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ORIGINAL PAPER

Biomarkers of Alzheimer's Disease in the Cerebrospinal Fluid of Spanish Patients With Mild Cognitive Impairment

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Abstract The study of biomarkers in the cerebrospinal fluid (CSF) of patients with mild cognitive impairment (MCI) is a technique used with increasing frequency in the early diagnosis of Alzheimers disease (AD). Our objectiv was to gain an own experience while evaluating the reliability, sensitivity, and reproducibility of this technique in Spanish patients. Thirty-seven patients with MCI and twenty-four control subjects were studied by means of AD biomarker analysis in CSF. xMAP Luminex and INNOBIA Alzbio3 reagents of Innogenetics were used. The study variables assessed were levels of A$\beta_{1–42}$, T-tau and P-tau$_{181p}$ proteins as well as the ratios of T-tau/A$\beta_{1–42}$ and P-tau$_{181p}$/A$\beta_{1–42}$. Samples from nineteen patients were examined twice. Intra-class correlation coefficients for the three biomarkers used showed values higher than 0.95. We observed significant differences between the control group and the MCI groups. In the 6 months following lumbar puncture (LP), eleven (29%) patients with MCI developed AD. These patients showed significant lower levels in A$\beta_{1–42}$ protein (276.35 ± 78 vs. 367.13 ± 123.49, $P < 0.03$) and higher ratios (T-tau/A$\beta_{1–42}$ [0.38 ± 0.2 vs. 0.22 ± 0.14, $P < 0.01$] and P-tau$_{181p}$/A$\beta_{1–42}$ [0.27 ± 0.13 vs. 0.16 ± 0.1, $P < 0.008$]) to those in the same group who remained stable. We obtained similar results to those in the most recent reliable literature with our ROC curves, especially with our P-tau$_{181p}$ values and T-tau/A$\beta_{1–42}$ ratio in order to differentiate between control and AD groups. Our experience showed that the analysis of CSF-AD biomarkers in patients with MCI is reliable, sensitive and reproducible. In our knowledge, this is the first experience in Spanish patients.

Keywords Alzheimer's disease · Mild cognitive impairment · CSF Biomarkers · A$\beta_{1–42}$ protein · T-tau protein · P-tau$_{181p}$ protein · T-tau/A$\beta_{1–42}$ ratio · P-tau$_{181p}$/A$\beta_{1–42}$ ratio · Sensitivity · Reliability · Reproducibility

Introduction

The study of biomarkers in the cerebrospinal fluid (CSF) of patients with Alzheimer's disease (AD) has shown conclusively the presence of anomalies consisting of lower levels of A$\beta_{1–42}$ protein, and higher levels of proteins T-tau and P-tau [1]. As such, they reflect the two pathophysiologies in AD given that there is no actual "in vivo" diagnostic test. Therefore, the low level of A$\beta_{1–42}$ protein is considered a biomarker for neuritic plaques, and the increase in tau proteins can be correlated to both the degree of neurofibrillar degeneration and Braaks state of the disease [2].

Many authors consider mild cognitive impairment (MCI) as the most important risk factor in the development of AD [3]. Therefore, 40–60% of patients with MCI

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develop AD in the five years following diagnosis of MCI [2], even though many others have a stable form of memory alteration. Since 2002, prospective studies have been performed of CSF in patients with MCI, in the first instance, comparing them with control subjects, and secondly, with patients with AD. In the majority of these, the alterations associated with MCI have been observed halfway between normality and AD [4–13].

Recently, we performed a meta-analysis, of studies published until September 2008, in patients with MCI where all or some of the biomarkers on CSF were analysed. The objective was to evaluate the diagnostic utility of this technique in predicting which patients will develop AD once a diagnosis of MCI is made. The results showed that the isolated alteration of T-tau and P-tau levels in the CSF are very sensitive in differentiating between those patients with MCI who will develop AD, and those who will remain stable [14]. Moreover, we saw that normal levels of biomarkers are reliable in excluding the possible development of AD in patients with MCI [14].

Given these results, we attempted to gain our own experience with these biomarkers in patients with MCI, following these patients for 6 months after lumbar puncture (LP), and comparing our results with the most recent and reliable literature [15].

Materials and Methods

Study Design

Observational study of cases and controls.

Study Subjects

Thirty-seven patients with a diagnosis of MCI as defined with Petersens criteria (2006) [16] attending the cognitive deterioration out-patients clinic (some of them for several years), of the General Hospital of Alicante were included.

They all underwent physical and neurological examination, neuropsychological studies, assessment of depression using the Yesavage scale, cerebral magnetic resonance imaging (MRI) (although in five cases, cerebral computer tomography (CT) was performed), blood tests and LP. Six months after LP, the patients were reviewed, and placed into two groups: MCI-stable (MCI-S), or deterioration to AD (MCI-D).

These patients are reviewed regarding development of AD every 6 months, using both the NINCDS-ADRDA criteria [17] and the global deterioration scale (GDS).

Twenty-four control subjects without subjective memory loss or known cognitive deterioration were included. These control subjects were patients who were to undergo spinal anaesthesia for traumatologic or urological non-malignant conditions. Clinical details, including blood test results were collected. Neuropsychological study was performed a few days after the relevant surgical procedure was undertaken. These patients were then invited to annually attend the cognitive impairment out-patients clinic for review.

Inclusion Criteria

Patients over the age of fifty-five years with concordant clinical and neuropsychological diagnosis. In the control group, no patient had subjective memory loss, all mini-mental state examination (MMSE) test results were above twenty-seven and the informant questionnaire on cognitive decline in the elderly (IQCODE) was under 78. The neuropsychological criteria for MCI group were a MMSE test among 26–23 and a IQCODE over 78. Decline over two points in MMSE test and/or increase in more than 7 points in IQCODE were considered as conversion to AD in MCI patients. Both before inclusion in this study, and before LP was perfomed, informed consent was obtained.

Exclusion Criteria

The presence of dementia or other neurological, psychiatric or medical disease which could provoke cognitive deterioration, anti-coagulant therapy, failure to obtain informed consent, and a score greater than five using the Yesavage scale of depression.

Procedures

The neurologist responsible for each patient made a diagnosis of amnestic (single or multiple domain) or non amnestic domain MCI in accordance with Petersen′s criteria [16]. Following this, a neuropsychological report enabled re-classification of the MCI patients into different diagnostic sub-groups.

The neuropsychological examination included: MMSE test, the IQCODE, Rey auditory verbal learning, California verbal learning, trail making test and the geriatric depression scale of Yesavage. With these tests, the evaluation of memory, language, executive function, attention, and visuo-constructive capacity were made. Alteration of one function was defined as a Z result of -1.5 or less, which was at least 1.5 standard deviations below the mean of the control subjects, in at least one of the tests used to evaluate that function. The neuropsychological tests done in control group were the same than in patients group.

Extraction and Analysis of CSF

This was performed between February 2008 and February 2009. The samples were obtained between 10 a.m. and 14 p.m.

In patients with MCI, the LP was performed by their own neurologist with a 20 × 3.5 gauge needle. The CSF sample was collected in standard tubes and centrifuged if little sanguinolent, before being frozen. Obvious sanguinolent CSF was discarded.

The CSF (±1 mL) of control subjects was obtained in the operating theatre by the anaesthetist performing spinal anaesthesia.

After LP, all patients were advised to avoid Valsalva manouevres for at least 3 days.

Quantification of Levels of $A\beta_{1–42}$, T-tau, and P-tau_{181p}

This was performed using xMAP Luminex technology and INNO-BIA Alzbio3 reagents of Innogenetics (Ghent, Belgium). The details of this reagent combination for immunoassay and analytic platform have been previously published [18].

All samples were simultaneously and blindly analysed with respect to the clinical details after recruitment was completed. The samples of nineteen patients were analysed twice on the same day and within the same kit of reagents.

Study Variables

Levels of $A\beta_{1–42}$, T-tau, and P-tau_{181p} proteins in the CSF, as well as the T-tau/$A\beta_{1–42}$, and P-tau/$A\beta_{1–42}$ ratios. These latter variables are being frequently used by many authors and appear to reflect the relationship between the two pathophysiological ways of the disease (amyloid and tau).

Statistical Analysis

The reliability of the technique was calculated using the intra-class correlation coefficient.

The Kolmogorov–Smirnov test was used to analyse the distribution of each variable.

Students t-test for parametric variables and the U-Mann–Whitney test for non-parametric variables were used for comparison between the two groups.

ANOVA was used for parametric variables and the Kruskal–Wallis test for non-paramentric variables for comparison between three groups.

Receiver operating characteristic (ROC) curve analysis was performed to determine the best cut-off values for measurement of variables. The best cut-off value was defined taking into account the highest sensitivity. Following this, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were all obtained for each variable and cut-off point. In all hypotheses, a p-value of less than 0.05 determined statistical significance. The statistical package SPSS version 10.0 was employed.

Ethical Criteria

The two pharmaceutical companies who contributed to this project had no role in either study design, drafting of the final report, or data interpretation and collection. This project was approved by the Ethical Committee in the Clinical Investigation Unit of the General Hospital of Alicante after civil liability insurance was obtained.

Results

Technique Reliability

In the nineteen samples analysed twice, the intra-class correlation coefficients were: 0.97 (CI 95%: 0.92–0.98) for $A\beta_{1–42}$ protein; 0.98 (IC 95%: 0.95–0.99) for T-tau protein, and 0.97 (IC 95%: 0.93–0.99) for P-tau_{181p} protein.

Control Versus MCI Group

The clinical characteristics and previous medical history of all patients is presented in Table 1. Even though there appears to be a difference in proportions of the sexes, there is no significant difference in age between MCI and control groups. However, we observed a higher proportion of cardiovascular risk factors in the control group (diabetes mellitus and arterial hypertension), and a higher proportion of patients with a history of depression. The evolution mean time before LP was 31.7 ± 25 months. 75% of MCI patients did not develop AD for over one year. There were no complications following LP.

The results of the variables studied in the MCI and control groups are shown together in Table 2. In MCI group, higher levels of T-tau and P-tau_{181p} proteins and higher T-tau/$A\beta_{1–42}$, and P-tau/$A\beta_{1–42}$ ratios were seen. In this group, there was a lower level of $A\beta_{1–42}$ protein with no statistical significance.

ROC curve analysis between control and MCI groups provides the achieved threshold concentrations for greater diagnostic sensitivity and specificity, as well as both PPV and NPV. The high percentages of sensitivity for the chosen cut-off values are highlighted (Table 3).

Comparison of MCI-S and MCI-D Groups

In the 6 months after LP was performed, eleven (29%) patients developed AD (MCI-D) and the rest remained

Table 1 Clinical and demographic characteristics of control and MCI groups

	Control group	MCI group	MCI-D-group
Number of cases	24	37	11
Sex (M/F)	10/14	13/24	2/9
Age (years)			
Mean ± SD	73.25 ± 8.34	73.43 ± 6.63	73.43 ± 6.63
Antecedents			
Diabetes	9/24 (37.5%)	7/37 (19%)	3/11 (27%)
Hypertension	18/24 (75%)	16/37 (43%)	6/11 (54%)
Hyperlipemia	8/24 (33,3%)	17/37 (46%)	3/11(27%)
Depression	0	14/37	2/11(18%)
Familial antecedents of dementia	3	8	0
Years of schooling (mean)	7	5	6
Start of symptoms (months)	–	1–12: 9	1–12: 4
		13–24: 13	13–24: 4
		25–36: 3	25–36: 2
		37–48: 3	37–48: 0
		49–60: 7	49–61: 1
		> 61: 2	>61: 0
Mean MMSE Folstein ± SD	28 ± 0.5	25 ± 2.4	23 ± 1.2
MCI-clinical		Amnestic: 33	Amnestic: 11
		Non amnestic: 4	Non amnestic: 0
Post-LP headache	0	0	0

MCI mild cognitive impairment, *SD* standard deviation, *MMSE* Mini-mental state examination

Table 2 CSF biomarker concentrations and ratios in control and MCI groups

	Control group (n = 24)	MCI group (n = 37)	S. L. ($P < 0.05$)
$A\beta_{1–42}$ (pg/ml) Mean ± SD	365.21 ± 112.53	340.14 ± 118.53	0.4
T-tau (pg/ml) Mean ± SD	53.21 ± 23.38	78.98 ± 37.57	0.004
P-tau_{181p} (pg/ml) Mean ± SD	31.25 ± 13.74	56.88 ± 25.65	0.0001
T-tau/$A\beta_{1–42}$ Mean ± SD	0.15 ± 0.08	0.27 ± 0.17	0.005
P-tau_{181p}/$A\beta_{1–42}$ Mean ± SD	0.09 ± 0.06	0.19 ± 0.12	0.001

Student t-test. $A\beta_{1–42}$, amyloid-β 1 to 42 peptide; p-tau_{181p}, tau phosphorylated at the theonine 181 position; SD, standard deviation. S.L., signification level

Table 3 Receiver operating characteristic (ROC) curve parameters for control versus MCI group

Parameters	T-tau	T-tau/$A\beta_{1–42}$	P-tau/$A\beta_{1–42}$	P-Tau
ROC AUC	0.73	0.71	0.76	0.81
Threshold value	48 pg/ml	0.11	0.66	28 pg/ml
Sensitivity (%)	84	89	86	86
Specificity (%)	59	46	50	55
PPV (%)	76	72	73	74
NPV (%)	70	73	71	72

$A\beta_{1–42}$, amyloid-β 1 to 42 peptide; p-tau_{181p}, tau phosphorylated at the theonine 181 position; ROC, receiver operating characteristic; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value

stable. No significant age differences were noted in either group. Of the MCI-D patients, 72% developed AD within two years since the start of MCI symptoms (Table 4).

Comparing the results of both MCI-S and MCI-D patients, lower levels of $A\beta_{1–42}$ protein, and higher ratios of T-tau/$A\beta_{1–42}$, and P-tau/$A\beta_{1–42}$ were seen in the latter group (Table 5).

ROC curve analysis between MCI-S and MCI-D for the given cut-off values, taking into account the sensitivity, shows that NPV and sensitivity values show higher percentages (Table 6).

Comparison Between Control and MCI-D Groups

No differences were observed in the age of either group, and the other clinical variables are shown in table 1.

Comparison of study variables shows a significant increase in T-tau and P-tau_{181p} proteins, and in the T-tau/$A\beta_{1–42}$, and P-tau/$A\beta_{1–42}$ ratios in the MCI-D group.

Table 4 Clinical and demographic characteristics of MCI-S and MCI-D groups, as defined 6 months after LP

	MCI-S groups	MCI-D groups
Number of cases	26	11
Sex (M/F)	11/15	2/9
Age (years)		
Mean ± SD	73.25 ± 8.34	73.43 ± 6.63
Antecedents		
Diabetes	4/26 (15%)	3/11 (27%)
Hypertension	10/26 (38%)	6/11 (54%)
Hyperlipemia	15/26 (57%)	3/11(27%)
Depression	12/26 (46%)	2/11 (18%)
Familial antecedents of dementia	8	0
Years of Schooling (mean)	4.3	6
Start of symptoms (months)	1–12: 5	1–12: 4
	13–24: 9	13–24: 4
	25–36: 1	25–36: 2
	37–48: 3	37–48: 0
	49–60: 6	49–60: 1
	>61: 2	>61: 0
Mean MMSE Folstein ± SD	25 ± 1.5	23 ± 1.2
MCI- clinic	Amnesic: 22	Amnesic: 11
	Non amnesic: 4	Non amnesic: 0

MCI mild cognitive impairment, *SD* standard deviation, *MMSE* Mini-mental state examination

In this latter group we also observed a lower level of $A\beta_{1–42}$ when compared to the control group (Table 5).

ROC curve analysis between the control and MCI-D groups, using Shaw et al. threshold values [15], reveals high sensitivity percentages for P-tau_{181p} protein, and the P-tau/$A\beta_{1–42}$ ratio. High specificity was observed for T-tau, $A\beta_{1–42}$ and T-tau/$A\beta_{1–42}$ ratio. Lastly, high percentages were obtained in NPV for $A\beta_{1–42}$, P-tau_{181p} proteins and both ratios (Table 7).

Table 6 Receiver operating characteristic (ROC) curve parameters for MCI-S versus MCI-D groups, as defined 6 months after LP

Parameters	$A\beta_{1–42}$	T-tau	P-tau	T-tau/$A\beta$	P-tau/$A\beta$
Threshold value	320	77.5	54.5	0.18	0.17
ROC AUC	0.72	0.72	0.70	0.75	0.76
Sensitivity (%)	82	72.7	82	91	82
Specificity (%)	62	70	58	50	66
PPV (%)	47	50	45	43	50
NPV (%)	89	86	88	93	89

$A\beta_{1–42}$, amyloid-β 1 to 42 peptide; p-tau, tau phosphorylated at the theonine 181 position; ROC, receiver operating characteristic; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value

Comparison of the Three Study Groups

Figure 1 shows how the three biomarkers and the two ratios relate to the control, MCI-S, and MCI-D groups. Using ANOVA, different significance levels were obtained for all variables except protein $A\beta_{1–42}$ where borderline significance was seen.

Discussion

This study presents our experience in the analysis of biomarkers in the CSF of patients with MCI. We observed significant differences between control and MCI subjects which supports the idea that this condition can be considered a risk factor for the development of AD [4]. We also note that those patients with MCI who have lower levels of $A\beta_{1–42}$ protein progress rapidly to AD. These results are consistent with most of the literature, including the meta-analysis published by our group recently [14].

Of our patients with MCI, 29% developed AD within the first 6 months after LP. This proportion, while greater than that described in the literature, can be explained by having

Table 5 CSF biomarker concentrations and ratios in control, MCI-S and MCI-D groups, as defined 6 months after LP

	MCI-S (n = 26)	S.L. ($P < 0.05$)	MCI-D (n = 11)	S.L. ($P < 0.05$)	Control group (n = 24)
$A\beta_{1–42}$ (pg/ml) Mean ± SD	367.13 ± 123.49	0.03	276.35 ± 78.00	0.02	365.21 ± 112.53
T-tau (pg/ml) Media ± DS	71.46 ± 33.97	0.06	96.74 ± 41.28	0.0001	53.21 ± 23.38
P-tau_{181p} (pg/ml) Media ± DS	51.89 ± 25.53	0.06	58.68 ± 22.85	0.0001	31.25 ± 13.74
T-tau/$A\beta_{1–42}$ Media ± DS	0.22 ± 0.14	0.01	0.38 ± 0.2	0.001	0.15 ± 0.08
P-tau_{181p}/$A\beta_{1–42}$ Media ± DS	0.16 ± 0.1	0.008	0.27 ± 0.13	0.0001	0.09 ± 0.06

Student t-test. $A\beta_{1–42}$, amyloid-β 1 to 42 peptide; p-tau_{181p}, tau phosphorylated at the theonine 181 position; SD, standard deviation. S.L., signification level

Table 7 Receiver operating characteristic curve parameters for control group versus MCI-D group

Parameters	T-tau	$A\beta_{1–42}$	P-Tau	T-tau/$A\beta_{1–42}$	P-tau/$A\beta_{1–42}$
ROC AUC	0.85	0.73	0.92	0.86	0.90
Threshold value	93 (pg/ml)	192 (pg/ml)	23 (pg/ml)	0.39	0.14
Sensitivity (%)	45	27	100	45	90.9
Specificity (%)	92	91	30	95	75
PPV (%)	56	53	77	59	62
NPV (%)	67	85	95	94	95

$A\beta_{1–42}$, amyloid-β 1 to 42 peptide; p-tau, tau phosphorylated at the theonine 181 position; ROC, receiver operating characteristic; AUC, area under the curve; PPV, positive predictive value; NPV, negative predictive value

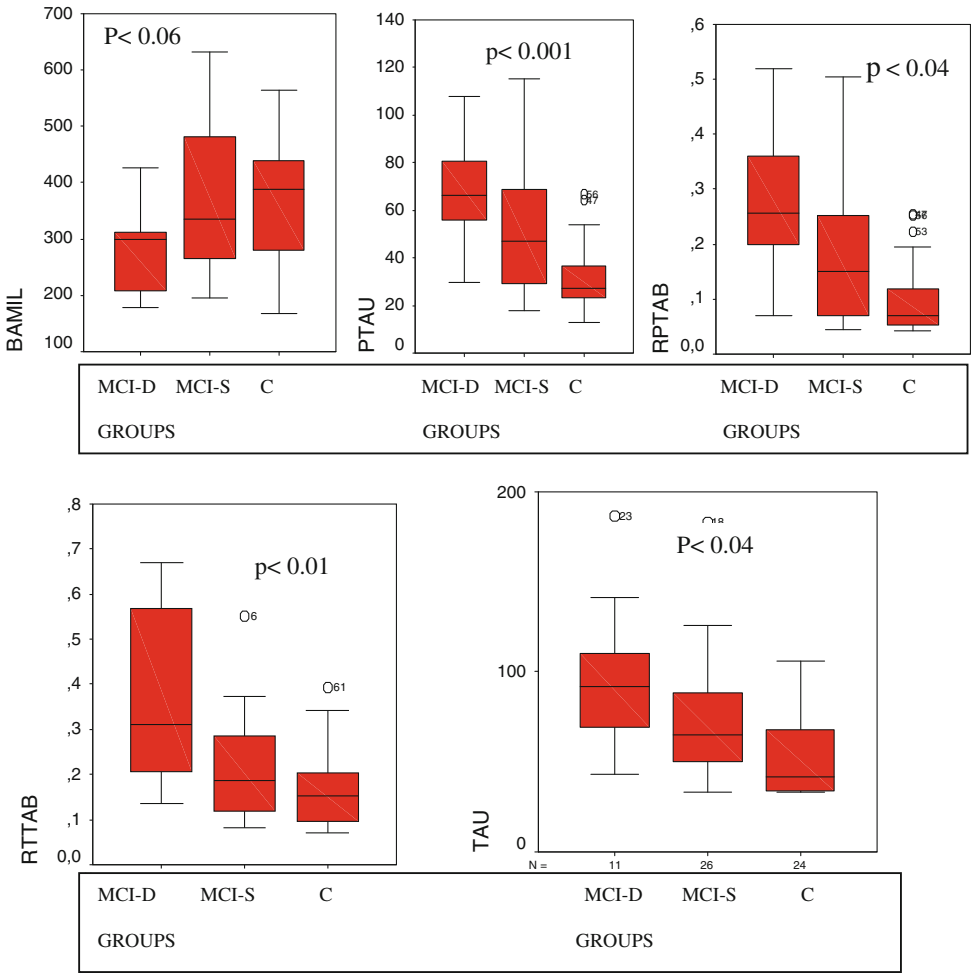

Fig. 1 Graphic representation of the CSF biomarker concentrations and ratios in control, MCI-S and MCI-D groups. Data on the y axis: BAMIL($A\beta_{1–42}$ protein) PTAU ($P\text{-}tau_{181p}$ protein) and TAU(T-tau protein) units: pg/ml; RPTAB ($P\text{-}tau_{181p}$ protein/$A\beta_{1–42}$ protein) and RTTAP (T-tau protein/$A\beta_{1–42}$ protein) are ratios

performed LP at the start of the study without taking into account the time for symptom development. If one considers that approximately 10% of patients with MCI develop AD annually, then our study results are consistent with this percentage, remembering that the average time scale from LP to development of AD was 31.7 ± 25 months.

Using similar methodology, Hansson et al. [4] demonstrated that 95% of patients with MCI who showed changes in some of these biomarkers developed AD in the five years following LP, while those who had normal levels of these biomarkers remained stable in 83% of cases. The association between pathological findings in the CSF and progression to AD was independent of other risk factors like age, sex, education, or the APOE genotype [4]. The review of our cohort will be continued to verify this.

In our patients who progressed to develop AD in the first 6 months after LP, the results are similar to previously

Table 8 Comparison between results of this study and Shaw et al. in P-tau/Aβ_{1-42} ratio

	Shaw et al. [15]	This study
ROC AUC	0.85	0.90
Threshold value	0.10	0.12
Sensitiviy (%)	91	91
Specificity (%)	71	75
PPV (%)	77	62
NPV (%)	88	95

Aβ_{1-42}, amyloid-β 1 to 42 peptide; p-tau, tau phosphorylated at the theonine 181 position; PPV, positive predictive value; NPV, negative predictive value. ROC, receiver operating characteristic; AUC, area under the curve

Table 9 Positive predictive value of the five variables combination taking as reference the Shaw et al. (2009) threshold values

	0	1	2	3	4 or 5
MCI-D	0%	9,1%	37.5%	33.3%	100%
Control group	100%	90.9%	62.5%	66.7%	0%

MCI-D Mild cognitive impairment developing dementia in the 6 months after lumbar puncture. *0* no abnormal variables, *1* one abnormal variable, *2* two abnormal variables, *3* three abnormal variables, *4* or *5* four or five abnormal variables

published data for patients with AD demonstrated at autopsy [15]. This was shown in a multi-centre American study arranged using the same methodology used by ourselves which again confirms the reliability and reproducibility of this technique. Using their cut-off points as the gold standard, we observed that in our patients, the P-tau$_{181p}$ protein showed a sensitivity and NPV of 100%, and the P-tau/Aβ_{1-42} ratio showed a sensitivity of 91% and a NPV of 94%. We also observed practically identical values for the P-tau/Aβ_{1-42} ratio in our results as compared to theirs. Therefore, a high level of reproducibility can be obtained with this technique (Table 8).

Lastly, it would seem that four altered variables are enough to achieve a PPV of 100% using the same criteria as Shaw et al. (Table 9).

The new criteria for investigation of AD propose that MRI, positron emission tomography (PET), and biomarkers in CSF allow the diagnosis of AD in patients with MCI [19]. Also the analysis of CSF shows advantages compared to the other two techniques. It is cheaper and easier to obtain than PET, which studies only the amyloid path for development of AD, and continues to be used in an extremely restricted manner. Furthermore, it is less time consuming than MRI volume assessment and CSF changes reflect AD pathology before volumetric analysis. Therefore, it appears to be particularly preferable for very early diagnosis [20].

Various studies have compared the predictive capability of this technique with other complementary tests in order to predict which MCI patients would develop AD [21, 22]. Vemuri et al. [22] within a multi-centre Alzheimer's disease neuroimaging initiative (ADNI) observed that a computerised MRI and CSF study provide additional predictive information regarding the time taken to convert from amnestic MCI to AD, and the combination of both provides a better predictive capability than either alone. Previously, Bouwman et al. [10] communicated that the study of CSF raised the predictive capability of manually analysed MRI, and that the presence of anomalies in either technique implied that 94% of patients with MCI would develop dementia.

From a methodological perspective, use of xMAP Luminex technology appears to present certain advantages with respect to analysis with ELISA. For example, the ability to simultaneously quantify the three biomarkers, and the small quantity of CSF required for analysis (50 µl for Luminex in front of 150 µl for ELISA). This facilitates the recruitment of control subjects and minimises the possible undesirable effects of LP. It also improves the technical management and quality control of the study according to some authors [23]. In our study, simultaneous measurement of all variables, the extraction of CSF during a definite time slot, as much as age identification in the compared groups considerably reduces any possible confounding factors.

In a recent study, the extensive use in Europe of biomarkers for dementia in the CSF was described [20], but this has not been the case until now in Spain, making the results of this study even more interesting.

The differences obtained between those patients who developed AD in the first 6 months after LP gives us a probable pattern of rapid change towards AD as described in recent literature [24] which would therefore advise the implementaion of this test for prognostic as well as diagnostic purposes. Even though it is an invasive test, no adverse effects were observed in the study population, which is in concordance with studies performed by other authors [25], especially when said test was performed under good conditions.

In the limitations of this study, we indicate that some patients were included several months or even years after the onset of clinical signs, however, control studies for LP have shown an important stability in the levels of CSF biomarkers [26]. We have not autopsy data and the number of subjects studies is small. From another perspective, we have had merely a short period of follow-up and review.

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