

**Title**

Discovery of simple blood-based test to predict early onset of Alzheimer's using standard Clinical Mass Spectrometry platforms

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**Summary**

Despite the increasing number of individuals affected by Alzheimer's disease (AD) every year, no effective therapy has been developed to treat this neurodegenerative disease yet. The current methods for AD diagnosis are effective for clinical confirmation of the disease only when symptoms become apparent, years after molecular damage started within the patients' brains. As higher expression of a conformationally altered p53 has been correlated with AD, we developed a mass spectrometry-based method for highly sensitive, specific, and reproducible quantification of a p53 conformational variant in plasma samples of patients with known clinical outcome. In particular, we tested the prognostic performance of an AD-specific 2D3A8-immunoselected p53 peptide (AZ 284™) in different sets of individuals progressing from both cognitively unimpaired (CU) and mild cognitive impairment (MCI) patients progressing to AD dementia. Our data showed that quantitative analysis of AZ 284™ is a reliable tool for predicting AD progression up to 6 years prior to dementia onset with AUC >90%. Taken together, these results support the implementation of p53 conformational variant quantification as an affordable and powerful diagnostic tool for early, non-invasive AD diagnosis.

**Keywords**

Alzheimer's disease; biomarker; prognosis; P53; mass spectrometry

## Introduction

AD is one of the most common causes of dementia (1) and its symptoms are anticipated by a long asymptomatic phase characterised by brain pathological molecular leading to neuronal dysfunction and ultimately neuronal death. This phase is often followed by symptomatic MCI before exacerbating to AD. The impact on affected individuals represents an economical burden at the healthcare and caregiver's level (2, 3). Up to this day, no effective therapies exist to stop the disease, and the existing ones attempt to slow onset and worsening of symptoms (4, 5). This is also because AD diagnosis is carried out too late, when symptoms appear.

Currently, AD diagnostic workflow includes a battery of neurophysiological tests followed by brain metabolism assessment via positron emission tomography with the Pittsburgh compound B (PiB-PET) and analysis of beta amyloids peptides ( $A\beta_{1-40}$  and  $A\beta_{1-42}$ ) and tau protein (total or phosphorylated) in the cerebrospinal fluid (CSF). As a genetic diagnostic marker, APOE  $\epsilon 4$  is tested for allele number as it correlates with a higher risk of developing AD. Recent studies attempted to identify blood-derived biomarkers for less invasive tests ( $A\beta_{1-40/42}$ , tau total and p-tau181 or p-tau217, microRNAs or neurofilament NfL), but the diagnosis is often in the shorter range of MCI, too late for a precocious intervention (6-10). Some biomarkers show promising earlier detection for neurodegeneration, as NfL, but lack specificity to AD.

Recently, investigations on contributing causes to AD development, reported a conformational variant of p53 in AD. p53 conformational changes could be triggered by post translational modifications and zinc atom chelation, essential for its native conformation, as a consequence of intracellular soluble  $A\beta$  peptides and/or a pro-oxidant environment (11-13). Altered p53 was observed in post-mortem AD brains and is detected in peripheral blood cells and its levels are increased in MCI or AD samples compared to age-matched controls (11, 14, 15). A conformational variant of p53 can differentiate between AD and other types of dementia (14): since MCI is not exclusive of AD, such specificity makes this a valuable biomarker. The p53 conformational variant specifically recognized by 2D3A8 antibody and here described was recently reported as a promising and early prognostic blood-based biomarker (16).

Lately an increasing use of mass spectrometry (MS) in clinical laboratories has been described (17, 18). This technology was initially considered too expensive for routine analyses, but its implementation led to abating costs and offering precise analyses – as for metabolomics and proteomics – making MS an essential element of clinical laboratories.

In this study, we show that quantitation of a conformational variant of p53 (U-p53<sup>Az</sup>) through AZ 284™ sequence peptide via MS-based detection (described as AlzoSure®) could represent a reliable biomarker for early AD diagnosis. We compared the predictive performance of AZ 284™ with that of canonical diagnostic tools (such as PiB-PET) or of risk factors (age, gender, and APOE  $\epsilon 4$ ), in a well characterized cohort assessed over time for progression to MCI and AD.

## Methods

### Subjects

Plasma was selected from 101 individuals from the Australian Imaging, Biomarkers, and Lifestyle (AIBL) longitudinal study of ageing. From these individuals, 107 plasma samples were used for this exploratory study. Of the 107 plasma samples, brain amyloid burden as measured by PiB-PET data was available in 103 samples. PiB-PET will be here used to refer to the amyloid status.

According to the NINCDS-ADRDA criteria the AIBL participants were classified as cognitively unimpaired (CU), with MCI or presenting symptomatic AD. In **Table 1**, demographics and characteristics of the cohort of this study are listed, subdivided by cognitive status: within CU two subgroups were defined, NMC (no memory complaints) and SMC (subjective memory complaints). For each cognitive status group, median age is reported and gender distribution are listed. As contributing factors, APOE  $\epsilon 4$  as well as PiB-PET status are reported. Within each diagnostic group, changes of cognitive status per follow-up visit (after 18 and 36 months) are reported.

### AlzoSure® method development

*Sample collection:* Blood samples collected by the biobank were kept at -80°C before undergoing chemical depletion with acetonitrile and analysis by AlzoSure® method.

*Sample preparation:* Samples were immunoprecipitated (IP) isolating U-p53<sup>AZ</sup> with the patented 2D3A8 MoAb (19), produced by Diadem SRL, coupled to Protein L magnetic beads (Thermofisher). Following IP, the isolated proteins were digested with trypsin and supplemented 0.2 fmol/1 µL of spiked labelled AZ 284™-was added right prior to spectrometric analysis. Labelled U-p53<sup>AZ</sup> 2 fmol/10 µL was added to a negative plasma sample as healthy standard control to monitor the process efficiency.

*Mass spectrometry:* To analyse the selected analytes, an Ultimate 3000 UPLC (Dionex, San Jose, CA, USA) liquid chromatography apparatus was used for analyte separation prior to MS analysis. The column was a Phenomenex Kinetex PFP 50x4.1 mm 2.6 µm. The mobile phases were A (bidistilled water, Sigma Aldrich, Italy, + 0.1% formic acid, VWR, Italy) and B (acetonitrile, Sigma Aldrich, Italy). A binary gradient was used: 2% of B was maintained for 2.5 minutes, in 0.5 minutes B was raised to 80%, and was maintained for 4 minutes. 2% of B was then reached in 1 minute and the column was re-equilibrated in starting conditions for 3 minutes. The chromatographic flow was 0.50 mL/minute at 20 µL injection volume.

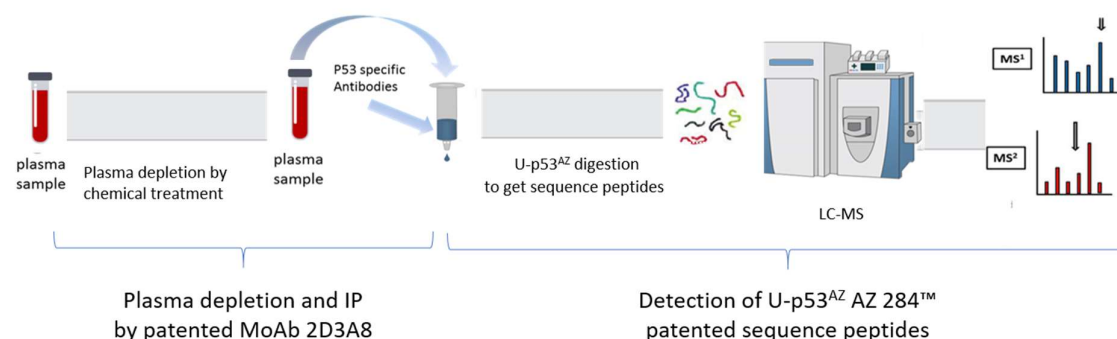
*Quantitative analysis of the peptide:* Calibration curve was performed in the range 0.1 – 10 fmol/10 µL using the progressive injection step with a 2x volume injection factor. The Limit of Detection (LOD) for the AlzoSure® method was 0.001 fmol/1 µL and Limit of Quantitation (LOQ; obtained considering a S/N ratio of 10) was 0.01 fmol/1 µL (20).

The instrumental calibration performance are fully reported in **Table S1**. The precision and accuracy error % were obtained considering also the space charge effect, that strongly affect the ion traps mass analyser (21). This effect is strongly reduced in SACI ionization conditions, due to the low amount of solvent charged species produced in the ion source (22).

Peptides sequences were analysed with a HCT Ultra Mass Spectrometer (Bruker, Breme, Germany) coupled to a surface-activated chemical ionization (SACI) source (23) operated in positive- and negative-ion modes. Full-scan spectra were acquired in the range 100–1200 m/z to detect the analytes. The ion source parameters were: capillary voltage: 1500 V; SACI surface voltage: 47 V; drying gas: 1 L/min; nebulizer gas: 75 psi; temperature: 320°C. Tandem mass spectrometry (MS/MS) experiments on plasma samples were performed with collision-induced dissociation using helium as the collision gas. An ion trap was used in profile low scan rate zoom scan mode to isolate and detect the analytes [M+H]<sup>+</sup> together with their specific isotopic cluster. Collision-induced dissociation was used to confirm the peptide identity (isolation windows, ±0.3 m/z, collision energy, 30% of its maximum value, which was 5 V peak to peak).

*Data analysis:* Each sample was validated through the PROSAD method (24), data elaboration was performed using SANIST-protein data elaboration platform.

**Figure 1. Method and analysis scheme.** Representative scheme for the blood sample processing as described in the methods.



IP: Immunoprecipitation; LC: Liquid Chromatography; MS: Mass Spectrometry

#### Statistical analysis:

Participants were classed as progressor (given progression from CU or MCI to symptomatic AD at different follow-ups), or a non-progressor, for stable status over the length of the study. To ascertain the likelihood of a participant being classed as a progressor, baseline levels of the peptide AZ 284™ were modelled with age, gender and APOE  $\epsilon 4$  allele status using logistic regression. To interpret predictive performance, the predicted probabilities were evaluated by Receiver Operating Characteristic (ROC) analyses using Youden's Index, extracting additional metrics such as Area Under the Curve (AUC), sensitivity, and specificity. DeLong's test was used to compare ROC performance.

**Table 1. Demographics and characteristics of individuals included in the study**

	CU			
	NMC	SMC	MCI	AD
<b>Number of samples, N</b>	24	19	49	15
<b>Age, median (IQR)</b>	74 (71-75)	77 (75-81)	76 (72-79)	76 (72-79)
<b>Female, N (%)</b>	14 (58)	11 (58)	20 (41)	6 (40)
<b>Clinical status in 18 months, N (%)</b>				
NMC	19 (79.2)	1 (5.3)	1 (2)	0 (0)
SMC	1 (4.2)	5 (26.3)	0 (0)	0 (0)
MCI	2 (8.3)	6 (31.6)	22 (44.9)	0 (0)
AD	2 (8.3)	6 (31.6)	25 (51)	11 (73.3)
Not assessed	0	1 (5.3)	1 (2)	4 (26.7)
<b>Clinical status in 36 months, N (%)</b>				
NMC	13 (54.2)	1 (5.3)	0 (0)	0 (0)
SMC	4 (16.7)	5 (26.3)	2 (4.1)	0 (0)
MCI	2 (8.3)	3 (15.8)	15 (30.6)	0 (0)
AD	2 (8.3)	7 (36.9)	12 (24.5)	7 (46.7)
Not assessed	3 (12.5)	3 (15.8)	20 (40.8)	8 (53.3)

<b>APOE <math>\epsilon</math>4, N (%)</b>				
0	17 (70.8)	8 (42.1)	30 (61.2)	4 (26.7)
1	6 (25)	10 (52.6)	12 (24.5)	10 (66.7)
2	1 (4.2)	1 (5.3)	7 (14.3)	1 (6.7)
<b>PiB-PET A<math>\beta</math> +, N (%)</b>	6 (25)	12 (63)	24 (49)	10 (67)
<b>[missing]</b>	[0]	[2]	[1]	[1]

AD: Alzheimer's disease; APOE  $\epsilon$ 4: Apolipoprotein E allele  $\epsilon$ 4; CU: Cognitively unimpaired; IQR: Interquartile range; MCI: Mild cognitive impairment; NMC: No memory complaints; PET: Positron emission tomography; PiB: Pittsburgh compound B; SMC: Subjective memory complaints

## Results

This study assesses the predictive power of the AZ 284™ through AlzoSure® in detecting the progression from CU or MCI to symptomatic AD. AZ 284™ is a peptide MS-identified from a conformation variant of p53 (U-p53<sup>Az</sup>) 2D3A8-immunoselected from plasma samples (**Figure 1**, method overview) (19).

107 plasma samples were tested for AZ 284™. Following an initial blood sampling and patient evaluation, the subjects were monitored from 18/36 months to a last follow-up of at least 6 years, with clinical assessment performed at each visit. To demonstrate the predictive value of AZ 284™, we used the initial blood sampling and the information derived from clinical outcomes assessed overtime. Progression of participants through diagnostic groups is reported in **Table 1**.

AUCs were compared for AZ 284™ alone, the base model alone, and their combination, with or without PiB-PET (Represented in **Figure 2** and listed in **Table 2**). At 18 months, the base model alone performed well (AUC nearing 85%), while the AUC for AZ 284™ alone was higher (AUC=97.64%). The base model paired with PiB-PET (AUC=90.94%) underperformed compared to AZ 284™ alone. Combining the base model with AZ 284™ further improved the predictive performance, increasing the AUC to 99.09%. Combination of PiB-PET, AZ 284™ and base model did not provide any relevant improvement compared to AZ 284™ plus base model (AUC=99.28%).

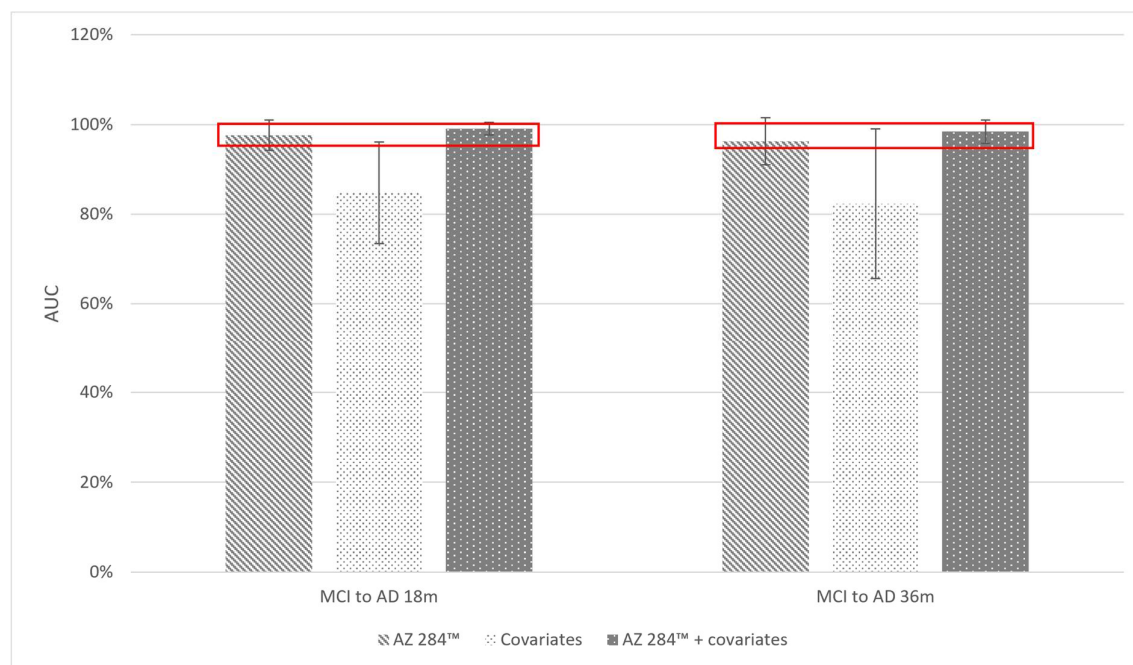
At 36 months, AZ 284™ alone showed a remarkable prognostic value (AUC=96.26%), higher than that of the base model alone (AUC=82.35%). Adding AZ 284™ to the base model increased the AUC to 98.40%. Combination of PiB-PET with base model and AZ 284™ slightly improved the predictive value compared to that of without PiB-PET (AUC=98.93%), whilst the base model with PiB-PET excluding AZ 284™ had poorer performance (AUC=83.96%).

**Table 2. AUC values described in the study**

	<b>AUC (95% CI) via Youden's Index</b>	
	<b>in 18 months</b>	<b>in 36 months</b>
<b>Progression MCI to AD</b>		
<b>AZ 284™</b>	97.64 (93.26 - 100)	96.26 (89.53 - 100)
<b>Base model</b>	84.78 ( 97.18 - 100)	82.35 (65.65 - 99.05)
<b>AZ 284™ + base model</b>	99.09 (97.18 - 100)	98.40 (94.86 - 100)
<b>Base model + PET</b>	90.94 (82.30 - 99.58)	83.96 (67.47 - 100)
<b>AZ 284™ + base model + PET</b>	99.28 (97.71 - 100)	98.93 (96.41 - 100)
<b>Progression CU to AD</b>	<b>in 36 months</b>	<b>in at least 6 years</b>

<b>AZ 284™</b>	87.90 (76.59 - 99.21)	91.61 (79.26 - 100)
<b>AZ 284™ + base model</b>	88.49 (77.61 - 99.29)	97.52 (93.63 - 100)
<b>Base model + PET</b>	95.60 (89.07 - 100)	95.47 (88.09 - 100)
<b>AZ 284™ + base model + PET</b>	95.37 (89.01 - 100)	97.10 (91.29 - 100)

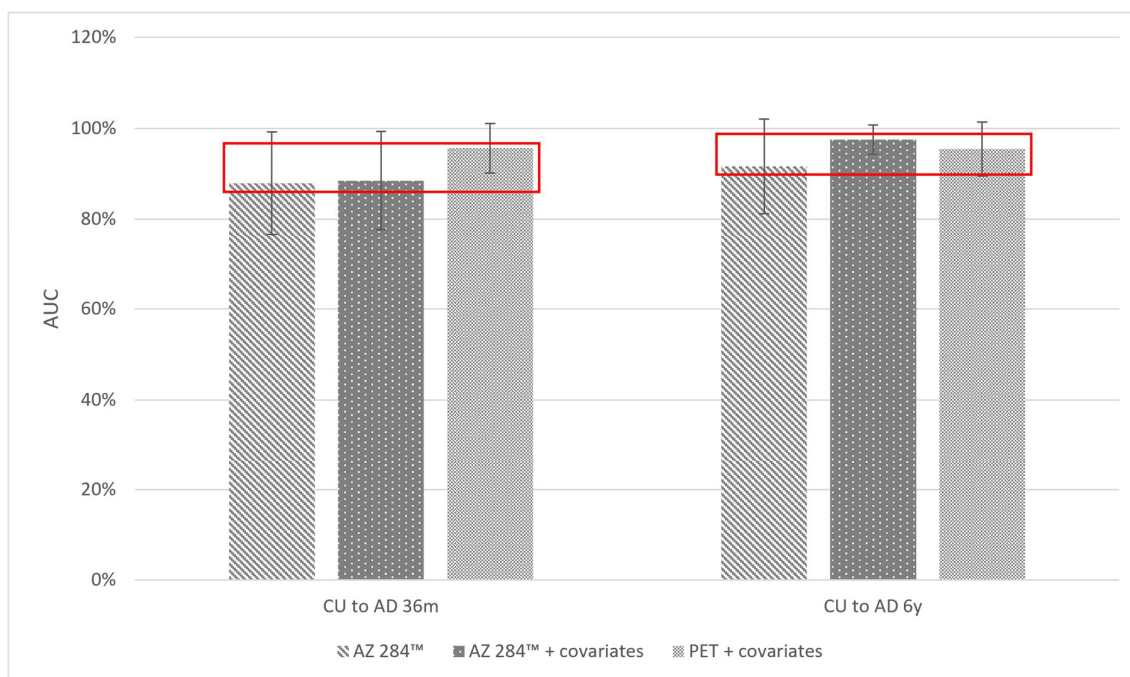
AD: Alzheimer's disease; AUC: Area Under Curve; CU: Cognitively unimpaired; MCI: Mild cognitive impairment; PET: Positron emission tomography



**Figure 2. Performance of AZ 284™ to predict subject progression from MCI to AD in 18 or 36 months.** Comparison of the performance of AZ 284™, base model and their combination. Each bar represents the AUC and 95% CI. In the left panel the performance refers to a time period of 18 months from the begin of the study, in the right panel the results after 36 months are shown.

The prognostic potential of U-p53<sup>AZ</sup> was also assessed in the CU groups progressing to AD in longer follow-ups. The prediction for CU-to-AD progression was assessed for AZ 284™ alone or with the base model, as well as for the base model paired with PiB-PET in presence or absence of AZ 284™ to compare more standard diagnostic tools.

The results, represented in **Figure 3** and reported in **Table 2**, show that at 36 months AZ 284™ alone had an AUC of 87.90%, comparable with that obtained combining the base model with AZ 284™ (AUC=88.49%). Notably, combination of the base model and PiB-PET showed strong performance (AUC=95.60%), and combination of the base model with PiB-PET and AZ 284™ scored similarly (AUC=95.37%). At the 6 years mark, combination of AZ 284™ (AUC=91.61%) with the base model improved the performance (AUC=97.52%), slightly albeit not significantly surpassing that of PiB-PET paired with the base model (AUC=95.47%). Combination of the base model, AZ 284™ and PiB-PET was similar although slightly lower in comparison to the predictive value of AZ 284™ combined with the base model alone (AUC=97.10%), showing that adding PiB-PET did not offer an added value in this time period.



**Figure 3. Performance of AZ 284™ to predict subject progression from CU to AD in 36 months and at least 6 years.** Comparison of the performance of AZ 284™, base model with AZ 284™ or PiB-PET, and the overall set of predictors. Each bar represents the AUC and 95% CI. Performance results refer to a time period of 36 months and at least 6 years after the begin of the study.

#### Discussion/Conclusion:

For the first time we present the performance of AZ 284™ quantification – through the developed AlzoSure® test – as a reliable biomarker for predicting AD progression. In particular, using a well-characterised longitudinal cohort, we demonstrated that AZ 284™ performs well in predicting AD progression from MCI (36 months earlier, AUC=96.3%), and even in CU subjects (6 years before conversion, AUC>90%). Combination of the base model with AZ 284™ detection further improved the AUC (AUC=98.4% in MCI 36 months earlier, AUC=97.5% for CU more than 6 years before), showing a promising performance compared to that of the base model paired with PET in MCI-to-AD at 36 months (AUC=83.96%) and a comparable to the CU-to-AD prediction in 6 years (AUC=95.47%).

In AIBL only the neuropsychological tests along with subjective assessment are used to define clinical status. The use of markers such as age, gender, APOE genotype and PET-Ab status are commonly used in clinical trials to enrich the sample for participants that are likely to decline within 36 months. Thus the addition of the AZ 284™ biomarker to this already strong predictor set as shown in this work provides extra confidence (~10-15%) that the chosen MCI participants will decline in the next 18-36 months (with this limited cohort of samples). The performance of the base model coupled with AZ 284™ and PiB-PET (compared to that obtained excluding PiB-PET) for progression CU-to-AD within 36 months was likely due to a steep decline that does not often present in AD (25). These preliminary data showed the promising performance of AZ 284™ detection even on a longer follow-up and represent an important advantage for the implementation of AlzoSure® as a test for early-stage or pre-clinical AD.

The current study has its limitations. Firstly, the sample sizes for the overall study, and the individual groups of progressors vs non-progressors, are quite small. Whilst these data come from a well characterised and phenotyped population, these preliminary results will need to be validated in another larger population. Secondly, despite the strong design for this study, follow-up of CU participants could be performed over a longer period of time. As a high proportion of those CU individuals with an appreciable accumulation of Amyloid will convert, a longer follow-up with a larger population may capture the predictive accuracy of CU-to-AD progression.

Identification of an early biomarker is pivotal to effectively counteract the disastrous effects of AD progression. Recently different strategies have been explored to select an effective disease-modifying agent able to tackle earlier the disease (26). Such studies aimed at targeting different pathological paths involving amyloid plaques and tau aggregations (5), and therapies tackling zinc dyshomeostasis. Classical biomarkers, even the highly sensitive blood-based recently characterised through MS (7, 9), are based on cerebral amyloidosis detection or prediction of amyloid positivity leading to a late diagnosis and hence a late therapeutic pharmacological interventions. U-p53<sup>AZ</sup> yields potential as prognostic tool for AD, showing a preliminary yet promising early diagnostic performance.

Another advantage of U-p53<sup>AZ</sup> is its availability in the blood cells, making the AlzoSure<sup>®</sup> test remarkably non-invasive and facilitating easier testing compared to CSF-based biomarkers. Combined with early and reliable prediction and its lower economic burden (for the affordability of MS), this opens the possibilities to an easy-to-perform test. Its implementation in non-invasive screenings to a larger population could facilitate: 1) precocious detection of AD in unaware individuals, 2) earlier detection could lead to better preparedness of the caregivers – which in a recent prospect EU-based was hypothesised to not be ready for an improvement in prediction and higher diagnosis rates (27) – 3) targeted therapies that could slow the disease, if not stop it directly. Currently, clinical trials suffer from a lack of early detection high-risk patients, reflected on inadequate stratification (28) and AlzoSure<sup>®</sup> may play a pivotal role in contributing to properly stratified clinical studies.

### Abbreviations

A $\beta$ : Amyloid beta; AD: Alzheimer's disease; AIBL: Australian Imaging, Biomarkers and Lifestyle; APOE  $\epsilon$ 4: Apolipoprotein E allele  $\epsilon$ 4; AUC: Area under curve; CI: Confidence interval; CSF: Cerebrospinal fluid; CU: Cognitively unimpaired; IP: Immunoprecipitation; MCI: Mild cognitive impairment; MS/MS: Tandem mass spectrometry; NfL: Neurofilament; NMC: No memory complaints; PET: Positron emission tomography; PiB: Pittsburgh compound B; PMF: peptide mass fingerprint; PROSAD: Progressive sample dosage; SACI: Surface activated chemical ionization; SMC: Subjective memory complaints; U-p53<sup>AZ</sup>: unfolded P53, conformational variant of p53 captured by the 2D3A8 mAb .

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### Authors' contribution

DU collaborated with SP to the study design, SP overviewed the development of the protocol, SC with ISB performed the MS testing. All authors contributed equally to the manuscript. All authors read and approved the final manuscript.

### Ethics approval and consent to participate

An appropriate institutional ethics committee approved the AIBL study, which followed relevant ethical regulations. Further information: Human Research Ethics Committee, Research Governance Unit, St Vincent's Healthcare, Australia (no. 028/06). Individuals registered in AIBL and could opt out of PiB-PET. Their plasma samples are stored in the biobank and a subset of them was requested for this exploratory study.

### Consent for publication

All authors agreed to the manuscript prior to submission.

### Availability of data and materials

Data are available from the authors upon request.

### Competing interests

SP is an employee of Diadem sr, Brescia, Italy; DU is co-founder and CSO of Diadem srl, Spin Off of Brescia University, Brescia, Italy. The ownership of the 2D3A8 antibody patent rights belongs to Diadem srl, Brescia, Italy. The Alzsure test was developed by Diadem srl, Brescia, Italy. SC (as ISB) stipulated a contract with Diadem srl to provide the technical support on MS testing, operated blindly with respect to the clinical outcomes.

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### Supplementary material.

**Table S1. Instrumental method calibration parameters.**

Parameter	Value
Linearity range	0.1 – 10 fmol/10 $\mu$ L
Precision error %	3-8 %
Accuracy error %	6-9 %