

“p53 POST-TRANSLATIONAL MODIFICATIONS AS MARKERS IN THE
DIAGNOSIS AND PROGNOSIS OF A NEURODEGENERATIVE DISEASE”

FIELD OF THE INVENTION

5 The present invention refers to p53 sequence and post translational modifications (PTMs)
and to their use as biomarkers in the diagnosis of a neurodegenerative disease and
cognitive decline to Alzheimer's disease and Alzheimer's disease and/or in the prognosis
of Alzheimer's disease at different stages and/or of neurodegenerative disease in a
biological sample. The invention also provides for a diagnostic method based on a highly
10 accurate mass spectrometry analysis for the diagnosis of neurodegenerative disease,
including Mild Cognitive Impairment (MCI), Alzheimer's disease (AD), fronto-temporal
dementia (FTD), Lewi's Body (LB), and vascular dementia (VD) in a subject, by
evaluating the changes (PTMs) to said p53 linear protein sequence specifically in a
biofluid sample. The invention also provides for a diagnostic method based on a highly
15 accurate mass spectrometry analysis for the prognosis of Alzheimer's disease (AD) at
asymptomatic and prodromal stages (MCI) by evaluating the changes of said PTMs to
the linear sequence of p53 protein specifically in a biofluid sample.

BACKGROUND ART

The confirmation of the presence of a large amount of altered conformational p53 isoform
20 as an early risk factor for Alzheimer's disease (shortly 'AD') have been demonstrated in
different published studies [1-3]. Initially, more than 400 subjects among AD, Mild
Cognitive Impairment, Parkinson Disease, other Dementia and healthy subjects were
enrolled in different independent studies and tested for Unfolded p53 by using different
techniques (immunoprecipitation experiments, FACS analysis, ELISA) with a
25 commercial conformational specific anti-p53 antibody [4-7]. In 2006 for the first time
Uberty et al. [8], demonstrated that fibroblasts from sporadic Alzheimer's disease (AD)
patients specifically expressed an anomalous and detectable conformational state of p53
that differentiate these cells from fibroblasts of age-matched non-AD subjects. In this
conformational altered state, p53 lost its ability to transactivate its target genes, and
30 consequently its biological functions [9-10]. The higher amount of unfolded p53 was also
confirmed in blood of AD compared to healthy-non demented subjects or patients
affected by other dementia and PD, as well as in MCI converted to AD.

Altogether these data suggested a direct association between Unfolded p53 and AD pathology.

In EP3201234B1, it has been reported the development of a new conformational specific anti-Up53 antibody named 2D3A8, that binds to an epitope (aa 282-297), accessible only
5 when p53 loses its wild type conformation towards an unfolded phenotype. Comparing to the commercial antibody used at the beginning of Unfolded p53 discovering in AD (PAb240, aa214-217), the 2D3A8 antibody showed higher sensitivity and specificity in identifying AD patients compared to healthy elderly in Oviedo cohort.

In particular, said immunodiagnostic method is able to identify immunocomplex in a
10 biological sample that are indicative of AD and to determine the predisposition of a subject affected by Mild Cognitive Impairment (MCI) to develop AD.

PCT/IB2019/051785 discloses a method based on the identification and quantification of the levels of specific p53 peptides, indicated as "P1" and "P2", that have been detected by mass spectrometry analysis in human plasma of patients affected by Alzheimer's
15 disease or patients that have symptoms that can predispose to the development of AD.

There is now the need of identifying new specific biological markers that can be used in the diagnosis and/or prognosis of Alzheimer's disease and of developing an accurate and sensible diagnostic method that can be used for the diagnosis and/or prognosis of AD, in particular at the pre-clinical and prodromal stages of the disease and for the differential
20 analysis of AD from other forms of dementia, such as Frontotemporal Dementia, Levy Body dementia and vascular dementia.

SUMMARY OF THE INVENTION

The object of the present invention has been achieved by identifying eleven main post-translation modifications (PTMs) in the amino acidic sequence of the p53 protein within
25 the region of amino acids 1-371, herein called PTM-1, PTM- 2, PTM-3, PTM- 4, PTM-5, PTM-6, PTM-7, PTM-8, PTM-9, PTM-10, PTM-11 and/or some truncated forms of the p53 protein in a biofluid sample.

An aspect of the present invention therefore relates to a diagnostic method based on the identification of said PTMs for use in the diagnosis of different forms of dementia and
30 cognitive decline and/or in the prognosis of Alzheimer's disease at different stages.

BRIEF DESCRIPTION OF THE DRAWINGS

The characteristics and the advantages of the present invention will become apparent from

the following detailed description and the working examples provided for illustrative purposes, as well as the annexed Figures, wherein:

Figure 1. Protein ubiquitination sites detected in samples of subjects affected by AD.

Figure 2. Protein ubiquitination sites detected in control samples (CU).

5 Figure 3. Protein ubiquitination sites detected in the samples of subjects affected by frontal dementia (FTD).

Figure 4. Protein ubiquitination sites detected in the samples of subjects affected by Lewy Body's dementia (LB).

10 Figure 5. Protein ubiquitination sites detected in the samples of subjects affected by vascular dementia (VD).

Figure 6. Protein ubiquitination sites detected in the samples of subjects affected by mild cognitive disorder (MCI).

Figure 7. Protein ubiquitination sites detected in the samples of cognitively healthy subjects (CU) who developed AD over a period of at least 18 months.

15 Figure 8. Protein ubiquitination sites detected in the samples of the subjects of the AD developed MCI group.

The sequences reported in the figures correspond to the linear sequence of SEQ ID N: 1.

DEFINITIONS

20 With the term "U-p53" it is meant to denote the region of amino acids 1-371 of the p53 protein, which involves the post translational modifications (PTMs), and in some cases also a truncation, on linear protein sequence as described below.

With term "p53" it is meant the wild-type protein p53 as following the Database "UniProtKB, Protein ID: P04637, amino acids: 1 – 393".

25 With the term "neurodegenerative disease" it is meant to denote a range of conditions that mainly affect the neurons in the human brain, also comprising forms of dementia, such as Mild Cognitive Impairment (MCI), fronto-temporal dementia (FTD), Lewi's Body (LB), and vascular dementia (VD), as well as the different stages of the said neurodegenerative diseases and cognitive decline to dementia, and Alzheimer's disease (AD) (including pre-clinical and prodromal stages).

30 DETAILED DESCRIPTION OF THE INVENTION

The invention therefore relates to a combination of p53 post translational modifications detected by a highly accurate mass spectrometry method that can be used as biomarkers

in an *in vitro* or *ex vivo* method for the diagnosis of a neurodegenerative disease. Said method is based on the identification of specific p53 modifications compared to its linear sequence, shortly referred to as 'PTMs', that have been detected by mass spectrometry analysis in a biofluid sample derived from patients affected by Alzheimer's disease or
5 patients that have symptoms that can predispose to the development of AD or to different forms of dementia.

In particular, first, p53 protein is captured by immunoprecipitation in a biofluid sample from patients at pre-clinical, prodromal clinical stages of Alzheimer's, Mild Cognitive Impairment (MCI) stable patients, and cognitive unimpaired subjects (CU),
10 Frontotemporal Dementia (FD), Vascular Dementia (VD) and Lewy Body Dementia (LB). Then, the post translational modifications of the captured protein are identified by protein sequencing with a highly sensitive selective mass spectrometry method. After sequencing, the post translational modifications are also identified by a database searching to check ones already described in literature.

15 The data obtained for each sample are then compared with PTMs detected in the biofluid samples from subjects with same clinical evidence showing a correlation between "PTMs and diagnosis", therefore demonstrating a strong evidence that the U-p53 PTMs can be considered as highly reliable biomarkers in the prognosis and diagnosis of a neurodegenerative disease.

20 Said method is advantageously fast, requires a small volume of biofluid sample and reliably identifies U-p53 PTMs in each sample analysed.

Furthermore, the method and the biomarkers identified can be used also in the diagnosis and prognosis of Alzheimer's disease in asymptomatic individuals and people suffering from MCI, thus allowing the access to the diagnostics market.

25 Furthermore, the method and the biomarkers identified can be used also for differentiating Alzheimer's disease, from other forms of dementia, such as LB, VD, FTD in demented patients. In fact, as it will be seen below, the U-p53 protein sequence in biofluid samples of patients affected by Alzheimer's disease shows a variability in terms of length within the region of amino acids 1-271, said variability including a truncation within the same
30 region. It should be appreciated that said variability and truncation are peculiar of Alzheimer's disease, as the same are not detected in biofluid samples of patients affected by other forms of dementia, much less in cognitive unimpaired subjects. At the same time,

a residual amount of U-p53 in the biofluid samples keeps its sequence length, whereon peculiar PTMs of Alzheimer's disease are detected. It follows that patients affected by Alzheimer's disease are unequivocally identified and distinguished from other dementia patients, insofar as the former show both a truncation in the U-p53 protein sequence and peculiar PTMs in the residual amount of untruncated U-p53 protein.

In addition, since said biomarkers can be used in the prognosis of cognitive decline to Alzheimer's Dementia in asymptomatic and MCI subjects and in the diagnosis of neurodegenerative disease as the dementia, said method advantageously allows the use of a U-p53 PTMs to select the subjects in clinical trials to enable success of the trial and to differentiate patients affected by AD from other forms of dementia as LB, VD, FTD.

The present invention thus relates to an *in vitro* or *ex vivo* method for the diagnosis or prognosis of a neurodegenerative disease, the method comprising the steps of:

a) analysing a biofluid sample for the presence of post-translation modifications (PTMs) in the region of amino acids 1-371 of the p53 protein (U-p53), said PTMs being:

- PTM-1 at the amino acid M1,
- PTM-2 at the amino acid K164,
- PTM-3 at the amino acid K370,
- PTM-4 at the amino acid L101,
- PTM-5 at the amino acid K120,
- PTM-6 at the amino acid K132,
- PTM-7 at the amino acid K139,
- PTM-8 at the amino acid K291,
- PTM-9 at the amino acid K357,
- PTM-10 at the amino acid S6,
- PTM-11 at the amino acid S33,

wherein the presence of at least two PTMs selected from PTM-2, PTM-7, PTM-8, and PTM-11 is indicative of a cognitive unimpaired subject (CU),

b) assessing the presence of:

- at least two PTMs selected from PTM-1, PTM-3, PTM-4, PTM-5, PTM-6, PTM-9, and PTM-10, and
- at least one PTM selected from PTM-2, PTM-7, PTM-8, and PTM-11,

as indicative of the occurrence or the risk of development of a neurological disease, said neurodegenerative disease being selected from Mild Cognitive Impairment (MCI), Alzheimer's disease (AD), Fronto-temporal dementia (FTD), Lewi's Body (LB), and vascular dementia (VD),

- 5 c) correlating the PTMs assessed in step b) with those identifying the corresponding neurodegenerative disease.

According to the present invention, preferably in the *in vitro* or *ex vivo* method:

- the post-translation modification PTM-1 has a group CO-CH₃ branched to the amino acid M1 of the p53 protein;
- 10 - the post-translation modification PTM-2 has a group CO-CH₃ branched to the amino acid K164 of the p53 protein;
- the post-translation modification PTM-3 has a group CO-CH₃ branched to the amino acid K370 of the p53 protein;
- the post-translation modification PTM-4 has a ubiquitination site [GG] branched
15 at the amino acid K101 of the p53 protein;
- the post-translation modification PTM-5 has a ubiquitination site [GG] branched at the amino acid K120 of the p53 protein, where [GG] denotes a lateral chain of two residues of "Glycine";
- the post-translation modification PTM-6 has a ubiquitination site [GG] branched
20 at the amino acid K132 of the p53 protein;
- the post-translation modification PTM-7 has a ubiquitination site [GG] branched at the amino acid K139 of the p53 protein;
- the post-translation modification PTM-8 has a ubiquitination site [GG] branched at the amino acid K291 of the p53 protein;
- 25 - the post-translation modification PTM-9 has a ubiquitination site [GG] branched at the amino acid K357 of the p53 protein;
- the post-translation modification PTM-10 has phosphorylation at the amino acid S6 of the p53 protein;
- the post-translation modification PTM-11 has phosphorylation at the amino acid S33 of
30 the p53 protein.

In a preferred embodiment, the *in vitro* or *ex vivo* method of the present invention is for differentiating Alzheimer's disease, from other forms of dementia, such as LB, VD, FTD

in demented patients. In fact, as said above, the assessment of following criteria are indicative of AD:

- a sequence variability in terms of length within the region of amino acids 1-271, said variability including a truncation within the same region, and
- 5 - the presence of at least two PTMs selected from PTM-1, PTM-3, PTM-4, PTM-5, and PTM-6, in a residual amount of untruncated sequence, preferably the presence of all PTM-1, PTM-3, PTM-4, PTM-5, and PTM-6.

Said truncation, mainly due to biological reactions, does not affect the detectability of PTMs in said residual amount of untruncated sequence.

- 10 As said above, it should be appreciated that said variability and truncation are peculiar of Alzheimer's disease, as the same are not detected in biofluid samples of patients affected by other forms of dementia. At the same time, a residual amount of U-p53 in the biofluid samples keeps its sequence length, whereon peculiar PTMs of Alzheimer's disease are detected. It follows that patients affected by Alzheimer's disease are unequivocally
- 15 identified and distinguished from other dementia patients, insofar as the former show both a truncation in the U-p53 protein sequence and peculiar PTMs in the residual amount of untruncated U-p53 protein.

- Preferably, in the *in vitro* or *ex vivo* method of the present invention, the presence of all PTM-2, PTM-7, PTM-8, and PTM-11 is indicative of a cognitive unimpaired subject
- 20 (CU).

Preferably, in the *in vitro* or *ex vivo* method of the present invention the presence of PTM-1, and PTM-10 is indicative of MCI.

- Preferably, in the *in vitro* or *ex vivo* method of the present invention the presence of at least two PTMs selected from PTM-4, PTM-5, and PTM-9 is indicative of an
- 25 asymptomatic subject having the prognosis of cognitive decline of Alzheimer's dementia (AD), more preferably the presence of all PTM-4, PTM-5, and PTM-9. In this regard, it should be appreciated that the method of the invention allows the cognitive unimpaired subject (CU) to be identified and distinguished from the asymptomatic subject having the prognosis of cognitive decline of Alzheimer's dementia, although both subjects are
- 30 formally asymptomatic and accordingly not distinguishable from each other through conventional cognitive tests.

Preferably, in the *in vitro* or *ex vivo* method of the present invention the presence of at

least two PTMs selected from PTM-1, PTM-3, PTM-5, PTM-6, and PTM-10 is indicative of MCI with a prognosis of cognitive decline of AD, more preferably the presence of all PTM-1, PTM-3, PTM-5, PTM-6, and PTM-10.

Preferably, in the *in vitro* or *ex vivo* method of the present invention the presence of PTM-5, and PTM-9 is indicative of FTD.

Preferably, *in vitro* or *ex vivo* method of the present invention the presence of PTM-5, and PTM-6 is indicative of LB.

Preferably, in the *in vitro* or *ex vivo* method of the present invention the presence of PTM-4, and PTM-5 is indicative of VD.

Preferably, said biofluid is blood, plasma, serum, saliva, urine, neuronal cells, blood cells or other types of cells.

According to a preferred embodiment, in the step a) of the *in vitro* or *ex vivo* method of the present invention, the p53 protein is captured in a biofluid sample by performing the following sub-steps of:

- (i) providing a biofluid sample;
- (ii) performing protein immunoprecipitation by an antibody that binds a p53 protein;
- (iii) performing protein fragmentation by trypsin;

and the step b) is performed by HPLC-mass spectrometry, Peptide Mass Fingerprint and Database Search.

In a preferred embodiment, the p53 protein in step a) is the U-p53 in a misfolded conformation.

Preferably, the antibody of sub-step (ii) is a conformationally specific antibody that binds to a p53 peptide, more preferably is a monoclonal/polyclonal antibody. In preferred embodiments, said monoclonal antibody is the antibody 2D3A8.

The amino acid sequences of the 2D3A8 antibody include the heavy chain (SEQ ID NO: 7) and light chain (SEQ ID NO: 8), heavy chain variable region (SEQ ID NO: 9) and light chain variable region (SEQ ID NO: 10), heavy chain CDRs 1, 2 and 3 (SEQ ID NOs: 11, 12 and 13, respectively) and light chain CDRs 1, 2 and 3 (SEQ ID NOs: 14, 15 and 16, respectively).

Preferably, the biological sample of step a) is subjected to protein plasma depletion by HPLC or chromatographic columns or chemical treatment, before performing step (ii).

In a preferred embodiment, in the step c) of the method of the present invention, the detected PTMs are correlated with the diagnosis/prognosis of Alzheimer's disease in a patient at different stages of the diseases or cognitive decline due to dementia.

Preferably, in the step c) the detected PTMs are correlated with the prognosis of cognitive
5 decline of Alzheimer's disease in asymptomatic individuals and subjects suffering from MCI.

In a further aspect, the present invention also relates to a diagnostic kit to be used for the implementation of the *in vitro* or *ex vivo* method above described, the kit comprising the reagent set to perform the immunoprecipitation including an antibody, the digestion of
10 the protein (preferably trypsin with/without Lys C), elution buffer to precipitate the protein captured by the antibody, and an injection buffer.

In further aspects, the present invention also relates to a method for detecting neurodegenerative disease or development of neurodegenerative disease in a subject by identifying the type of post-translational modifications (PTMs) in the region of amino
15 acids 1-371 of the p53 protein (U-p53) present in a sample from said subject, the method comprising the steps of:

- a. subjecting said sample to immunoprecipitation with an antibody that binds to an amino acid sequence defined by amino acids 282-297 of U-p53;
- b. subjecting said immunoprecipitated sample of step (a) to protease digestion;
- 20 c. detecting the presence of post-translation modifications (PTMs) in the region of amino acids 1-371 of the p53 protein (U-p53) in said digested sample of step (b) and classifying the PTM as PTM-1, PTM-2, PTM-3, PTM-4, PTM-5, PTM-6, PTM-7, PTM-8, PTM-9, PTM-10 and PTM-11,

wherein said PTM-1 is at the amino acid M1 of said U-p53, said PTM-2 is at the
25 amino acid K164 of said U-p53, said PTM-3 is at the amino acid K370 of said U-p53, said PTM-4 is at the amino acid L101 of said U-p53, said PTM-5 is at the amino acid K120 of said U-p53, said PTM-6 is at the amino acid K132 of said U-p53, said PTM-7 is at the amino acid K139 of said U-p53, said PTM-8 is at the amino acid K291 of said U-p53, said PTM-9 is at the amino acid K357 of said U-p53, said PTM-10 is at the amino
30 acid S6 of said U-p53, and said PTM-11 is at the amino acid S33 of said U-p53,

wherein the presence of at least two PTMs selected from PTM-1, PTM-3, PTM-4, PTM-5, PTM-6, PTM-9, and PTM-10, and the presence of at least one PTM selected

from PTM-2, PTM-7, PTM-8, and PTM-11 is indicative of neurogenerative disease or development of neurodegenerative disease,

wherein said neurodegenerative disease is Alzheimer's disease, cognitive decline to Alzheimer's disease (AD), Mild cognitive impairment (MCI), Mild cognitive impairment (MCI) with a prognosis of cognitive decline to AD, Frontotemporal dementia (FTD), and/or Lewy Body's Dementia (LB), and vascular dementia (VD).

According to the present invention, preferably in said method said PTM-1 has a group CO-CH₃ branched to the amino acid M1 of the p53 protein; said PTM-2 has a group CO-CH₃ branched to the amino acid K164 of the p53 protein; said PTM-3 has a group CO-CH₃ branched to the amino acid K370 of the p53 protein; said PTM-4 has a ubiquitination site [GG] branched at the amino acid K101 of the p53 protein; said PTM-5 has a ubiquitination site [GG] branched at the amino acid K120 of the p53 protein; said PTM-6 has a ubiquitination site [GG] branched at the amino acid K132 of the p53 protein; said PTM-7 has a ubiquitination site [GG] branched at the amino acid K139 of the p53 protein; said PTM-8 has a ubiquitination site [GG] branched at the amino acid K291 of the p53 protein; said PTM-9 has a ubiquitination site [GG] branched at the amino acid K357 of the p53 protein; said PTM-10 has phosphorylation at the amino acid S6 of the p53 protein; and said PTM-11 has phosphorylation at the amino acid S33 of the p53 protein.

Preferably in said method, said at least two PTMs detected in step (c) are selected from the group consisting of PTM-1, PTM-3, PTM-4, PTM-5, and PTM-6, said detection being indicative of Alzheimer's disease (AD) or prognosis of AD.

Preferably in said method, said at least two PTMs detected in step (c) are selected from the group consisting of PTM-1, and PTM-10, said detection being indicative of MCI.

Preferably in said method, said sample is from a subject who exhibits no symptoms of AD, wherein said at least two PTMs detected in step (c) are selected from the group consisting of PTM-4, PTM-5, and PTM-9, said detection being indicative of a prognosis of cognitive decline to AD.

Preferably in said method, said at least two PTMs detected in step (c) are selected from the group consisting of PTM-1, PTM-3, PTM-5, PTM-6, and PTM-10, said detection being indicative of MCI with a prognosis of cognitive decline to AD.

Preferably in said method, said at least two PTMs detected in step (c) are selected from

the group consisting of PTM-5, and PTM-9, said detection being indicative of FTD.

Preferably in said method, said at least two PTMs detected in step (c) are selected from the group consisting of PTM-5, and PTM-6, said detection being indicative of LB.

Preferably in said method, said at least two PTMs detected in step (c) are selected from
5 the group consisting of PTM-4, and PTM-5, said detection being indicative of VD.

Preferably in said method, said sample is selected from the group consisting of blood, plasma, serum, saliva, urine, neuronal cells.

Preferably in said method, said protease is trypsin.

Preferably in said method, said detection of step (c) is performed by one or more of
10 HPLC-mass spectrometry, Peptide Mass Fingerprint and Database search.

Preferably in said method, said antibody is a monoclonal antibody, more preferably it is 2D3A8.

Preferably in said method, said sample is subjected to protein plasma depletion by HPLC or chromatographic columns or chemical treatment, prior to performing steps (a) to (c).

15 In further aspects, the present invention also relates to a kit for detecting neurodegenerative disease or development of neurodegenerative disease in a subject, the kit comprising a reagent set to perform immunoprecipitation, said reagent set comprising an anti-human p53 antibody capable of binding to an amino acid sequence defined by amino acids 282-297 of U-p53, preferably wherein said anti-human p53 antibody being
20 a monoclonal antibody, more preferably said monoclonal antibody being 2D3A8.

It should be also understood that all the combinations of preferred aspects of the peptides of the invention, as well as of the preparation processes, kit and methods using of the same, as above reported, are to be deemed as hereby disclosed.

All combinations of the preferred aspects of the PTMs of the invention, preparation
25 processes, kit and methods disclosed above are to be understood as herein described.

Below are working examples of the present invention provided for illustrative purposes.

MATERIALS AND METHODS

Isolation and identification of the U-p53 protein sequences and of its post-translational modifications

30 The analysis relates to the identification of the U-p53 protein sequence and of its post translational modifications when extracted from plasma of cognitive unimpaired subjects (CU), of patients affected by AD, of other forms of dementia (FTD, LB and VD) and

from individuals with Mild Cognitive Decline (MCI), from MCI patients with a prognosis of cognitive decline of AD (MCI to AD) and from patients with a prognosis of cognitive decline of an asymptomatic AD (CU to AD).

Sample preparation

5 1. Buffers

- Buffer A: Tris 25 mM, Sodium Chloride (NaCl) 0.15 mM, Tween-20 50 mM; Preparation: Tris (303 mg), Sodium Chloride (NaCl; 885 mg) and Tween-20 (5.5 g) are collected. Bidistilled water is added so to reach 100 mL final volume. Note: The solution must be fresh prepared for each analytical section.

10 - Buffer B: Glycine 0.1 M pH 2.0. Preparation: Glycine (750 mg) Glycine is treated with bidistilled water. 100 mL solution was obtained. HCl 0.1 M is added to obtain pH 3 value. Note: The solution must be fresh prepared for each analytical section.

- Ammonium bicarbonate (NH₄HCO₃) 0.4 g are solubilized in 100 mL of Bidistilled Water. Note: solution pH should be checked before to proceed with the analysis. pH must
15 be lower than 8 to obtain a reproducible digestion.

2. Reagent preparation

- Dithiothreitol (DTT) 180 mM in 50 mM AmBic. Procedure: DTT 0.3 g are solubilized in 0.5 mL of bidistilled water. 10 mL of 50 mM ammonium bicarbonate (NH₄HCO₃) are added. Solubilize the mixture by using vortex. Note: The solution must be fresh prepared
20 for each analytical section.

- Iodoacetamide (IAA) 400 mM in 50 mM AmBic. Procedure: Iodoacetamide (IAA) 0.7 g are solubilized in 10 mL of 50 mM ammonium bicarbonate (NH₄HCO₃) solution. Solubilize the mixture by using vortex. Note: The solution must be fresh prepared for each analytical section.

25 - 25 ng/μL Trypsin solution. Procedure: 20 μg of trypsin are solubilized 800 μL of 50 mM NH₄HCO₃. Solubilize the mixture by using vortex. Note: The solution must be fresh prepared for each analytical section.

3. Bead-antibody binding

Protein magnetic bead L 50 μL (0.5 mg) are collected in a Vial;

30 150 μL Buffer A are added. Vortex is applied;

Magnetic surface is used to discard the supernatant.

Buffer A 1 mL is added. Vortex is applied for 1 minute;

Magnetic surface is used to discard the supernatant;

Antibody solution (200 µL, 0.05 µg/ µL corresponding to 10 µg) is added to ProteinL magnetic bead;

The solution is mixed for 2 hours;

- 5 Magnetic surface is used to discard the supernatant;

Buffer A 500 µL is added;

Magnetic surface is used to discard the supernatant;

Wash and discard the supernatant again;

Buffer A 1mL is added.

- 10 The solution is stored at room temperature.

4. Plasma chemical contaminants depletion and immune precipitation

Samples extracted from the different categories of patients are thawed at room temperature under laminar flow cabinet for 30 min.

The sample is spiked in 25 µL aliquots. They are separately processed.

- 15 The remaining material is stored at -20 °C for retesting purpose.

5 µL of CH₃CN are added to 25 µL of plasma.

The acetonitrile spike is repeated every 1 minute since to reach a mixture volume of 50 µL. Apply vortex for 5 minutes until when white deposit is observed.

The sample centrifugation takes place at 13000 g for 10 minutes. 40 µL of supernatant is

- 20 added to the bead-antibody complex. Vortex is weakly applied.

The mixture is incubated at room temperature for 1 hour and then at 4° overnight.

A magnetic surface is used to remove the supernatant.

Buffer A 500 µL are added and the mixture was vortexed.

A magnetic plane is used to remove the supernatant.

- 25 Buffer B 45 µL are added to the pellet. After mixing, to incubate for 10 minutes at room temperature.

A magnetic surface is used to collect the eluate (40 µL) that is enzymatically digested.

5. Enzymatic Digestion of the immunocaptured p53 protein

2,15 µl of Dithiothreitol (DTT) 180 mM are added to 40 µL of the eluate.

- 30 The mixture is incubated for 15 min at 50 °C and at room temperature for 30 minutes;

2,15µl of Iodoacetamide (IAA) 400 mM are added 42.15 µL of the mixture.

The obtained mixture is incubated for 15 minutes at room temperature.

2,15 μL of AmBic 50 mM are added 44.30 μL of the obtained mixture.

1 μL of trypsin (25 ng / μL) containing Lys-c (50 ng / μL) and AmBic 50mM is added to 46.45 μL of the obtained mixture.

Incubation takes place at 37 °C for 3.5 hours followed by 57°C for 30 minutes.

- 5 1 μL of Formic Acid (HCOOH) is added to 47.45 μL of the obtained mixture to stop the enzymatic digestion. pH value is checked and it has to be in the range 1-4. If it is higher than 4 progressive volume (1 μL) of Formic Acid is added to obtain a pH value between 1 and 4. 10 μL of the obtained sample are analysed.

6. Detection of PTMs by LC-SACI-MS

- 10 HPLC Ultimate 3000 (ThermoFisher, USA) with a Phenomenex Kinetex PFP 50x4.1 mm 2.6 μm are used to perform the chromatographic analysis. Binary gradient is used: Phase A (H_2O +0.2 % Formic Acid (HCOOH)) and Phase C acetonitrile (CH_3CN). The gradient is reported in the table below. 10 μL of sample are injected.

LTQ Orbitrap XL is used for the data acquisition. SACI ionization source is employed.

- 15 The potential surface is 47 V, Gas nebulizer pressure is 75 Psi and dry gas flow is 1.0 L/min. 350 °C of nebulizer temperature was employed together with 320 °C of dry gas one. SACI peptide adduct profile mode is employed for data acquisition (Cristoni et al. Rapid Commun Mass Spectrom. 2003;17(17):1973-81.).

Table 1: Chromatographic gradient.

Gradient		
Time (minute)	% C	Flow (mL/min)
0	2%	0.250
2.5	2%	0.250
3	80%	0.250
7	80%	0.250
8	2%	0.250

- 20 7. Data extraction and protein characterization

Protein sequence and PTM data is obtained using the SANIST-prot tool operating in bottom up conditions.

Correlation between p53 sequence peptide and AD diagnosis.

The plasma samples of 7 patients affected by AD, 5 cognitive unimpaired (CU), 2 patients affected by MCI, 6 frontal dementia (FD), 1 patient with vascular dementia (VD) and 1 patient with Lewy Body dementia (LB) and 6 patients with MCI to AD and 6 patients CU

to AD have been treated with the experimental protocol based on protein L to isolate protein p53 disclosed above. Said protein has been exposed to double enzymatic digestion (Lys-C + trypsin) in order to maximize the peptide recovery.

Sample ID*	N	Diagnosis
1-AD; 2-AD; 3-AD; 4-AD; 5-AD; 4-S; 7-S	7	AD
1-C; 2-C; 3-C; 4-C; 5-C	5	CU
9D-MCI; 10D-MCI	2	MCI
1-S; 2-S; 5-S; 8-S; 10-S; 13-S	6	CU to AD
3-S; 6-S; 9-S; 11-S; 12-S; 14-S	6	MCI to AD
1D-FD; 2D-FD; 3D-FD; 4D-FD; 5D-FD; 6D-FD	6	FTD
8D-FD V	1	VD
7D-FD	1	LB

*Sample ID is a mere code exclusively used to label the samples and, as such, have no correlation to the subsequent diagnosis of corresponding patients

Results obtained

1. U-p53 protein immunocaptured from subjects AD

The p53 protein extracted from AD individuals results truncated in the region of amino acid 1-248 with respect to the wt p53 protein (SEQ ID NO: 1) Database: UniProtKB, Protein ID: P04637, amino acids: 1 - 393). Different mistakes of enzymatic digestion have been reported that lead to the presence of variable regions, inter-subjects, between the residuals 249-371 of the truncated protein.

In Table 2 are reported the p53 linear sequences identified in AD patients and the respective molecular weight (MW).

Table 2.

Sample ID	Sequence	SEQ ID	MW (Da)
1-AD	EVRVCACPGRRRTEENLR KKGEPHHELPPGSTRALPN NTSSSPQPKKKPLDGEYFTL QIRGRERFEMFRELNEALEL KDAQAGKEPGGSRAHSSHLKS	SEQ ID NO: 2	11425
2-AD	RPILTIITLEDSSGNLLGRN SFEVRVCACPGRRRTEEN LRKKGEPHHELPPGSTRAL PNNTSSSPQPKKKPLDGEYF TLQIRGRERFEMFRELNEAL ELKDAQAGKEPGGSRAHSSH	SEQ ID NO: 3	13823

	LKS		
3-AD	SGNLLGRNSFEVRVCACPGR DRRTEENLRKKGEPHHELP PGSTKRALPNNTSSSPQPKK KPLDGEYFTLQIRGRERFEM FRELNEALELKDAQAGKEPG GSRAHSSHLKS	SEQ ID NO: 4	12471
4-AD	TLEDSSGNLLGRNSFEVRVC ACPGRDRRTEENLRKKGEP HHELPPGSTKRALPNNTSSS PQPKKKPLDGEYFTLQIRGR ERFEMFRELNEALELKDAQA GKEPGGSRAHSSHLKS	SEQ ID NO: 5	13016
5-AD	EVRVCACPGRDRRTEENLR KKGEPHHELPPGSTKRALPN NTSSSPQPKKKPLDGEYFTL QIRGRERFEMFRELNEALEL KDAQAGKEPGGSRAHSSHLKS	SEQ ID NO: 2	11425
4-S	EVRVCACPGRDRRTEENLR KKGEPHHELPPGSTKRALPN NTSSSPQPKKKPLDGEYFTL QIRGRERFEMFRELNEALEL KDAQAGKEPGGSRAHSSHLKS	SEQ ID NO: 2	11425
7-S	RPILTIITLEDSSGNLLGRN SFEVRVCACPGRDRRTEEN LRKKGEPHHELPPGSTKRAL PNNTSSSPQPKKKPLDGEYF TLQIRGRERFEMFRELNEAL ELKDAQAGKEPGGSRAHSSH LKS	SEQ ID NO: 3	13823
MW-average			12432

2. U-p53 immunocaptured from Cognitive unimpaired (CU) and Cognitive unimpaired to AD patients.

The linear sequence of p53 extracted from 5 Cognitive unimpaired patients and 6 Cognitive unimpaired later declined to AD correspond to the entire sequence with 1-371 amino acids (SEQ ID N. 6), with a molecular weight of 41134 Da. No residuals corresponding to the region 372-391 have been identified. Table 3 reports the linear sequences obtained from the Cognitive unimpaired and Cognitive unimpaired to AD patients.

10 Table 3.

Sample ID	Sequence	SEQ ID	MW (Da)
1-C; 2-C; 3-C; 4-C; 5-C; 1-S; 2-S; 5-S; 8-S; 10-S; 13-S	MEEPQSDPSVEPPLSQETFSDLWKLLPENNVL SPLPSQAMDDLMLSPDDIEQWFTEDPGPDEAP RMPEAAPPVAPAPAAPTPAAPAPAPSWPLSSS VPSQKTYQGSYGFRGLHSGTAKSVTCTYSP ALNKMFCQLAKTCPVQLWVDSTPPPGTRVRA MAIYKQSQHMTEVVRRCPHHERCSDSDGLAP PQHLIRVEGNLRVEYLDDRNTFRHSVVVPYEP PEVGSDCTTIHYNMCMSSCMGGMNRRPILTI ITLEDSSGNLLGRNSFEVRVCACPGRRRTEE ENLRKKGEPHHELPPGSTKRALPNNTSSSPQP KKKPLDGEYFTLQIRGRERFEMFRELNEALEL KDAQAGKEPGGSRAHSSHLKS	SEQ ID NO: 6	41139
MW-average			41139

3. U-p53 protein immunocaptured from subjects affected by fronto-temporal dementia, Lewy Body's Dementia, Vascular Dementia, Mild Cognitive Decline (MCI) and MCI to AD

The results obtained from 16 subjects (6 with frontotemporal dementia, 1 with vascular dementia, 1 with Lewy Body's dementia, 2 MCI subjects and 6 MCI who developed AD) report the presence of the whole protein of 1-371 residuals. Table 4 reports the linear protein sequences of the tested subjects.

Table 4.

Sample ID	Sequence	SEQ ID	MW (Da)
1D-FD; 2D-FD; 3D-FD; 4D-FD; 5D-FD; 6D-FD; 7D-FD; 8D-FD V; 9D-MCI; 10D-MCI; 3-S; 6-S; 9-S; 11-S 12-S 14-S	MEEPQSDPSVEPPLSQETFSDLWKLLPEN NVLSPLPSQAMDDLMLSPDDIEQWFTED PGPDEAPRMPEAAPPVAPAPAAPTPAAPA PAPSWPLSSSVPSQKTYQGSYGFRGLH SGTAKSVTCTYSPALNKMFCQLAKTCPV QLWVDSTPPPGTRVRAMAIYKQSQHMTE VVRRCPHHERCSDSDGLAPPQHLIRVEGN LRVEYLDDRNTFRHSVVVPYEPPEVGSD CTTIHYNMCMSSCMGGMNRRPILTIITL EDSSGNLLGRNSFEVRVCACPGRRRTEE ENLRKKGEPHHELPPGSTKRALPNNTSSS PQPKKKPLDGEYFTLQIRGRERFEMFREL NEALELKDAQAGKEPGGSRAHSSHLKS	SEQ ID NO: 6	41139
MW-average			41139

4. Description of the PTMs observed from the immunocaptured protein

The extracted and sequenced p53 protein from the different clinical groups in addition to a different linear sequence, corresponding accordingly to different molecular weight, also showed post-transductional modifications (PTMs), mainly characterized by ubiquitination, acetylation and phosphorylation on specific amino acid residues. The samples belonging to the same clinical group also showed a highly homogeneity in the PTMs, which in combination with the same protein sequence represent an element characterizing the clinical group to which they belong.

In Figures 1-8 the ubiquitination sites observed are reported.

4.1. AD subjects

Under-expressed peptide sequences belonging to the amino acid region 1-248 were detected in AD patients. Given their low abundance, they could derive from whole sequence of p53 proteins that are believed to be weakly interacting with the antibody. The protein sequence has several ubiquitination sites indicated with the notation "*" in Fig. 1.

4.2. Cognitive unimpaired (CU) subjects

The ubiquitination sites detected in cognitive unimpaired samples are reported in Fig. 2.

4.3. Subjects affected by fronto-temporal dementia (FTD)

The ubiquitination sites detected in FTD samples are reported in Fig. 3.

4.4. Subjects affected by Lewy Body's dementia (LB)

The ubiquitination sites detected in LB samples are reported in Fig. 4.

4.5. Subjects affected by vascular dementia (VD)

The ubiquitination sites detected in VD samples are reported in Fig. 5.

4.6. Subjects affected by MCI

The ubiquitination sites detected in MCI samples are reported in Fig. 6.

4.7. Samples of cognitive unimpaired subjects (CU) who developed AD

The protein ubiquitination sites detected in the samples of cognitively healthy subjects who developed AD over a period of 18-72 months are shown in Fig. 7.

4.8. Samples of MCI subjects who developed AD

The ubiquitination sites detected in MCI subjects who developed AD are reported in Fig.

8.

From the data obtained we can observe that there were cumulatively 11 PTMs spanning the full sequence of the protein. Peptides spanning the protein up 371 residues were

detected in all samples, however the peptides belonging to the region 1-248 residues AD patients seemed to be cut from the protein not as consequence of enzymatic digestion due to the analytical protocol but due to biological process of full p-53 protein. Amino acids in the region from 372 to the end of the p-53 protein was missing in all samples belonging to different clinical groups.

The PTMs observed in the different patients are disclosed in Table 5 (Y=detected; N= not detected)

Table 5.

	AC-M1	AC-K164	AC-K370	U- K101	U-K120	U-K132	U-K139	U-K291	U-K357	Phospho-S6	Phospho-S33	Truncation
CU	N	Y	N	N	N	N	Y	Y	N	N	Y	N
MCI	Y	N	N	N	N	N	Y	Y	N	Y	Y	N
CU to AD	N	N	N	Y	Y	N	N	Y	Y	N	N	N
MCI to AD	Y	N	Y	N	Y	Y	Y	Y	N	Y	Y	N
AD	Y	Y	Y	Y	Y	Y	N	Y	N	N	Y	Y
FTD	N	N	N	N	Y	N	N	Y	Y	N	N	N
LB	N	Y	N	Y	Y	Y	N	Y	N	N	N	N
VD	N	N	N	Y	Y	N	N	Y	N	N	N	N

REFERENCES

1. Stanga, S. et al., 2010. Unfolded p53 in the pathogenesis of Alzheimer's disease: Is HIPK2 the link? *Aging*, 2(9), pp.545–554.
2. Lanni, C. et al., 2007. Unfolded p53: A potential biomarker for Alzheimer's disease. In *Journal of Alzheimer's Disease*. pp. 93–99.
3. Uberty, D. et al., 2008. Conformationally altered p53: a putative peripheral marker for Alzheimer's disease. *Neuro-degenerative diseases*, 5(3-4), pp.209–11.
4. Lanni, C. et al., 2008. Conformationally altered p53: a novel Alzheimer's disease marker? *Molecular psychiatry*, 13(6), pp.641–7.
5. Lanni, C., Racchi, M., et al., 2010. Unfolded p53 in blood as a predictive signature signature of the transition from mild cognitive impairment to Alzheimer's disease. *Journal of Alzheimer's disease: JAD*, 20(1), pp.97–104.
6. Buizza, L. et al., 2012. Conformational altered p53 as an early marker of oxidative

stress in Alzheimer's disease. PloS one, 7(1), p.e29789

7. Arce-Varas N, et al. Comparison of extracellular and intracellular blood compartments highlights redox alterations in Alzheimer's and Mild Cognitive Impairment patients. Current Alzheimer Research 2017; 14(1): 112-122.

5 8. Uberti, D. et al., 2006. Identification of a mutant-like conformation of p53 in fibroblasts from sporadic Alzheimer's disease patients. Neurobiology of aging, 27(9), pp.1193–201.

9. Lanni, C., Nardinocchi, L., et al., 2010. Homeodomain interacting protein kinase 2: a target for Alzheimer's beta amyloid leading to misfolded p53 and inappropriate cell survival. PloS one, 5(4), p.e10171.

10 10. Lanni, C. et al., 2008. Pharmacogenetics and Pharmagenomics, Trends in Normal and Pathological Aging Studies: Focus on p53. Current Pharmaceutical Design, 14(26), pp.2665–2671.

11. Peptide Mass Fingerprint (PMF; Cristoni S. et al Expert Rev Proteomics. 2004 Dec;1(4):469-83)

SEQUENCE LISTING

SEQ ID NO: 1

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
 5 1 5 10 15
 Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
 20 25 30
 Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp
 35 40 45
 10 Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
 50 55 60
 Arg Met Pro Glu Ala Ala Pro Pro Val Ala Pro Ala Pro Ala Ala Pro
 65 70 75 80
 Thr Pro Ala Ala Pro Ala Pro Ala Pro Ser Trp Pro Leu Ser Ser Ser
 15 85 90 95
 Val Pro Ser Gln Lys Thr Tyr Gln Gly Ser Tyr Gly Phe Arg Leu Gly
 100 105 110
 Phe Leu His Ser Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser Pro
 115 120 125
 20 Ala Leu Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro Val Gln
 130 135 140
 Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val Arg Ala Ala
 145 150 155 160
 Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg Cys Pro
 25 165 170 175
 His His Glu Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro Gln His
 180 185 190
 Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp Asp Arg
 195 200 205
 30 Asn Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro Glu Val
 210 215 220
 Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser Ser

225 230 235 240
 Cys Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr Leu
 245 250 255
 Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu Val Arg
 5 260 265 270
 Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn Leu
 275 280 285
 Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr Lys
 290 295 300
 10 Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys Lys
 305 310 315 320
 Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg Glu Arg
 325 330 335
 Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys Asp Ala
 15 340 345 350
 Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser His Leu
 355 360 365
 Lys Ser Lys Lys Gly Gln Ser Thr Ser Arg His Lys Lys Leu Met Phe
 370 375 380
 20 Lys Thr Glu Gly Pro Asp Ser Asp
 385 390

SEQ ID NO: 2

Glu Val Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu
 25 1 5 10 15
 Glu Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly
 20 25 30
 Ser Thr Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro
 35 40 45
 30 Lys Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly
 50 55 60
 Arg Glu Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu

65 70 75 80
 Lys Asp Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser
 85 90 95
 Ser His Leu Lys Ser
 5 100

SEQ ID NO: 3

Arg Pro Ile Leu Thr Ile Ile Thr Leu Glu Asp Ser Ser Gly Asn Leu
 1 5 10 15
 10 Leu Gly Arg Asn Ser Phe Glu Val Arg Val Cys Ala Cys Pro Gly Arg
 20 25 30
 Asp Arg Arg Thr Glu Glu Glu Asn Leu Arg Lys Lys Gly Glu Pro His
 35 40 45
 His Glu Leu Pro Pro Gly Ser Thr Lys Arg Ala Leu Pro Asn Asn Thr
 15 50 55 60
 Ser Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp Gly Glu Tyr Phe
 65 70 75 80
 Thr Leu Gln Ile Arg Gly Arg Glu Arg Phe Glu Met Phe Arg Glu Leu
 85 90 95
 20 Asn Glu Ala Leu Glu Leu Lys Asp Ala Gln Ala Gly Lys Glu Pro Gly
 100 105 110
 Gly Ser Arg Ala His Ser Ser His Leu Lys Ser
 115 120

25 SEQ ID NO: 4

Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu Val Arg Val Cys Ala
 1 5 10 15
 Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn Leu Arg Lys Lys
 20 25 30
 30 Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr Lys Arg Ala Leu
 35 40 45
 Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys Lys Pro Leu Asp

50 55 60
 Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg Glu Arg Phe Glu Met
 65 70 75 80
 Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys Asp Ala Gln Ala Gly
 5 85 90 95
 Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser His Leu Lys Ser
 100 105 110

SEQ ID NO: 5

10 Thr Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu
 1 5 10 15
 Val Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu
 20 25 30
 Asn Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser
 15 35 40 45
 Thr Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys
 50 55 60
 Lys Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg
 65 70 75 80
 20 Glu Arg Phe Glu Met Phe Arg Glu Leu Asn Glu Ala Leu Glu Leu Lys
 85 90 95
 Asp Ala Gln Ala Gly Lys Glu Pro Gly Gly Ser Arg Ala His Ser Ser
 100 105 110
 His Leu Lys Ser
 25 115

SEQ ID NO: 6

Met Glu Glu Pro Gln Ser Asp Pro Ser Val Glu Pro Pro Leu Ser Gln
 1 5 10 15
 30 Glu Thr Phe Ser Asp Leu Trp Lys Leu Leu Pro Glu Asn Asn Val Leu
 20 25 30
 Ser Pro Leu Pro Ser Gln Ala Met Asp Asp Leu Met Leu Ser Pro Asp

35 40 45
 Asp Ile Glu Gln Trp Phe Thr Glu Asp Pro Gly Pro Asp Glu Ala Pro
 50 55 60
 Arg Met Pro Glu Ala Ala Pro Pro Val Ala Pro Ala Pro Ala Ala Pro
 5 65 70 75 80
 Thr Pro Ala Ala Pro Ala Pro Ala Pro Ser Trp Pro Leu Ser Ser Ser
 85 90 95
 Val Pro Ser Gln Lys Thr Tyr Gln Gly Ser Tyr Gly Phe Arg Leu Gly
 100 105 110
 10 Phe Leu His Ser Gly Thr Ala Lys Ser Val Thr Cys Thr Tyr Ser Pro
 115 120 125
 Ala Leu Asn Lys Met Phe Cys Gln Leu Ala Lys Thr Cys Pro Val Gln
 130 135 140
 Leu Trp Val Asp Ser Thr Pro Pro Pro Gly Thr Arg Val Arg Ala Met
 15 145 150 155 160
 Ala Ile Tyr Lys Gln Ser Gln His Met Thr Glu Val Val Arg Arg Cys
 165 170 175
 Pro His His Glu Arg Cys Ser Asp Ser Asp Gly Leu Ala Pro Pro Gln
 180 185 190
 20 His Leu Ile Arg Val Glu Gly Asn Leu Arg Val Glu Tyr Leu Asp Asp
 195 200 205
 Arg Asn Thr Phe Arg His Ser Val Val Val Pro Tyr Glu Pro Pro Glu
 210 215 220
 Val Gly Ser Asp Cys Thr Thr Ile His Tyr Asn Tyr Met Cys Asn Ser
 25 225 230 235 240
 Ser Cys Met Gly Gly Met Asn Arg Arg Pro Ile Leu Thr Ile Ile Thr
 245 250 255
 Leu Glu Asp Ser Ser Gly Asn Leu Leu Gly Arg Asn Ser Phe Glu Val
 260 265 270
 30 Arg Val Cys Ala Cys Pro Gly Arg Asp Arg Arg Thr Glu Glu Glu Asn
 275 280 285
 Leu Arg Lys Lys Gly Glu Pro His His Glu Leu Pro Pro Gly Ser Thr

290 295 300
 Lys Arg Ala Leu Pro Asn Asn Thr Ser Ser Ser Pro Gln Pro Lys Lys
 305 310 315 320
 Lys Pro Leu Asp Gly Glu Tyr Phe Thr Leu Gln Ile Arg Gly Arg Glu
 5 325 330 335
 Arg

SEQ ID NO: 7

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 10 1 5 10 15
 Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 20 25 30
 Val Met His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 15 Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe
 50 55 60
 Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
 20 85 90 95
 Ala Arg Gly Gly Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser
 100 105 110
 Val Thr Val Ser Ser Glu Ser Gln Ser Phe Pro Asn Val Phe Pro Leu
 115 120 125
 25 Val Ser Cys Glu Ser Pro Leu Ser Asp Lys Asn Leu Val Ala Met Gly
 130 135 140
 Cys Leu Ala Arg Asp Phe Leu Pro Ser Thr Ile Ser Phe Thr Trp Asn
 145 150 155 160
 Tyr Gln Asn Asn Thr Glu Val Ile Gln Gly Ile Arg Thr Phe Pro Thr
 30 165 170 175
 Leu Arg Thr Gly Gly Lys Tyr Leu Ala Thr Ser Gln Val Leu Leu Ser
 180 185 190

Pro Lys Ser Ile Leu Glu Gly Ser Asp Glu Tyr Leu Val Cys Lys Ile
 195 200 205
 His Tyr Gly Gly Lys Asn Arg Asp Leu His Val Pro Ile Pro Ala Val
 210 215 220
 5 Ala Glu Met Asn Pro Asn Val Asn Val Phe Val Pro Pro Arg Asp Gly
 225 230 235 240
 Phe Ser Gly Pro Ala Pro Arg Lys Ser Lys Leu Ile Cys Glu Ala Thr
 245 250 255
 Asn Phe Thr Pro Lys Pro Ile Thr Val Ser Trp Leu Lys Asp Gly Lys
 10 260 265 270
 Leu Val Glu Ser Gly Phe Thr Thr Asp Pro Val Thr Ile Glu Asn Lys
 275 280 285
 Gly Ser Thr Pro Gln Thr Tyr Lys Val Ile Ser Thr Leu Thr Ile Ser
 290 295 300
 15 Glu Ile Asp Trp Leu Asn Leu Asn Val Tyr Thr Cys Arg Val Asp His
 305 310 315 320
 Arg Gly Leu Thr Phe Leu Lys Asn Val Ser Ser Thr Cys Ala Ala Ser
 325 330 335
 Pro Ser Thr Asp Ile Leu Thr Phe Thr Ile Pro Pro Ser Phe Ala Asp
 20 340 345 350
 Ile Phe Leu Ser Lys Ser Ala Asn Leu Thr Cys Leu Val Ser Asn Leu
 355 360 365
 Ala Thr Tyr Glu Thr Leu Asn Ile Ser Trp Ala Ser Gln Ser Gly Glu
 370 375 380
 25 Pro Leu Glu Thr Lys Ile Lys Ile Met Glu Ser His Pro Asn Gly Thr
 385 390 395 400
 Phe Ser Ala Lys Gly Val Ala Ser Val Cys Val Glu Asp Trp Asn Asn
 405 410 415
 Arg Lys Glu Phe Val Cys Thr Val Thr His Arg Asp Leu Pro Ser Pro
 30 420 425 430
 Gln Lys Lys Phe Ile Ser Lys Pro Asn Glu Val His Lys His Pro Pro
 435 440 445

Ala Val Tyr Leu Leu Pro Pro Ala Arg Glu Gln Leu Asn Leu Arg Glu
 450 455 460
 Ser Ala Thr Val Thr Cys Leu Val Lys Gly Phe Ser Pro Ala Asp Ile
 465 470 475 480
 5 Ser Val Gln Trp Leu Gln Arg Gly Gln Leu Leu Pro Gln Glu Lys Tyr
 485 490 495
 Val Thr Ser Ala Pro Met Pro Glu Pro Gly Ala Pro Gly Phe Tyr Phe
 500 505 510
 Thr His Ser Ile Leu Thr Val Thr Glu Glu Glu Trp Asn Ser Gly Glu
 10 515 520 525
 Thr Tyr Thr Cys Val Val Gly His Glu Ala Leu Pro His Leu Val Thr
 530 535 540
 Glu Arg Thr Val Asp Lys Ser Thr Gly Lys Pro Thr Leu Tyr Asn Val
 545 550 555 560
 15 Ser Leu Ile Met Ser Asp Thr Gly Gly Thr Cys Tyr
 565 570

SEQ ID NO: 8

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
 20 1 5 10 15
 Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Ser Asn Tyr
 20 25 30
 Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile
 35 40 45
 25 Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
 50 55 60
 Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln
 65 70 75 80
 Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Tyr
 30 85 90 95
 Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys Arg Ala Asp Ala Ala
 100 105 110

Pro Thr Val Ser Ile Phe Pro Pro Ser Ser Glu Gln Leu Thr Ser Gly
 115 120 125
 Gly Ala Ser Val Val Cys Phe Leu Asn Asn Phe Tyr Pro Lys Asp Ile
 130 135 140
 5 Asn Val Lys Trp Lys Ile Asp Gly Ser Glu Arg Gln Asn Gly Val Leu
 145 150 155 160
 Asn Ser Trp Thr Asp Gln Asp Ser Lys Asp Ser Thr Tyr Ser Met Ser
 165 170 175
 Ser Thr Leu Thr Leu Thr Lys Asp Glu Tyr Glu Arg His Asn Ser Tyr
 10 180 185 190
 Thr Cys Glu Ala Thr His Lys Thr Ser Thr Ser Pro Ile Val Lys Ser
 195 200 205
 Phe Asn Arg Asn Glu Cys
 210

15

SEQ ID NO: 9

Glu Val Gln Leu Gln Gln Ser Gly Pro Glu Leu Val Lys Pro Gly Ala
 1 5 10 15
 Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr
 20 20 25 30
 Val Met His Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile
 35 40 45
 Gly Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe
 50 55 60
 25 Lys Gly Lys Ala Thr Leu Thr Ser Asp Lys Ser Ser Ser Thr Ala Tyr
 65 70 75 80
 Met Glu Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
 85 90 95
 Ala Arg Gly Gly Tyr Tyr Ala Met Asp Tyr Trp Gly Gln Gly Thr Ser
 30 100 105 110
 Val Thr Val Ser Ser
 115

SEQ ID NO: 10

Asp Ile Gln Met Thr Gln Thr Thr Ser Ser Leu Ser Ala Ser Leu Gly
5 1 5 10 15
Asp Arg Val Thr Ile Ser Cys Arg Ala Ser Gln Asp Ile Ser Asn Tyr
20 25 30
Leu Asn Trp Tyr Gln Gln Lys Pro Asp Gly Thr Val Lys Leu Leu Ile
35 40 45
10 Tyr Tyr Thr Ser Arg Leu His Ser Gly Val Pro Ser Arg Phe Ser Gly
50 55 60
Ser Gly Ser Gly Thr Asp Tyr Ser Leu Thr Ile Ser Asn Leu Glu Gln
65 70 75 80
Glu Asp Ile Ala Thr Tyr Phe Cys Gln Gln Gly Asn Thr Leu Pro Tyr
15 85 90 95
Thr Phe Gly Gly Gly Thr Lys Leu Glu Ile Lys
100 105

SEQ ID NO: 11

20 Ser Tyr Val Met His
1 5

SEQ ID NO: 12

Tyr Ile Asn Pro Tyr Asn Asp Gly Thr Lys Tyr Asn Glu Lys Phe Lys
25 1 5 10 15
Gly

SEQ ID NO: 13

Gly Gly Tyr Tyr Ala Met Asp Tyr
30 1 5

SEQ ID NO: 14

Arg Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn

1 5 10

SEQ ID NO: 15

5 Tyr Thr Ser Arg Leu His Ser

1 5

SEQ ID NO: 16

10 Gln Gln Gly Asn Thr Leu Pro Tyr Thr

1 5

CLAIMS

1. An *in vitro* or *ex vivo* method for the diagnosis or prognosis of a neurodegenerative disease, the method comprising the step of:

- 5 a) analysing a biofluid sample for the presence of post-translation modifications (PTMs) in the region of amino acids 1-371 of the p53 protein (U-p53), said PTMs being:
- PTM-1 at the amino acid M1,
 PTM-2 at the amino acid K164,
 10 PTM-3 at the amino acid K370,
 PTM-4 at the amino acid L101,
 PTM-5 at the amino acid K120,
 PTM-6 at the amino acid K132,
 PTM-7 at the amino acid K139,
 15 PTM-8 at the amino acid K291,
 PTM-9 at the amino acid K357,
 PTM-10 at the amino acid S6,
 PTM-11 at the amino acid S33,

wherein the presence of at least two PTMs selected from PTM-2, PTM-7, PTM-8, and
 20 PTM-11 is indicative of a cognitive unimpaired subject (CU),

b) assessing the presence of:

- at least two PTMs selected from PTM-1, PTM-3, PTM-4, PTM-5, PTM-6, PTM-9, and PTM-10, and
- at least one PTM selected from PTM-2, PTM-7, PTM-8, and PTM-11,

25 as indicative of the occurrence or the risk of development of a neurological disease, said neurodegenerative disease being selected from Mild Cognitive Impairment (MCI), Alzheimer's disease (AD), Fronto-temporal dementia (FTD), Lewi's Body (LB), and vascular dementia (VD),

c) correlating the PTMs assessed in step b) with those identifying the
 30 corresponding neurodegenerative disease,
 wherein

- the presence of PTM-1, and PTM-10 is indicative of MCI;

- the presence of at least two PTMs selected from PTM-4, PTM-5, and PTM-9 is indicative of a prognosis of cognitive decline to AD of an asymptomatic subject;
 - the presence of at least two PTMs selected from PTM-1, PTM-3, PTM-5, PTM-6, and PTM-10 is indicative of MCI with a prognosis of cognitive decline to AD;
 - 5 - the presence of PTM-5, and PTM-9 is indicative of FTD;
 - the presence of PTM-5, and PTM-6 is indicative of LB;
 - the presence of PTM-4, and PTM-5 is indicative of VD,
- or wherein said *in vitro* or *ex vivo* method is for differentiating Alzheimer's disease, from other neurodegenerative diseases, wherein in step b) the assessment of following criteria
- 10 are indicative of AD:
- a sequence variability in terms of length within the region of amino acids 1-271, said variability including a truncation within the same region, and
 - the presence of at least two PTMs selected from PTM-1, PTM-3, PTM-4, PTM-5, and PTM-6, in a residual amount of untruncated sequence.
- 15
2. The *in vitro* or *ex vivo* method of claim 1, wherein:
- the post-translation modification PTM-1 has a group CO-CH₃ branched to the amino acid M1 of the p53 protein;
 - the post-translation modification PTM-2 has a group CO-CH₃ branched to the
 - 20 amino acid K164 of the p53 protein;
 - the post-translation modification PTM-3 has a group CO-CH₃ branched to the amino acid K370 of the p53 protein;
 - the post-translation modification PTM-4 has a ubiquitination site [GG] branched at the amino acid K101 of the p53 protein;
 - 25 - the post-translation modification PTM-5 has a ubiquitination site [GG] branched at the amino acid K120 of the p53 protein;
 - the post-translation modification PTM-6 has a ubiquitination site [GG] branched at the amino acid K132 of the p53 protein;
 - the post-translation modification PTM-7 has a ubiquitination site [GG] branched
 - 30 at the amino acid K139 of the p53 protein;
 - the post-translation modification PTM-8 has a ubiquitination site [GG] branched at the amino acid K291 of the p53 protein;

- the post-translation modification PTM-9 has a ubiquitination site [GG] branched at the amino acid K357 of the p53 protein;

- the post-translation modification PTM-10 has phosphorylation at the amino acid S6 of the p53 protein;

5 - the post-translation modification PTM-11 has phosphorylation at the amino acid S33 of the p53 protein.

3. The *in vitro* or *ex vivo* method of claim 1 or 2, said *in vitro* or *ex vivo* method being for differentiating Alzheimer's disease, from other neurodegenerative diseases, wherein in
10 step b) the assessment of following criteria are indicative of AD:

- a sequence variability in terms of length within the region of amino acids 1-271, said variability including a truncation within the same region, and

- the presence of all PTM-1, PTM-3, PTM-4, PTM-5, and PTM-6.

15 4. The *in vitro* or *ex vivo* method of claim 1 or 2, wherein the presence of all PTM-4, PTM-5, and PTM-9 is indicative of a prognosis of cognitive decline to AD of an asymptomatic subject.

5. The *in vitro* or *ex vivo* method of claim 1 or 2, wherein the presence of all PTM-1,
20 PTM-3, PTM-5, PTM-6, and PTM-10 is indicative of MCI with a prognosis of cognitive decline to AD.

6. The *in vitro* or *ex vivo* method of any one of claims 1-5, wherein said biofluid is blood, plasma, serum, saliva, urine, neuronal cells, preferably blood, in particular, plasma.

25

7. The *in vitro* or *ex vivo* method of any one of claims 1-6, wherein in the step a), the p53 protein is captured in a biofluid sample by performing the following sub-steps of:

(i) providing a biofluid sample;

(ii) performing protein immunoprecipitation by an antibody that binds a p53
30 protein;

(iii) performing protein fragmentation by trypsin;

and the step b) is performed by HPLC-mass spectrometry, Peptide Mass Fingerprint and

Database Search.

8. The *in vitro* or *ex vivo* method of claim 7, wherein the immunoprecipitation of sub-step (ii) is performed with a monoclonal/polyclonal antibody that binds to a p53 peptide,
5 where preferably, said monoclonal antibody is the antibody 2D3A8.

9. The *in vitro* or *ex vivo* method of claim 7 or 8, wherein the biological sample of step a) is subjected to protein plasma depletion by HPLC or chromatographic columns or chemical treatment, before performing the step (ii).

10

10. Use of a diagnostic kit for the implementation of the *in vitro* or *ex vivo* method of any one of claims 1-9, the kit comprising a reagent set to perform the immunoprecipitation including an antibody, the digestion of the protein (preferably trypsin with/without Lys C), elution buffer to precipitate the protein captured by the antibody, and an injection
15 buffer.