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RESEARCH ARTICLE

A biomarker for severity of Alzheimer's disease: ^{1}H-NMR resonances in cerebrospinal fluid correlate with performance in mini-mental-state-exam

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Abstract

Context: There is no valid biomarker for severity of Alzheimer's disease (AD) available until now.

Objective: Therefore, we investigated ^{1}H-NMR spectroscopy for specific resonances as biomarkers for severity of AD.

Materials and methods: Cerebrospinal fluid (CSF) of patients with diagnosed AD and healthy control subjects was analysed by one-dimensional water-suppressed ^{1}H-NMR spectroscopy. The resonances were correlated with the cognitive performance of the patients and controls.

Results: Specific ^{1}H-NMR resonances showed higher intensities in AD patients than in control subjects. Mini-mental-state-exam scores correlated with ^{1}H-NMR resonances in AD patients.

Discussion and conclusion: ^{1}H-NMR resonances of CSF are obviously valid biomarker for severity of AD, despite the lack of knowledge of the underlying molecular structure. Successful isolation and identification of these substances will most likely provide details to the pathophysiology of AD.

Keywords: Diagnostic marker, dementia, metabonomics, proteomics

Introduction

The incidence of dementias such as Alzheimer's disease (AD) is increasing. Estimates suggest 35.6 million people with dementia in 2010, doubling every 20 years (Prince, Jackson, and Ferri 2009).

Early diagnosis is of great importance as patients can only be treated symptomatically because all known and established therapies for AD only slow down progression of this disease (McGeer and McGeer 2003). Sufficient treatment in the early phase of AD helps keeping patients in an autonomous state as long as possible (Leifer 2003), while decreasing health care costs and increasing quality of life for patients and caregivers (Relkin 2000).

In early-stage diagnostics, a combination of CSF biomarkers including total τ-protein, phosphorylated τ-protein, and the 42 amino acid residue form of β-amyloid provide sensitivity and specificity figures around 80% (Blennow and Zetterberg 2009). Other biological markers such as CSF levels of neurotransmitters, cytokines, or superoxide dismutase appear to be of minor diagnostic value (Tarawneh and Holtzmann 2010). The apolipoprotein epsilon 4 allele depicts a risk factor for AD but not a diagnostic marker (Green et al. 2009) as many individuals with epsilon 4 do not develop the disease. Above all, none of these biomarkers have been validated as marker for severity of AD (Olson and Humpel 2010).

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We have already established a metabonomics approach to screen CSF of AD patients for potential biomarkers of early AD by one-dimensional water-suppressed proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. We found that ^{1}H-NMR spectroscopy is capable of detecting substances in CSF of AD patients that were not detectable in CSF of healthy controls and that can be used as a biomarker for diagnosing AD (Kork et al. 2009).

In this study, we hypothesized that substance concentrations in CSF are associated with cognitive performance, namely severity of disease. As ^{1}H-NMR resonance intensities reflect substance concentration, we analysed the data investigating the association of ^{1}H-NMR resonance intensity and cognitive performance by means of neuropsychological testing in AD patients.

Methods

Patients

The local ethics committee of the Charité (Berlin, Germany) approved the study (ek.211-18) and written informed consent was obtained from all patients. A total of 20 patients with clinically apparent dementia due to AD were recruited. A total of 27 neurologically healthy control subjects underwent CSF puncture because of back pain complaints, exclusion of meningitis or headaches in the neurological department. CSF punctures were conducted after thorough skin disinfection; CSFs were not contaminated with blood and immediately stored at –80°C. Laboratory parameters were gathered using standard analyser techniques.

AD was diagnosed according to DSM IV, ICD 10, and NINCDS-ADRDA criteria (McKhann et al. 1984) conducting a structured medical history given by patients and caregivers, a complete physical examination, blood and CSF testing for 24S-OH-cholesterol, α-1-antichymotripsin, homocysteine, vitamin A, B6, B12, C, E, folic acid and homocysteine concentrations, apoliprotein E genotyping. HIV or syphilis testing (Teunissen et al. 2002), magnetic resonance imaging (MRI; Essig and Schoenberg 2003; Kantarci and Jack 2003), and a CSF puncture (Kantarci and Jack 2003) was conducted to rule out other possible causes of dementia.

Patients underwent the cognitive testing including mini-mental-state-exam (MMSE; Folstein, Folstein and McHugh 1975), CERAD (The Consortium to Establish a Registry for Alzheimer's Disease, German version; Morris et al. 1988), Wechsler Memory Scale Revised Logical Memory (Härting et al. 2000), Trail Making Test (Reitan and Wolfson 2004), Clinical Dementia Rating (Morris et al. 1993), Alzheimer's Disease Assessment Scale (Morris et al. 1988), Free and Cued Selective Reminding Test (Grober et al. 1997), A structured interview for the diagnosis of the Alzheimer type (SIDAM; Busse et al. 2002), Clocktest (Shulman 2000), Bayer Activities of Daily Living (B-ADL) Scale (Hindmarch et al. 1998), and Frontal Behaviour Interview (Dubois et al. 2000). The MMSE was conducted twice, at first physician contact within the primary evaluation of the patient at admission and a second time as part of the CERAD test battery during detailed neurocognitive testing. Based on the results of these tests, all patients included in the study fulfilled the criteria of AD diagnosis. Patients classified as AD patients did neither show clinical signs of Lewy Body Dementia (e.g. scenic hallucinations), Fronto-Temporal Dementia (e.g. massive disinhibition), or vascular diseases (e.g. stroke, focal neurological signs) nor did their cranial MRI show signs of severe vascular disease or isolated frontotemporal atrophy.

NMR spectroscopy

A Bruker AMX 500 FT spectrometer (Bruker Analytische Messtechnik, Bremen, Germany) was used to accumulate water-suppressed ^{1}H-NMR specta. 50µL of 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP) and 50µL of deuterium oxide (D_2O) were added to 500µL CSF and mixed thoroughly for 3 min. Spectrometer configuration was conducted as described previously (Kork et al. 2009). ^{1}H-NMR resonances were referenced to TSP at 0 ppm. Intensities of the resonances were normalized to the intensity of the TSP resonance resulting in relative intensity. If resonance intensities were not discriminable from noise, intensities were rated as 0. All substances were purchased from Sigma-Aldrich (Taufkirchen, Germany).

Protein denaturation and membrane filtration

Protein denaturation was conducted using perchloric acid. Samples were adjusted to a concentration of 0.6 mol/L perchloric acid and centrifuged at 4,000 rpm for 4 minutes at 10°C. Supernatants were adjusted to pH 9.5 with potassium hydroxide (both Sigma-Aldrich, Taufkirchen, Germany). Membrane filtration was conducted using an Amicon Ultra centrifugal filter for 3kD and 10kD filter from Sigma-Aldrich, Taufkirchen, Germany.

Statistics

Normally distributed variables are presented as mean ± standard deviation and were compared using the *t*-test. Not normally distributed variables are presented as median and quartiles and were compared using the Mann-Whitney-U-Test. Correlation was computed using Pearson's ρ for normally distributed variables and Spearman's ρ for nonnormally distributed variables. All tests have been conducted using IBM SPSS Statistics 19.0 (IBM, Somers, NY, USA), the post-hoc power analysis was conducted using G*Power (University of Düsseldorf, Germany). All probabilities < 0.05 were considered significant and marked with an asterisk in the corresponding figures.

Results

The biochemical and clinical characteristics of the AD patients and the control subjects are shown in Table 1.

Table 1. Biochemical and clinical characteristics of the AD patients and healthy control subjects. The indexes (Q) were calculated dividing CSF/serum concentrations.

	AD ($n=20$)	Control ($n=27$)	p
Age (years)	70.2 ± 2.3	51.4 ± 3.7	0.00*
Male/female	10/10	8/19	0.26
Creatinine (µmol/L)	82.3 ± 4.9	64.8 ± 5.9	0.90
Leucocytes (nL^{-1})	8.1 ± 0.4	7.4 ± 0.6	0.20
Pulse rate (min^{-1})	75.7 ± 3.9	68.7 ± 5.2	0.29
Systolic BP (mmHg)	121.7 ± 6.5	125.7 ± 6.3	0.81
Diastolic BP (mmHg)	70.0 ± 3.7	78.0 ± 3.4	0.19
CSF protein (mg/dL)	44.5 ± 4.2	36.6 ± 1.7	0.12
Serum protein (mg/dL)	7811 ± 128	7558 ± 163	0.38
CSF albumin (mg/dL)	25.8 ± 2.3	20.6 ± 1.0	0.06
Serum albumin (mg/dL)	4159 ± 70	3992 ± 194	0.88
Q_{Alb}	6.3 ± 0.6	4.8 ± 0.3	0.05*
CSF IgG (mg/dL)	2.8 ± 0.2	2.2 ± 0.1	0.03*
Serum IgG (mg/dL)	1055 ± 53	997 ± 46	0.45
Q_{IgG}	2.8 ± 0.3	2.3 ± 0.1	0.27
Index	0.44 ± 0.01	0.46 ± 0.01	0.26

Note: * indicates all probabilities < 0.05.
BP, blood pressure; CSF, cerebrospinal fluid, IgG: Immune globulin G.

Table 2. Intensity of ^{1}H-NMR-resonances in arbitrary units (AU) in AD patients and healthy controls.

Chemical shift (ppm)	AD (AU × 10^{-3})	Control (AU × 10^{-3})	p
1.44	0.49 [0.26–.83]	1.00 [0.40–2.07]	0.02*
1.47	1.78 [1.31–2.28]	0.00 [0.00–1.88]	<0.01*
1.92	3.02 [2.20–4.20]	5.10 [4.25–6.30]	<0.01*
6.87	0.30 [0.00–.39]	0.00 [0.00–0.25]	0.02*
6.89	0.31 [0.04–.35]	0.00 [0.00–0.19]	<0.01*
7.03	0.25 [0.02–.28]	0.00 [0.00–0.00]	<0.01*
7.19	0.24 [0.03–.32]	0.00 [0.00–0.25]	<0.01*
7.33	0.23 [0.04–.27]	0.00 [0.00–0.21]	<0.01*
7.43	0.18 [0.00–.24]	0.00 [0.00–0.00]	0.02*
7.44	0.00 [0.00–.07]	0.00 [0.00–0.00]	0.02*
7.73	0.23 [0.00–.28]	0.00 [0.00–0.16]	0.02*
7.91	0.18 [0.02–.35]	0.00 [0.00–0.00]	<0.01*
8.46	0.32 [0.18–.44]	0.39 [0.35–0.57]	<0.01*

Note: * indicates all probabilities < 0.05.
AD, Alzheimer's disease.

NMR spectroscopy

^{1}H-NMR spectra of CSF from AD patients showed resonances that were detected with different intensities in control subjects. ^{1}H-NMR spectra of CSF from AD patients showed ^{1}H-NMR resonances with lower intensity at 1.44 ppm ($p=0.02$) and 1.92 ppm ($p<0.01$) and a resonance that had a higher intensity at 1.47 ppm ($p<0.01$) compared to healthy control subjects (Table 2). The strongest differences were detected in the range of 6.60 ppm and 8.60 ppm. Figure 1 shows a characteristic ^{1}H-NMR spectrum of CSF from an AD patient (Figure 1A) and a healthy control subject (Figure 1B) in the range of 6.60 ppm and 8.60 ppm. ^{1}H-NMR spectra of CSF from AD patients showed multiple ^{1}H-NMR resonances with different intensities compared to healthy control subjects (Table 2).

Neuropsychological testing

AD patients had median score of 22.0 [18.25 to 24.25] in the MMSE at time of first physician contact. AD patients' CERAD results in detail: verbal fluency –1.90 [–3.09 to –0.85], Boston Naming Test 0.72 [–1.11 to 1.31], MMSE –3.95 [–5.39 to –2.30], word list learning –3.13 [–4.48 to –2.03], word list delayed recall –2.50 [–2.50 to –1.87], word list savings –2.38 [–3.80 to –1.71], figures copy –1.30 [–1.52 to 0.53], figures delayed recall–2.55 [–3.10 to –1.92], figures savings –2.57 [–2.80 to –1.33] (median and quartiles of age, sex and education corrected z-values).

Correlation between ^{1}H-NMR resonances and neuropsychological testing

The data show that CSF of AD patients contains substances that are associated with cognitive performance. In the ^{1}H-NMR spectra of CSF from the AD patients, the intensity of the resonance at 7.03 ppm ($p < 0.01$) showed a strong correlation with performance in the MMSE at time of first physician contact. Intensities of the resonances at 6.87 ppm ($p \leq 0.01$), 7.19 ppm ($p = 0.03$), 7.33 ppm ($p < 0.01$), and 7.73 ppm ($p = 0.04$) showed a correlation with performance in the MMSE at time of first physician contact (Table 3, Figure 2). The post-hoc power analysis confirmed this results showing for the significant resonances ($p < 0.01$) at 6.87 ppm a power of 0.83 (FPC) and 0.95 (z-values), at 7.03 of 0.99 (FPC) and 0.94 (z-values) and at 7.33 of 0.85 (FPC).

This correlation could not be shown for the CERAD subitems. Some ^{1}H-NMR resonance intensities showed a significant difference with random subitems. In accordance to the correlation between the resonance intensities and the MMSE values, the z-value of MMSE testing within the CERAD test battery correlated with the intensity of the resonance at 1.44 ppm ($p = 0.04$), and strongly with the resonances at 6.87 ppm ($p < 0.01$) and 7.03 ppm ($p < 0.01$; Table 3, Figure 2, Figure 3).

Protein denaturation, membrane filtration and comparison to spectra of known biomarkers

Comparing ^{1}H-NMR spectra of τ-protein and β-amyloid revealed no similar resonances with the resonances described in this experiment. For further characterization of the underlying molecules, we filtrated CSF of AD patients through a 3kD and a 10kD membrane ($n=2$) as well as conducted protein denaturation using perchloric acid ($n=2$). After those procedures, we performed a secondary ^{1}H-NMR spectroscopy. The described resonances could still be detected in ^{1}H-NMR spectroscopy in the filtrates and in the supernatants after protein denaturation.

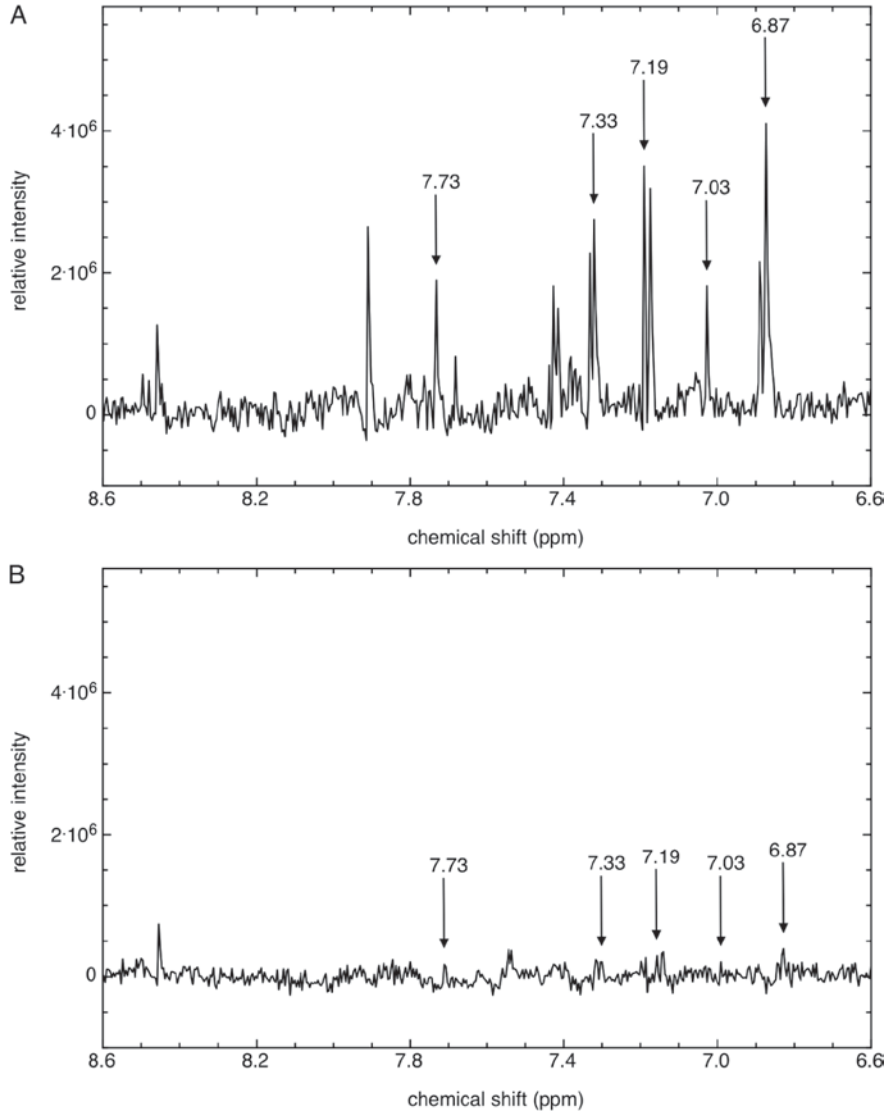

Figure 1. Typical water-suppressed ^{1}H-500-MHz-NMR spectra of CSF in an AD patient (A) and a healthy control subject (B) (Time domain [TD] = 32 K; size [SI] = 16 K; pulse 1 [P1] = 6.2 μs; number of scans [NS] = 128) from 6.6 ppm to 8.6 ppm.

Table 3. Correlation between ^{1}H-NMR-resonances and MMSE at first physician contact (FPC) and *z*-values of the MMSE subtest within the CERAD in AD patients.

Chemical shift (ppm)	Correlation (MMSE FPC)	*p*	Correlation (MMSE *z*-values)	*p*
1.44	−.354	0.15	−.569	0.04*
1.47	−.121	0.63	−.012	0.97
1.92	−.180	0.46	−.042	0.89
6.87	−.620	<0.01*	−.697	<0.01*
6.89	−.231	0.36	−.063	0.84
7.03	−.746	<0.01*	−.687	<0.01*
7.19	−.501	0.03	−.525	0.07
7.33	−.631	<0.01*	−.541	0.06
7.43	−.273	0.27	−.372	0.21
7.44	−.203	0.42	−.027	0.93
7.73	−.499	0.04*	−.396	0.21
7.91	.270	0.28	.382	0.20
8.46	−.395	0.11	−.385	0.19

Note: * indicates all probabilities < 0.05.
AD, Alzheimer's disease, MMSE, mini-mental-state-exam.

Discussion

These results indicate that CSF from patients with AD shows ^{1}H-NMR resonances with higher intensities than CSF of healthy control subjects. The intensities of these ^{1}H-NMR resonances correlate negatively with cognitive performance measured by the MMSE and with age, sex, and education corrected *z*-values of the CERAD sub-item for MMSE in AD patients. Although the substances causing the resonances have not been identified, their concentration could be correlated negatively with the performance in the MMSE.

There is no valid biomarker for severity of AD. The only CSF AD biomarkers used in clinical routine are τ-protein and β-amyloid. These biomarkers and their common derivatives have repeatedly been reported not to correlate with the severity of dementia (Haense et al. 2008; Kahle et al. 2000; Lee et al. 2008; Stefani et al. 2009). The resonances detected in this study did not match the spectra of τ-protein and β-amyloid but

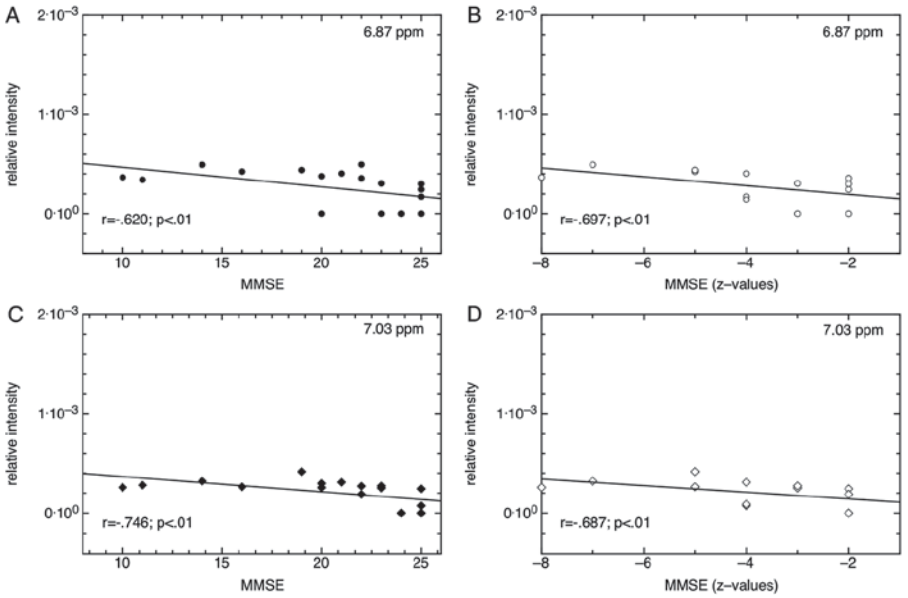

Figure 2. Scatter plots and regression lines of the relative ^{1}H-NMR resonance intensities that significantly correlate with both MMSE (6.87 ppm (A); 7.03 ppm (C)) and CERAD derived *z* values (6.87 ppm (B); 7.03 ppm (D)).

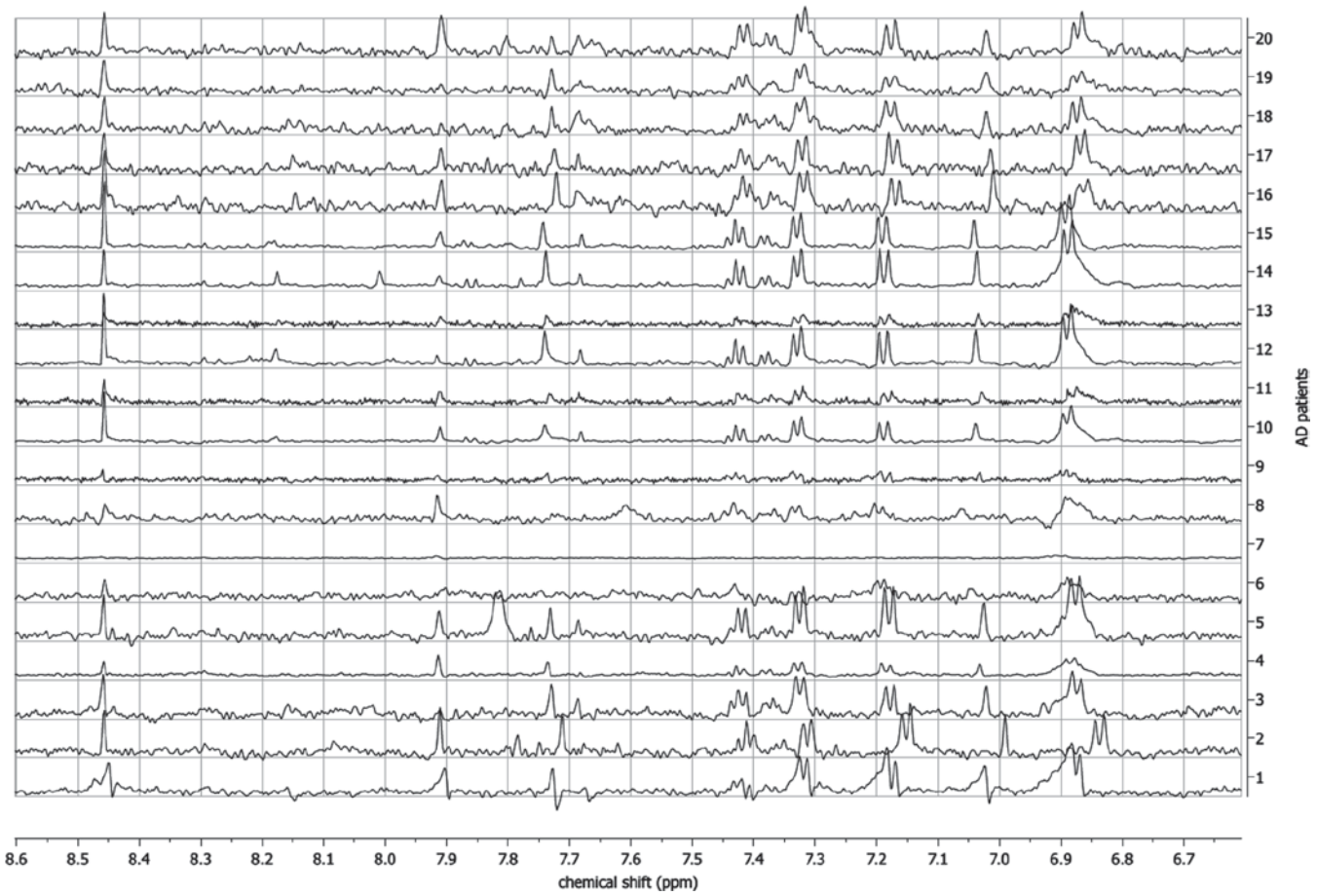

Figure 3. Water-suppressed ^{1}H-500-MHz-NMR spectra of the AD Patients.

in fact did correlate with severity of AD. The substances detected in this study did pass through an exemplary conducted 3kD and 10kD membrane filtration. In addition, the substances withstood exemplary conducted denaturation by perchloric acid. Therefore, the underlying substances causing the resonances between 6.60 ppm and 8.60 ppm presumably occur due to small peptides or modifications in small peptides in the CSF

of patients suffering from AD or by low molecular substances that often show resonances within the chemical shift above 6 ppm. That would concur with findings indicating protein cyclation, oxidation, and nitration adduct residues and free adducts is increased in the CSF of AD patients (Ahmed et al. 2005).

In this study, age differed in AD patients and healthy control subjects. We correlated age and the ^{1}H-NMR resonances to rule out that we detected a phenomenon of age. Age and the detected ^{1}H-NMR resonances did not correlate significantly ($r < .39$, $p > 0.10$). Moreover, the age-, sex-, and education-corrected *z*-values of the MMSE scores within the CERAD test showed the same correlation with ^{1}H-NMR resonances as the corresponding, noncorrected MMSE scores, albeit for some resonances correlation did not reach statistical significance. Lesser correlations between the resonance intensities and the CERAD-MMSE than with the MMSE at admission could be caused by higher patient activation due to clinical testing within the diagnostic process.

It has been shown that AD patients more frequently suffer from impairment of the blood brain barrier (BBB) than patients without AD. It has in fact been suggested that BBB dysfunction modifies disease progression but it has also been shown that BBB dysfunction only occurs in a small subgroup of AD patients (Bowman et al. 2007). In this sample, the CSF/serum albumin index (Q_{Alb})—an ordinary measure of BBB dysfunction—did differ between AD patients and controls. Yet, Q_{Alb} was well beneath the commonly accepted threshold for BBB impairment at $Q_{Alb} > 9$ for both groups and BBB in AD has been associated with substantially higher Q_{Alb} (Hansson et al. 2009) than in our sample. Moreover, a micro-BBB impairment has been discussed in AD (Pluta 2007) and may be causative for the crossing of low molecular substances such as small peptides or other low molecular substances.

Although the number of AD patients and healthy control subjects included in this study is limited, 21 patients have been calculated to be sufficient to conduct conclusive analyses using a proteomic approach investigating 500 proteins simultaneously (Zellner, Veitinger and Umlauf 2009). Furthermore, the post-hoc power analysis confirmed these results.

In summary, the results of this study indicate that ^{1}H-NMR spectroscopy is suitable as a valid biomarker for severity of AD, even without knowledge of the substances or substance modifications causing these ^{1}H-NMR resonances. Yet the successful isolation and identification of these substances will most likely provide details to the pathophysiology of AD. Especially, as to why higher concentrations of these substances correlate with a decline in cognitive performance, a primary symptom of AD. This may ultimately lead to the identification of new therapeutic targets in what the identified substances may also act as a marker of therapy efficiency.

Conclusions

These results indicate that CSF from patients with AD contains components in higher concentrations than in CSF of healthy control subjects that correlate negatively with cognitive performance measured by the MMSE. Thus, these ^{1}H-NMR resonances are valid biomarkers for severity of Alzheimer's disease.

Acknowledgments

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Declaration of interest

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