay based on the existence of a DHEA synthesis alternative pathway in the brain can be successfully used as an early diagnostic tool for AD. We demonstrate that the results of the assay are tightly correlated with cognitive deficits of AD patients and allow one to discriminate MCI from AD. Such an assay could be used to diagnose AD at a very early stage and monitor the effect of therapeutic modalities on the evolution of the disease.

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Authors' disclosures available online (<http://www.j-alz.com/disclosures/view.php?id=721>).

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