ORIGINAL ARTICLE

D-serine levels in Alzheimer's disease: implications for novel biomarker development

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Alzheimer's disease (AD) is a severe neurodegenerative disorder still in search of effective methods of diagnosis. Altered levels of the NMDA receptor co-agonist, D-serine, have been associated with neurological disorders, including schizophrenia and epilepsy. However, whether D-serine levels are deregulated in AD remains elusive. Here, we first measured D-serine levels in post-mortem hippocampal and cortical samples from nondemented subjects ($n = 8$) and AD patients ($n = 14$). We next determined D-serine levels in experimental models of AD, including wild-type rats and mice that received intracerebroventricular injections of amyloid-β oligomers, and APP/PS1 transgenic mice. Finally, we assessed D-serine levels in the cerebrospinal fluid (CSF) of 21 patients with a diagnosis of probable AD, as compared with patients with normal pressure hydrocephalus ($n = 9$), major depression ($n = 9$) and healthy controls ($n = 10$), and results were contrasted with CSF amyloid-β/tau AD biomarkers. D-serine levels were higher in the hippocampus and parietal cortex of AD patients than in control subjects. Levels of both D-serine and serine racemase, the enzyme responsible for D-serine production, were elevated in experimental models of AD. Significantly, D-serine levels were higher in the CSF of probable AD patients than in non-cognitively impaired subject groups. Combining D-serine levels to the amyloid/tau index remarkably increased the sensitivity and specificity of diagnosis of probable AD in our cohort. Our results show that increased brain and CSF D-serine levels are associated with AD. CSF D-serine levels discriminated between nondemented and AD patients in our cohort and might constitute a novel candidate biomarker for early AD diagnosis.

INTRODUCTION

Alzheimer's disease (AD) is a complex neurological disorder characterized by progressive memory loss and cognitive impairment. AD neuropathology includes brain deposition of amyloid plaques, neurofibrillary tangles and significant synapse loss.[1,2] AD remains as a largely idiopathic disorder, although mounting evidence suggests that levels of amyloid-β oligomers (AβOs) build up in patient brains to cause synapse failure and memory loss.[3,4]

Currently, diagnosis of probable AD is based on neuropsychological testing, fluid biomarker assessment and brain imaging,[5–9] but diagnosis of the earliest stages of AD, before major brain damage takes place, is still challenging. In order to improve diagnostics and to allow treatment to be initiated at the earliest possible stage, there is an urgent need to incorporate biomarkers capable of detecting disease onset or at early stages. In this context, cerebrospinal fluid (CSF) levels of amyloid-β_{1-42} (Aβ42), total tau protein and hyperphosphorylated tau (p-tau) have now been included in diagnostic guidelines.[6,10] Such CSF biomarkers have been advocated for research purposes, but sensitivity and specificity issues have generally raised concerns about their widespread clinical application.[5,6,11]

Aberrant activation of glutamate receptors of the *N*-methyl-D-aspartate subtype (NMDARs) has been associated with synapse dysfunction and neurotoxicity in AD.[12–17] Accordingly, memantine (an open-channel blocker of NMDARs) has been approved for clinical use in patients with moderate-to-severe AD.[18] D-serine is the main co-agonist at NMDARs in frontal brain areas[19–21] and has been implicated in NMDAR-mediated neurotoxicity.[22] Consistent with a possible role in pathological states, *in vitro* studies have shown increased release of D-serine from both glial and neuronal cells and NMDAR activation under injury, in particular in AD model systems.[23,24] On the other hand, exogenous D-serine administration may improve behavioral deficits in experimental models[25,26] and may act as a NMDAR antagonist under some circumstances.[27]

Literature reports on changes in D-serine levels in AD brains have been controversial. Early studies reported unaltered D-serine levels in the frontal and parietal cortices of AD patients,[28–30] whereas another study found increased D-serine levels in the CSF of AD patients compared with healthy controls.[31]

The goal of the current study was to investigate whether D-serine levels are deregulated in AD, and to assess its potential as a novel biomarker in AD. We initially investigated D-serine levels in post-mortem AD brains in comparison with brains from

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cognitively intact control subjects. To determine whether there was a causal relationship between AβO toxicity and D-serine levels, we next studied D-serine levels in cellular and animal models of AD. Finally, we collected CSF and measured D-serine levels in patients with probable AD, major depression or hydrocephalus and healthy controls. Results showed elevated D-serine levels in brain tissue from AD patients in comparison with controls. In experimental models, we found that Aβ oligomers caused elevations in D-serine levels, likely via upregulation of serine racemase (SR). Further, we found increased D-serine levels in the CSF of patients with probable AD. Incorporation of D-serine measurements into the amyloid-tau biomarker index significantly increased diagnostic sensitivity and specificity in our cohort, suggesting that CSF D-serine determination may constitute a simple and effective manner to improve in vivo diagnosis of AD.

MATERIALS AND METHODS

Study approval
Experiments using human samples were approved by local ethics committees from each participating institution. All study subjects or their next-of-kin (in the case of post-mortem samples) provided written informed consent for study participation. Experiments in animals were approved by the Institutional Animal Care and Use Committee of the Federal University of Rio de Janeiro (protocols # IBqM 022, 041 and 055).

Post-mortem samples
Post-mortem tissue samples were obtained from the Brain Bank of the Brazilian Aging Brain Study Group,[32] School of Medicine of the University of Sao Paulo. Brains were obtained from the Sao Paulo Autopsy Service, after written informed consent. We studied 17 cases with a neuropathological diagnosis of AD confirmed for the presence of pathological hallmarks by an experienced neuropathologist, and 12 cases without neuropathological changes. The clinical dementia rating (CDR) was determined by a validated interview conducted with the informant caregiver.[33,34] The control group consisted of cases with CDR = 0, whereas the AD group included cases with CDR ranging from 1 to 3. Demographic characteristics of those groups are presented in Table 1. More detailed information on individual subjects is provided in Supplementary Table 1.

CSF samples
Twenty-one patients with probable AD were recruited from the AD Center at the Institute of Psychiatry of the Federal University of Rio de Janeiro (IPUB/UFRJ). Patients with probable AD were diagnosed according to the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS-ADRDA) and the Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria, using a combination of clinical evaluation, neuropsychological testing and biomarker (Aβ and total tau protein) assessment, as described.[35] Nine nondemented patients diagnosed with major depression according to Diagnostic and Statistical Manual of Mental Disorders, 4th edition criteria were also recruited at IPUB/UFRJ. Ten healthy control subjects and nine patients with normal pressure hydrocephalus, diagnosed according to the International Classification of Diseases, 10th edition, were recruited at the Neurolife Laboratory, a private clinic specialized in CSF analysis in the city of Rio de Janeiro. Patients were

subjected to the mini-mental state exam to assess cognitive performance. All patients included in the study were older than 60 years of age. Exclusion criteria for all groups included psychiatric and neurological diagnoses other than AD and major depression, any unstable clinical diagnoses, cigarette smoking (more than 10 packs per year) and alcohol abuse. CSF samples were collected through lumbar puncture in the L3–4 or L4–5 interspace and were immediately stored at − 80 °C. All lumbar punctures were performed around 1100 hours in order to minimize possible circadian fluctuations in the concentrations of analytes.

Demographic characteristics for the four subject groups are presented in Table 1. More detailed information on individual subjects is provided in Supplementary Table 2. Studied groups were significantly different in terms of gender distribution. However, D-serine levels were similar between males and females across diagnostic groups ($c^2 = 0.23$; $P = 0.63$) (Supplementary Figure 1).

Psychotropic medication used by probable AD patients included rivastigmine (47.6%; $n = 10$), risperidone (38.1%; $n = 8$), memantine (28.6%; $n = 6$), donepezil (23.8%; $n = 5$), clonazepam (19.0%; $n = 4$), citalopram (4.8%; $n = 1$), trazodone (4.8%; $n = 1$), biperiden (4.8%; $n = 1$), escitalopram (4.8%; $n = 1$) and mirtazapine (4.8%; $n = 1$). Two (9.5%) patients with probable AD were not taking any medication at the time of the study. None of the medications used showed any significant effect on CSF D-serine levels (Supplementary Table 3).

Biochemical analyses of human samples
Post-mortem tissue was homogenized in buffer containing 20 mM Tris-HCl (pH 7.4), 2 mM EDTA and a cocktail of protease inhibitors (Roche complete mini, Basel, Switzerland). D-serine, L-serine and glycine levels in tissue homogenates, CSF and culture media were measured by high-performance liquid chromatography as previously described.[36,37] Amino-acid concentrations were expressed per gram of total proteins in tissue homogenates or as actual concentration in CSF and culture media.

CSF levels of p-tau, total tau protein and Aβ42 were measured using commercially available enzyme-linked immunosorbent assays (ELISA INNOTEST p-tau181, INNOTEST htau, INNOTEST β-amyloid(1-42) kits, respectively; Innogenetics, Gent, Belgium) according to manufacturer's instructions. The INNOTEST amyloid/tau index (IATI) was calculated as:

$$IATI = A\beta 42/(240 + 1.18 * t-tau) \qquad (1)$$

Soluble AβOs
AβOs were prepared weekly from synthetic Aβ$_{1-42}$ (American Peptide, Sunnyvale, CA, USA), and were routinely characterized by size-exclusion chromatography and, occasionally, by western immunoblots and transmission electron microscopy, as previously described.[38,39] Oligomers were stored at 4 °C and were used within 48 h of preparation.

Mature hippocampal cultures
Primary rat embryo hippocampal neuronal cultures, prepared and developed in Neurobasal medium supplemented with B27 (Invitrogen, Carlsbad, CA, USA) and antibiotics according to established procedures,[16] were used after 18–21 days in vitro. Cultures were exposed for 24 h to 500 nM AβOs or an equivalent volume of vehicle (2% dimethyl sulfoxide in phosphate-buffered saline) at 37 °C.

Animals
C57Bl/6 wild-type (WT) mice were obtained from the animal facility at CECAL/FIOCRUZ (Rio de Janeiro, Brazil). Three-month-old mice received a single intracerebroventricular injection of either vehicle (2% dimethyl sulfoxide in phosphate-buffered saline) or Aβ oligomers (10 pmol total Aβ, corresponding to 45 ng) as described.[40,41] After 8 days, animals were killed and had their hippocampi collected for D-serine analysis.

APPSwe/PS1ΔE9 transgenic mice on a C57Bl/6 background[42] were obtained from the Jackson Laboratories (Bar Harbor, ME, USA). WT littermates were used as controls. For D-serine assessment in APP/PS1 brains, nine animals aged 14–16 months were used per experimental group.

Surgical procedures in adult male Wistar rats were performed as described,[43] with slight modifications. A sagittal incision was made on the scalp and a small craniotomy was performed unilaterally to implant a cannula in the hippocampus (A/P: − 3.0 mm; L: 1.5 mm; D/V: 3.5 mm). After 7–10 days of recovery from surgery, animals were chronically injected with 1 μg of AβOs or vehicle (2% dimethyl sulfoxide in saline; 3–5 μl per

Table 1. Demographic characteristics of individual subjects in post-mortem analysis

	Control	Alzheimer's disease	Statistics
Age, years (range)[a]	74.7 (11.5)	80.9 (4.7)	2.60 (0.09)
Sex, male/female[b]	7/5	6/11	1.56 (0.46)
Post-mortem Interval, h[a]	14.2 (3.2)	12.4 (3.5)	1.20 (0.31)

Abbreviation: ANOVA, analysis of variance. Values are presented as means (s.d.). Statistical significance is given by [a]One-way ANOVA, F (P-value). [b]X^2-test (P-value).

injection) once a week for 5 weeks. Rats were euthanized and samples collected 3 days after the last AβO injection.

In all experiments, animals were caged in groups of five with controlled room temperature and humidity. Animals had free access to food/water and were under a 12-h light/dark cycle.

Biochemical analyses in experimental models

Homogenates from hippocampi or primary neuronal cultures were prepared in RIPA buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA and 1% Triton X-100) containing protease and phosphatase inhibitor cocktails (Thermo-Pierce, Rockford, IL, USA). Protein concentration was determined by the BCA assay (Thermo-Pierce). Homogenates or conditioned media were treated with trichloroacetic acid (5% final concentration) to precipitate proteins and extract free amino acids. Samples were centrifuged (20 000 g for 5 min), the supernatants were extracted three times with water-saturated diethyl ether to remove trichloroacetic acid and amino acids were measured by high-performance liquid chromatography, as described.[36,37,44]

For western blot analysis, soluble lysates (30 μg protein applied per lane) were resolved on 10% SDS-polyacrylamide gel electrophoresis, electro-transferred onto nitrocellulose membranes and probed using anti-serine racemase antibody (BD Biosciences, San Jose, CA, USA; 1:500). β-actin (Abcam, Cambridge, UK; 1:50 000) was used as a loading control. Immunoblots were developed with SuperSignal West Femto Maximum Sensitivity substrate (Thermo Scientific, Waltham, MA, USA) and imaged on photographic film.

For determination of SR messenger RNA levels in primary neuronal cultures exposed to vehicle or AβOs, total RNA was extracted with Trizol (Life Technologies, Carlsbad, CA, USA). RNA characterization and complementary DNA synthesis were performed as described.[38,41] Quantitative reverse transcription-PCR protocols were performed using specific primers for SR (forward 5′-TAGCGGGACAAGGGACAATT-3′; reverse 5′-TGCATACTT GATTTCATCTTCCGTG-3′) and β-actin (forward 5′-GTCTTCCCCTCCATCG TG-3′; reverse 5′-AGGATGCCTCTCTTGCTCTG-3′), as described.[41] Results were analyzed according to the $2^{-(\Delta\Delta Ct)}$ method[45] and are shown normalized by levels in vehicle-treated cultures.

Statistical analyses

Results from human samples are presented as means ± s.d., except for analysis of covariance results, which are presented as means ± s.e. The distributions of D-serine and L-serine levels were evaluated for normalcy, and winsorized means were calculated if outliers were present. For D-serine, we adjusted one outlier in the data set for the hippocampus and two outliers in the data set for the occipital cortex in the AD group. For L-serine, we adjusted one outlier in the CSF study data set in the AD group and two outliers in the hydrocephalus group; one outlier in the hippocampus data set, one in the parietal cortex data set and one in the occipital cortex in the AD group. For glycine, we adjusted one outlier in the CSF study data set in the AD group. Statistical significances of differences between groups were determined by analysis of covariance followed by Bonferroni's multiple comparison tests. Associations between measures were analyzed by Pearson's bivariate correlation. Effect size was measured by Cohen's test, and post hoc statistical power analysis was performed using G Power (University of Dusseldorf; available at http://www.gpower.

hhu.de[46]) to determine the minimal sample size that would be required for a duplication effect under our conditions.

In experimental studies, analyses were performed with GraphPad Prism (La Jolla, CA, USA) and data sets were assessed for normality prior to significance determination. Values are expressed as means ± s.e.m., unless otherwise stated. Significance was set at 5% in two-sided tests.

RESULTS

Brain D-serine levels in neuropathologically confirmed AD

We initially investigated D-serine levels in post-mortem samples from three brain regions: hippocampus, parietal and occipital cortices. Cases were divided in two groups: controls (cases without clinical dementia or neuropathology) and AD (cases with clinical signs of dementia and neuropathology typical of full-blown AD). Demographic characteristics of cases are presented in Table 1. Because the AD group was significantly older (80.9 ± 4.7 years) than the control group (74.7 ± 11.5 years), age was entered as a covariate in the analyses.

After adjustment for age, D-serine levels were significantly higher in AD brains compared with controls in both the hippocampus (Figure 1a) and parietal cortex (Figure 1b), two regions severely affected by AD. In contrast, D-serine levels were not significantly different between groups in the occipital cortex, an area more resistant to AD pathology (Figure 1c). Glycine levels were unchanged in the hippocampus and occipital cortex, but were fairly elevated in the parietal cortex of AD patients in comparison with controls (Table 2). No significant differences between groups were observed in brain levels of L-serine and total serine (Table 2). Results demonstrate that D-serine levels are elevated in post-mortem brain regions affected by AD.

Soluble AβOs increase D-serine levels in hippocampal cultures

To gain insight into the mechanism underlying elevated D-serine levels in AD brains, we examined the impact of soluble AβOs, which accumulate in AD brains and are thought to trigger synapse failure, on D-serine levels in cultured hippocampal neurons. Exposure of cultures to AβOs (500 nM) for 24 h significantly increased D-serine levels in conditioned medium compared with vehicle-treated cultures (Figure 2a). We next looked at messenger RNA and protein levels of SR, the enzyme responsible for synthesis of D-serine.[47,48] AβOs increased SR at both messenger RNA (Figure 2b) and protein levels (Figure 2c) in hippocampal cultures, likely explaining the increase in D-serine release to the medium in such cultures.

D-serine is elevated in the brains of AβO-injected rodents and of APP/PS1 transgenic mice

To determine whether AβO – induced upregulation of D-serine levels occurs in vivo, we chronically injected AβOs into rat
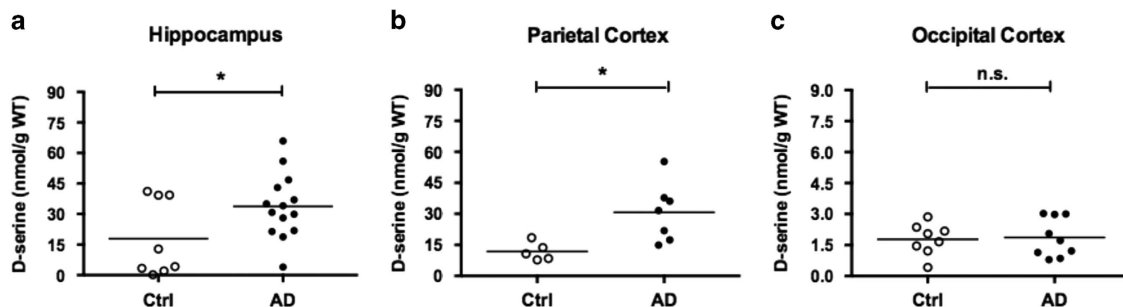

Figure 1. D-serine levels are increased in Alzheimer's disease (AD) post-mortem brain tissue. D-serine levels in the hippocampus (**a**), parietal cortex (**b**) and occipital cortex (**c**) of post-mortem samples from control (Ctrl) and AD subjects. Values are presented as nmol of D-serine per g of wet tissue (WT). Horizontal lines represent mean values for each diagnostic group. Data points correspond to individual values. Statistical significance is given by the Student's *t*-test (*P < 0.05; NS, not significant).

Amino acid (nmol g^{-1} WT)	Control	AD	ANCOVA F (P-value)
Hippocampus			
ᴅ-serine	18.1 (5.1)	33.0 (4.0)[a]	9.00 (0.001)*
ʟ-serine	1371 (234)	1061 (175)	0.56 (0.58)
Total serine	1388 (243)	1270 (182)	0.11 (0.90)
Glycine	1191 (255.6)	1017 (249)	1.05 (0.13)
Parietal cortex			
ᴅ-serine	11.8 (5.44)	36.2 (4.43)[a]	6.08 (0.013)*
ʟ-serine	2453 (390)	2434 (308)	0.54 (0.60)
Total serine	2465 (388)	2473 (307)	0.54 (0.60)
Glycine	3679 (57.2)	4058 (140.1)	6.00 (0.0005)*
Occipital cortex			
ᴅ-serine	1.73 (0.34)	1.76 (0.29)	0.49 (0.62)
ʟ-serine	75.0 (17.1)	110.5 (14.4)	2.53 (0.10)
Total serine	76.7 (22.2)	128.4 (18.7)	2.02 (0.16)
Glycine	194 (43.7)	284.5 (202.2)	21.42 (0.23)

Table 2. Amino-acid levels in post-mortem brain tissue samples

Abbreviations: AD, Alzheimer's disease; ANCOVA, analysis of covariance; WT, wet tissue. Values are presented as means (s.e.). P-values were given by ANCOVA using age as covariate, followed by the Bonferroni adjustment for multiple comparisons. Asterisks indicate statistically significant differences. [a]AD significantly different from control ($P < 0.05$).

hippocampi for 5 weeks (see Materials and methods). AβOs increased hippocampal ᴅ-serine levels compared with levels measured in hippocampi of vehicle-injected rats (Figure 3a). We next examined WT C57Bl/6 mice that received a single intracerebroventricular injection of either vehicle or 10 pmol AβOs, recently shown to cause memory/behavioral impairments.[40,41] We found significantly increased ᴅ-serine levels in the hippocampi of AβO-injected mice compared with vehicle-injected animals (Figure 3b). Importantly, levels of ʟ-serine (Supplementary Figure 2a) and glycine (Supplementary Figure 2b), another NMDAR co-agonist, were unaltered by AβO injection, likely not reflecting a global deregulation of amino-acid levels triggered by AβOs.

Further, we measured ᴅ-serine levels in the brains of APPSwe, PS1ΔE9 (APP/PS1) mice, which harbor transgenes for human amyloid precursor protein (APP) bearing the Swedish mutation and a mutant form of presenilin 1 (PS1). These animals display elevated Aβ levels and develop age-related cognitive
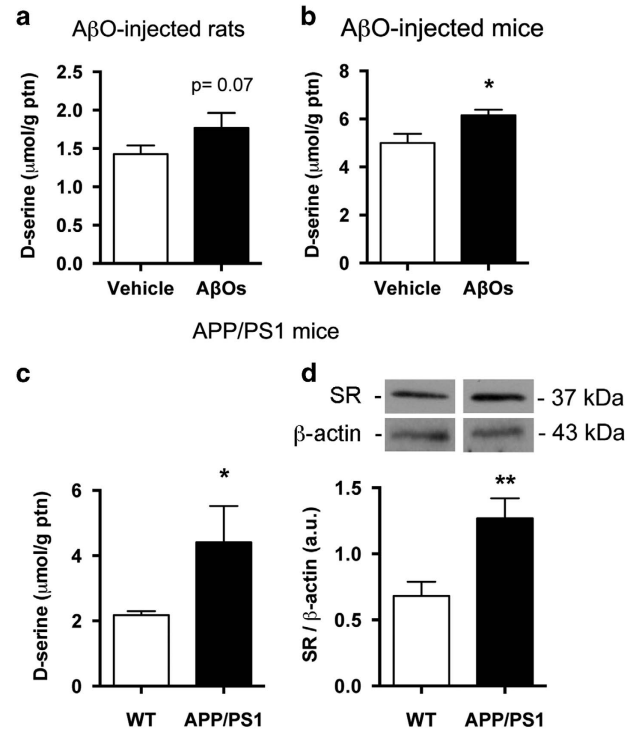

Figure 3. Amyloid-β oligomers (AβOs) increase hippocampal ᴅ-serine and serine racemase (SR) levels *in vivo*. (**a**) AβOs increased ᴅ-serine levels in hippocampal homogenates of rats that received intrahippocampal injections of AβOs (1 μg) or vehicle (2% dimethyl sulfoxide in phosphate-buffered saline) once a week for 5 weeks ($n = 8$ veh; $n = 7$ AβOs), as analyzed 3 days after the last injection. (**b**) ᴅ-serine content is increased in the hippocampi of mice that received a single intracerebroventricular injection of AβOs (10 pmol, or 45 ng). ᴅ-serine levels were measured 8 days post injection ($n = 10$ per group). (**c** and **d**) Thirteen- to fourteen-month-old APPSwe/PS1ΔE9 (APP/PS1) transgenic mice showed increased hippocampal levels of ᴅ-serine (**c**) and SR (**d**) compared with wild-type (WT) mice ($n = 8$ per group). ᴅ-serine was measured by high-performance liquid chromatography and its values were corrected by total protein (ptn) content in the analyzed samples. SR protein levels were detected by western blotting using β-actin as the loading control. *$P < 0.05$; **$P < 0.01$ (Student's *t*-test), statistical significances were assessed in comparison with controls. Results are presented as means ± s.e.m. of individuals.
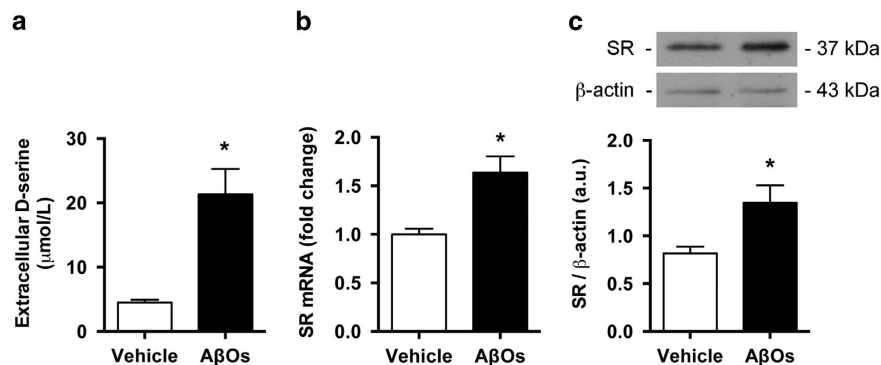
Figure 2. Amyloid-β oligomers (AβOs) increase ᴅ-serine and serine racemase (SR) levels in hippocampal cultures. Primary rat hippocampal neuronal cultures were exposed to 500 nᴍ AβOs or vehicle (2% dimethyl sulfoxide in phosphate-buffered saline) for 24 h. (**a**) AβOs increased extracellular levels of ᴅ-serine. (**b** and **c**) AβOs increased total levels of SR messenger RNA (mRNA) (**b**) and protein (**c**). ᴅ-serine was measured by high-performance liquid chromatography and its values were corrected by total protein content in the analyzed samples. SR protein levels were detected by western blotting, using β-actin as a loading control. *$P < 0.05$ (Student's *t*-test), statistical significance was assessed in comparison with control. Results are expressed as means ± s.e.m. of three independent experiments (each carried out in triplicate wells) with different neuronal cultures.
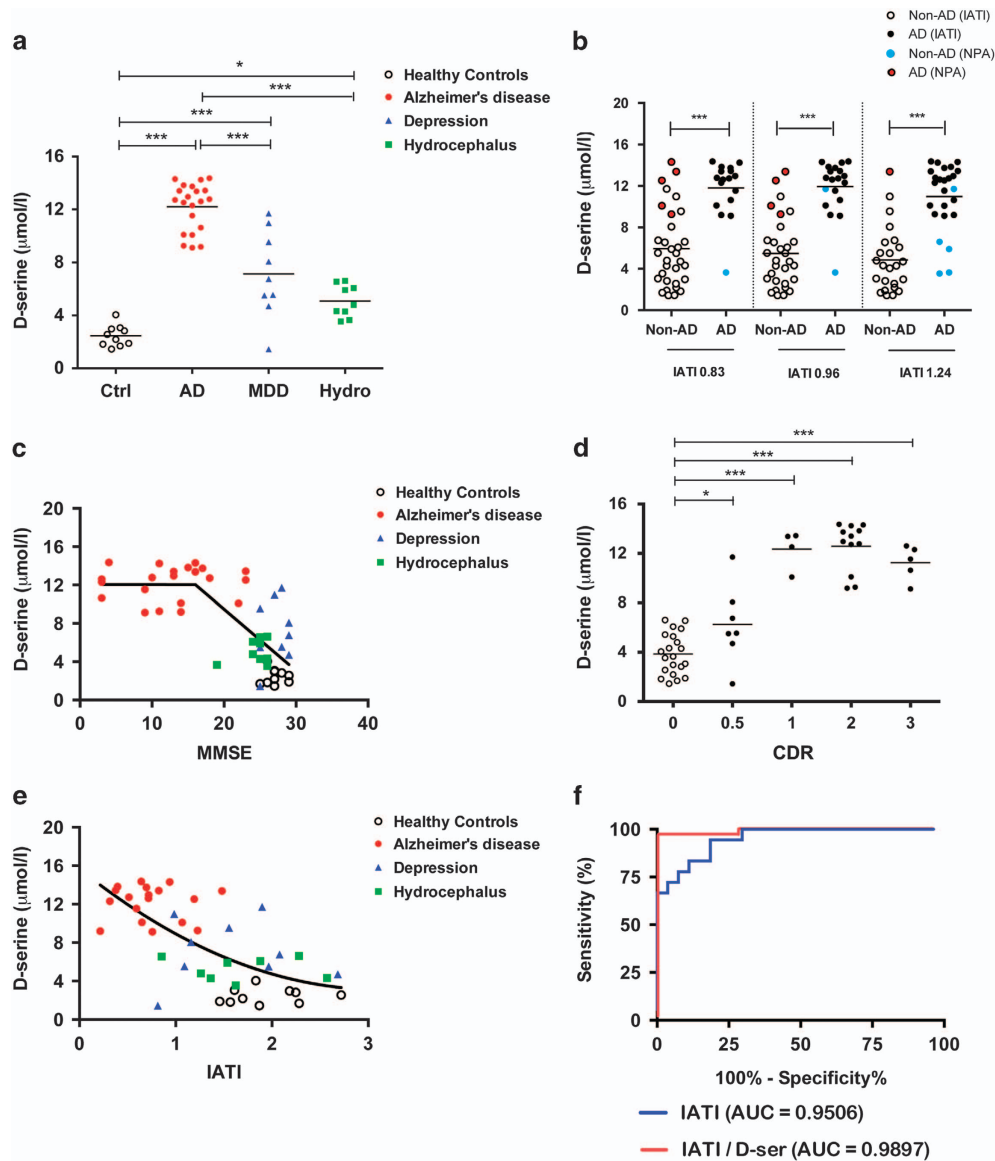
Figure 4. Increased cerebrospinal fluid (CSF) levels of D-serine in patients with probable Alzheimer's disease (AD). (**a**) CSF levels of D-serine in healthy controls (Ctrl) and in patients with probable AD, major depressive disorder (MDD) or hydrocephalus (Hydro). Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Bonferroni adjustment for multiple comparisons. (**b**) CSF D-serine levels (μmol l^{-1}) in non-AD (white circles for INNOTEST amyloid/tau index (IATI)-based classification; blue circles for neuropsychological assessment) and AD cases (black circles for IATI-based classification; red circles for neuropsychological assessment) using different IATI cutoffs (0.83; 0.96; 1.24). Horizontal bars represent mean values for each group. ***$P < 0.001$; Student's t-test. (**c**) CSF levels of D-serine as a function of the mini-mental state exam (MMSE). (**d**) CSF levels of D-serine as a function of CDR scores. Horizontal lines represent mean values for each CDR group. Data points correspond to individual values. Statistical significance is given by one-way ANOVA followed by Bonferroni adjustment for selected groups: CDR 0.5, 1, 2 and 3 versus CDR 0. (**e**) CSF levels of D-serine as a function of IATI across subject groups. (**f**) Receiver-operating characteristic curves for diagnostic based on IATI alone (blue line) or IATI/D-ser levels (red line), showing increased sensitivity and specificity when D-serine is added to the calculation. *$P < 0.05$; ***$P < 0.001$. CDR, clinical dementia rating score; NPA, neuropsychological assessment.

deficits.[42,49,50] In harmony with our hypothesis, we found markedly increased hippocampal D-serine levels in APP/PS1 mice compared with WT littermates (Figure 3c). This was accompanied by increased SR levels in the hippocampus (Figure 3d). Levels of L-serine (Supplementary Figure 2c) and glycine (Supplementary Figure 2d) were similar in WT and APP/PS1 mice. Results thus establish that AβOs instigate increases in hippocampal D-serine and SR levels *in vivo*.

CSF levels of D-serine in patients with probable AD
Given our findings in post-mortem brains and in experimental models of AD, we next investigated whether CSF levels of D-serine

were altered in a group of patients with probable AD, as compared with healthy controls, patients with major depression or patients with hydrocephalus. Remarkably, mean D-serine levels in probable AD patients were approximately fivefold higher than in healthy controls, and about twofold higher than in the depression and hydrocephalus patient groups (Figure 4a and Table 3), yielding an effect size (d, Cohen's test) of 7.1 between controls and probable AD subjects. Mean D-serine levels in the major depression and hydrocephalus groups were also significantly higher than in healthy controls (Figure 4a and Table 3). Further, when biomarker combination (IATI) was used to separate AD from non-AD cases (using either 0.83, 0.96 or 1.246 as cutoffs,

Table 3. Amino-acids levels in CSF samples

Amino acid (μmol l^{-1})	Control	Alzheimer's disease	Depression	Hydrocephalus	ANOVA F (P-value)
D-serine	2.45 (0.65)	12.32 (0.44)[a]	5.14 (3.28)[b]	5.08 (1.22)[c]	68.79 (0.0001)*
L-serine	27.52 (9.28)	30.86 (4.99)	35.75 (11.68)	29.27 (13.15)	1.40 (0.26)
Total serine	29.97 (9.02)	43.07 (4.22)[d]	42.88 (11.24)[e]	34.36 (12.43)	6.70 (0.001)*
Glycine	291.6 (68.36)	336.9 (39.21)	322.9 (44.95)	258.2 (18.11)[f,g]	7.22 (0.0005)*

Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of covariance; CSF, cerebrospinal fluid. Values are presented as means (s.d.). P-values were given by one-way ANOVA followed by the Bonferroni adjustment for multiple comparisons. Asterisks indicate statistically significant differences. [a]AD significantly different from control, depression and hydrocephalus ($P=0.0001$). [b]Depression significantly different from control ($P=0.0001$). [c]Hydrocephalus significantly different from control ($P=0.03$). [d]AD significantly different from control ($P=0.002$). [e]Depression significantly different from control ($P=0.01$). [f]Hydrocephalus significantly different from AD ($P=0.005$). [g]Hydrocephalus significantly different from depression ($P=0.005$).

Table 4. Demographic, clinical and biomarker characteristics of CSF donor subjects

	Control	Alzheimer's disease	Depression	Hydrocephalus	Statistics
Age, years	70.7 (6.3)	72.1 (8.4)	69.8 (5.8)	74.6 (7.4)	0.71 (0.55)
Sex, male/female	2/8	9/12	0/9	5/4	5.90 (0.01)*
Education, years	7.9 (5.1)	4.8 (4.8)	2.7 (2.6)	7.6 (5.7)	2.66 (0.06)
MMSE	27.1 (1.3)	12.7 (6.2)[a]	24.4 (2.2)	27.2 (1.8)	39.66 (0.0001)*
Disease duration, months	NA	44.8 (28.2)	NA	24.7 (13.6)	NA
IATI	1.95 (0.40)	0.74 (0.34)[a]	1.58 (0.62)	1.67 (0.56)	18.29 (0.0001)*

Abbreviations: ANOVA, analysis of variance; CSF, cerebrospinal fluid; IATI, INNOTEST amyloid tau index; MMSE, mini-mental state examination; NA, not applicable. Values are presented as means (s.d.). Statistical significance presented as F (P-value) based on one-way ANOVA followed by the Bonferroni adjustment for multiple comparisons, except for sex, which is given by the X^2-test (P-value). Asterisks indicate statistically significant differences. [a]AD significantly different from control, hydrocephalus and depression groups ($P=0.0001$).

in accordance with previous studies that used cutoffs of < 0.8, < 1.0 or < 1.2 for AD diagnosis[51–54]), CSF D-serine levels were again significantly different from non-AD patients (Figure 4b). Remarkably, cases clinically diagnosed as probable AD by neuropsychological assessment had higher CSF D-serine levels (Figure 4b) than nondemented patients. In addition, total serine levels (that is, D-serine+L-serine) were higher in AD and major depression compared with healthy controls (Table 3), whereas L-serine levels did not differ significantly between groups (Table 3). CSF glycine levels were not significantly different between AD and control groups (Table 3).

As expected, mean mini-mental state exam scores were significantly lower in the AD group compared with the other patient groups (Table 4). Interestingly, individual D-serine levels across all subjects, regardless of diagnosis, were negatively correlated to the mini-mental state exam score (Figure 4c). We further investigated how D-serine levels correlated to dementia by separating subjects into groups stratified by CDR scores. The CDR 0 (cognitively normal) group showed significantly lower levels of D-serine than the groups with CDR 0.5, 1, 2 and 3 (Figure 4d). It is interesting to note that D-serine levels were already elevated in patients with a CDR score of 0.5, typically associated with pre-dementia mild cognitive impairment.

CSF D-serine versus amyloid/tau biomarkers of AD
IATI is a score that combines CSF Aβ42 and total tau protein levels, and has been proposed as a biomarker to assist in the diagnosis of AD.[51] As expected, the mean IATI score was significantly lower in the AD group than in the other three groups (Table 4). Interestingly, individual D-serine levels were negatively associated with IATI scores (Figure 4e).

To explore the potential of D-serine as a biomarker, we compared CSF D-serine levels and IATI scores on their specificity and sensitivity for the diagnosis of probable AD in our cohort. Using the cutoff of 1.246, IATI showed 81.4% sensitivity and 94.4% specificity (in good agreement with published values[55]), whereas determination of CSF D-serine levels (using a cutoff of

9.82 μmol l^{-1}) afforded 92.9% sensitivity and 85.7% specificity for AD. Remarkably, combined use of both IATI and CSF D-serine levels (that is, calculating an IATI/ D-serine ratio, and using a cutoff of 0.14) resulted in significantly increased sensitivity (96.3%) and specificity (100%) for the diagnosis of probable AD. Increased sensitivities and specificities were also obtained by inclusion of D-serine determination using lower IATI cutoff values (Supplementary Table 4). Receiver-operating characteristic curves for IATI alone and IATI/ D-serine are shown in Figure 4f.

DISCUSSION
We identified elevated levels of D-serine in post-mortem samples from brain regions involved in disease progression in neuropathologically confirmed AD cases. Pathological changes in AD usually spread from limbic structures, comprising the hippocampal formation, to associative areas such as the posterior parietal cortex, and only later reach primary neocortical areas, such as the striate cortex in the occipital lobe.[56,57] Consistent with this pattern, we found increased D-serine levels in the hippocampus and parietal cortex of AD brains compared with nondemented controls, but no differences in the occipital cortex. Noteworthy, three early studies using post-mortem frontal or temporal cortical tissue failed to detect altered D-serine levels between AD and controls.[28–30] Those findings, when contrasted to our results stratified by regions and CDR, raise the possibility that D-serine elevation is not a widespread event in AD brains, but rather occurs in a region-specific manner according to disease progression. These observations are consistent with a scenario in which regional elevations in D-serine levels trigger localized deregulation of NMDAR-dependent synaptic plasticity and, ultimately, NMDAR-related excitotoxicity and neuronal injury, culminating in cognitive decline in AD.

To gain insight into the underlying mechanisms leading to increased D-serine levels in the AD brain, we next investigated D-serine levels in AD model systems. AβOs build up in AD brains[58–60] and are thought to trigger toxic mechanisms leading to synapse

failure in AD,[3] including aberrant NMDAR function,[15–17] increased glutamate release[17,24] and impaired synaptic plasticity.[13,61] We now report that D-serine is elevated in the extracellular medium of hippocampal cultures exposed to AβOs and in the hippocampi of rodents that received brain injections of AβOs. Furthermore, APP/PS1 mice, which display age-dependent AD pathology and memory impairment, showed markedly increased brain D-serine levels. Consistently, increased SR levels were also verified in both AβO-exposed cultures and transgenic rodent brains likely explaining elevated D-serine content *in vitro* and *in vivo*. Importantly, elevations in D-serine content do not appear to reflect a general deregulation of neuroactive amino-acid levels, as levels of neither L-serine nor of the NMDAR co-agonist, glycine, were altered in the hippocampus and CSF of AD cases and in AβO-injected mice or APP/PS1 mice. These observations suggest a causal relationship between AβO accumulation and D-serine increases in AD brains.

Abnormal NMDAR function is thought to underlie, at least in part, AD pathogenesis,[4,12,17,62,63] and AβOs impair NMDAR-dependent synaptic plasticity and instigate NMDAR-mediated synapse loss and oxidative stress.[13,15,16,61,64] Thus, our finding of elevated levels of D-serine, the main co-agonist at NMDARs in frontal brain regions, points to a novel mechanism by which AβOs may trigger synapse dysfunction and memory impairment.

Importantly, AD brains present reduced levels of NMDAR[65–67] in areas that are relevant for disease progression. Therefore, it is tempting to speculate that increased D-serine levels could comprise an initial adaptive response to maintain proper neurotransmission. However, given that NMDAR function appears to be overactivated in AD,[12] elevated D-serine could contribute to an excitotoxic scenario, worsening AD neuropathological outcomes.

Whether and how increased levels of D-serine participate in cognitive and behavioral outcomes in AD is still unknown. D-serine administration has been shown to promote synaptogenesis and to have memory-enhancing effects.[25,68,69] Conversely, excessive D-serine levels were shown to mediate NMDAR-dependent late-phase apoptosis[70] and to contribute to neurological insults, such as excitotoxicity and brain ischemia.[22] Further, a recent report suggested that D-serine might act as a NMDAR antagonist under some circumstances.[27] Therefore, a fine regulation of D-serine levels is required for maintaining proper synaptic homeostasis and function. Future experimental studies may unravel the role of D-serine in synapse dysfunction and neurotoxicity in AD.

The main finding of the current study is that CSF levels of D-serine are significantly higher in patients diagnosed with probable AD than in nondemented subjects, suggesting that D-serine could be a novel AD biomarker. Importantly, the significant correlation between increased D-serine content in the CSF and poorer cognitive performance in the mini-mental state exam suggests that D-serine determination could comprise a powerful diagnostic tool in conjunction with assessment of cognitive decline. As CSF D-serine is already significantly elevated in patients with CDR 0.5, this might facilitate diagnosis of AD-related cognitive decline at an early stage of cognitive impairment, and permit interventional approaches at a phase in which disease could still be modifiable.

The fact that CSF levels of D-serine are also elevated in depression and hydrocephalus, compared with control subjects, might suggest that D-serine would not be a specific AD biomarker. We note, however, that D-serine levels in AD are clearly and significantly higher than in depression or hydrocephalus, allowing definition of a cutoff value that discriminates between those disorders. Further, combination of CSF D-serine measurements with the validated IATI biomarker yielded 100% specificity in our cohort. Our results suggest that CSF D-serine levels adequately discriminate AD from non-AD cases when used in combination with different IATI cutoff values, and correlate well with neuropsychological diagnosis. Results thus indicate that determination of CSF D-serine levels could improve diagnostic accuracy in AD. Nonetheless, as a pilot study, replication of these findings in other cohorts would make a strong case for the incorporation of D-serine levels in a panel of CSF biomarkers for AD.

It is interesting to note that elevated D-serine levels in depression might be related to late development of AD. Depression is often clinically associated with AD.[71,72] It has recently been shown that AD and depression share common mechanisms, and that both memory loss and depressive-like symptoms are instigated by AβOs in mice.[40] Because AβOs increase brain D-serine levels, it is plausible that elevated levels of D-serine in AD and depression are both consequences of the neurotoxic impact of Aβ. This interesting hypothesis deserves further investigation and might open new avenues for studying the pathogenesis of AD-linked disorders.

In conclusion, our results show that AD patients have increased levels of D-serine in the CSF and in specific brain regions, and that this appears to be triggered by the action of soluble AβOs in the brain. CSF D-serine levels are strongly correlated with memory impairment and could constitute an effective diagnostic tool for probable AD. A limitation of this study is the cohort size and heterogeneity, although these factors did not compromise robustness of our findings, as indicated by statistical power analyses with adequate sample sizes. Our present study constitutes a novel and encouraging effort toward improved AD diagnostics. Nevertheless, despite the current statistically robust results, we acknowledge that this is a pilot investigation and, thus, a larger prospective clinical study is warranted to extend the validity of our results.

The field of AD diagnostics still suffers from the lack of accurate and efficient biomarkers. This may be attributed, in part, to the complex nature of this disease. Nonetheless, very recent approaches have provided advances in establishing clearer and more efficient strategies for biomarker-based diagnosis. For example, a recent paper by Lehmann et al.[55] proposed a new diagnostic scale that might be translated into clinical applications in the near future. Furthermore, other recent efforts have unveiled a number of promising CSF biomarkers, including AβOs, double-stranded RNA-dependent protein kinase and other neuronal injury markers.[73–77] Combined use of those biomarkers and others currently in development with determination of CSF D-serine concentrations could constitute a valuable strategy to increase sensitivity and specificity in the diagnosis of AD.

CONFLICT OF INTEREST

CM, STF and RP are inventors. The remaining authors declare no conflict of interest.

DISCLAIMER

Funding agencies had no role in study design, collection, analysis, interpretation of data, writing of the report or the decision by the authors to submit the paper for publication. The Federal University

of Rio de Janeiro has intellectual property rights in the use of D-serine as a biomarker in Alzheimer's disease.

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