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Unfolded p53 in Blood as a Predictive Signature Signature of the Transition from Mild Cognitive Impairment to Alzheimer's Disease

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Abstract. Mild cognitive impairment (MCI) is a syndrome defined as cognitive decline, but not sufficient to meet the criteria for any specific dementia. Although subjects with MCI may have an increased risk to develop AD, this clinical state encompasses several subtypes of cognitive dysfunction of different etiologies, none of which necessarily progresses to AD. The current inability of clinical criteria to accurately identify this at-risk group for AD development is fuelling the interest in biomarkers able to supplement clinical approaches. We recently described a blood-based cytofluorimetric method for conformationally altered p53 protein detection that allows the discrimination of AD patients from control subjects and patients affected by other dementias. The same protein also predicted progression to AD in preclinical patients with MCI two years before clinical diagnosis of AD was made. Herein, we describe these findings and discuss the potential of the test in diagnosing AD.

Keywords: cytofluorimetric approach, mild cognitive impairment, risk factor, unfolded blood p53

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by irreversible cognitive and physical deterioration. AD is the most common cause of dementia of the elderly population and its incidence doubles every five years between 65 and 85 years of age. It also represents a growing public health problem as life expectancy increases and the number of people with AD is expected to increase dramatically from approximately 24 million people in 2001 up to 81 million worldwide by 2040 [1]. The treatment of AD remains a major challenge because of an incomplete understanding of the events that lead to the selective neurodegeneration typical of AD brains. In view of existing and emerging therapeutic compounds, the focus has increasingly shifted to accurate detection of the earliest phase of illness.

The degenerative process in AD probably starts 20–30 years before the clinical onset of the disease [2]. This clinical phase, between prodromal and established AD, lies within the boundaries of the diagnosis of mild cognitive impairment (MCI), during which subjects have

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measurable cognitive deficits, but not sufficient to fulfill criteria for any specific dementia [3,4]. In particular, MCI subjects show the presence of subjective memory impairments, objective memory performance changes, and declining financial skills [5], whereas other cognitive functions and daily activities remain normal [6].

Although it is documented that subjects with MCI have an increased risk of AD, this clinical state encompasses several subtypes of cognitive dysfunction of varied etiologies, none of which necessarily progresses to AD [7]. In particular, MCI subclassifications include an amnestic form (aMCI), characterized by isolated memory impairments, and one non-amnestic (naMCI) in which other cognitive functions rather than memory are mostly impaired [8]; within these groups, aMCI subjects showed 8.6-fold higher odds of developing AD [9]. Furthermore, it has been determined that patients suffering from aMCI progress to AD at a rate of 12 to 28% per year [3,10], whereas other MCI subjects improve, reverting to a normal level of cognitive functioning [11] and some die [12], thus underlining multiple etiologies for MCI. Hence, within this context, there is a need to reliably predict which patients with MCI will progress to AD. The current inability of clinical criteria to accurately identify this at-risk group is fuelling the emerging interest in biomarkers to potentially supplement clinical approaches.

Within this reference frame, we studied p53 in aged controls and demented patients finding that the conformational state of p53 protein in blood cells allows the differentiation of AD patients from control subjects and patients affected by other dementias. The notions about p53 come mainly from oncology. p53 has been described as a the guardian of the genome [13]. It is activated in response to certain stressful situations, inducing either cell cycle arrest and DNA repair or apoptosis [14]. The full-length p53 molecule comprises three major domains; the N terminus transactivation domain, the core DNA-binding domain, that is stabilized by a Zn^{2+} atom coordinated by a histidine and four cysteines, and the C terminus tetramerization domain. p53 is mutated in about half of all human cancers, with 95% of these mutations occurring in the core domain [15]. In particular, the core domain is endowed with high flexibility, and certain missense mutations inside this central domain can affect tertiary structure of the protein. Thermodynamic studies of p53 cancer mutations have identified three major phenotypes classified as: i) mutation affecting DNA contacts that have little impact on protein folding; ii) mutation disordering local structure leaving more than 85% of the protein in folded conformation; and iii) mutations globally denaturating the core domain, thus resulting in more than 50% of unfolded isoform [16–19]. This third phenotype is recognized by PAb240 antibody, since its epitope is cryptic when p53 is in wild type conformation and becomes accessible only when the core domain is denatured. It should be stressed that conformational changes of p53 can also be due to posttranscriptional modifications in the absence of specific mutations [20–23].

Table 1
Demographic and clinical variables and genotype frequency of the APOE polymorphisms of all the subjects. N: number; M: male; F: female; L.O.I.: length of illness; MMSE: Mini-Mental State Examination. Data are expressed as mean ± standard deviation. For the genotype frequency values are expressed as number (%)

	CTR	AD	MCI
N (M;F)	45 (20;25)	45 (9;36)	28 (5;23)
mean age ± SD	75 ± 12.6	81 ± 7.2	69 ± 12.4
L.O.I. (month)		52 ± 23	19 ± 6
MMSE	28.4 ± 1.6	14 ± 5.5	27.3 ± 2
Genotype	CTR	AD	MCI
$\varepsilon2/\varepsilon3$	5 (11)	2 (4)	3 (10.7)
$\varepsilon3/\varepsilon3$	35 (78)	25 (56)	17 (60.7)
$\varepsilon3/\varepsilon4$	5 (11)	14 (31)	7 (25)
$\varepsilon2/\varepsilon4$	0	0	1 (3.6)
$\varepsilon4/\varepsilon4$	0	4 (9)	0

Within this context, the presence of an altered unfolded p53 isoform was found enhanced in AD patients in comparison with control and other types of dementia [24]. Notably, the best sensitivity and specificity of this marker was observed at ages $\leqslant$ 70 years [24], suggesting the idea that unfolded p53 could also be detected in the early stage of the disease.

On this basis, the purpose of our study was to further investigate whether conformationally altered p53 expression may be applied to those patients falling in the ill-defined category of MCI and in particular to predict which subjects among MCI patients will progress to AD.

MATERIALS AND METHODS

Subjects

Venous blood samples from healthy people and from patients affected by MCI and AD were obtained from Sant'Orsola Hospital in Brescia, Italy. This population consisted in a group of 45 patients with sporadic AD, 45 healthy age-matched controls (CTR), and 28 patients with MCI (Table 1).

The protocol of the study, including the follow-up visits, was approved by the Ethical Committee and a

written consent was obtained from all subjects or, where appropriate, their caregivers.

All the subjects were examined by a senior neurologist or geriatrician and diagnosis of dementia was made according to DSM-IV and the NINCDS-ADRDA criteria. All MCI subjects met the original Petersen/Mayo criteria for MCI [3,7]. Dementia was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT scan) investigation. Cognitive status was quantified using the Mini Mental State Examination (MMSE). All AD patients fulfilled the criteria for probable AD [25] and were classified as "sporadic" on the basis that they lacked a familial history of the disease, as acquired from interviews with first degree relatives. Control subjects were aged individuals with no clinical signs of neurological or psychiatric diseases, mostly enrolled among spouses of the AD group of patients. None of the subjects selected in this study was affected by neoplastic or autoimmune disease when the blood samples were taken. For each subject, the count of leukocytes was within the regular interval clinical frame.

After two years from the recruitment, all 28 MCI subjects were recalled and examined by the geriatrician. The progression to AD was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (CT Scan) investigation.

Flow cytometry

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll Hipaque density gradient from Na+/citrate samples (Eurobio, Italy) and fixed in 2% formaldehyde in PBS. Rinsed cells were permeabilized with 0.2% saponin in PBS solution and incubated in ice for 30 min with a primary monoclonal antibody recognizing unfolded p53 (clone PAb240; NeoMarkers, Fremont, CA) (4 μg/ml in PBS/1% BSA solution). In particular the PAb240 antibody recognizes the epitope 211–217 aa inside the core domain, which is accessible only when the core domain is denatured and the protein assumes an unfolded tertiary structure, thus discriminating the so called "mutant-like" isoform [22]. Cells rinsed in PBS/1% BSA were incubated for 30 min in ice with a goat anti-mouse IgG antibody phycoerythrin (PE)-conjugated (DakoCytomation, Denmark; 1:40 in PBS/1% BSA). After rinsing, few microliters of cell suspension were deposed on a glass slide and observed with a fluorescence microscope Olympus BX51 with blue excitation (BP450–480 nm, DM 500, and barrier filter 515 nm) equipped with a Camedia digital camera. Samples were observed at 40X magnification. Pictures were taken with the same instrumental setting. Orange-red fluorescence from AD positive cells was compared with the background fluorescence of control samples as a technical control of the experimental setting. The percentage of PAb240 positive cells was quantified by cytofluorimetric analysis. Cell suspension was analyzed with a flow cytometer Partec PASII (Partec, Germany). PBMC population was identified by forward and side angle scatter (FSC, SSC) and mutant p53 emission was detected in the FL-2 channel (535–580 band pass filter). For each sample, data from 20000 events were recorded in list mode, displayed on logarithmic scales and analyzed using WinMDI 2.8 software.

Molecular genetic analysis

Genomic DNA was extracted from peripheral leukocytes by proteinase K digestion and standard phenol/chlorophorm extraction procedure. The APOE gene polymorphisms were determined by Hha I restriction endonuclease digestion of PCR products, according to Hixson and Vernier [26].

Statistical analysis

The data were analyzed by analysis of variance (ANOVA) followed when significant by an appropriate post hoc comparison test. Differences were considered significant when a p-value $\leqslant 0.05$ was attained.

RESULTS

118 subjects were enrolled among those referring to the Department of Geriatric Medicine, S. Orsola Fatebenefratelli Hospital in Brescia, Italy. Control subjects and AD and MCI patients were comparable as far as age and gender distribution (see Table 1). The distribution of $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles of APOE was 0.022/0.733/0.244 in AD cases, and 0.056/0.889/0.056 in control subjects; the frequency of the APOE $\varepsilon 4$ allele was, as expected, higher in AD population than in control subjects. MCI showed a distribution of $\varepsilon 2/\varepsilon 3/\varepsilon 4$ alleles of APOE of 0.071/0.786/0.143.

Blood mononuclear cells derived from AD and MCI patients and healthy subjects were subjected to cytofluorimetric analysis using the conformational specific antibody, PAb240, which discriminates unfolded p53 ter-

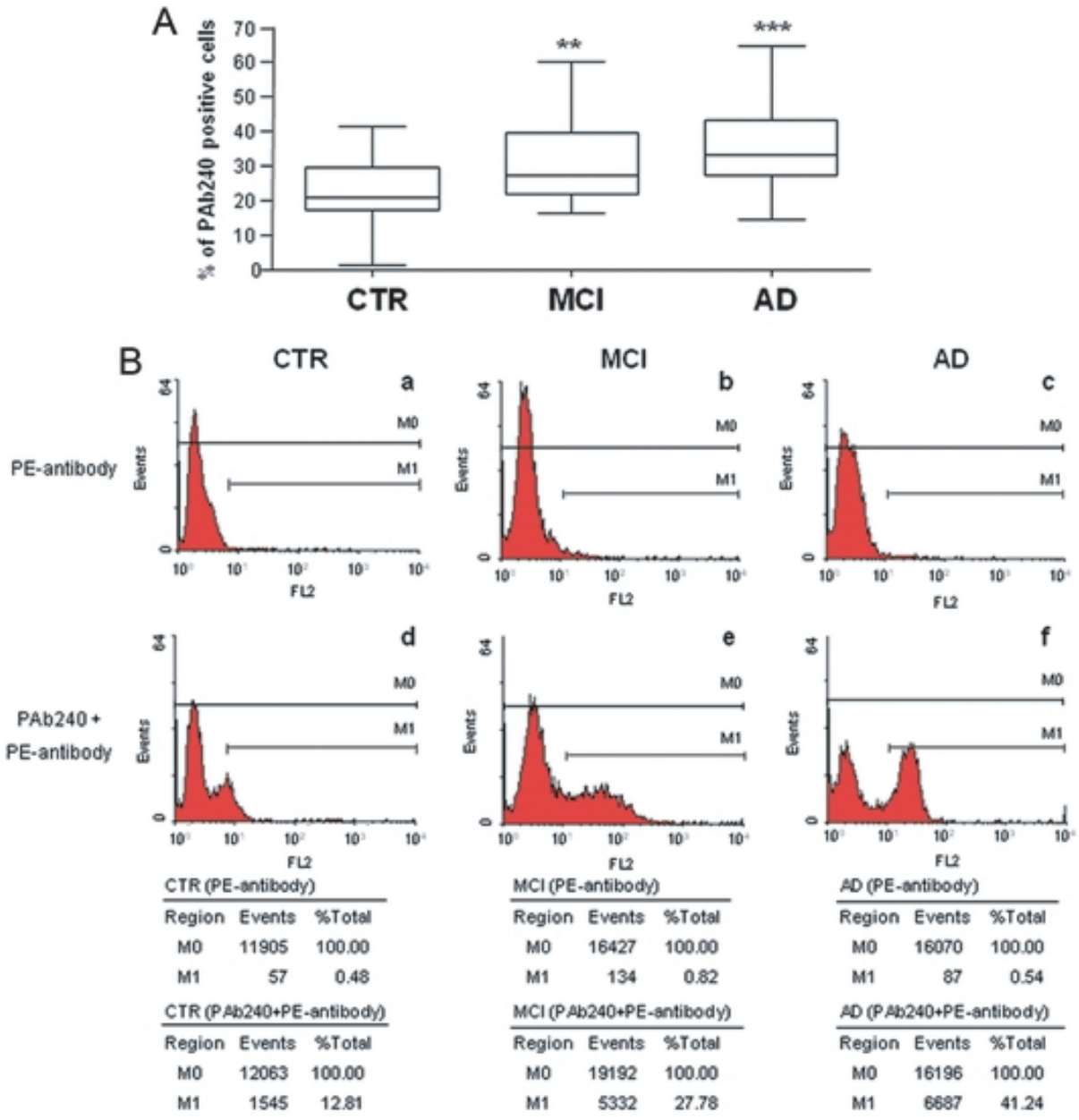

Fig. 1. p53 protein expression in blood cells from AD, MCI, and non-AD patients. (**A**) Box plot reporting the amount of conformationally altered p53 (%) expressed in peripheral blood cells for each subject enrolled in this study. Patients are divided according to the diagnostic group. Tukey-Kramer Multiple Comparisons Test has been used for statistical analysis. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. control. (**B**) Quantitative analysis of conformationally altered p53 expression after flow-cytometric analysis. Panels a, b, c, d, e, f are histograms reporting the fluorescence due to conformationally altered p53 expression (FL-2) vs the number of reacting cells (events). For each histogram the correspondent statistical analysis is reported.

tiary structure. We only analyzed this population, since we previously demonstrated by immunofluorescence staining the presence of false positive cells, identified as granulocytes, showing under fluorescence microscope, a light diffuse mostly non specific signal, compared to the strong positivity to PAb240 antibody in monocytes and lymphocytes [24]. A highly statistically significant difference was found in the percentage of PAb240 positive cells when comparing controls with patients affected by MCI or AD subjects (percent of PAb positive

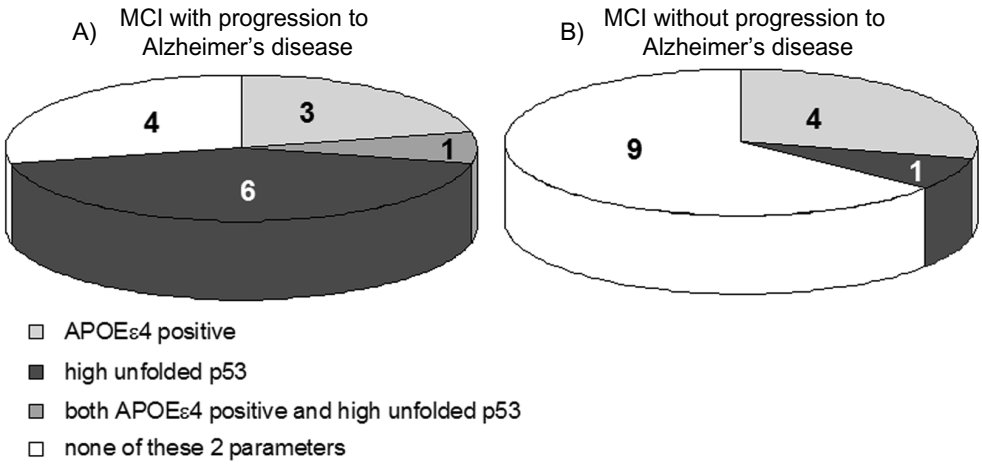

Fig. 2. Scheme of converted and not-converted MCI patients based on unfolded p53 analysis and APOEε4 genotype. Twenty-eight MCI (5 males and 23 females) with a mean $\pm$ standard deviation age of 69 $\pm$ 12.4 and MMSE score of 27.3 $\pm$ 2 were examined. Cognitive status was quantified using the Mini Mental State Examination (MMSE). None of the subjects selected in this study was affected by neoplastic or autoimmune disease when the blood samples were taken. Patients were divided in three groups according to APOEε4 variant and high unfolded p53. Two years later, patients were re-evaluated and classified as converted and non-converted in AD (A, B).

cells, mean $\pm$ SD; control subjects: 23.4 ± 11.9; MCI: 30.6 ± 10.7; AD: 35.1 ± 11.3; MCI versus control $P < 0.01$, AD versus control $P < 0.001$) (Fig. 1).

To better characterize the nature of this different expression among AD, MCI, and control subjects, we evaluated whether the expression of conformational mutant p53 showed a correlation with some parameters linked to AD. As previously reported [24], a statistically significant correlation was observed only when the expression of mutant conformational p53 and the age of both control subjects and AD patients were considered, thus confirming in another independent population that conformationally altered p53 is an age-dependent factor (data not shown).

Focusing on MCI group (28 patients), we found that 8 of them expressed APOEε4 allele. In addition, measurement of the percentage of PBMC expressing unfolded p53 of MCI patients defined two well separated groups. The division in two groups was made considering one standard deviation over control mean (calculated reference value 35.3%), consistently with other previous data from literature [27].

The first group ($n = 20$) had percentages of unfolded p53 values ranging from 19.5% to 29.1% and the other group ($n = 8$) expressed values ranging 38.2% to 60.3%. The latter was candidate at high risk to develop AD, according to our previous indications [13]. Thus, according to the predicted criteria, 16 patients (8 with APOEε4 and 8 with high unfolded p53) were at high risk to develop AD.

Interestingly, the follow-up of MCI patients at two years from recruitment revealed that 14 out of 28 MCI patients converted to AD (Fig. 2). Among these, six had high values of unfolded p53, three expressed APOEε4 variant, and one had both high unfolded p53 and APOEε4. Four of the novel AD diagnoses were not predicted either by the APOE genotype or by p53 conformational state (Fig. 2A). Among the non-converted group (14 patients), four expressed the ε4 variant of APOE gene and only one had high unfolded p53 (Fig. 2B). Moreover, in the MCI converted group, the expression of conformational altered p53 was independent from distribution of ε4 allele of APOE (Fig. 2). This is also consistent with our previous work [28], demonstrating that the presence of the ε4 allele of APOE does not influence the level of conformational mutant p53 expression. The combination of APOEε4 allele and unfolded p53 yielded a sensitivity of 71.4% and a specificity of 84%, comparable with data reported by Zetterberg et al. [29] on cerebrospinal fluid (CSF) biomarkers.

DISCUSSION

Recent research suggested that onset of AD is commonly preceded by an interim phase known as MCI. Since MCI diagnosis may include patients in a transitional state between prodromal and full blown AD, it is considered a window of therapeutic opportunity for treating patients prior to symptom onset [3], even if the

presently available treatments are mostly symptomatic. On the other hand, important new pharmacotherapeutic options will likely become available only over the next decade, considering the variety of drug targets and mechanisms of action identified and the total number of compounds under investigation [30]. In view of existing and emerging therapeutic compounds, there is increasing interest to develop techniques allowing an accurate detection of the earliest phase of the disease. The introduction of biological markers in the clinical management of AD will not only improve diagnosis relating to early detection of neuropathology with underlying molecular mechanisms but also provide tools for the assessment of objective treatment benefits.

Measurement of p53 conformational status in blood cells has been found to discriminate AD cases from normal aging, Parkinson's disease, and other dementias [24]. In particular, the measurement of conformationally altered p53 has been demonstrated to be highly sensitive mainly in young patients with a sensitivity of 90% in subjects below 70 years of age [24]. Since p53 conformational changes found in AD cells have been demonstrated to be independent of gene mutations [31], it is reasonable to wonder about the mechanism by which p53 changes its conformational state. This issue is actually under investigation in our laboratory. On the other hand, a pathogenic link between p53 conformational changes and AD was indirectly suggested by the effects of low soluble concentrations of amyloid-β (Aβ) on p53 tertiary structure in fibroblasts [32] and other cell lines [33]. It is noteworthy that Aβ and pro-oxidant conditions may induce conformational changes in p53. The effect itself of Aβ on p53 misfolding can be counteracted by vitamin E [33]. Indeed, pro-oxidant conditions in MCI and AD patients [34] may have driven the observed changes in PBMC. In addition the possibility that the p53 misfolding in MCI and AD patients reflects an overall propensity to protein misfolding cannot be ruled out.

In this study, to assess whether p53 conformational changes are an early event in AD pathology, we followed up patients falling in the MCI category for two years. Analysis of unfolded p53 was performed at the beginning of the enrollment and was used to predict which subjects among MCI patients will progress to AD. The results obtained in the present study confirm and extend the previous observations on AD patients. Furthermore, we found that a cytofluorimetric approach for conformationally altered p53 protein was able to predict progression to AD in a small cohort of preclinical patients with MCI two years before clinical diagnosis for AD was made. The rate of progression of MCI to AD was comparable with the mean reported in population studies [3,10]. In fact we found that 50% of MCI patients converted to AD after two years from the recruitment. The high expression of unfolded p53 may be considered as high risk factor for the conversion to AD (among 8 patients with elevated unfolded p53 seven converted). In fact, in the MCI converted group, 50% was predicted based on elevated levels of conformationally altered p53, whereas only 28% was predicted based on APOE status. Noteworthy, higher levels of mutant conformational p53 and APOEε4 appeared to be independent risk factors for AD even if numbers are small to be strong at this point. In line with our previous data [28], complexively the mutant conformational p53 may be considered as a marker useful in the ε4 negative younger patients ($\leqslant$ 70 years).

Interestingly enough, when we combined the data on p53 and APOEε4, we obtained a calculated sensitivity of 71.4% and a specificity of 84% for the predictive test. Intriguingly, this accuracy was comparable with data reported by Zetterberg et al. [29] on combined CSF biomarkers.

The fact that p53 was able to identify only a subset of converting patients is consistent with the idea that single biomarkers may not have sufficient sensitivity to detect all cases of the disease or adequate specificity to distinguish one pathology fingerprint from other disease profiles. For this reason is important to identify a battery of determinations to increase the accuracy of the diagnosis. In this context, several studies have showed that selected combinations of preclinical markers may predict this conversion [35–37]. The combined use of cognitive tests, APOE genotype and a neuroradiological technique has been proposed as the best option for prediction, showing an accuracy $>$ 90% [38]. Other research groups focused on CSF, total-tau/Aβ_{1-42}, and magnetic resonance imaging (MRI) biomarkers, such as hippocampal, entorhinal, and ventricular volumes and brain atrophy rates, finding that their combination provides better prediction than either source of data alone [39,40], with a diagnostic accuracy of 80–90%. However, it is difficult to prepare a patient for all these analyses and sequential studies appear difficult to perform, making routine testing not practical in elderly at-risk patients. This emphasizes the need for simple not invasive tests, useful to provide biomarkers that can identify MCI patients declining to AD. Search of biomarkers in the blood compartment has seen the significant contribution of the groups directed by Wyss-Coray. They developed a proteomic analysis of 18 pro-

teins in the plasma of patients able to predict with 90% accuracy the diagnosis of AD [41]; furthermore these 18 proteins showed a potential in identifying patients with MCI who progress to AD [42]. However, the proteomic approach lacks of the identification of post-transcriptional protein modifications deriving from biochemical and metabolic events linked to the development of the pathology.

Here we found that high values of unfolded blood p53, which has been linked to AD pathology, may be considered as high risk factor for the conversion to AD and, when associated with APOEε4 genotype, it yielded sensitivity and specificity values comparable to those obtained by a combination of CSF biomarkers. We are aware that the sample size of our study is too small to reach a definitive conclusion and the definition of conformationally altered p53 as predictive signature for MCI to AD requires a further investigation in larger and independent populations of patients, which is at the moment under investigation. Anyway, we suggest that measurement of conformational p53 state can be useful as an easy accessible adjunctive diagnostic tool in identifying those in the at-risk group of MCI patients who progress to AD.

Finally, it is important to underline that we cannot speculate at this time whether conformational altered p53 found in AD peripheral cells is also present in the brain of AD patients and the relevance of such impairment in terms of neuronal function. Studies in this direction are now in progress in our laboratory. There are, however, previous postmortem studies suggesting an involvement of p53 in degenerating neurons in AD. These include de la Monte and colleagues [43] showing increased p53 and Fas expression in specific populations of cortical neurons; Kitamura et al. [44] showing increased amount of p53 in temporal cortex, mainly localized in glial cells; and Seidl et al. [45] showing higher levels of p53 in frontal and temporal lobe from Down syndrome patients. On the other hand it is not known whether the increase in p53 observed in the quoted papers occurs in degenerating neurons and/or reflects the expression of a conformational altered isoform of p53, as we detected in blood cells and fibroblasts from AD patients [24,31,32].

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