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Performance of a non-invasive blood test for a conformational variant of p53 to predict Alzheimer's disease within 6 years of clinical diagnosis

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Abstract

Background: Research continues to search for blood-based biomarkers sensitive to Alzheimer's disease (AD) pathology during the initial stages when symptoms of cognitive decline are not yet apparent. A blood-based biomarker candidate is metalloprotein p53, the conformation of which was previously found to be altered in peripheral cells from individuals with mild cognitive impairment (MCI) and AD, presenting as an unfolded p53 (U-p53) conformational variant.

Methods: Plasma samples from the well-characterized Australian Imaging, Biomarkers, and Lifestyle (AIBL) cohort were used to identify the clinically relevant AZ 284® peptide, specifically present in samples from individuals with symptomatic AD. The AZ 284® peptide, which is a marker of the U-p53 conformational variant (U-p53^{AZ}), was identified by immunoprecipitation (IP) with a novel U-p53 conformational variant-specific antibody followed by liquid chromatography (LC) tandem mass spectrometry (MS/MS) and protein sequencing. Using IP-LC surface-activated chemical ionization (SACI) MS/MS analysis, the prognostic and diagnostic performance of U-p53^{AZ} were examined in the longitudinal AIBL cohort, including 252 plasma samples derived from 214 elderly individuals. For the prognostic analyses, U-p53^{AZ} levels were assessed at 36, 72, and 90 months after baseline assessment.

Results: The prognostic performance of U-p53^{AZ} to predict the progression to AD from preclinical or prodromal AD was high, with area under the receiver operating characteristic curve (AUC) values close to or above 0.90. Furthermore, U-p53^{AZ} predicted the progression to AD more than 6 years prior to symptom onset with positive and negative predictive values of about 90%. Additionally, the estimated prognostic performance of U-p53^{AZ} was superior to other main risk factors (i.e., age, sex, and number of *APOE* ε4 alleles) either alone or in

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combination with amyloid status. Furthermore, U-p53^{AZ} had high diagnostic performance to differentiate cognitively normal individuals from those with AD (AUC values >0.88).

Conclusion: These findings support the use of U-p53^{AZ} as a prognostic blood-based biomarker accurately predicting the progression to AD dementia during the preclinical and prodromal stages at least 6 years before receiving the clinical diagnosis of AD dementia.

Keywords: Alzheimer's disease, AD, blood-based biomarker, p53, unfolded p53, U-p53

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Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder, and the most common cause of dementia, affecting more than 47 million people worldwide (1). The disease spans a clinical continuum starting with a preclinical phase lasting up to two decades. Although cognitive symptoms are not yet apparent, biomarker studies show that the neuropathological features of AD develop during this initial preclinical stage (2, 3). After the period of normal cognition, the individuals will progress to prodromal AD, also referred to as mild cognitive impairment (MCI) due to AD, and ultimately progress to receive a diagnosis of AD dementia (4, 5). Putative disease-modifying treatments have failed so far to prevent the progressive neurodegeneration associated with AD (6-8). One of the potential reasons for these failures is absence of reliable and minimally-invasive biomarkers to detect the disease during the preclinical stage (9). Such early biomarkers could stratify patients in clinical trials who are at high risk to develop AD dementia, years before irreversible neurodegeneration has occurred (9, 10).

Currently, the diagnostic workflow developed by the National Institute on Aging and Alzheimer’s Association (NIA-AA) consists of a battery of neurophysiological tests followed by brain imaging and cerebrospinal fluid (CSF) sampling to identify amyloid (A) peptides, tau (T) protein, and markers of neurodegeneration (N) (11). However, imaging and CSF sampling are costly and invasive, limiting use of these methods for large-scale screening of patients with initially normal cognition (11). Blood-based biomarkers offer the advantage of being non-invasive and more readily accessible for screening purposes (12). Besides many studies documenting the diagnostic performance of blood-based biomarkers, ongoing research is aiming to identify prognostic plasma biomarkers for predicting subsequent AD early during the preclinical stage (12).

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During this preclinical stage, early pathological changes may initially induce compensatory responses, including antioxidant responses counteracting the increased reactive oxygen and nitrogen species (ROS/RNS) production that gradually becomes inefficient, exacerbating oxidative stress (13). This was supported by studies demonstrating a deficiency of endogenous antioxidant capacity and an increase in ROS/RNS in both the brain and peripheral tissues of patients throughout the AD continuum (14-16). Notably, redox post-translational modifications (redox-PTMs) were shown to modify the native structure of p53, resulting in an unfolded p53 conformational variant (U-p53), as shown in immortalized lymphocytes derived from individuals with AD (17). Due to redox-PTMs, p53 had lost its canonical biological function, which may contribute to the underlying pathology of AD (18). Furthermore, *in vitro* experiments also demonstrated that nanomolar $A\beta_{1-40}$ and $A\beta_{1-42}$ concentrations caused similar changes in the p53 tertiary protein structure (19-21). Increased peripheral levels of the p53 conformational variant were confirmed in AD by using different cohorts enrolled in different studies (22-24). Recently, a novel antibody (2D3A8) was able to recognize the conformational variant in plasma samples from individuals across the AD continuum (25). Using this antibody, Abate et al., demonstrated the promising prognostic performance of the plasmatic U-p53 variant (herein described as "U-$p53^{AZ}$") in predicting the AD likelihood risk in the preclinical and prodromal stages with an overall accuracy of 86.67%, suggesting it as a potential biomarker candidate of AD dementia (25).

The present study aimed to further explore the potential of the U-$p53^{AZ}$ variant as a blood-based biomarker to accurately distinguish different diagnostic groups and, more importantly, to predict the predisposition of cognitively normal (CN) individuals and patients with MCI to subsequently develop AD years before dementia onset.

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Materials & Methods

Subjects

The Australian Imaging, Biomarkers, and Lifestyle (AIBL) longitudinal cohort study was initiated in 2006 and has progressively enrolled over 1,000 participants in Melbourne and Perth, Australia (26). Participants entered the study between the ages of 60-85 years, with enrolment criteria excluding comorbidities, such as uncontrolled diabetes and vascular disease, as well as severe depression and psychiatric illness. Participants underwent 2 hours of neuropsychological testing and fasting blood collection over two to three visits every 18 months. In addition to basic information on sex and age, data on the clinical diagnosis, amyloid burden as determined by positron emission tomography with the labeled Pittsburgh compound B (PiB-PET), Clinical Dementia Rating (CDR), and Mini-Mental State Examination (MMSE) were available during follow-up. Participants were classified using the NINCDS-ADRDA criteria, covering the following classifications: CN, MCI, and presenting symptomatic AD. The CN group was further divided into two subgroups: no memory complaints (NMC) and subjective memory complaints (SMC). A clinical panel blinded to biomarker data of apolipoprotein E (*APOE*) allele status and PET-amyloid provided a confirmed clinical classification on every MCI and AD dementia case. The study was approved by the appropriate institutional ethics committee and was performed following all relevant ethical regulations (for a detailed description, see the Human Research Ethics Committee, Research Governance Unit, St Vincent's Healthcare, Australia (no. 028/06)). Based on availability of samples and their longitudinal data, 262 plasma samples derived from 224 individuals were included. Plasma samples were collected from participants at different stages of dementia development, including also 10 samples collected from 10 participants with types of dementia other (OD) than AD.

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Blood collection and apolipoprotein E (*APOE*) genotype

Blood fractions were processed under AIBL standard operating procedures at room temperature and the EDTA collection tubes had pre-added prostaglandin-E1 to produce a final concentration of 33 ng/mL of whole blood preventing platelet activation. Samples were processed within 3 hours and subsequently stored as aliquots in vapor phase liquid nitrogen. Genotyping was performed as previously described (27).

Antibody preparation

The novel 2D3A8 antibody was previously shown to specifically bind a linear epitope of the p53 conformational variant (U-p53^{AZ}), which is exposed in samples derived from patients during the AD continuum (28). The antibody recognizes the linear epitope of the sequence RRTEEENLRKKGEPHH located between the amino acid positions 282-297 of human p53, and was prepared using conventional immunization and hybridoma techniques (28). The antigen used was the following peptide: CRTEEENLRKKGEPHH conjugated with bovine serum albumin by the glutaraldehyde method. For immunization, 6/8-week-old BALB/C mice, which were healthy and disorder-free, received 3-weekly inoculations of 50 mg of the antigen with Freund's Adjuvant. Antibody titers were measured by spectrophotometric reading after the third inoculation, yet the mice received further inoculation to obtain higher titers. The splenocytes showing the highest titers were fused with mouse myeloma cells (SP2/O cell line). Subsequently, fusion products were screened by enzyme-linked immunosorbent assay (ELISA), and the clones underwent further selection into 24-well plates and culture flasks.

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Immunoprecipitation (IP) and nanoflow electrospray ionization tandem mass spectrometry (MS/MS)

AD continuum clinically relevant peptides were identified by U-p53^{AZ} protein sequencing, performed at MyomicsDX Inc., Towson, MD, USA. Briefly, IP was carried out on high abundance protein-depleted plasma samples using two different antibodies. The first IP was performed using the 2D3A8 antibody (10 µg/sample), while the second IP was performed using a mixture of antibodies specific for p53 (DO11:DO12:SAPU:KJC12 at volume ratios of 1:1:2:2 with final concentration of 1 µg/µl and used as 10 µg/sample) (29, 30). After IP, the eluted peptides were fractionated on an Agilent 1290 Infinity II liquid chromatography (LC) system. Next, data-dependent MS/MS analyses of the tandem mass tag labeled peptides were carried out on a Thermo Scientific™ EASY-nLC 1000™ HPLC system and Thermo Scientific EASYSpray™ source with an analytical nanoflow column system. Samples were analyzed on a Thermo Scientific™ Q Exactive Mass Spectrometer and survey full scan MS spectra (m/z 350−1800) were acquired in the Orbitrap with 35,000 resolution after accumulation of ions to a 3 × 106 target value based on predictive automated gain control from the previous full scan. The 10 most intense multiply-charged ions ($z \geq 2$) were sequentially isolated and fragmented in the Axial Higher energy Collision-induced Dissociation (HCD) cell using normalized HCD collision energy at 30% with an automatic gain control target of 1e5 and a maximal injection time of 400 ms at 35,000 resolution. Mass spectrometry raw files were automatically processed through Proteome Discoverer 2.2 software using Xtract in addition to default spectrum selector node. The searches were performed using Mascot search engine together with Sequest HT interfaced with different processing nodes of Proteome Discoverer 2.2. The final dataset was reprocessed through the MyProt-QuantiR (MyOmicsDx Inc.) software package, identifying the sequence peptide AZ 284® as the most clinically relevant peptide in samples from individuals with AD.

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Measurement of the U-p53^{AZ} peptide in plasma samples using the AlzoSure® test

AlzoSure® test was carried out at Ion Source Biotechnologies srl, Bresso, Italy using IP-LC surface-activated chemical ionization (SACI) MS/MS analysis to detect U-p53^{AZ} by the AZ 284® peptide at one timepoint in plasma samples from the AIBL cohort. The test was performed while being blinded of the clinical and cognitive data. Briefly, U-p53^{AZ} from 25 µL protein-depleted plasma samples was immunoprecipitated with the 2D3A8 antibody using Protein L magnetic beads (Invitrogen). Samples were proteolyzed with trypsin for 3 hours 30 min. at 37°C and then 30 min. at 57°C. The peptides were analyzed on an HPLC Ultimate 3000 (Thermo Fisher Scientific). Bi-phase solvent gradient consisted of 0.2% formic acid with increasing levels of 90% acetonitrile. SACI and the spectra were acquired by SACI peptide adducts profile. The peptides quantization was carried out by PROSAD method and the data analysis was performed by SANIST (31). The performance of the method was assessed by testing 262 samples in duplicates by 11 analytical sessions and repeating this for 36 samples, confirming the test's reliability.

Statistical analyses

Both diagnostic and prognostic analyses were conducted. Prognostic analyses for individuals in the preclinical stage of AD were conducted to assess the biomarker's ability to predict AD on participants who were clinically diagnosed as having SMC or who were clinically diagnosed as CN with CDR=0 at the time of baseline biomarker assessment. Additionally, prognostic analyses were conducted for individuals in the prodromal stage on participants who were clinically diagnosed as having MCI or who were clinically diagnosed as MCI with CDR=0.5 at the time of baseline biomarker assessment. Analyses of diagnostic accuracy were based on the biomarker data and the clinical diagnosis based on the participant's phenotype, assessed at a single time point. Most of these analyses were based on biomarker data and

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diagnostic information at the first time when the biomarkers were measured (i.e., baseline diagnostic performance), but some included the biomarker data and diagnostic information at a subsequent time to increase the sample size for some diagnostic groups (e.g., when data of PiB-PET were needed). Additionally, the diagnostic and prognostic criteria included neuropsychologically diagnosed AD dementia with CDR ≥1 and further with confirmation by a positive PiB-PET scan (herein described as "AD dementia – PET positive") versus individuals who were neuropsychologically diagnosed as CN with CDR 0 and who were negative on PiB-PET (herein described as "CN – PET negative"). To determine the predictive and diagnostic performance, time-dependent receiver operating characteristic (ROC) curves and the corresponding area under the receiver operating characteristic curve (AUC) values, sensitivity, and specificity were estimated by using the cutoffs as determined by the Youden's Index. The DeLong test was used to compare ROC performances. Further, time-dependent positive predictive values (PPV) and negative predictive values (NPV) were computed by assuming an incidence rate of AD dementia of 30% for CN at baseline and 50% for participants diagnosed with MCI at baseline (32, 33).

Results

Demographics and characteristics of the AIBL cohort

Out of the 214 elderly individuals (median age: 75 years), 180 had known PET-amyloid status and 213 were screened for the presence of the *APOE* ε4 allele. A total of 252 samples were evaluated from these elderly individuals across all diagnostic groups (i.e., NMC, SMC, MCI, and AD) of whose clinical diagnosis was confirmed at baseline and over the follow-up, including individuals who showed a cognitive decline to AD dementia. The number of participants included in the prognostic analyses are summarized in **Table 1** and **Table 2**.

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Table 1. Number of participants included in the prognostic analyses.

	CN		
	NMC	**SMC**	**MCI**
Number of patients, N	40	94	66
Progression to			
AD	5/40	21/94	40/66
AD dementia – PET pos; CDR ≥1	1/40	7/94	16/66

AD: Alzheimer's disease; CDR: clinical dementia rating; CN: cognitively normal; MCI: mild cognitive impairment; NMC: no memory complaints; PET pos: positron emission tomography positive; SMC: subjective memory complaints

Table 2. Number of participants included in the prognostic analyses by CDR classification.

	CN-CDR=0	**MCI-CDR=0.5**
Number of patients, N	122	84
Progression to		
AD	/	32/84
AD dementia – PET pos; CDR ≥1	7/122	19/84
AD-CDR ≥1	14/122	34/84

AD: Alzheimer's disease; CDR: clinical dementia rating; CN: cognitively normal; MCI: mild cognitive impairment; PET pos: positron emission tomography positive

Diagnostic performance of U-p53^{AZ} to differentiate between AD stages

The samples from the AIBL cohort were used to assess U-p53^{AZ} levels and determine its diagnostic performance to differentiate CN individuals from those with symptomatic AD. In individuals diagnosed with AD at baseline, average levels of U-p53^{AZ} were approximately

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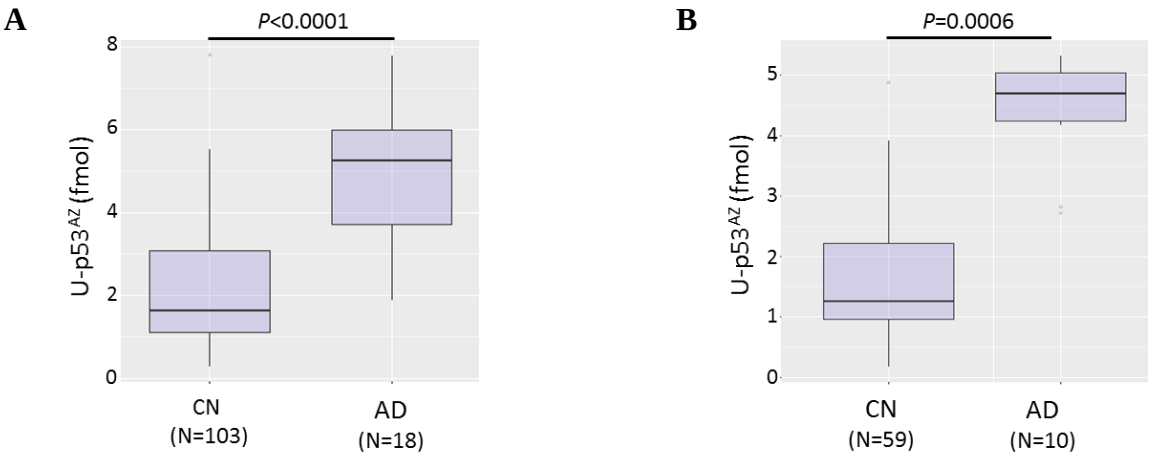
three times higher as compared with individuals who were CN (i.e., NMC and SMC) at baseline (P<0.0001) (**Figure 1A**). The difference between U-p53^{AZ} levels at baseline was more pronounced when comparing participants who were CN throughout the whole study duration with those who were diagnosed with AD at some point over the follow-up (P=0.0006) (**Figure 1B**). Average baseline levels of U-p53^{AZ} were also higher in participants whose diagnosis of AD dementia was confirmed by PiB-PET (P<0.0001) (**Figure 1C**). Additionally, the average baseline levels of U-p53^{AZ} were significantly increased in those diagnosed with MCI at some point over the follow-up as compared with individuals who were CN throughout the study duration (P=0.0002) (**Figure 1D**).

Figure 1. Boxplots showing average baseline levels of U-p53^{AZ} in different diagnostic groups. A) CN versus AD at baseline. B) Stable CN versus those who progressed to AD at some point over the study follow-up. C) CN – PET negative versus AD dementia – PET positive at baseline. D) Stable CN versus those who progressed to MCI at some point over the study follow-up.



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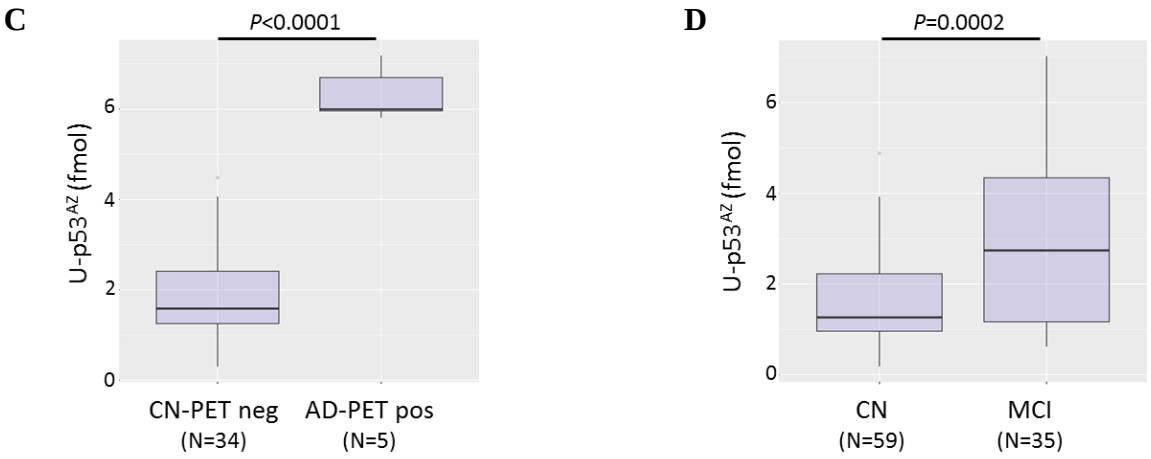

AD: Alzheimer's disease; CN: cognitively normal; MCI: mild cognitive impairment; PET: positron emission tomography

Accordingly, the diagnostic performance of U-p53^{AZ} to differentiate CN individuals from those with AD was high, with AUC values above 0.88 (**Table 3**).

Table 3. Diagnostic performance of U-p53^{AZ} to differentiate cognitively normal (CN) individuals from those with AD.

CN versus AD (at baseline)	
AUC (95% CI)	0.89 (0.82-0.96)
CN – PET negative versus AD dementia – PET positive (at baseline)	
AUC (95% CI)	0.95 (0.90-1.00)
Stable CN versus those who progressed to AD	
AUC (95% CI)	0.95 (0.91-0.98)

AD: Alzheimer's disease; AUC: area under the receiver operating characteristic curve; CI: confidence interval; CN: cognitively normal; PET: positron emission tomography

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Prognostic performance of U-p53^{AZ} to predict the progression to AD at the preclinical stage

Baseline levels of plasma U-p53^{AZ} were used to predict clinical symptoms in accordance with the clinical diagnosis assessed at 36, 72, and 90 months after plasma collection (**Table 4**). Time-dependent ROC analysis showed a high performance of U-p53^{AZ} in predicting conversion to AD among those with SMC at baseline. The estimated AUC values were all above 0.90 at 36, 72, and 90 months after plasma collection (**Table 4**). Notably, even at the longer follow-up of 90 months, U-p53^{AZ} showed a valuable prognostic value expressed by an estimated sensitivity of 82%, specificity of 92%, PPV of 81%, and NPV of 92% for predicting the progression from SMC to AD at 90 months after baseline assessment. Similarly, baseline U-p53^{AZ} levels reliably predicted progression from SMC to AD dementia confirmed by PiB-PET (i.e., AD dementia – PET positive) at 72 months after baseline assessment (**Table 4**). Additionally, the prognostic performance of U-p53^{AZ} in CN individuals with CDR=0 for predicting conversion to a CDR ≥1 with a diagnosis of AD 36, 72 and 90 months after baseline was still high with AUC values all above 0.90 (**Table 4**). At 90 months after baseline assessment, U-p53^{AZ} showed an estimated sensitivity, specificity, PPV, and NPV of 77%, 97%, 99%, and 91%, respectively, in predicting if CN individuals (CDR=0) would progress to AD (CDR ≥1).

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Table 4. Prognostic performance of U-p53^{AZ} to predict the progression to AD at the preclinical stage.

Progression SMC to AD	**in 36 months**	**in 72 months**	**in 90 months**
AUC (95% CI)	0.92 (0.85-0.99)	0.95 (0.88-1.00)	0.95 (0.85-1.00)
Progression SMC to AD dementia – PET positive	**in 36 months**	**in 72 months**	**in 90 months**
AUC (95% CI)	0.92 (0.75-1.00)	0.96 (0.80-1.00)	NA
Progression CN with CDR=0 to AD with CDR ≥1	**in 36 months**	**in 72 months**	**in 90 months**
AUC (95% CI)	0.98 (0.94-1.00)	0.99 (0.96-1.00)	0.98 (0.95-1.00)

AD: Alzheimer's disease; AUC: area under the receiver operating characteristic curve; CDR: clinical dementia rating; CI: confidence interval; NA: not available; PET: positron emission tomography; SMC: subjective memory complaints

The prognostic performance of U-p53^{AZ} was compared with the performance of age and sex (i.e., basic model) in combination with the number of *APOE* ε4 alleles and assessment of amyloid status. At 36 months after baseline assessment, the prognostic performance of U-p53^{AZ} to predict the progression from SMC to AD or CN with CDR=0 to AD with CDR ≥1 was significantly higher than the screening for amyloid positivity (**Table 5**). Additionally, assessment of U-p53^{AZ} was superior to the basic model in combination with the number of *APOE* ε4 alleles and/or amyloid status in predicting the progression from SMC to AD or CN with CDR=0 to AD with CDR ≥1 within 72 months before receiving a clinical diagnosis (**Table 5**).

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Table 5. Prognostic performance of U-p53^{AZ} to predict the progression to AD dementia at the preclinical stage in comparison with the basic model (i.e., age and sex) in combination with the number of *APOE* ε4 alleles (i.e., e4) and/or amyloid status.

	36 months		72 months	
Progression SMC to AD	**AUC (95% CI)**	***P*-value**	**AUC (95% CI)**	***P*-value**
U-p53^{AZ}	0.92 (0.85-0.99)	/	0.95 (0.88-1.00)	/
Basic + e4	0.76 (0.18-1.00)	0.08	0.84 (0.21-1.00)	0.04
Basic + amyloid	0.80 (0.27-1.00)	0.03	0.87 (0.32-1.00)	0.01
Basic + e4 + amyloid	0.79 (0.17-1.00)	0.06	0.87 (0.24-100)	0.02

	36 months		72 months	
Progression CN with CDR=0 to AD with CDR ≥1	**AUC (95% CI)**	***P*-value**	**AUC (95% CI)**	***P*-value**
U-p53^{AZ}	0.98 (0.94-1.00)	/	0.99 (0.96-1.00)	/
Basic + e4	0.69 (0.16-1.00)	0.17	0.91 (0.29-1.00)	0.01
Basic + amyloid	0.68 (0.42-0.94)	0.01	0.88 (0.63-1.00)	<0.0001
Basic + e4 + amyloid	0.78 (0.50-1.00)	<0.0001	0.95 (0.66-100)	<0.0001

AUC: area under the receiver operating characteristic curve; CDR: clinical dementia rating; CI: confidence interval; e4: *APOE* ε4 allele; SMC: subjective memory complaints

Prognostic performance of U-p53^{AZ} to predict the progression to AD at the prodromal stage

The prognostic performance of plasma U-p53^{AZ} to predict the progression from MCI to AD was determined at 72 months after baseline assessment. ROC analysis demonstrated that U-p53^{AZ} was able to reliably predict whether or not individuals with MCI would convert to AD within 72 months after study initiation (AUC=0.95; 95% CI: 0.89-1.00) (**Table 6**). Similarly, U-p53^{AZ} predicted the progression from MCI with CDR=0.5 to AD dementia confirmed by

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PiB-PET (i.e., AD dementia – PET positive) with an AUC of 0.95 (95% CI 0.57-1.00) (**Table 6**). At 72 months after baseline assessment, the estimated sensitivity and specificity of U-p53^{AZ} to predict the progression from MCI (classification based only on neuropsychiatric assessment without confirmation by biomarkers or cognitive evaluation by MMSE or CDR value) to AD were 70% and 90%, respectively, with a PPV of 88% and a NPV of 75%. Additionally, the progression from individuals clinically classified as MCI and showing a CDR value of 0.5 to AD confirmed by PiB-PET was predicted by U-p53^{AZ} at 72 months after baseline assessment with a sensitivity of 91%, specificity of 90%, PPV of 90%, and NVP of 91%.

Table 6. Prognostic performance of U-p53^{AZ} to predict the progression to AD at the prodromal stage.

Progression MCI to AD	**in 72 months**
AUC (95% CI)	0.95 (0.89-1.00)
Sensitivity, %	70
Specificity, %	90
PPV, %	88
NPV, %	75
Progression MCI with CDR=0.5 to AD dementia – PET positive	**in 72 months**
AUC (95% CI)	0.95 (0.57-1.00)
Sensitivity, %	91
Specificity, %	90
PPV, %	90
NPV, %	91

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AD: Alzheimer's disease; AUC: area under the receiver operating characteristic curve; CDR: clinical dementia rating; CI: confidence interval; NPV: negative predictive value; PET: positron emission tomography; PPV: positive predictive value; SMC: subjective memory complaints

Plasma U-p53^{AZ} did predict the progression from MCI/prodromal AD to AD better than the basic model with or without the number of *APOE* ε4 alleles and with or without amyloid PET but differences were not statistically significant (**Table 7**). The wide confidence intervals in the basic model group combined with genetic testing for *APOE* ε4 carrier status and/or amyloid positivity may have contributed to this outcome.

Table 7. Prognostic performance of U-p53^{AZ} to predict the progression to AD at the prodromal stage in comparison with the basic model (i.e., age and sex) in combination with the number of *APOE* ε4 alleles (i.e., e4) and/or amyloid status.

	72 months	
Progression MCI to AD	**AUC (95% CI)**	***P*-value**
U-p53^{AZ}	0.95 (0.89-1.00)	/
Basic + amyloid	0.65 (0.15-1.00)	0.25
Basic + e4 + amyloid	0.67 (0.17-1.00)	0.18

AD: Alzheimer's disease; AUC: area under the receiver operating characteristic curve; CI: confidence interval; e4: APOE ε4 allele; MCI: mild cognitive impairment

Discussion

During the past decades, there has been a continuous quest for blood-based biomarkers that could identify individuals who are at risk to develop AD dementia before the onset of irreversible cognitive deterioration (10). However, no blood-based biomarker has currently been included in the diagnostic algorithm screening for patients who are at risk to develop

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AD dementia later in life (11). Due to suboptimal diagnostic pathways, more than 50% of people in Europe and the United States with dementia do not receive a formal diagnosis in primary care, often leading to inappropriate disease management (10). Additionally, lack of easily accessible and reliable prognostic biomarkers limits successful enrolment of individuals who are prone to develop AD dementia in clinical intervention trials. Therefore, it would be desirable to have non-invasive and effective blood-based biomarkers to increase diagnostic accuracy, recognize AD dementia in earlier stages, and enrich clinical trial populations while reducing the need for CSF sampling and brain imaging (9, 10).

Although p53 is mostly known for its role as a tumor suppressor, it is becoming well-established that alterations in p53 activity affect cell fate decisions, thus contributing to a variety of diseases beyond cancer (34). A mounting body of evidence supports p53 playing a neuroprotective role by mediating DNA damage repair, protecting against oxidative stress, stimulating neuronal outgrowth and axon regeneration, controlling synaptic function, and even repressing the expression of the β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) (35-39). A conformational variant of p53, herein described as U-p53, has been detected in peripheral cells derived from individuals with MCI and AD (22-24). A link between p53 and amyloid beta has been well described (40, 41). For example, $A\beta_{1-40}$ and $A\beta_{1-42}$ at nanomolar concentrations induced p53 conformational changes towards an open variant unable to preserve its neuroprotective activity (19-21). This was also confirmed by several studies describing how altered p53 functioning in the brain can contribute to AD pathology (42, 43). Accordingly, detection of an unfolded conformational variant of p53 in peripheral cells was highly predictive of the conversion from the prodromal stage to AD dementia (23, 24). However, initial studies only included a limited number of patient samples and assessed the presence of the

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unfolded conformational variant of p53 by conventional techniques, such as flow cytometry and ELISA, with commercially available antibodies (23, 24). Very recently, Abate et al. introduced the novel 2D3A8 antibody to measure the U-p53 conformational variant, clinically relevant in AD (U-p53^{AZ}), showing for the first time its high performance as a blood-based biomarker to predict the progression to AD dementia already during the preclinical and prodromal stages (25).

In this study, 2D3A8-based IP followed by LC-SACI/MS/MS sequential technique was used to identify and subsequently quantify the AZ 284® peptide from U-p53^{AZ} in plasma samples derived from the well-characterized longitudinal AIBL cohort, including individuals across the AD continuum. Mass spectrometry has been used in several studies examining blood-based biomarkers for AD dementia, with its major strength its high specificity in comparison to other techniques, such as ELISA (44). Upon quantification of the AZ 284® peptide, the current study aimed to examine if U-p53^{AZ} (AlzoSure®) could be used as a predictive signature of AD dementia, identifying individuals during the preclinical and prodromal stages who are at high risk of developing symptomatic AD within a defined timeframe. Based on the samples derived from the AIBL cohort, U-p53^{AZ} was shown to accurately predict if individuals would experience cognitive decline to symptomatic AD with AUCs all above 0.90, regardless if U-p53^{AZ} was assessed during the preclinical or prodromal stages and regardless of which diagnostic criteria were used. Notably, the prognostic performance of U-p53^{AZ} during the preclinical stage was particularly high when levels were assessed 90 months after receiving the clinical diagnosis of AD dementia. At 90 months after baseline assessment, PPV and NPV values were both in the range of 90%, underscoring the potential of U-p53^{AZ} as a reliable blood-based prognostic biomarker of AD risk. When comparing its prognostic performance to the performance of the number of *APOE* ε4 alleles and/or amyloid status in combination with

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the basic model (i.e., age and sex), U-p53^{AZ} clearly showed superiority to predict cognitive decline to AD dementia during the preclinical and prodromal stages. Remarkably, the prognostic performance of the basic model and the number of *APOE* ε4 alleles was not substantially increased by the addition of amyloid status and was therefore still inferior to the use of U-p53^{AZ} as a prognostic biomarker. Although the use of U-p53^{AZ} is focused on its potential as blood-based prognostic biomarker, it also has the ability to differentiate CN individuals those with AD with AUC values above 0.88.

When comparing to other studies, the prognostic performance of U-p53^{AZ} alone was within the same range as previously has been described for the combination of magnetic resonance imaging (MRI), PET, CSF, and covariates (45). However, being a blood-based biomarker, U-p53^{AZ} offers the advantage of being non-invasive and more readily accessible than imaging and CSF sampling. Although ATN blood-based biomarkers were originally designed for diagnostic purposes, they were recently shown to accurately predict the clinical progression to AD dementia in non-demented adults and patients with MCI (46). Especially, plasma phospho-tau181 has received interest in predicting future AD dementia correlating with CSF phospho-tau181 levels (47, 48). Another emerging blood-based biomarker is the misfolded variant of Aβ, predicting the progression of individuals with SMC to clinical AD 6 years in advance with an AUC similar to U-p53^{AZ} (49). It will be interesting to examine in future studies if the specificity and prognostic performance of U-p53^{AZ} can be even further increased when used in conjunction with one or more of these ATN blood-based biomarkers.

Although the results from this study demonstrated the high prognostic performance of U-p53^{AZ}, the current results are based on a discovery cohort requiring confirmation of the presented results in a replication cohort. A follow-up study is being conducted to determine whether the findings of the AIBL cohort are generalizable defining a biomarker

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cutoff that can be used in clinical practice to stratify CN individuals based on their risk to develop AD dementia later in life. Another limitation of the current study is the limited number of converters and participants with PiB-PET data. As a result, no biomarker data beyond 72 months after baseline assessment could be included in the prognostic analyses for the conversion from MCI/prodromal AD to AD. Additionally, the study only included 10 samples from individuals with OD with limited follow-up data, which was not enough to make any conclusions on the specificity of U-p53^{AZ} for other neurodegenerative disorders. Nevertheless, the average level of U-p53^{AZ} in the samples from the individuals with OD was about half of the level detected in samples from those with AD dementia confirmed by CDR and PiB-PET. Furthermore, evidence from other studies has previously confirmed its specificity, as the expression of the unfolded p53 conformational variant was significantly higher in peripheral cells from patients with AD compared with individuals affected by either Parkinson's disease or other types of dementia (22). Recently, the specificity of the 2D3A8 antibody was also demonstrated by Abate et al., showing that the levels of the U-p53 conformational variant in plasma from patients with cancer, cardiovascular, inflammatory, and metabolic diseases were comparable to the levels detected in plasma from stable CN individuals (25). This is an important finding as p53 is known for its multiple roles in many conditions, including the above-described diseases.

The ongoing follow-up study of this discovery cohort includes more longitudinal data to evaluate how the levels of U-p53^{AZ} change throughout the AD continuum and how these levels correlate with markers of AD pathology in the brain, such as tau deposits. These longitudinal data could also be used to determine the effectiveness of disease-modifying interventions, assuming that halting the neuropathological disease progression would also prevent the increase in U-p53^{AZ} levels.

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Conclusions

This study confirmed that the AZ 284® peptide, representing the U-p53 conformational variant (U-p53^{AZ}), is a clinically relevant peptide for predicting in asymptomatic individuals the progression to AD at least 6 years before receiving a clinical diagnosis of AD. Quantification of U-p53^{AZ} (by AlzoSure®) could be implemented as an initial step into the diagnostic pathway of AD, aiding clinicians to decide at the first visit if more expensive and invasive diagnostic testing through imaging and CSF sampling is warranted. Finally, incorporating a reliable and effective blood-based biomarker of AD dementia early into the diagnostic algorithm may better inform patient stratification into clinical intervention trials aimed at halting or reducing dementia progression.

Abbreviations

Aβ: amyloid beta; AIBL: Australian Imaging, Biomarkers, and Lifestyle; AUC: area under the receiver operating characteristic curve; AD: Alzheimer's disease; APOE ε4: apolipoprotein E ε4 allele; BACE1: β-site amyloid precursor protein-cleaving enzyme 1; CI: confidence interval; CN: cognitively normal; CSF: cerebrospinal fluid; ELISA: enzyme linked immunosorbent assay; HCD: higher energy collision-induced dissociation; HPLC: high performance liquid chromatography; IP: immunoprecipitation; MCI: mild cognitive impairment; MRI: magnetic resonance imaging; MS/MS: tandem mass spectrometry; NMC: no memory complaints; OD: other types of dementias; PiB-PET: positron emission tomography utilizing Pittsburgh compound B; ROC: receiver operating characteristic; PTMs: posttranslational modifications; RNS/ROS: reactive nitrogen/reactive oxygen species; SACI: surface-activated chemical ionization; SMC: subjective memory complaints; U-p53: unfolded p53

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Competing interests

The ownership of the 2D3A8 antibody patent rights belongs to Diadem srl, Brescia, Italy. AlzoSure® Predict was developed by Diadem srl, Brescia, Italy. SP and PK are employees of Diadem srl, Brescia, Italy. DU is co-founder and CSO of Diadem srl, Spin Off of Brescia University, Brescia, Italy. GBF and AMF are part of Diadem's Medical Advisory Boards. CX is a consultant of Diadem srl.

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