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Validation of Alzheimer's disease CSF and plasma biological markers: The multicentre reliability study of the pilot European Alzheimer's Disease Neuroimaging Initiative (E-ADNI) ☆

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ABSTRACT

Background: Alzheimer's Disease Neuroimaging Initiatives ("ADNI") aim to validate neuroimaging and biochemical markers of Alzheimer's disease (AD). Data of the pilot European-ADNI (E-ADNI) biological marker programme of cerebrospinal fluid (CSF) and plasma candidate biomarkers are reported.
Methods: Six academic EADC centres recruited 49 subjects (healthy controls, subjects with mild cognitive impairment (MCI) and AD). We measured CSF β-amyloid 42 (CSF Aβ42), total tau-protein (t-tau), phosphorylated tau-proteins (P-tau181, P-tau231), plasma β-amyloid 40 and 42 (Aβ40/Aβ42). Immediate fresh shipment was compared to freezing and later shipment on dry ice.
Results: CSF T-tau (fresh samples) was increased in AD versus controls ($p = 0.049$), CSF Aβ42 (frozen samples) was decreased in MCI and AD ($p = 0.02$), as well as plasma Aβ40 (fresh and frozen samples) in AD ($p = 0.049$ and $p = 0.016$). Pooled values of neurochemical parameters and ratios thereof were different between centres ($p < 0.005$). Analysis of frozen samples yielded higher diagnostic accuracy than immediate fresh shipment with 100% (fresh: 100%) correctly classified in control subjects, 100% (78%) in MCI, 91% (91%) in AD.
Conclusion: The use of frozen rather than fresh samples renders higher diagnostic accuracy within a multicentre context. We confirmed the feasibility of a multicentre AD biomarker programme for future clinical trials.

Abbreviations: AD, Alzheimer's disease; MCI, mild cognitive impairment; ADNI, Alzheimer's Disease Neuroimaging Initiative; E-ADNI, European-ADNI; EADC, European Alzheimer's Disease Consortium; neuGRID, FP7 "A grid-based e-infrastructure for data archiving/communication and computationally intensive applications in the medical sciences"; FDG PET, fluorodeoxyglucose positron emission tomography; CSF, cerebrospinal fluid; ANOVA, analysis of variance; LSD, least significant difference; SD, standard deviation; ELISA, enzyme-linked immunosorbent assay; NINDS-AIREN, National Institute for Neurological Disorders and Stroke – Association Internationale pour la Recherche et l'Enseignement en Neurosciences; MMSE, mini-mental state examination; ADAS-Cog, Alzheimer's Disease Assessment Scale-Cognitive subscale; DRadas, delayed recall test from the ADAS-Cog; DNA, deoxyribonucleic acid; PI, principal investigator; Aβ40, β-amyloid 40; Aβ42, β-amyloid 42; t-tau, total tau-protein; P-tau181, phosphorylated tau-protein 181; P-tau231, phosphorylated tau-protein 231.

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1. Introduction

The Alzheimer's Disease Neuroimaging Initiative (ADNI) aims to collect core feasible neuroimaging and biochemical marker data in the US and Canada to validate these markers for use in AD diagnostic and treatment trials. A major goal of the pilot European-ADNI (E-ADNI) programme of the European Alzheimer's Disease Consortium (EADC) is to test the ability of European expert centres to implement the data acquisition procedures of the large-scale NIH funded US-ADNI and use them on the US-ADNI clinical target groups (healthy aging, MCI subjects, and patients with Alzheimer's disease) (Frisoni et al., 2008).

In the US, the ADNI has recruited large groups of Alzheimer's and MCI subjects and normal controls in about 60 clinical and research centres and collected imaging, clinical, and biological data in a standardized and centralized fashion that allows for pooled cross-sectional and prospective analyses (Mueller et al., 2005).

Similarly in Europe, 50 clinical and research centres of the EADC are currently running Europe wide clinical trials which collect clinical, imaging, and biological information in a standardized fashion while harmonization and centralized collection are cared for by external agencies. Further evidence of coordinated multisite AD studies in Europe include the recently completed six-year large-scale German Competence Network, the Swedish network on Alzheimer's disease, and a multicentre fluorodeoxyglucose positron emission tomography (FDG PET) imaging study funded by the EC (Herholz, 2003; Herholz et al., 2002).

The aim of this pilot project is to demonstrate that the core ADNI methodology, i.e. standardized and centralized collection of magnetic resonance (MR) imaging, clinical data, blood, and CSF samples can be adopted by European expert academic Alzheimer's centres to collect reliable, accurate and robust data. Few test sites and subjects were involved to collect single time point data. Once collected by the participating centres, data and specimens were sent to central repositories through conventional means (CD for images; e-mail attachments for the clinical data; express courier for the biological samples). The whole infrastructure for centralized data collection is under development (FP7 ''neuGRID: a Grid-based e-infrastructure for data archiving/communication and computationally intensive applications in the medical sciences", www.neuGRID.eu). Thus, neuGRID and the present pilot E-ADNI are providing information whose integration will facilitate the implementation of a future larger study within the EC.

In the current article, we present the results of the E-ADNI biological marker programme's CSF and plasma studies for the first time, obtained within the ''Pilot E-ADNI" study (coordinated by the PI's Harald Hampel, Germany/Ireland and Kaj Blennow, Sweden). We place a special emphasis on the assessment of the core feasible biochemical marker candidates (as defined by the NIA Biological Marker Working Group (Frank et al., 2003)) CSF Aβ42, t-tau and P-tau181 and P-tau231 and of plasma Aβ40 and Aβ42. Alterations of these biomarkers in AD and MCI subjects compared to healthy elderly controls have largely been shown (Blennow and Hampel, 2003; Hampel et al., 2008) and they have been suggested core biological markers for AD (Frank et al., 2003). Therefore, these markers were investigated in the current pilot feasibility study.

2. Materials and methods

2.1. Centres

Centres for the E-ADNI study were selected mostly from the EADC based on scientific expertise, demonstrated activity within the Consortium, and geographic representativeness. CSF and plasma samples for neurochemical analysis were obtained from the following E-ADNI centres: Dept. of Psychiatry, Ludwig-Maximilian University, Munich, Germany; IRCCS Centro San Giovanni Di Dio Fatebenefratelli, Brescia, Italy; Huddinge Hospital, Huddinge, Sweden; MDRU, Rigshospitalet, Copenhagen, Denmark; Ospedale S Giovanni Calibita, Isola Tiberina, Roma; Centre Hospitalier Universitée de Toulouse, France (provided plasma samples only). Responsibility for clinical issues including adaptation of the US-ADNI case report form and collection of the clinical variables was in Toulouse (Centre Hospitalier Universitée de Toulouse, France); the centre for imaging issues including installation of ADNI sequences, scanner qualification, image quality control, image collection, and analysis was located in Amsterdam (VU Medical Centre, Amsterdam, The Netherlands); CSF issues including adaptation of the US-ADNI CSF collection protocol, centralized collection of samples, and assaying were handled in Munich (Dept. of Psychiatry, Ludwig-Maximilian University, Munich, Germany); plasma issues including adaptation of the US-ADNI plasma collection protocol, centralized collection of samples, and assaying were handled in Gothenburg (Dept. of Clinical Neuroscience, University of Gothenburg, Sweden). The centre in Brescia (IRCCS Centro San Giovanni Di Dio Fatebenefratelli, Brescia, Italy) was responsible for the overall project management, training of personnel in enrolment sites, monitoring of data, image, and sample collection, as well as for reporting.

2.2. Patients

Each centre was asked to enrol 3 consecutive newly diagnosed patients with Alzheimer's disease, 3 patients with MCI (single and multiple domain amnestic MCI (Petersen and Touchon, 2005), and 3 normal age-matched controls. Controls were older patients undergoing prostate or hip surgery with spinal anaesthesia (Brescia and Rome), true volunteers, usually patients' spouses (Stockholm, Toulouse and Copenhagen), and persons with memory complaints with normal results in clinical and instrumental exams diagnosed as functional complaints (Munich). The subjects were enrolled between January 1st and March 31st 2007. All subjects underwent MR scan and lumbar puncture (LP) under routine clinical conditions and the same data, image, and sample collection procedures. Patients and controls were explained the aim of the project, and gave informed consent. The study was reviewed and approved first by the Ethics Committee of the coordinating site (CEIOC – Comitato Etico delle Istituzioni Ospedaliere Cattoliche), then by Ethics Committees of all other sites. None of the subjects fulfilled one or more of the National Institute for Neurological Disorders and Stroke – Association Internationale pour la Recherche et l'Enseignement en Neurosciences (NINDS-AIREN) criteria for vascular dementia.

2.3. Clinical and neuropsychological data collection

The clinical core module aimed to adapt the ADNI clinical data collection form to the E-ADNI centres. For the purely clinical data (sociodemographics, disability, behaviour, comorbility, etc.), translation was performed into local languages – the usual procedure of translation and back translation was applied for some scales that were unavailable in local versions.

Adaptation of neuropsychological tests tapping language functions, memory, and attention that use a linguistic strategy was a most sensitive issue in the study. The greatest problem for European multicentre studies on cognition is the equivalence of language-based tests in the different idioms and, consequently, normative data. Local validated versions of the neuropsychological tests were used in all E-ADNI sites. The Rey Auditory Verbal Learning Test was not included in the pilot

E-ADNI battery in order to avoid interference with word list recall test of the Alzheimer's Disease Assessment Scale-Cognitive Subscale (ADAS-Cog) as all cognitive tests were done in a single assessment. The Digit Span Forward and Backward were not included for time constraints. The North American Reading Test was not done because correspondent versions in local idioms were unavailable.

2.4. MR-imaging

MR-imaging was a part of the pilot E-ADNI study and data will be provided in a separate manuscript.

2.5. Blood, plasma, and CSF

Blood, plasma, and CSF collection and storage procedures were adapted from the ADNI-protocol, agreed between the biological PI centres (Munich and Gothenburg), and transferred to E-ADNI centres in order to create blood, plasma, and CSF repositories.

Blood and CSF were drawn and pre-processed at each of the centres according to an ADNI modified protocol, and sent to the biological principal investigator (PI) centres (Munich for CSF and Gothenburg for plasma). Serum analyses were not included in the pilot study, because if plasma analyses work well, so will serum analyses.

Obtained biological fluids were used to evaluate the levels of following parameters: Aβ42, t-tau, and phosphorylated tau-protein (P-tau181 and P-tau231) in CSF; Aβ42 and Aβ40 in plasma; DNA was not shipped and analyses were performed for APOE genotyping in the participating centres.

Data on patient recruitment, data collection, and clinical, neuropsychological, and imaging features have been described elsewhere in greater detail (Frisoni et al., 2008); the English version of the Case Report Form used in the present study can be downloaded from http://www.centroAlzheimer.it/E-ADNI_project.htm.

2.5.1. Procedures for collection, processing, aliquotation, storage, and shipment of samples of biological fluids

All samples were collected in the morning (8–11 am) to avoid diurnal variations.

2.5.1.1. Cerebrospinal fluid. Lumbar punctures were performed with the patient either lying down or sitting. CSF was obtained using a small gauge needle to avoid headache. The first 1 ml (2 ml in case of bleeding at the puncture) of CSF was discarded. Approximate volume of collected CSF was 8 ml (5 ml for the study and 1–3 ml for standard tests). CSF was collected in polypropylene tubes to avoid adsorbance of peptides and proteins to the test tube wall. The obtained sample was immediately sent (<30 min) to the local laboratory, where it was mixed thoroughly by hand and 1–3 ml was taken off for standard tests, i.e. cell count, protein analyses, and others. The CSF sample intended for the study was centrifuged in the original polypropylene tube at 2000*g* for 10 min at +4 °C, to eliminate cells and other insoluble material. One 1.0 ml aliquot of CSF was immediately sent in an appropriate box at room temperature to the biological PI centre in Munich (Germany) by courier mail. Remaining 4 ml were aliquoted in 8 × 0.5 ml portions, frozen at −80 °C, and stored until all patients at the centre were included and samples were sent in one batch. The samples were then sent, on dry ice in an appropriate box to Munich by courier mail.

2.5.1.2. Plasma. Plasma samples were collected by venipuncture in tubes containing EDTA as anticoagulant. After centrifugation, plasma samples were aliquoted in polypropylene tubes and stored at −80 °C pending biochemical analyses.

2.5.2. Analysis of samples

All measurements of CSF Aβ42, CSF t-tau and P-tau181 in CSF were performed in Munich, Germany (biological PI centre). These parameters were measured in duplicates with commercially available enzyme-linked immunosorbent assays (ELISAs) (Innotest b-amyloid1-42, Innotest hTAU-Ag, Innogenetics, Innotest PHOSPHO-TAU (181P) Zwjindrecht, Belgium, Art. No. K-1080, Art No. K-1032, and Art No. K-1120). Levels of CSF P-tau231 in CSF were measured using a sandwich ELISA developed by Applied NeuroSolutions, Inc. (Vernon Hills, IL).

Quantification of β-amyloid isoforms in plasma was performed in the biological PI centre in Gothenburg, Sweden, using the high sensitivity INNOTEST Aβ1-42 ELISA (Innogenetics, Ghent, Belgium), as previously described (Vanderstichele et al., 2000). β-amyloid 40 levels were determined using the hAmyloid β40 ELISA Highly Sensitive kit (The Genetics Company, Schlieren, Switzerland). This assay employs antibody W02 (epitope 5–8 in the Aβ sequence) for capture and the Aβ40 end-specific antibody G2-10 for detection (Hansson et al., 2007).

APOE genotyping was performed according to standard procedures by local laboratories of the E-ADNI centres.

2.6. Statistical analysis

In order to document differences between the groups and centres clinical, neuropsychological and laboratory variables as well as ratios of "frozen/fresh biomarker" were compared using nonparametric Kruskal–Wallis one-way analysis of variance (ANOVA). The homogeneity of variances was assessed with Levene and Brown-Forsythe tests. The Bonferroni least significant difference (LSD) test was used for post hoc analysis. Discriminant analysis was performed in order to determine which method of sample storage contributes to better classification of patients to diagnostic groups. Spearman's rank and Pearson correlation coefficients were used to assess the correlations between different biomarkers, as well as between diagnoses and biomarker levels. All data are presented as means ± standard deviation (SD). Differences were considered significant at $p < 0.05$. Data were analysed using the Statistical Package for Social Sciences (SPSS for Windows, version 15.0, SPSS Inc., 2006).

3. Results

3.1. Demographics

A total of 49 subjects were recruited with a mean age of 70.2 ± 10.6 (range 42–87) years. In the study participated 26 (53%) male and 23 (47%) female subjects. The distribution of number of patients in the centres is shown in the Fig. 1.

Over all centres were recruited 15 (31%) healthy older controls, 17 (35%) subjects with MCI, and 17 (35%) AD-patients. The distribution of number of cases with particular diagnosis in every centre was rather uniform, although the precise statistical verification is not possible because of small sample sizes (for detailed information see Fig. 1).

3.2. Cognitive testing

Mean Mini-Mental State Examination (MMSE) score [points] in healthy controls was 29.0 ± 0.7, in MCI subjects 27.1 ± 2.1, and in AD-patients 23.0 ± 3.3. The MMSE score was statistically different in healthy controls versus MCI ($p = 0.027$) and AD-patients ($p < 0.0005$), as well as in MCI-subjects versus AD-patients ($p < 0.0005$).

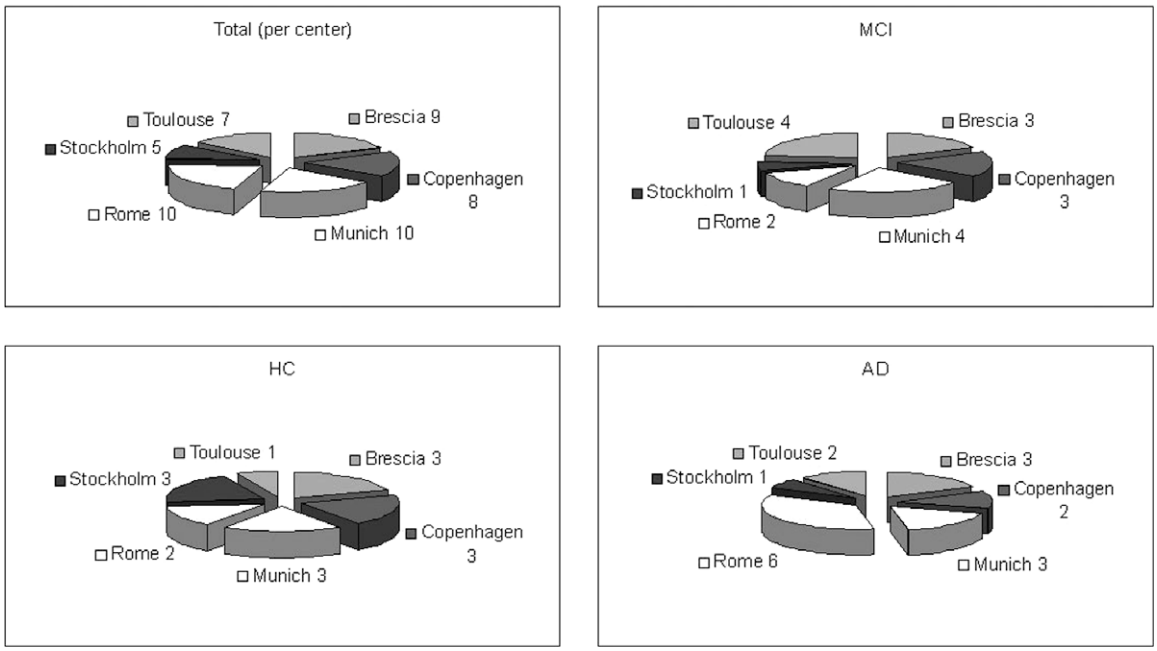

Fig. 1. Distribution of number of cases (healthy controls, MCI-, and AD-patients, total) in the centres.

Mean delayed recall test from the ADAS-Cog (DRadas) score in healthy controls was 6 ± 2.4 points, in MCI subjects 3 ± 2.6 points, in AD-patients 1 ± 1.6 points. The DRadas score in healthy controls significantly differed from that of patients with MCI ($p = 0.002$) and AD ($p < 0.0005$), and in MCI subjects differed from that of AD-patients ($p = 0.028$).

3.3. Laboratory data

The mean levels of neurochemical parameters measured in CSF and plasma within centres are presented in the Table 1.

There are no statistically significant differences between means of parameters measured in different centres.

We also performed a statistical analysis of differences of overall means of neurochemical parameters depending on diagnosis and without consideration of centres (Table 2).

We found statistically significant differences of CSF t-tau value (fresh samples) in healthy controls versus AD-patients (healthy controls <AD; $p = 0.049$), CSF Aβ42 value (frozen samples) in healthy controls versus MCI-subjects and AD-patients (healthy controls > MCI > AD; $p = 0.02$), plasma Aβ40 (fresh samples) in healthy controls versus AD-patients (healthy controls > AD; $p = 0.049$), and plasma Aβ40 value (frozen samples) in healthy controls versus AD-patients (healthy controls > AD; $p = 0.016$). Differences of other neurochemical parameters between groups did not reach statistical significance, although their change showed an expected trend, namely the values of CSF t-tau measured in frozen samples and CSF P-tau181 and 231 (fresh and frozen samples) were higher in MCI- and AD-patients than in normal controls, and in AD-patients versus MCI-subjects; the values of CSF Aβ42 (fresh and frozen samples) were lower in AD-patients than in MCI-patients, as well as plasma Aβ40 (fresh and frozen samples) were lower in AD-patients compared to MCI-patients and in MCI-patients versus healthy controls. In addition, the values of plasma Aβ42 (fresh and frozen samples) were lower in AD and MCI-patients compared to healthy controls and in AD-patients than in MCI-patients.

In order to evaluate the influence of sample storage and shipment on the results of tau-proteins and beta-amyloid measurements, the "fresh/frozen marker" ratios between the centres were calculated (Table 3).

We also performed an analysis of cumulative values of neurochemical parameters with and without freezing without consideration of centres. We have fond no significant differences between cumulative biomarker values measured in fresh and frozen samples, but strong positive correlations between biomarker levels in fresh and frozen samples (see Table 4). Discriminant analysis was performed in order to determine which method of sample storage contributes to better classification of patients to diagnostic groups. We introduced the demographic (age, gender) and neuropsychological (MMSE, DRadas) variables together with the biomarker levels measured in fresh or frozen samples. The use of fresh samples resulted in 100% correctly classified cases in the healthy controls group, 77.8% in MCI- and 90.9% in AD-group compared to 100% in healthy controls and MCI-groups, and 90.9% in AD-group when using frozen samples.

Positive correlations (Spearman's correlation analysis) between the levels of plasma Aβ40 (fresh samples) and plasma Aβ42 ($r = 0.296$, $p = 0.041$ for fresh samples, and $r = 0.285$, $p = 0.05$ for frozen samples); the same is true for the plasma Aβ40 measured in frozen samples and plasma Aβ42 values ($r = 0.330$, $p = 0.022$ for fresh samples, and $r = 0.382$, $p = 0.007$ for frozen samples) were found. There are no correlations between the levels of fresh or frozen Aβ40 and 42 measured in plasma and in CSF.

The Spearman's correlation analysis of diagnosis with different neurochemical parameters showed weak positive correlations of normal cognitive status with the level of CSF Aβ42 measured in fresh samples ($r = 0.323$, $p = 0.042$), level of plasma Aβ40 measured in frozen samples ($r = 0.287$, $p = 0.045$), and the level of plasma Aβ42 measured in frozen ($r = 0.348$, $p = 0.015$) and in fresh samples ($r = 0.293$, $p = 0.041$), as well as weak negative correlation with the level of CSF t-tau measured in fresh samples ($r = -0.364$, $p = 0.018$).

3.4. Genetic analysis

All patients were assessed for APOE genotype. APOEε2 allele in heterozygosity was found in 5 (10.2%) subjects (1 healthy patient, 2 MCI-subjects and 2 AD-patients). APOEε3 allele in heterozygosity

Table 1
Mean levels of measured neurochemical parameters within centres (pg/ml).

Centre	Cerebrospinal Fluid								Plasma			
	Total tau-protein		Phospho tau-protein 181		Phospho tau-protein 231		Beta-amyloid 42		Beta-amyloid 40		Beta-amyloid 42	
	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen	Fresh	Frozen
Brescia	379 ± 285	408 ± 287	60 ± 30	62 ± 31	28 ± 39	37 ± 41	425 ± 196	458 ± 179	172 ± 55	184 ± 58	23 ± 7	23 ± 8
Copenhagen	394 ± 275	398 ± 236	61 ± 27	60 ± 23	22 ± 40	26 ± 45	538 ± 250	636 ± 370	169 ± 41	198 ± 44	26 ± 8	26 ± 7
Munich	413 ± 311	430 ± 304	63 ± 16	51 ± 15	–	18 ± 21	626 ± 256	503 ± 184	150 ± 43	186 ± 48	20 ± 10	24 ± 9
Rome	243 ± 134	274 ± 150	40 ± 18	40 ± 19	11 ± 19	13 ± 21	439 ± 157	367 ± 152	172 ± 42	174 ± 49	17 ± 5	20 ± 6
Stockholm	377 ± 64	440 ± 45	63 ± 5	63 ± 6	32 ± 13	44 ± 12	400 ± 74	441 ± 91	174 ± 93	187 ± 87	16 ± 4	18 ± 6
Toulouse	–	–	–	–	–	–	–	–	152 ± 41	172 ± 16	20 ± 6	22 ± 9

was found in 23 (46.9%) subjects (8 healthy controls, 9 MCI-subjects, 6 AD-patients), the same allele in homozygosity was found in 21 (42.9%) subjects (7 healthy controls, 7 MCI-subjects, 7 AD-patients). APOEε4 allele in heterozygosity was found in 20 (40.8%) subjects (7 healthy controls, 7 MCI-subjects, 6 AD-patients), the same allele in homozygosity was found in 4 (8.2%) subjects (1 MCI-subject and 3 AD-patients). We found no differences between the groups in APOE genotype as well as no correlations between diagnosis and APOE genotype.

4. Discussion

The main finding of the study reported here is that use of frozen rather than fresh samples renders higher diagnostic accuracy within a multicentre context. Moreover, we show that the cooperation between different European centres within international multicentre MCI and AD studies is feasible.

Six E-ADNI centres succeeded to recruit normal controls, MCI- and AD-patients for clinical and neuropsychological assessment, as well as to collect and correctly transfer blood and CSF samples to the reference centres. This fact permitted us to perform statistical analysis of differences of overall means of neurochemical parameters depending on diagnosis without consideration of centres. In order to exclude Type I error post hoc analysis was controlled with the help of Bonferroni least significant difference test. We found significant differences of CSF t-tau value (fresh samples) in healthy controls versus AD-patients, of CSF Aβ42 value (frozen samples) in healthy controls versus MCI-subjects and AD-patients, of plasma Aβ40 (fresh samples) in healthy controls versus AD-patients, and of plasma Aβ40 value (frozen samples) in healthy controls versus AD-patients; another evidence of the fact that these significances are not a consequence of randomness is that other neurochemical parameters also showed an expected trend, although the statistical significance was not achieved probably due to small sample sizes.

In order to determine the best way of sample storage and shipment for future studies, we assessed the influence of delayed or immediate freezing on the results of protein measurements and calculated the ratios of fresh/frozen protein levels. We have also performed an analysis of pooled values of neurochemical parameters with and without freezing, again without taking into consideration the centres. We found no significant differences in the pooled values of CSF and plasma biomarkers measured in frozen versus fresh samples. Although, statistically significant differences of some described ratios stipulate in the future the need of unified method of blood and CSF samples storage and transportation, despite the fact of strong positive correlations between pooled values of neurochemical parameters with and without freezing. This statement is confirmed by the results of the discriminant analysis showing that the use of frozen samples for biomarker assessment contributes to better classification of patients to diagnostic groups. We can conclude that the use of frozen samples shipped on the dry ice would be preferable for future multicentre biomarker studies.

The analysis of the pilot E-ADNI study also permitted to find correlations of cognitive status with the levels of CSF Aβ42, plasma Aβ40 and 42, and CSF t-tau. This finding is in line with some earlier studies (de Leon et al., 2006; Tapiola et al., 2000), but is not a consistent finding (Buerger et al., 2002a; Hampel et al., 2001) probably due to differences in age and dementia severity in the populations studied.

The main limitation of the present pilot study is the small size of the samples. As a result we have found few statistically significant differences of the neurochemical parameters, although many showed the expected trend. Furthermore, we have found no

Table 2
Overall means ± SD of different neurochemical parameters depending on diagnosis.

	Parameter (pg/ml)		Healthy controls	MCI-subjects	AD-patients
CSF	Total tau-protein	Fresh[a]	262 ± 114	325 ± 243	474 ± 291
		Frozen	291 ± 126	365 ± 261	485 ± 261
	Phospho tau-protein 181	Fresh	50 ± 15	59 ± 28	60 ± 23
		Frozen	48 ± 15	56 ± 29	58 ± 21
	Phospho tau-protein 231	Fresh	8 ± 13	30 ± 37	29 ± 34
		Frozen	12 ± 18	28 ± 36	36 ± 35
	Beta-amyloid 42	Fresh	588 ± 193	457 ± 256	440 ± 145
		Frozen[a]	615 ± 265	438 ± 199	390 ± 145
PLASMA	Beta-amyloid 42	Fresh	23 ± 8	21 ± 6	18 ± 8
		Frozen	24 ± 7	23 ± 7	20 ± 8
	Beta-amyloid 40	Fresh[a]	187 ± 63	157 ± 45	152 ± 36
		Frozen[a]	208 ± 52	178 ± 49	166 ± 42

[a] Statistical significant differences between patients groups on post hoc analysis with Bonferroni LSD at $p < 0.05$.

Table 3
Ratios between fresh and frozen analytes (ratios are represented as means ± SD).

Centre	CSF			PLASMA	
	Phosphorylated tau-protein[a]	Total tau-protein	Beta-amyloid 42[a]	Beta-amyloid 40[a]	Beta-amyloid 42[a]
Brescia	98 ± 8	90 ± 12	90 ± 17	95 ± 9	99 ± 9
Copenhagen	100 ± 6	100 ± 23	90 ± 18	85 ± 5	98 ± 14
Munich	124 ± 9	95 ± 9	125 ± 9	81 ± 13	83 ± 13
Rome	103 ± 17	91 ± 17	125 ± 39	104 ± 40	84 ± 10
Stockholm	100 ± 3	86 ± 10	92 ± 17	91 ± 7	92 ± 6
Toulouse	–	–	–	87 ± 17	92 ± 8

Remarks: Phosphorylated tau-protein ratio: Munich vs. Brescia, Copenhagen, Rome, and Stockholm ($p = 0.001$).
CSF beta-amyloid 42 ratio: Brescia vs. Munich ($p = 0.002$), vs. Rome ($p = 0.003$); Copenhagen vs. Munich ($p = 0.002$), vs. Rome ($p = 0.004$); Rome vs. Stockholm ($p = 0.014$); Stockholm vs. Munich ($p = 0.011$).
Plasma beta-amyloid 40 ratio: Munich vs. Rome ($p = 0.015$).
Plasma beta-amyloid 42 ratio: Brescia vs. Munich ($p = 0.002$), vs. Rome ($p = 0.004$); Copenhagen vs. Munich ($p = 0.006$), vs. Rome ($p = 0.011$).
[a] Statistical significant differences between centres in Kruskal-Wallis ANOVA with on post hoc Bonferroni LSD test at $p < 0.05$.

Table 4
Correlations of the cumulative values of neurochemical parameters with and without freezing without consideration of centres.

	Marker (pg/ml)	Fresh samples	Frozen samples	*r* Value
CSF	Total tau-protein	357 ± 242	383 ± 235	0.974 ($p < 0.0005$)
	Phosphorylated tau-protein 181	56 ± 23	54 ± 22	0.948 ($p < 0.0005$)
	Phosphorylated tau-protein 231	22 ± 5	26 ± 5	0.969 ($p < 0.0005$)
	Beta-amyloid 42	498 ± 209	484 ± 228	0.855 ($p < 0.0005$)
PLASMA	Beta-amyloid 42	20 ± 8	22 ± 8	0.936 ($p < 0.0005$)
	Beta-amyloid 40	165 ± 50	184 ± 50	0.818 ($p < 0.0005$)

relationship of neurochemical parameters with APOE genotype and no correlation between diagnosis and APOE genotype, which is probably again due to the small size of samples. While this pilot study was powered for feasibility rather than testing biological hypotheses, future large-scale multicentre European studies will require much larger sample size.

The core feasible biomarkers of AD should fulfill the criteria established by the expert consensus conference (Anon, 1998). The potential marker should reflect a neuropathological characteristic of AD, should be validated in patients with neuropathologically confirmed diagnosis, reach the sensitivity of at least 85%, and specificity to differentiate AD from age-matched controls and other dementias of at least 75%. Currently, the regulatory authorities, such as FDA and EMEA, recommend validation of core feasible biomarkers of AD as primary end-points in upcoming phase II and III treatment trials of potential disease-modifying therapeutics (Hampel et al., 2008). Several CSF biomarkers, such as t-tau, p-tau and Aβ42, were already studied or are being evaluated within controlled multicentre phase IIb studies (Buerger et al., 2002b; Ewers et al., 2007; Hampel et al., 2004), other new potential biomarkers (BACE1 and isoprostanes) are undergoing phase I or IIa studies (Zhong et al., 2007). The thorough validation of core feasible biomarkers, however, can be achieved only as a result of a collaboration between leading academic centres. The results of the pilot E-ADNI study prove the feasibility of the multicentre biomarker measurement in the future large-scale EU studies.

The pilot E-ADNI study was aimed to act as a springboard to prepare a more extensive longitudinal study in the EU as a companion or complement to the US-ADNI. The more extensive longitudinal EU study also benefits from another effort – the FP7-funded neuGRID study, aimed to develop the infrastructure for clinical data and image collection of a large E-ADNI study. Thus, neuGRID together with the pilot E-ADNI (aimed at testing the feasibility of data collection) should provide a formidable thrust for the preparation of the larger EU study.

5. Disclosure statement for authors

The authors have no conflicts of interest to disclose. The industry sponsors had no role in the analysis or interpretation of these data nor in the content of the paper. Appropriate approval procedures were used concerning human subjects.

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