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ORIGINAL ARTICLE

Conformationally altered p53: a novel Alzheimer's disease marker?

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The identification of biological markers of Alzheimer's disease (AD) can be extremely useful to improve diagnostic accuracy and/or to monitor the efficacy of putative therapies. In this regard, peripheral cells may be of great importance, because of their easy accessibility. After subjects were grouped according to diagnosis, the expression of conformationally mutant p53 in blood cells was compared by immunoprecipitation or by a cytofluorimetric assay. In total, 104 patients with AD, 92 age-matched controls, 15 patients with Parkinson's disease and 9 with other types of dementia were analyzed. Two independent methods to evaluate the differential expression of a conformational mutant p53 were developed. Mononuclear cells were analyzed by immunoprecipitation or by flow-cytometric analysis, following incubation with a conformation-specific p53 antibody, which discriminates unfolded p53 tertiary structure. Mononuclear cells from AD patients express a higher amount of mutant-like p53 compared to non-AD subjects, thus supporting the study of conformational mutant p53 as a new putative marker to discriminate AD from non-AD patients. We also observed a strong positive correlation between the expression of p53 and the age of patients. The expression of p53 was independent from the length of illness and from the Mini Mental State Examination value.
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Keywords: Alzheimer's disease; p53; protein tertiary structure; human peripheral cells; flow cytometry; age dependence

Introduction

Alzheimer's disease (AD) cannot be diagnosed until dementia appears, thus the detection of early disease-related biomarkers is crucial to facilitate the development of new diagnostic tools and drug therapies.[1] Candidate biochemical markers for AD should be molecules representing some of the cerebral pathogenetic processes typical of AD. Alternatively, they should represent altered metabolic or cellular processes in the brain or in peripheral tissues from affected patients.[2] Currently, three cerebrospinal fluid (CSF) biomarkers have shown the highest diagnostic potential: total-tau, phospho-tau and Abeta 1–42.[3–5]

An intriguing correlation between p53 and AD has been recently demonstrated by Uberti *et al.*,[6] who showed an impairment of the p53 signaling pathway in AD fibroblasts. This finding was based on new putative pathogenetic processes contributing to disease by altering the cellular responsiveness to noxious stimuli. In particular, fibroblasts from sporadic AD patients specifically express an anomalous and detectable conformational state of p53 (mutant-like p53) that allowed to differentiate these from fibroblasts of age-matched non-AD subjects.[7]

The protein p53 is known to respond to a variety of cellular stresses and may induce cell cycle arrest or apoptosis.[8–10] Recently, p53 has also been found to be involved in neurodegenerative processes.[11–15] Post-transcriptional modification can alter p53 tertiary structure and prevent it from binding to specific DNA sequences, making p53 a lookalike to mutant phenotype, although in AD we did not find evidence of genetic mutations.[7]

We used immunoprecipitation and flow-cytometric analysis to investigate the different expression of conformationally altered p53 between AD and non-AD subjects on peripheral blood cells. Our results

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highlight the possibility to use the analysis of conformational mutant p53 to improve the early diagnosis of AD.

Materials and methods

Subjects
Three independent groups of samples were collected, in different periods and from different locations and analyzed by independent techniques.

Samples processed by immunoprecipitation. The first venous blood samples (3–4 ml) from 14 healthy people, 16 patients affected by AD and 6 with other types of dementia were collected during 2004 in Sant'Orsola Hospital in Brescia, Italy (Table 1a) and processed by immunoprecipitation. The group 'Other dementia' included also one patient affected by Parkinson's disease (PD) and dementia.

An independent group of patients came from Poland, Department of Neurology, MSWiA Hospital, Warsaw. This population consisted of 13 AD and 12 controls (CTRs) (Table 1b), and was collected at the International Institute of Molecular and Cell Biology in Warsaw. Lymphocytes from these patients were immortalized as described earlier,[16] grown in Roswell Park Memorial Institute (RPMI) with 10% fetal bovine serum, 2% glutamine, 1% *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonicacid and 1% penicillin–streptomycin solution and used for immunoprecipitation experiments.

Samples analyzed by flow-cytometric analysis. From March 2005, a third and more consistent group of venous blood samples from healthy people and from patients affected by AD, PD and other dementias was obtained from the Institute 'Fondazione Casimiro Mondino' in Pavia and from Sant'Orsola Hospital in Brescia, Italy. This population consisted of a group of 75 patients with sporadic AD, 66 healthy age-matched CTRs, 15 patients affected by PD and 3 patients with other dementias (Table 1c).

The protocol of the study was approved by the Ethical Committees of the respective Institutions and a written informed 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 Diagnostic and Statistical Manual of Mental Disorders (DSM-IV) and the National Institute of Neurological and Communicative Disorder and Stroke—Alzheimer Disease and Related Disorder Association (NINCDS-ADRDA) criteria. Dementia was diagnosed based upon interview, objective and neurological examination, cognitive evaluation, laboratory and radiological (computerized

Table 1 Demographic and clinical variables of all the subjects. (a) Italian samples processed by immunoprecipitation; (b) Polish samples processed by immunoprecipitation; (c) Italian subjects analyzed by flow-cytometric analysis

	AD	*CTR*	*PD*	*Other dementias*[a]
(a)				
n (M; F)	16 (7; 9)	14 (4; 10)		6 (1; 5)
Mean age ± s.d. (years)	82 ± 9	82 ± 10		83 ± 4.5
LOI (months)	51 ± 13			38 ± 25
MMSE	15 ± 6	28 ± 1.2		13 ± 2.8
	AD	*CTR*	*PD*	*Other dementias*
(b)				
n (M; F)	13 (9; 4)	12 (1; 11)		
Mean age ± s.d. (years)	65 ± 5.5	76 ± 14		
LOI (months)	55 ± 31			
MMSE	12 ± 10	28 ± 2.6		
	AD	*CTR*	*PD*	*Other dementias*[b]
(c)				
n (M; F)	75 (20; 55)	66 (33; 33)	15 (10; 5)	3 (2; 1)
Mean age ± s.d. (years)	79 ± 9	78 ± 10	72 ± 10	84 ± 4
LOI (months)	53 ± 26		75 ± 17	36 ± 33
MMSE	14 ± 6	28 ± 1.6	26 ± 0.8	14 ± 7

Abbreviations: AD, Alzheimer's disease; CTR, control; F, female; LOI, length of illness; M, male; MMSE: Mini Mental State Examination; *n*, number; PD, Parkinson's disease.
Data are expressed as mean ± s.d.
[a]Two Lewy body dementia; one Parkinson's disease with dementia; one normotensive hydrocephalus and two vascular dementia.
[b]Two vascular dementia and one progressive supranuclear palsy.

tomography scan) investigation. Cognitive status was quantified using the Mini Mental State Examination (MMSE). All AD patients fulfilled the criteria for probable AD[17] 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. PD patients were diagnosed in accordance with the UK Parkinson's Disease Society Brain Bank. The score at the Hoehn and Yahr scale ranged between 1 and 2.[18,19] None of these patients was demented (see Table 1c). CTR 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.

Immunoprecipitation analysis

p53 conformational state was analyzed by immunoprecipitation as detailed previously.[7] Briefly, cells were lysed in immunoprecipitation buffer (10 mM Tris, pH 7.6; 140 mM NaCl; and 0.5% NP40 including protease inhibitors); 100 μg of protein extracts was used for immunoprecipitation experiments performed in a volume of 500 μl with 1 μg of the conformation-specific antibodies PAb1620 (wild-type specific) or PAb240 (mutant specific).

Immunofluorescence and flow cytometry

Peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on a Ficoll Hypaque density gradient from Na^+/citrate samples (Eurobio Laboratories, Paris, France) and fixed in 2% formaldehyde in phosphate-buffered saline (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 mutant p53 (clone PAb240; NeoMarkers, Fremont, CA, USA) (4 μg/ml in PBS/1% bovine serum albumin (BSA) solution). Cells rinsed in PBS/1% BSA were incubated for 30 min in ice with a goat anti-mouse immunoglobulin (Ig) G antibody phycoerythrin-conjugated (DakoCytomation, Glostrup, Denmark; 1:40 in PBS/1% BSA). After rinsing, few microliters of cell suspension were deposited on a glass slide and observed with a fluorescence microscope Olympus BX51 (Olympus Optical Co., GmbH, Hamburg, Germany) with blue excitation (BP450–480 nm, DM 500 and barrier filter 515 nm) equipped with a Camedia digital camera. Samples were observed at $\times 40$ magnification. Pictures were taken with the same instrumental setting. Orange-red fluorescence from AD-positive cells was compared with the background fluorescence of CTR samples. Cell suspension was then analyzed with a flow cytometer Partec PASII (Partec, GmbH, Munster, Germany). PBMC population was identified by forward and side-angle scatter and mutant p53 emission was detected in the FL-2 channel (535–580 bandpass filter). For each sample, data from 20 000 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–chloroform extraction procedure. The APOE gene polymorphisms were determined by *Hha*I restriction endonuclease digestion of PCR products, according to Hixson and Vernier.[20]

Statistical analysis

Following analysis of variance, Student's *t*-test was used to compare values in different groups. Data were analyzed by simple linear regression (Pearson correlation coefficient). Differences were considered significant when a *P*-value <0.05 was attained. Odds ratio (OR) as the estimates of relative risk for disease was calculated using 95% confidence interval (CI).

Results

Italian and Polish samples, processed by immunoprecipitation, were comparable as far as age and gender distribution are concerned; MMSE score and length of illness were also examined (Table 1a and b).

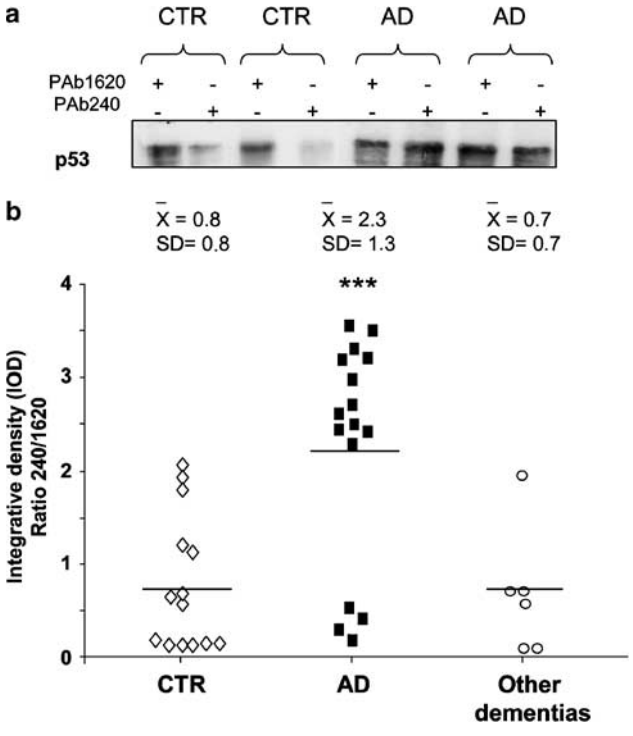


Figure 1 Immunoprecipitation of blood cells from Italian controls (CTRs) and AD patients. (**a**) Representative experiment on mononuclear blood cells from two CTR and two AD patients with the PAb240 and PAb1620 antibodies. (**b**) Ratio between the intensity of PAb240 and PAb1620 immunoreactive bands. Bars represent mean value of the respective group. Data are expressed as mean $\pm$ s.d. $***P=0.0003$ vs CTR.

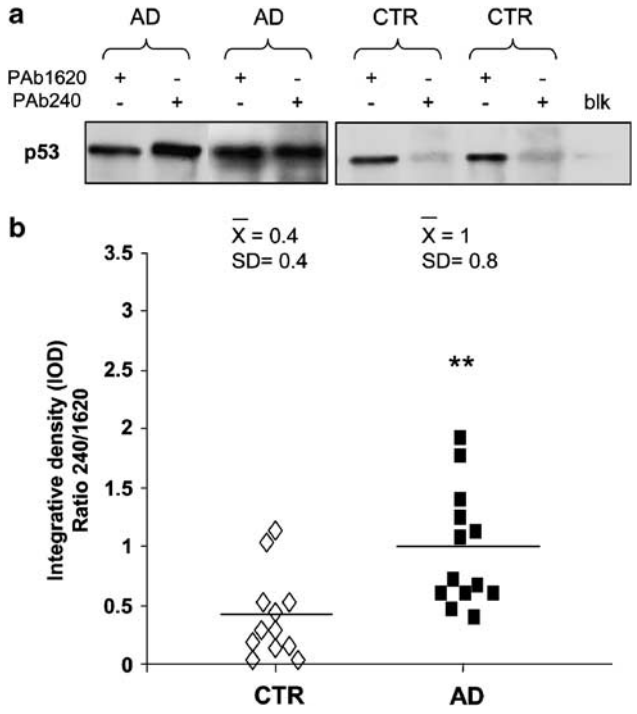


Figure 2 Immunoprecipitation of blood cells from Polish controls (CTRs) and AD patients. (**a**) Representative experiment on lymphocytes from two CTR and two AD patients. (**b**) Ratio between the intensity of PAb240 and PAb1620 immunoreactive bands. Bars represent mean value of the respective group. Data are expressed as mean ± s.d. $^{**}P = 0.0031$ vs CTR.

The representative immunoblots (Figures 1a and 2a) show that blood cells from CTR subjects expressed wild-type p53 exclusively as demonstrated by the reactivity with PAb1620, and the virtual absence of the reactivity to PAb240. In contrast, samples from AD patients expressed markedly higher levels of conformationally altered p53 (recognized by PAb240).

The calculated ratio between PAb240 and PAb1620 immunoreactivity was significantly higher in blood cells from AD compared to non-AD subjects (Figures 1b and 2b). Patients affected by other types of dementia showed no statistically significant difference when compared to CTR subjects (Figure 1b).

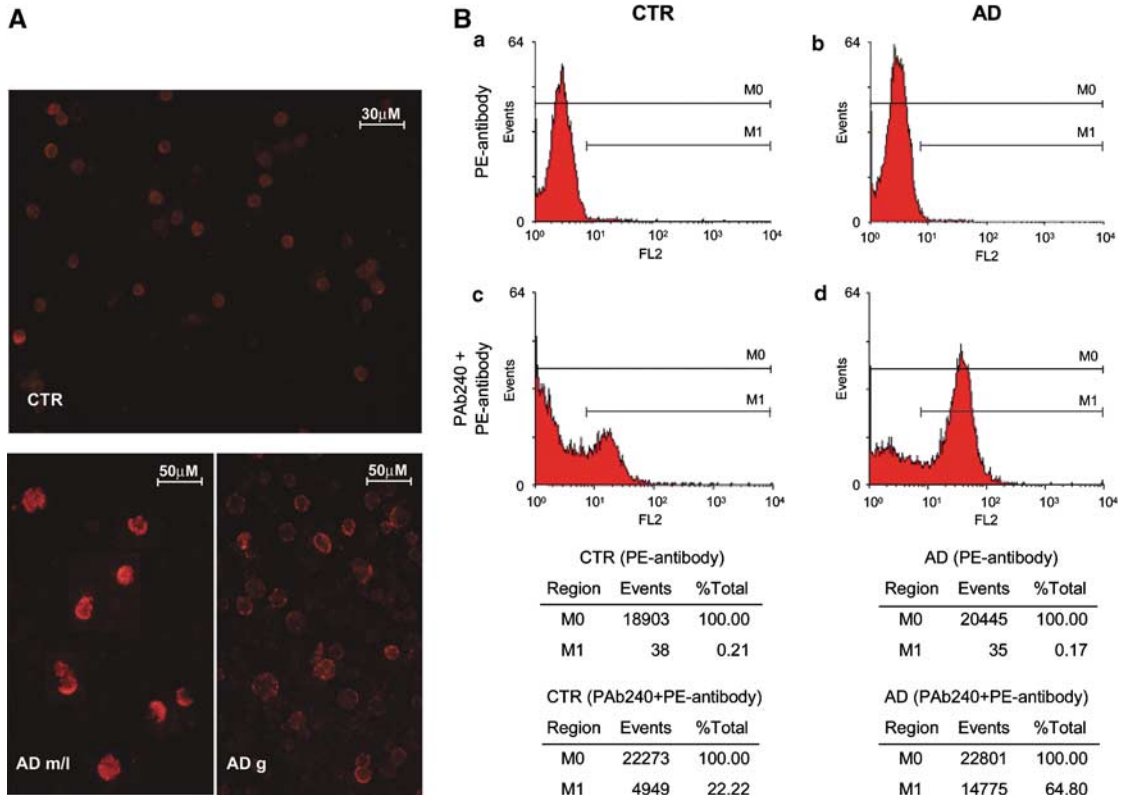
On the basis of these results, to confirm the different expression of conformationally altered p53 between AD and non-AD subjects, we set up a flow-cytometric approach, that allowed us to analyze quantitatively a more consistent number of samples compared to immunoprecipitation assay.

Within this context, another group of CTR subjects, AD and non-AD (other dementias and PD) patients comparable for age and gender distribution (see Table 1c) was collected. The distribution of ε2/ε3/ε4 alleles of APOE was 0.05/0.53/0.37 in AD cases, and 0.07/0.80/0.12 in CTR subjects; as expected the APOE ε4 allele was strongly associated with AD.

Mononuclear cells were processed for immunofluorescence experiments, to better define the positivity of cells to altered p53, using the same antibody, the PAb240, used for immunoprecipitation (Figure 3a). Immunofluorescence staining highlighted the presence of false-positive cells, identified as granulocytes, showing under fluorescence microscope, as a light diffuse mostly non-specific signal, compared to the strong positivity to PAb240 antibody in monocytes and lymphocytes (Figure 3a); these data are of great importance, because they indicate that the accidental presence of granulocytes, recognized on the basis of their physical properties, had to be excluded from the subsequent flow-cytometric analysis.

Upon quantitative flow-cytometric analysis, peripheral blood cells from AD and non-AD subjects showed a different expression of mutant-like p53. In particular, mononuclear cells from AD patients express a higher amount of conformational mutant p53 than blood cells from non-AD subjects (Figure 3b).

A statistically significant difference was found in the percentage of PAb240-positive cells between patients affected by AD and CTR subjects (median ± s.d.; CTR subjects: 21.1 ± 12.3%; AD: 34.5 ± 15.1%; AD vs CTR $P < 0.0001$). Patients affected by PD and other types of dementia showed no statistically significant difference when compared with CTR subjects (median ± s.d.; CTR subjects: 21.1 ± 12.3%; PD subjects: 21.2 ± 8.8%; other dementias: 27.5 ± 2.5%; PD vs CTR $P = 0.8349$; other dementias vs CTR $P = 0.6158$) (Figure 4). To better characterize the nature of this different expression between AD and CTR subjects, we evaluated whether the expression of conformational mutant p53 showed a correlation with some parameters linked to AD, such as age, MMSE score and length of illness. A statistically significant correlation was observed when the expression of mutant conformational p53 and the age of both CTR subjects and AD patients were considered (Figure 5a), thus demonstrating that altered p53 is an age-dependent factor. On the other hand, in AD patients, the expression of mutant conformational p53 was independent from the length of illness and from the MMSE score (data not shown), suggesting that p53 could be considered, within specific age interval segments, an early marker. Because p53 showed age-associated changes, we subdivided CTR subjects and AD patients in specific age interval segments and worked out the related cut-off points by linear regression, taking as reference the linear fit of CTRs (Figure 5b). In addition, for each age interval, sensitivity and specificity were calculated to evaluate the diagnostic performance of conformationally altered p53 as AD biomarker. The sensitivity to discriminate AD from non-demented aged individuals up to 70 years of age was 90% at a specificity level of 77%, compared to the other groups of age (sensitivity, specificity; between 71 and 80 years: 77%, 70%; older than 80 years: 85%, 50%). In addition, among the three age interval segments, we observed that individuals up to 70 years of age presenting increased p53 showed a higher risk of



CTR (PE-antibody)

Region	Events	%Total
M0	18903	100.00
M1	38	0.21

AD (PE-antibody)

Region	Events	%Total
M0	20445	100.00
M1	35	0.17

CTR (PAb240+PE-antibody)

Region	Events	%Total
M0	22273	100.00
M1	4949	22.22

AD (PAb240+PE-antibody)

Region	Events	%Total
M0	22801	100.00
M1	14775	64.80

Figure 3 Immunofluorescence and flow-cytometric analysis of blood cells from controls (CTRs) and AD patients. (**A**) Fluorescence micrographs of mononuclear cells from CTR and AD patients. In the micrograph AD m/l, the fluorescence intensity of mononuclear cells (monocytes and lymphocytes, m/l) is evident as compared to the very faint background of CTR sample. Figure ADg also showed some non-specific red fluorescence from polymorphonuclear cells (granulocytes, g). (**B**) Quantitative analysis of mutant-like p53 expression after flow-cytometric analysis. Panels (a–d) are histograms reporting the fluorescence due to mutant-like p53 expression (FL-2) vs the number of reacting cells (events). For each histogram, the correspondent statistical analysis is reported.

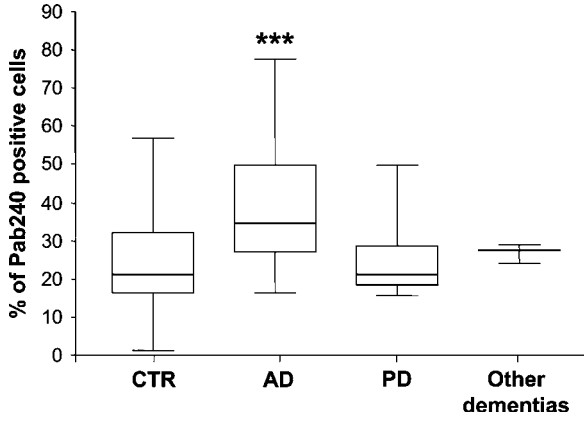


Figure 4 p53 protein expression in blood cells from AD and non-AD patients. Box plot reporting the amount of conformational mutant p53 (%) expressed in peripheral blood cells. Patients are divided according to the diagnostic group. Bars represent the median value of the respective group. ***$P<0.0001$ vs CTR.

sporadic AD (OR 29.2, 95% CI, 2.78–306.82), than older subjects (between 71 and 80 years: 8.1, 95% CI, 2.36–28.15; older than 80 years: 5.5, 95% CI, 1.54–19.60).

Discussion

This is the first study demonstrating a role for conformationally altered p53 as a novel candidate biomarker for AD. The main finding of the present study is that peripheral blood cells from sporadic AD patients specifically express an anomalous and detectable conformational state of p53 that makes these cells distinguishable from cells of age-matched non-AD subjects, thus supporting the existence of a new putative marker for AD.

In our previous study, we demonstrated that in fibroblasts from AD patients, a significant amount of total p53 assumes an unfolded tertiary structure, thus compromising p53 response to different cytotoxic injuries.[7] The results obtained in the present study confirm and extend the previous observations. We used blood mononuclear cells derived from CTRs, non-AD and AD patients, as a more accessible cellular model than fibroblasts. To replicate the results in independent populations, we collected our samples in three different locations and time frames. We initially used immunoprecipitation analysis and showed that, similarly to fibroblasts,[7] mononuclear cells from AD patients significantly express a higher

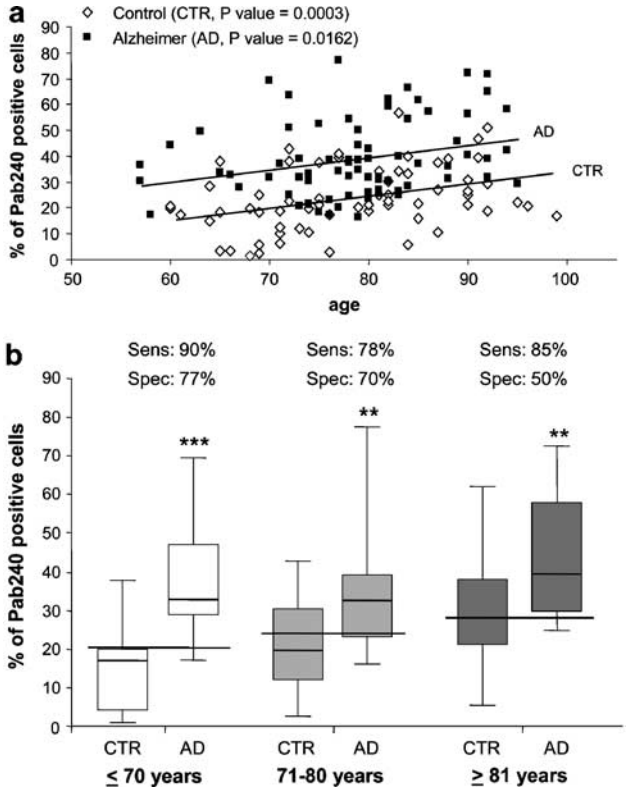


Figure 5 p53 is an age-dependent factor. (**a**) Correlation between the age of non-AD and AD patients vs amount of conformational mutant p53 (%). Correlation lines related to AD and CTR distribution are indicated. (**b**) Box plot of the amount of conformational mutant p53 (%) of all the subjects classified within specific age interval segments. Bold bars represent cut-off points of the specific age interval segments. Sensitivity and specificity values are reported. $***P<0.0001$, $**P<0.001$ vs control (CTR).

level of mutant-like p53 compared to cells from CTR subjects and from people affected with other forms of dementia.

Aware of the fact that immunoprecipitation and western blot analysis are semiquantitative methods, and are prone to errors due to possible over- or underestimation of the bands, we developed a rapid, easy and quantitative flow-cytometric approach for the discrimination of conformational mutant p53-bearing cells from AD patients compared to non-AD CTRs, using small volumes of blood.

The literature on the evaluation of AD biomarkers has consolidated the significance of the measurement of total-tau, phospho-tau and Abeta 1–42 in the CSF.[3,4] Search of biomarkers in the blood compartment has seen the significant contribution of Di Luca *et al*. and Rosenberg *et al.*, both demonstrating that platelets of AD patients express differential levels of APP isoforms, and these differences are sensitive to disease progression and treatment.[21–26] We propose here an assay that can be performed on low volumes of blood with an analytical procedure that is simple to perform and uses a flow-cytometric method, widely diffused both in hospitals and in diagnostic laboratories.

We observed that the level of conformationally altered p53, both in CTRs and in AD, is an age-dependent event, while it is independent from the length of illness and from the MMSE score. This would suggest that it is a trait marker rather than a state marker. In addition, the linear correlation with age would also suggest that its significance would be different within specific age interval segments. Although we are aware that the subdivision in age intervals of our clinical sample is rather arbitrary, we wanted to determine the diagnostic performance of conformationally altered p53 as an AD trait marker. We calculated sensitivity and specificity within different age intervals and found that these values were more significant in subjects up to 70 years of age compared with the corresponding values for individuals older than 70 years. Within this specific age interval ($\leqslant$70 years), we worked out a sensitivity of 90% to discriminate AD patients from non-demented aged individuals at a specificity value of 77%. Sensitivity and specificity values were strongly reduced at increasing ages, because of the age effect on p53. A comparison of our sensitivity and specificity values with those published in several studies, which evaluated the diagnostic power of CSF markers for AD (total-tau, phospho-tau and Abeta 1–42), reveal that p53 measurement is more sensitive (90% compared to respectively 81.4, 81.3 and 85.9%), but less specific (77% compared to respectively 91.5, 91.2 and 88.5%).[3,4]

Although the age stratification is admittedly a *post hoc* analysis, it serves to indicate that the putative marker proposed appears to be extremely important in the younger patients, also considering that the positivity to p53 conformational mutant induced a calculated OR of 29.2 for AD. The fact that the most significant differences are observed in the youngest patients indicate that the measurement of conformational mutant p53 may be usefully applied to detect AD at the early stages, perhaps applied to those patients falling in the ill-defined category of mild cognitive impairment (MCI). In fact, a marker, which could allow doctors to predict if an MCI patient will progress to AD with dementia or show a benign form of MCI as part of the normal aging process, is still eagerly awaited.[26,27]

Whether this different expression of conformationally altered p53 will be suitable as an adjunctive diagnostic tool in early stage AD in larger and independent populations of patients is under investigation.

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