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(54) Title: HSP27 AS BIOMARKER FOR ALZHEIMER'S DISEASE

(57) Abstract: The present invention relates to a method for assessing Alzheimer's disease in vitro comprising measuring the level of HSP27, or a variant thereof in a sample collected from an individual, wherein an altered level of said protein, or a variant thereof, is indicative that said individual suffers from Alzheimer's disease. Furthermore, the present invention relates to a method for monitoring the progression of the Alzheimer's disease in vitro comprising measuring the level of HSP27, or a variant thereof in a sample collected from an individual, wherein an altered level of HSP27 compared with an earlier measurement of the level of HSP27, or a variant thereof, is indicative for the progression of Alzheimer's disease of said individual.



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HSP27 AS BIOMARKER FOR ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a complex neurodegenerative dementing illness. It is histopathologically characterized by the deposition of extracellular amyloid plaques mainly consisting of amyloid- β protein ($A\beta$) and the intracellular accumulation of hyperphosphorylated tau into neurofibrillary tangles.

5 In clinical practice, the diagnosis of AD is still largely based on cognitive tests and behavioral analysis. One of the most promising sources of biomarkers in AD is the cerebrospinal fluid (CSF). The CSF is, compared to the brain, more easily accessible and is in direct contact with the extracellular space of the central nervous system where biochemical changes in the brain could potentially be reflected. Therefore, a number of
10 putative biological markers of AD have been suggested, like CSF total tau (Andreasen, N., Vanmechelen, E., Van de Voorde, A., Davidsson, P., Hesse, C., Tarvonen, S., Raiha, I., Sourander, L., Winblad, B., and Blennow, K. (1998) J Neurol Neurosurg Psychiatry 64, 298-305) and phospho-tau levels (Andreasen, N., Sjogren, M., and Blennow, K. (2003) World J Biol Psychiatry 4, 147-155), CSF A β 1-42 levels (Ida, N., Hartmann, T., Pantel, J., Schroder, J., Zerfass, R., Forstl, H., Sandbrink, R., Masters, C. L., and Beyreuther, K.
15 (1996) J Biol Chem 271, 22908-22914) or a combination thereof (Shoji, M., Matsubara, E., Kanai, M., Watanabe, M., Nakamura, T., Tomidokoro, Y., Shizuka, M., Wakabayashi, K., Igeta, Y., Ikeda, Y., Mizushima, K., Amari, M., Ishiguro, K., Kawarabayashi, T., Harigaya, Y., Okamoto, K., and Hirai, S. (1998) J Neurol Sci 158, 134-140). Yet, a
20 definitive diagnosis of AD can still only be made by a postmortem assessment of brain neuropathology and there is clearly a need for a reliable biomarker for AD.

Therefore, the present invention provides a method for assessing Alzheimer's disease in vitro comprising measuring the level of HSP27, or a variant thereof in a sample collected from an individual wherein an altered level of HSP27 or a variant thereof, is
25 indicative that said individual suffers from Alzheimer's disease.

Preferably, measured level of HSP27 or a variant thereof, is increased in an AD patient.

A sample may be a brain tissue sample (e.g. gray matter brain tissue) or a body fluid sample. A body fluid is e.g. blood (whole blood, serum, plasma) or cerebrospinal fluid
30 (CSF). Preferably, the sample is a body fluid sample. More preferably, protein of interest

is detected in CSF or blood. Methods for collecting tissue or fluid sample from an individual are well known to the skilled in the art.

The term "variants" in this context relates to proteins or peptides substantially similar to said proteins. The term "substantially similar" is well understood by the person skilled in the art. In particular, a variant may be an isoform or allele which shows amino acid exchanges compared to the amino acid sequence of the most prevalent peptide isoform in the human population. Preferably, such a substantially similar peptide has a sequence similarity to the most prevalent isoform of the protein or peptide of at least 80%, preferably at least 85%, more preferably at least 90%, most preferably at least 95%. Substantially similar are degradation products, e.g., proteolytic degradation products, which are still recognized by the diagnostic means or by ligands directed against the respective full-length protein or peptide. The term "variants" is also meant to relate to splice variants.

The term "variant" also relates to a post-translationally modified protein such as glycosylated protein. A "variant" is also a peptide which has been modified after collection of the sample, for example by covalent or non-covalent attachment of a label, particularly a radioactive or fluorescent label, to the protein.

The term "protein of interest" as used herein refers to HSP27 (SEQ. ID NO: 1) or a variant thereof. Heat shock protein 27 is also known as Heat shock protein beta 1 (HspB1) and Stress-responsive protein 27 (SRP27). HSP27 is an ATP-independent chaperone that confers protection against apoptosis through various mechanisms, including a direct interaction with cytochrome c.

An altered protein level is a level of protein which is either decreased or increased compared to a reference level. A reference level can be a level measured in one or more healthy individual or a level measured in a sample collected at an earlier point in time.

The invention also includes the measuring of HSP27 in combination with other biomarkers, simultaneously or non-simultaneously.

The present invention further provides a method for monitoring the progression of the Alzheimer's disease in vitro comprising measuring the level of HSP27 or a variant thereof in a sample collected from an individual, wherein an altered level of HSP27, or a variant thereof compared with an earlier measurement of the level of HSP27, or a variant thereof is indicative for the progression of Alzheimer's disease in said individual.

The sample can be a brain tissue sample (preferably, gray matter brain tissue) or a body fluid sample. Preferably, the sample is a body fluid sample. More preferably, the sample is cerebrospinal fluid (CSF) or blood sample.

The term "progression" of Alzheimer's disease refers to alteration of the stage of the disease. The stage of Alzheimer's disease is usually described in Braak stages. Braak has described six stages in Alzheimer's disease, the so-called Braak Stages I - VI, wherein Braak Stage I is the weakest manifestation of the disease and Braak stage VI is the most advanced stage (see H. Braak, E. Braak: Neuropathological staging of Alzheimer-related changes. *Acta Neuropathologica*, 1991, 82: 239-259; H. Braak, E. Braak, J. Bohl: Staging of Alzheimer-related cortical destruction. *European Neurology*, Basel, 1993, 33: 403-408). An alteration from e.g. Braak stage II to Braak stage IV means that the disease became worse. An alteration from e.g. Braak stage IV to Braak stage II means in improvement of the patient's condition.

If a protein is down-regulated in an AD patient, the measured protein level will further decrease (compared with an earlier measurement of the protein level) when the disease becomes more severe or worse. If a protein is up-regulated protein in a AD patient, the measured protein level will increase (compared with an earlier measurement of the protein level) when the disease becomes more severe. Preferably, the protein of interest is up-regulated in an individual suffering of Alzheimer's disease and its protein level increases when the disease becomes worse. The above described method may also be used to test the efficacy of a treatment for Alzheimer's disease.

The term "earlier measurement" of the level of a protein as used herein refers to a measured level of a protein, or variant thereof, in a Alzheimer patient to an earlier time point.

25

In a preferred embodiment, a relative increase of the level of the protein of interest indicates that the disease becomes worse and a relative decrease of the level of the protein of interest indicates that the disease becomes less severe. The term "relative increase" as used herein refers to the comparative difference between a first measurement of a protein, or a variant thereof and second measurement of the protein, or a variant thereof, wherein the measured level of the second measurement is higher than the level of the first measurement. The term "relative decrease" means the comparative difference between a first measurement of the level of a protein, or a variant thereof, and a second measurement of the protein, or a variant thereof, wherein the measured level of the second measurement is lower than the protein level of the first measurement.

35

Furthermore, the present invention provides the use of HSP27, or a variant thereof as biomarker for Alzheimer's disease and/or for the progression of Alzheimer's disease of an individual.

5 The term "biomarker" as used herein refers to molecules in an individual which are differentially present (i.e. present in increased or decreased levels) depending on presence or absence of a certain condition, disease, or complication. In particular, biochemical markers are gene expression products which are differentially present (e.g., through increased or decreased level of expression or turnover) in presence or absence of a certain
10 condition, disease, or complication. A biomarker of the invention is a biochemical marker. A biochemical marker is a protein, polypeptide or peptide. The level of a suitable biomarker can indicate the presence or absence of a particular condition, disease, or risk, and thus allow diagnosis or determination of the condition, disease or risk.

 The term "normal level" as used herein refers to the normal range of the level of
15 protein of interest in a body fluid sample of a control. A control is one or more individuals not suffering from Alzheimer's disease. Preferably, the number of individuals is higher than 100, more preferably more than 500, most preferably more than 1000. The normal range is determined by methods well known to the skilled person in the art. A preferred method is for example to determine the range of the values between quantile
20 2.5 and quantile 97.5, which leaves 5% of "normal" values outside the normal range or in other words, it covers 95% of all values of the control.

 The pathological status is defined as deviation from the normal status. According to the invention this pathological status is indicated by a decreased or increased level of a biomarker. The term "decreased level" as used herein refers to the level of protein of
25 interest in a body fluid sample which is significantly lower than the normal level. Significantly lower means that the level is lower and that the difference to the normal level is statistically relevant ($p \leq 0.05$, preferably, $p \leq 0.01$). The term "increased level" as used herein refers to the level of protein of interest in a body fluid sample which is significantly higher than the normal level. Significantly higher means that the level is
30 higher and that the difference to the normal level is statistically relevant ($p \leq 0.05$, preferably, $p \leq 0.01$).

 The person skilled in the art is familiar with different methods of measuring the level of a peptide or polypeptide. The term "level" relates to amount or concentration of a
35 peptide or polypeptide in an individual or a sample taken from an individual.

In the context of the present invention, amount also relates to concentration. It is evident, that from the total amount of a substance of interest in a sample of known size, the concentration of the substance can be calculated, and vice versa.

The term "measuring" according to the present invention relates to determining the
5 amount or concentration, preferably semi-quantitatively or quantitatively. Measuring can be done directly or indirectly. Indirect measuring includes measuring of cellular responses, bound ligands, labels, or enzymatic reaction products.

Measuring can be done according to any method known in the art. Preferred methods are described in the following:

10 In a preferred embodiment, the method for measuring the level of a protein of interest, comprises the steps of (a) contacting a cell with the protein for an adequate period of time wherein said cell is capable of a cellular response to the protein, (b) measuring the cellular response.

In another preferred embodiment, the method for measuring the level of a
15 polypeptide of interest comprises the steps of (a) contacting a protein with a specifically binding ligand, (b) (optionally) removing non-bound ligand, (c) measuring the amount of bound ligand.

Preferably, the protein is contained in a body fluid sample, and the amount of the protein in the body fluid sample is measured.

20 A body fluid is e.g. blood (whole blood, blood serum, blood plasma) and cerebrospinal fluid (CSF). Preferably, body fluid is cerebrospinal fluid. Samples of body fluids can be obtained by any method known in the art.

If necessary, the samples may be further processed. Particularly, proteins may be purified from the sample according to methods known in the art, including filtration,
25 centrifugation, or extraction methods such as chloroform/phenol extraction.

For measuring cellular responses, the sample or processed sample is added to a cell culture and an internal or external cellular response is measured. The cellular response may include the expression of a reporter gene or the secretion of a substance, e.g., a protein, peptide, polypeptide, or a small molecule.

30 Other preferred methods for measurement may include measuring the amount of a ligand binding specifically to the protein, peptide or polypeptide of interest. Binding according to the present invention includes both covalent and non-covalent binding.

A ligand according to the present invention can be any protein, peptide, polypeptide, nucleic acid, or other substance binding to the protein of interest. It is well known that proteins, if obtained or purified from the human or animal body, can be modified, e.g., by glycosylation. A suitable ligand according to the present invention may
5 bind the protein exclusively or additionally via such sites.

Preferably, the ligand should bind specifically to the protein to be measured. "Specific binding" according to the present invention means that the ligand should not bind substantially to ("cross-react" with) another protein or substance present in the sample investigated. Preferably, the specifically bound protein or isoform should be
10 bound with at least 3 times higher, more preferably at least 10 times higher and even more preferably at least 50 times higher affinity than any other relevant protein, peptide or polypeptide.

Non-specific binding may be tolerable, particularly if the investigated protein can still be distinguished and measured unequivocally, e.g., according to its size (such as on a
15 Western Blot), or by its relatively higher abundance in the sample.

Binding of the ligand can be measured by any method known in the art. Preferably, the method is semi-quantitative or quantitative. Suitable methods are described in the following.

First, binding of a ligand may be measured directly, e.g., by NMR or surface
20 plasmon resonance.

Second, the ligand may be coupled covalently or non-covalently to a label allowing detection and measurement of the ligand.

Labeling may be done by direct or indirect methods. Direct labeling involves coupling of the label directly (covalently or non-covalently) to the ligand. Indirect
25 labeling involves binding (covalently or non-covalently) of a secondary ligand to the first ligand. The secondary ligand should specifically bind to the first ligand. Said secondary ligand may be coupled with a suitable label and/or be the target (receptor) of tertiary ligand binding to the secondary ligand. The use of secondary, tertiary or even higher order ligands is often used to increase the signal. Suitable secondary and higher order
30 ligands may include antibodies, secondary antibodies, and the well-known streptavidin-biotin system (Vector Laboratories, Inc.)

The ligand may also be "tagged" with one or more tags as known in the art.

Such tags may then be targets for higher order ligands. Suitable tags include biotin, digoxigenin, His-Tag, Glutathion-S-Transferase, FLAG, GFP, myc-tag, influenza A virus haemagglutinin (HA), maltose binding protein, and the like. In the case of a peptide or polypeptide, the tag is preferably at the N-terminus and/or C-terminus.

- 5 Suitable labels are any labels detectable by an appropriate detection method. Typical labels include gold particles, latex beads, acridan ester, luminol, ruthenium, enzymatically active labels, radioactive labels, magnetic labels ("e.g., magnetic beads", including paramagnetic and superparamagnetic labels), and fluorescent labels.

- Enzymatically active labels include e.g., horseradish peroxidase, alkaline
10 phosphatase, beta-Galactosidase, Luciferase, and derivatives thereof. Suitable substrates for detection include di-amino-benzidine (DAB), 3,3'-5,5'-tetramethylbenzidine, NBT-BCIP (4-nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate, available as readymade stock solution from Roche Diagnostics), CDP-10 Star™ (Amersham Biosciences), ECF™ (Amersham Biosciences). A suitable enzyme-substrate
15 combination may result in a colored reaction product, fluorescence or chemoluminescence, which can be measured according to methods known in the art (e.g., using a light-sensitive film or a suitable camera system).

- Typical fluorescent labels include fluorescent proteins (such as GFP and its derivatives), Cy3, Cy5, Texas Red, Fluorescein, and the Alexa dyes (e.g., Alexa 568).
20 Further fluorescent labels are available, e.g., from Molecular Probes (Oregon). Also the use of quantum dots as fluorescent labels is contemplated.

Typical radioactive labels include ³⁵S, ¹²⁵I, ³²P, ³³P and the like. A radioactive label can be detected by any method known and appropriate, e.g., a light-sensitive film or a phosphor imager.

- 25 Suitable measurement methods according the present invention also include precipitation (particularly immunoprecipitation), electrochemiluminescence (electro-generated chemiluminescence), RIA (radioimmunoassay), ELISA (enzyme-linked immunosorbent assay), sandwich enzyme immune tests, electrochemiluminescence sandwich immunoassays (ECLIA), dissociation-enhanced lanthanide fluoro immuno
30 assay (DELFIA), scintillation proximity assay (SPA), turbidimetry, nephelometry, latexenhancedturbidimetry or nephelometry, solid phase immune tests, and mass spectrometry such as SELDI-TOF, MALDI-TOF, or capillary electrophoresis-mass spectrometry (CEMS).

- Further methods known in the art (such as gel electrophoresis, 2D gel
35 electrophoresis, SDS polyacrylamid gel electrophoresis (SDS-PAGE), Western Blotting),

can be used alone or in combination with labelling or other detection methods as described above.

Preferred ligands include antibodies, nucleic acids, proteins, peptides or polypeptides, and aptamers, e.g., nucleic acid or peptide aptamers. Methods to such
5 ligands are well-known in the art. For example, identification and production of suitable antibodies or aptamers is also offered by commercial suppliers. The person skilled in the art is familiar with methods to develop derivatives of such ligands with higher affinity or specificity. For example, random mutations can be introduced into the nucleic acids, proteins, peptides or polypeptides. These derivatives can then be tested for binding
10 according to screening procedures known in the art, e.g., phage display.

Aptamers are chemically synthesized (usually short) strands of oligonucleotides (DNA or RNA) that can adopt highly specific three-dimensional conformations. Aptamers are designed to have appropriate binding affinities and specificities towards certain target molecules.

15 The term "antibody" as used herein includes both polyclonal and monoclonal antibodies, as well as any modifications or fragments thereof, such as Fv, Fab and F(ab)₂ fragments that are capable of binding antigen or hapten.

The present invention also relates to a kit comprising a means or an agent for measuring HSP27 or a variant thereof.

20 Such means or agent may be any suitable means or agent known to the person skilled in the art. Examples for such means or agents as well as methods for their use have been given in this specification. For example, a suitable agent may be any kind of ligand or antibody specific for measuring said biomarkers. The kit may also comprise any other components deemed appropriate in the context of measuring the level(s) of the respective
25 biomarkers, such as suitable buffers, filters, etc.

Optionally, the kit may additionally comprise a user's manual for interpreting the results of any measurement(s) with respect to determining whether an individual suffers from Alzheimer's disease and/or monitoring the progression of Alzheimer's disease of an individual. Particularly, such manual may include information about what measured
30 level corresponds to a decreased level or increased level.

The proteins of interest may also be used as target. Therefore, the present invention provides a method of screening for a compound which interacts with HSP27, or a variant thereof. Such methods are well known in the art.

A suitable method is for example the method of screening for a compound which interacts with HSP27, or a variant thereof, comprising a) contacting HSP27, or a variant thereof with a compound or a plurality of compounds under conditions which allow interaction of said compound or a plurality of compounds with HSP27, or a variant thereof; and b) detecting the interaction between said compound or plurality of compounds with HSP27, or a variant thereof.

The protein of interest may be immobilized prior step a) or between step a) and step b).

Having now generally described this invention, the same will become better understood by reference to the specific examples, which are included herein for purpose of illustration only and are not intended to be limiting unless otherwise specified, in connection with the following figures.

Figures

Figure 1 shows histograms of protein identifications in dependence of the rank correlation P-value for the data with randomized label.. X-axis: the distribution of log(P)-values; black: real data with randomized labels; white: simulated data set with randomized labels. The high rank correlation values cannot be produced by chance in a dataset that contains all the real data that has randomly re-labeled.

Figure 2 shows a graphical representation of the Braak-stage dependent increase of peptide counts of HSP27. (y-axis: number of peptide counts, x-axis: Braak stage)

Examples:

Commercially available reagents referred to in the examples were used according to manufacturer's instructions unless otherwise indicated.

5 Example 1:

Sample preparation

Grey matter brain tissue from Alzheimer patients with different Braak Stages (II; IV; V; VI) two in each case and grey matter brain tissue of two healthy controls, were investigated. For each brain, the following sample preparation procedure was carried out:

- 10 1. Grey matter tissue from frozen brain sections of the occipital association gyrus was mechanically dissected, and dried.
2. The tissue was solubilized in 2% SDS; 66mM Na₂CO₃,
3. A BCA Protein concentration test was performed to determine the protein concentration of each sample.
- 15 4. Proteinseparation step: After reduction and alkylation 38 µg Protein per Sample was loaded on a 4-12% Bis Tris Gel
Each Protein lane was cut into 19 sections. Each section was digested using an established in-gel digest procedure by which tryptic digests of the proteins were obtained and dried in the lyophilisator.
- 20 5. A fused silica column (75µm ID, 280 µm OD) packed with Magic C18 material was used as both the LC separation column and the electrospray tip.
6. The Peptides of each gel section were injected twice. They were concentrated on a C18 Trap Colomn and separated via nanoflow over the Magic C18 column by a Dionex Ultimate HPLC.
- 25 7. The separated peptides were analyzed on the fly by a Thermo LTQ Iontrap. Each MS Scan was set to acquire a full mass spectrum between 400 and 1500 Da followed by seven MS/MS scans between 400 and 2000 Da of the top seven ions from the preceding MS scan. Relative collision energy for CID was set to 35%. Dynamic exclusion was enabled with a repeat count of 2, a repeat duration of 60 seconds, and
30 an exclusion duration of 60 seconds. Altogether 38 LC-MS runs were performed for each Sample. Each run took 150 minutes.

Data Evaluation

Mass spectra data annotation

The acquired MS/MS spectra were searched by SEQUEST against a Human
5 Genome derived Protein Database (humangp) containing more than 48000 different
proteins, pig trypsin peptides were added as well. All accepted peptides had to have a Δcn
of at least 0.1 and a xCorr of 2.0 for +1 2.5 for +2 and 3.2 for +3 charges. At least two
different peptides passing the above filter criteria have to be seen to consider a protein as
a valid identification. Furthermore only proteins which have been identified in both
10 duplicates have been taken into account.

Peptide counts as protein quantifier

It has been attempted to use counts of detected peptides per protein identity as
indicators for protein quantity. This approach has been applied for proteomics data
generated from a mouse model of AD, and a similar approach has been recently
15 published in peer-reviewed literature (Quantitative methods for proteomics data analysis:
Lu P, Vogel C, Wang R, Yao X, Marcotte EM. Absolute protein expression profiling
estimates the relative contributions of transcriptional and translational regulation. Nat
Biotechnol. 2007 Jan. 25(1):117-24; Benjamini, Yoav; Hochberg, Yosef (1995).
"Controlling the false discovery rate: a practical and powerful approach to multiple
20 testing". Journal of the Royal Statistical Society, *Series B (Methodological)* 57 (1): 289–
300.). An estimation of the false discovery rate was used by simulation of random datasets
by permutation of the labels in the experimental data. Marker candidates have to have P-
values larger than $10^{-2.5}$ and need to be at least one standard deviation separated from the
bulk of the random P-values distribution as calculated by the FDR simulation.

25 A reasonable estimate for a FDR statistic requires at least three samples in order to
be able to estimate P-Values for T statistics. Thus, the samples per group could not be
compared (as each group contains exactly two samples). However, the light could be
combined to moderate AD pathology and the severe pathology groups to try to find the
differences caused by disease severity.

30 Rank order changes as robust estimator of changes

While peptide counts are a useful tool for the elucidation of expression differences
in sets of experiments that have appropriate sample numbers, the experiment was

conducted to include few repeats but many (four) categories of severity. Proteins that are robustly changing with disease severity were mainly interesting.

Spearman-rank order correlation coefficients were used to estimate the connection between Braak stage and protein expression (as estimated by peptide counts). The
5 significance of a non-zero value of the Spearman rank order correlation coefficient were given by a t-Statistic (Rank order statistics: Press WH, Teukolky SA, Vetterling WT, Flannery BP. Numerical recipes in C – Second Edition. p 609-656, Cambridge University Press 1992), and as usual, a simulation of the dataset with randomized labels allowed estimating an empirical false discovery rate at a given significance level (see Figure 1).

10

Results:

The histogram for the distribution of log(P)-value for the real data and a simulated data set with randomized labels showed that log(P) values above 4 are almost absent from the randomly labeled dataset (figure 1). A protein with a log(P) value above 4 is therefore
15 considered as significant. HSP27 (SEQ. ID NO: 1) has a log(P) value of 6.47. The peptide counts show a stage-dependent increase (see Figure 2).

Claims

1. A method for assessing Alzheimer's disease in vitro comprising measuring the level of HSP27, or a variant thereof in sample collected from an individual, wherein an altered level of said protein, or a variant thereof, is indicative that said individual suffers from Alzheimer's disease.
2. The method according to claim 1 wherein the level of said protein, or a variant thereof, is increased.
3. Method for monitoring the progression of the Alzheimer's disease in vitro comprising measuring the level of HSP27 or a variant of said protein in a sample collected from an individual, wherein an altered level of HSP27 or a variant of of said protein compared with an earlier measurement of the level of HSP27, or a variant thereof, is indicative for the progression of Alzheimer's disease of said individual.
4. The method according to claim 3, wherein the level of HSP27 or a variant of said protein is increased compared with the earlier measured level.
5. The method according to any one of the claims 1 to 4, wherein the sample is a body fluid sample, preferably a cerebrospinal fluid sample or a blood sample.
6. Use of HSP27, or a variant thereof as biomarker for Alzheimer's disease and/or for the progression of Alzheimer's disease.
7. A kit comprising a means or an agent for measuring of HSP27, or a variant thereof.
8. The kit according to claim 7 wherein the kit further comprises a user's manual for interpreting the results of any measurement with respect to determining the risk of an individual suffering from Alzheimer's disease and/or monitoring the progression of Alzheimer's disease of an individual.
9. Use of a kit according to claim 7 or 8 in a method according to any of claims 1 to 5.
10. Methods, uses and kits substantially as herein before described especially with reference to the foregoing examples

Figure 1:

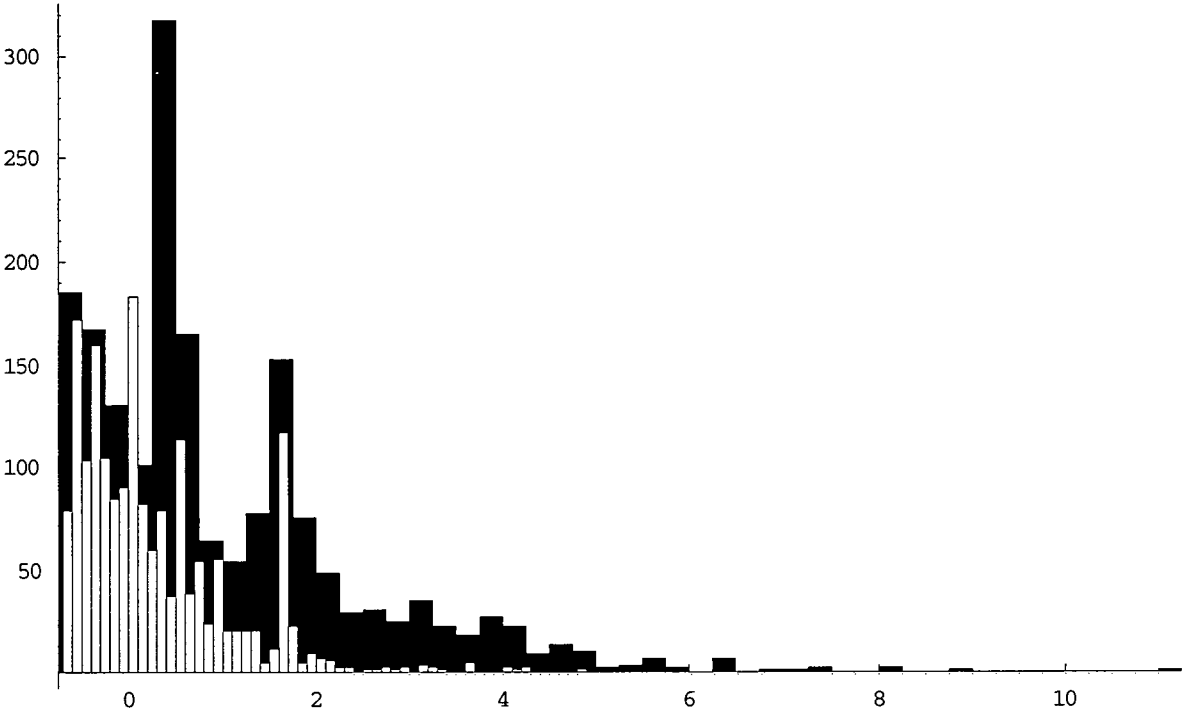
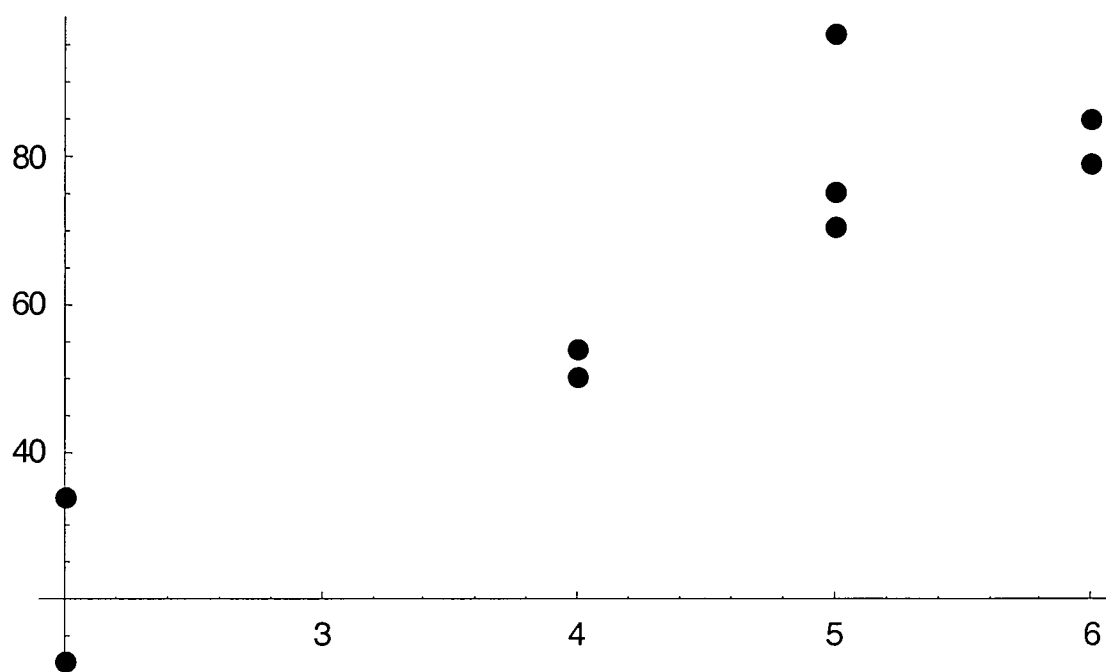


Figure 2:



INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/004235

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, INSPEC, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RENKAWEK K ET AL: "Expression of small heat-shock protein hsp 27 in reactive gliosis in Alzheimer disease and other types of dementia" ACTA NEUROPATHOLOGICA, SPRINGER VERLAG, BERLIN, DE, vol. 87, no. 5, 1 January 1994 (1994-01-01), pages 511-519, XP008095466 ISSN: 0001-6322 the whole document In particular: Title and abstract; Materials and methods; Fig. 2; p. 514, col. 2, paragraphs 2-3 and p. 516, col. 1, first paragraph. ----- -/--	1-9

☒ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 September 2008

Date of mailing of the international search report

19/09/2008

Name and mailing address of the ISA/

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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/004235

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RENKAWEK ET AL: "Immunohistochemical expression of alpha-B-crystallin and heat shock protein 27 in the brain in Down syndrome" DEVELOPMENTAL BRAIN DYSFUNCTION, KARGER, BASEL, CH, vol. 8, no. 1, 1 January 1995 (1995-01-01), pages 35-39, XP008095465 ISSN: 1019-5815 the whole document In particular: title and abstract; Fig. 4a and 4b	1-9
X	RENKAWEK K ET AL: "Increased expression of heat-shock protein 27 kDa in Alzheimer disease: A preliminary study" NEUROREPORT, RAPID COMMUNICATIONS OF OXFORD, OXFORD, vol. 5, no. 1, 25 October 1993 (1993-10-25), pages 14-16, XP008095468 ISSN: 0959-4965 the whole document In particular: Abstract and results.	1-9
X	RENKAWEK K ET AL: "Dementia, gliosis and expression of the small heat shock proteins hsp27 and alphaB-crystallin in Parkinson's disease" NEUROREPORT, RAPID COMMUNICATIONS OF OXFORD, OXFORD, vol. 10, no. 11, 2 August 1999 (1999-08-02), pages 2273-2276, XP008095467 ISSN: 0959-4965 the whole document In particular: Abstract and results.	1-9
X	ARRIGO ET AL: "Hsp27 (HspB1) and alphaB-crystallin (HspB5) as therapeutic targets" FEBS LETTERS, ELSEVIER, AMSTERDAM, NL, vol. 581, no. 19, 24 April 2007 (2007-04-24), pages 3665-3674, XP022191492 ISSN: 0014-5793 the whole document In particular: p. 3668-3669, item 2. -/--	1-9

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2008/004235

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	STEGE G J ET AL: "The molecular chaperone alphaB-crystallin enhances amyloid beta neurotoxicity" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS 19 AUG 1999,, vol. 262, no. 1, 19 August 1999 (1999-08-19), pages 152-156, XP002494274 the whole document In particular: abstract; p. 155, last paragraph. -----	1-9
A	IMURA T ET AL: "Differential expression of small heat shock proteins in reactive astrocytes after focal ischemia: possible role of beta-adrenergic receptor" THE JOURNAL OF NEUROSCIENCE : THE OFFICIAL JOURNAL OF THE SOCIETY FOR NEUROSCIENCE 15 NOV 1999,, vol. 19, no. 22, 15 November 1999 (1999-11-15), pages 9768-9779, XP002494275 the whole document -----	1-9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2008/004235

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 9
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers allsearchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.2

Claims Nos.: 9

Present claim 10 relates to a product/method defined (inter alia) by reference to the following unusual parameter: "substantially as herein before described especially with reference to the foregoing examples".

The use of this unusual parameter in the present context is considered to lead to a lack of clarity because the claim does not clearly identify the product/method encompassed by it as the parameters cannot be clearly and reliably determined by indications in the description or by objective procedures which are usual in the art. This makes it impossible to compare the claim/s to the prior art. As a result, the application does not comply with the requirement of clarity under Article 6 PCT.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.2), should the problems which led to the Article 17(2)PCT declaration be overcome.