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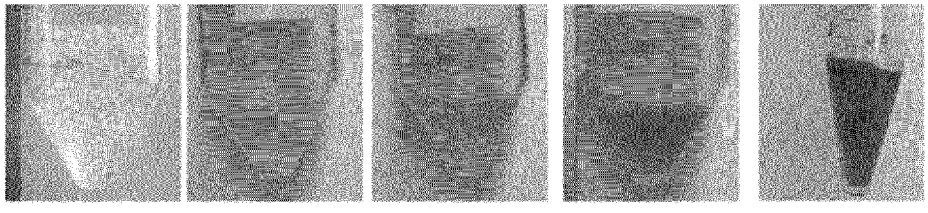
(54) Title: METHOD FOR DIAGNOSING NEURODEGENERATIVE DISEASES

Figure 1



(57) Abstract: The invention relates to an in vitro method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject. Said A/T/N classification subsequently may be used, but is not limited to, the diagnosis of Alzheimer's disease (AD), the diagnosis of SNAP or the exclusion of AD.

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Method for diagnosing neurodegenerative diseases

The invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject. Said A/T/N classification subsequently may be used, but is not limited to, the diagnosis of Alzheimer's disease (AD), the diagnosis of SNAP or the exclusion of AD.

BACKGROUND OF THE INVENTION

Neurodegenerative diseases represent a major threat to human health. These disorders are becoming increasingly prevalent, in part because the elderly population has increased in recent years. Examples of neurodegenerative diseases are Alzheimer's disease (AD) and suspected non-Alzheimer pathophysiology (SNAP). AD is the most prevalent neurodegenerative disease comprising 50% to 60% of dementia cases. With an aging population worldwide the number of subjects with dementia is expected to quadruple by 2050 (Brookmeyer (2007), Alzheimers Dement. 3, 186-191). It is now accepted that AD (through the accumulation of neurogenerative proteins) begins at least 15 to 20 years before symptom onset (Bateman (2012), N. Engl. J. Med. 367, 795-804). SNAP is a biomarker-based concept for AD-like neurodegeneration in subjects without β-amyloidosis (Jack (2016), Nat. Rev. Neurol. 12(2), 117-124).

Several marker proteins/biomarkers are reported to correlate with the diagnosis of AD and SNAP, namely beta amyloid (particularly Aβ42 and/or Aβ40), pTau (particularly pTau181 and/or pTau231) and tTau (Scheltens (2016), Lancet, 388(10043), 505-517). Amyloid Precursor Protein (APP) is a transmembrane protein that can be proteolytically cleaved by several enzymes, including α-, β-, and γ-secretases. Cleavage by β-, and γ-secretases results in beta amyloid peptides ranging in 38 to 43 amino acids. Of these peptides, 1 to 42 is the most amyloidogenic, due to its high propensity to aggregate. Although Aβ40 constitutes the majority of the expressed beta amyloid peptides, due to changes in aggregation dynamics caused by the last two amino acids of Aβ42, Aβ42 is far more toxic (Selkoe (1999), Nature,

399, A23-A31). Under physiological conditions, Tau binds and stabilizes microtubules, an integral part of a cell's internal skeleton. In AD, Tau becomes hyperphosphorylated, in particular on amino acid 181 (pTau181) and 231 (pTau231) and 217 (pTau217) and is no longer able to bind the microtubules. This results in intracellular aggregation of the Tau proteins into higher molecular weight species or filaments and then subsequently neurofibrillary tangles. The presence of the aggregates and disruption of the cytoskeleton results in synaptic dysfunction and neuronal death. Tau is then released from the brain tissue into CSF resulting in increased levels in the CSF (Medeiros (2011), CNS Neurosci. Ther., 17(5), 514-524). So far reliable detection of said marker proteins in the context of AD was only possible in cerebrospinal fluid (CSF). CSF, the fluid surrounding the brain and the spinal cord of all vertebrates, has two major functions. It supplies the brain with vital nutrients and removes metabolites and waste products produced in the brain and the spinal cord of all vertebrates, thus maintaining organ homeostasis.

Aβ42 monomers accumulate to form di-, trimeric and larger oligomeric structures (quaternary structures) in the brain and subsequently react as toxic agents to the brain. The accumulation leads to malfunction of synapses with consecutive cell death over time and increased concentrations of Aβ42 aggregates in the brain. It was observed that the pathological process of accumulation of Aβ42 monomers to larger n-mers in the brain results in a decrease of the total amount of Aβ42 protein detectable in CSF (Sunderland (2003), JAMA, 289(16), 2094-2103).

AD is a chronic disease that takes place silently first at a cellular level decades before symptom onset and then followed by neuronal loss in conjunction with cognitive decline (Hulette (1998), J. Neuropathol. Exp. Neurol., 57(12), 1168-1174; Serrano-Pozo (2011), Cold Spring. Harb. Perspect. Med., 1(1)). Formerly, AD was diagnosed based only on clinical symptoms (McKhann (1984), Neurology, 34(7), 939-944). Currently, the revised definition of Alzheimer's Disease AD is also based on the determination of disease-causing neurotoxic proteins, and in particular Aβ42, Aβ40, pTau (particularly pTau181 and/or pTau231 and/or pTau217) and tTau (Deutsche Gesellschaft für Neurologie, (2016), S3-Leitlinie "Demenzen", S3

Leitlinie Demenzen; Dubois (2021), Lancet Neurol., 20(6), 484-496; Jack (2018), Alzheimers. Dement., 14(4), 535-562).

The above-mentioned marker proteins are determined either in the CSF (obtained via lumbar puncture) with laboratory immunoassays or alternatively through imaging procedures (e.g. amyloid tracer or tau tracer PET-CT scans) in the brain. The analysis of CSF has significant advantages over imaging modalities because each of the key relevant proteins can be individually quantified by objective means. Amyloid-and tau-tracer PET Scans are inferior to CSF because, first of all, a radioactively labeled antibody has to be injected into the subject's venous system in order to allow PET scan-based measurements. Secondly, the readout of the generated signal in the amyloid-and tau-PET Scans is not fully quantitatively nor standardized, since the signal allows no comprehensive discrimination between Aβ42 and Aβ40, since sometimes these two proteins also tend to form accumulations. Same applies for Aß42 and tau conglomerates. The rating is mainly based on the subjective experience of the rating physician and the results vary between different tracers, machines and rating physicians.

The research framework for AD diagnosis, developed under the auspices of the National Institute on Aging and Alzheimer's Association (Jack (2018), Alzheimers. Dement., 14(4), 535-562), proposes to categorize individuals based on biomarker evidence of pathology, using the so-called A/T/N (amyloid, tau, neurodegeneration) classification system (see **Figure 10**). According to the ATN system, each individual is rated for the presence of pathological levels (i) Aβ42 (CSF Aβ42 or amyloid positron emission tomography-computed tomography (PET-CT): "A"), (ii) hyperphosphorylated Tau (CSF pTau181 or Tau PET-CT: "T"), and (iii) neurodegeneration (atrophy on structural magnetic resonance imaging (MRI), fluordeoxyglucose (FDG), PET-CT, or CSF total tau (tTau): "N"), resulting in 8 possible classification combinations (**Figure 10**). The main relevant classification consists of the biomolecular-based classification of the amyloid-pathology by distinguishing "A+" from "A-" subjects, because this differentiation is crucial for deciding on the subsequent diagnosis, e.g. determining whether a subject is in the AD continuum or not according to the NIA-AA criteria as well as different treatment pathways of the subject. Subject stratification for drug-based therapies targeting the amyloid pathology requires a positive amyloid test (A+), including therapy eligibility (see appropriate use criteria for Lecanemab (Leqembi®)).

In CSF samples, the AD biomarker proteins are typically detected with standard laboratory assays with Enzyme-linked Immunosorbent Assay (ELISA-)- or Chemiluminescence immunoassay (CLIA-)-based methods. These methods always use an antibody that is specific for the protein to be measured and has specific properties. These antibodies usually recognize either very specific oligomers or monomers of the respective protein. Mostly, however, they are not 100% specific for the respective oligomeric form but rather recognize the oligomeric forms in a ratio of an estimated 80% (e.g. oligomers) to 20% (monomers), for example.

After antibody epitope recognition, a signal is generated. This signal is then counted, summed up and then back calculated into a concentration using calibrator curves. As a result, the value 1000 pg/ml could therefore, for example, be composed of 100% monomeric forms of the protein or 100% oligomeric forms or a ratio of 50% oligomers and 50% monomers and so on.

However, based on the results from these methods, it is not possible to know the exact composition and the amount of the quaternary isoforms in the individual case. By adding oligomer-destroying substances after an initial measurement, the presence of oligomers can be indirectly indicated if the subsequent result reveals increased levels. If, for example, a value of 1000 pg/ml increases to 1500 pg/ml after the addition of “oligomer-destroying” substances, then it is reasonable to assume that more binding sites have become free due to the destruction of the larger oligomers, and thus the value has increased. Indirectly, the question of the presence of oligomers can be answered in the affirmative. However, it cannot be definitively clarified whether all previously present oligomers were broken down into e.g. monomers or whether other oligomers that additionally might be present have not yet reacted. This indirect test also cannot clarify or answer the question of the exact concentration of each different oligomeric species having different sizes in relation to monomers that were present in the respective sample. As described above, the choice of the antibody also influences the probability of selecting more oligomers or more monomers, and the numerical result is biased by the fact that these antibodies are never 100% specific for recognizing either one or the other species. In addition, again the problem arises that oligomers of different sizes are very likely to be detected as similar or equivalent ones and thus no statement is possible about the exact composition of the respective sample.

In summary, such methods are not able to exactly differentiate between a monomeric and an oligomeric structure of the biomarker proteins, nor can they inform about their molecular weight or quaternary structure, and they are further not able to exactly quantify the different quaternary structures individually or in combination. This information is especially relevant for the Aβ42, pTau, such as pTau181, pTau231 and/or pTau217, as well as for tTau since it is especially the state of oligomerization and the quantity of oligomers or monomers and or the ratio thereof that plays a major role in the pathological process of AD and enables to stage the disease accordingly.

Therefore, the current state of the laboratory procedures for diagnosing AD lacks a very vital piece of information about the exact quantities of the quaternary forms of each analyte and therefore do not allow to display e.g. changes in the quaternary structure of the proteins of interest during the course of the disease. The same holds true for the amyloid-and tau-PET-CT procedures and plasma-based measurements which are not able to exactly describe, differentiate or quantify different monomerization and oligomerization states as well.

Using CSF to determine the quaternary structure (oligomeric forms) of the AD associated biomarkers (e.g. as a protein specific biomarker signature or via a classification specific score) and the distribution between the different forms is disadvantageous and has also not been successfully performed, due to i) the high dilution of the different proteins of interest in their different oligomerization states in the total volume of CSF, ii) the impact of different diffusion kinetics of monomers and oligomers, depending on their molecular weight, which would change the ratios between the proteins of interest and scores as a function of the distance between the brain and the site of sampling in the lumbar area at L4/L5. Furthermore, any diagnostic procedure requiring a lumbar puncture (to obtain CSF) or PET-CT scans (by applying a radioactive substance) are very burdensome to the subject, expensive and associated with a non-negligible risk of side-effects. Hence, they can be applied only to already symptomatic subjects and are not suitable for any early detection before onset of clinical symptoms or for a targeted screening process nor can they be applied frequently over the course of the disease to monitor changes e.g. in the overall amounts of the proteins or in the different oligomeric

stages of the disease under the presence and absence of influencing factors, like drugs that especially aim to destroy the toxic oligomeric forms of the proteins.

Due to the limitations of CSF based analysis, alternative sample materials were studied for the diagnosing of neurodegenerative diseases. A large number of studies have attempted to detect Aβ42 and Aβ40 in plasma samples to diagnose patients with AD (A+). To allow detection in the blood, the brain- or CSF-derived biomarkers must cross the blood-brain barrier. The biomarker then subsequently gets "diluted" in high blood volume; for example, beta amyloid concentrations are tenfold lower in plasma than in CSF. Extra-cerebral production (e.g. by platelets) and consumption (proteolytic degradation, metabolization, clearance) of biomarkers leads to poor correlation with CSF levels and reduced effect sizes. Additionally, plasma or blood is a complex, high-protein matrix compared to CSF. Possible interferences and protein-binding might result in epitope masking, or strong matrix effects. Diurnal changes and external influences such as of medications seem to be more pronounced in blood leading to large intra- and interindividual variation. Also, a small effect size, meaning only small differences of plasma beta amyloid levels between cases and controls of only 10 to 15% were problematic. Thus, small pre-analytical or measurement errors can reduce the clinical performance to a level where it is no longer clinically useful. Additional inconsistent results over different technologies (Chong (2021), J. Neurol. Neurosurg. Psychiatry, 92(11), 1231-1241) and the lack of validation (in large, independent cohort) and standardization (e.g. pre-analytical procedure, analytical platform) as well as the missing of a prospective validation under routine conditions yielding in the result that up to now no successful correlation of plasma-based beta amyloid measurements towards CSF based or amyloid-PET scan-based A/T/N classification could be achieved. Nevertheless, as mentioned above, a three-dimensional analysis in plasma samples, with the possibility to exactly differentiate each quaternary structure and to exactly quantify these quaternary structures, is thought to increase the diagnostic performance of the analytical procedures in plasma as well. It is to be assumed that especially the misfolded and aggregated pathological forms of e.g. toxic oligomeric species might be hidden in the overall amount of plasma Aβ42. Again, current technologies do not differentiate between different oligomeric or monomeric structures that are being present in the body fluids. Additionally, as mentioned before, Aß42 proteins, for

example, must cross the blood-brain barrier, concentrations are tenfold lower, extra-cerebral production (e.g. by platelets) and consumption alternate the protein composition and possible interferences and protein-binding might additionally result in epitope masking or strong matrix effects. By separating each individual Aß42 quaternary structures, the specific toxic species might be recognized, quantified and would theoretically increase effect size, since it would allow to distinguish between the brain-derived toxic species from the peripheral non-toxic species and thus result in increased diagnostic overall performance. Nevertheless, plasma is due to the above-mentioned alterations not the most suitable peripheral body fluid to examine brain pathologies and there are other body fluids, like nasal secretion, that are particularly interesting for the analysis and diagnosis of brain pathologies like AD and other forms of neurodegeneration or oncological diseases.

The brain-nose interface (BNI) facilitates the exchange of substances between the brain and the nasal cavity. Cradling the olfactory bulb at the skull base and the upper nasal cavity, it contains olfactory neurons extending through the cribriform plate, directly linking the central nervous system (CNS) and the external environment, bypassing the blood-brain barrier. Olfaction, immune response, and the glymphatic drainage of cerebrospinal fluid (CSF) to cervical lymph nodes are key functions of the BNI. In olfaction, odorants in the external environment are detected by olfactory receptors within the olfactory cleft, initiating a cascade of neuronal signaling that is transmitted directly to the brain via the BNI. Neurons in the BNI are vital for triggering local immune reactions that help defend against infections. Lastly, the BNI provides a route for CSF to exit the subarachnoid space of the CNS and drain into cervical lymph nodes via lymphatic vessels in the nasal cavity. This clearance process prevents the accumulation of waste in the CNS and is important for brain health and function.

Yoo et al. already suggested that soluble Aβ can be detected in subjects' nasal discharge to discriminate between AD and nonAD patients (Yoo (2020), Sci. Rep., 10:11234). Yoo et al. used the pan-Aβ antibody 6E10 to detect Aβ oligomers and correlated the results with the levels to cognitive performance alone. They did not demonstrate the presence or absence of AD based on the biochemical A/T/N classification which according to the consensus definition from the NIA-AA (Jack (2018), Alzheimers. Dement., 14(4), 535-562) is the relevant definition of AD.

Previous work has additionally always been hampered by the problem of extremely limited amounts of Aβ42, pTau and tTau detectable in nasal secretions combined with the need for very highly sensitive, complex and costly techniques.

For example, in the studies of Kim et al. two oligomeric forms of pan-Aβ could be measured in nasal secretion. They were either indirectly indicated by a capacitance change index developed by the authors (Kim (2019), Sci. Rep., 9:4966) or via Western Blot. They could show two bands, the 70 kDa band for pan-Aβ and the 100kD band, detected by the pan-Aβ antibody 6E10. By applying an oligomerization disrupting agent and by showing then changes in the oligomerization bands in AD subjects, the authors indirectly concluded that there are increased oligomeric Aβ in nasal secretion from AD subjects.
To our understanding, the effect of the oligomer disrupting agent was not consistent between the AD and cognitively unimpaired samples. In addition, the pan-Aβ antibody 6E10 also recognizes sAPPα which has an apparent molecular weight around 100 kDa; it is therefore not clear that the 100 kDa band is in fact beta amyloid.
All of this was taken as an indirect indication of the presence of high molecular weight structures of beta-amyloid, with no precise information about the level of beta-amyloid at different oligomerization states or different quaternary structures nor the exact distribution at the different oligomers in the sample. While some subjects in the AD group were classified as amyloid positive based on CSF or PET-CT scans, some subjects without amyloid pathology were included in the AD group. Finally, they were not able to comprehensively classify subjects according to the ATN classification system using their nasal secretion-based measurement system.

Additionally, nasal secretion is a body fluid that up to now has only been used to answer scientific questions and there is little to no prior art about composition of nasal secretion nor pre-treatments. Nasal secretion also contains endogenous substances which can interfere with the assay procedure. Such inferences can originate from mucins or other analyte-scavenging components in nasal secretion. They could encapsulate proteins of interest and inhibit protein recognition by antibodies. A harsh pre-treatment of the collected nasal secretion sample would be required prior to the immunological detection procedure, which

might interfere with an ELISA- or CLIA-based procedure, if used without additional, complex procedures prior to analysis.

In conclusion, prior art procedures suffered from several and serious disadvantages when used for the A/T/N-based subject classification of neurodegenerative diseases such as AD and/or SNAP.

Thus, there is a need for a laboratory-based diagnostic procedure to evaluate neurodegenerative diseases in accordance with the A/T/N system as basis to diagnose AD and/or SNAP, which is rapid, inexpensive and minimally invasive and allows for the testing of a wide range of the population, including pre-symptomatic subjects.

SUMMARY OF THE INVENTION

This need is addressed by the present invention by providing the embodiments as defined in the claims. In particular, the invention provides an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject.

In the appended Examples the inventors unexpectedly and surprisingly demonstrate that A/T/N classification can be performed in nasal secretion samples. Specifically, size separation of the higher three-dimensional molecular structures (quaternary structure like oligomers or monomers) of protein isoforms and the exact quantification of each three dimensional structure of protein isoforms yields in higher diagnostic performance, higher precision and reproducibility and allows performance of the globally accepted A/T/N classification in nasal secretion samples to aid in the diagnosis of AD, SNAP and to exclude pathologies as well. In other words, the inventors showed for the first time that the relevant biomarkers can be reliably detected, differentiated, and distinguishably quantified in nasal secretion samples in amounts that allow A/T/N classification. Thus, the present invention provides a minimally invasive method that allows A/T/N classification. The present invention allows to diagnose or exclude a neurodegenerative disease such as AD with a minimally invasive method and, thus,

without the risk of significant side effects. Accordingly, the method provided by the present invention allows to test subjects/patients for the presence of a neurodegenerative diseases such as AD even before symptoms of e.g. AD are apparent in the subjects/patients (preclinical stage). Thus, neurodegenerative diseases such as AD can be diagnosed very early and a corresponding therapy can be started in a timely manner. Due to its minimally invasive character, longitudinal measurements of the key relevant biomarkers and distinguished quantification of the different monomeric and oligomeric quaternary structures of each protein isoform can be done allowing to monitor the biological behavior of individual patient profiles under the presence and absence of influencing factors like e.g. anti-amyloid drug therapies. In the future, based on these profiles drug efficacy and individual pharmacokinetic effects (side effects, drug response, non-response) can be monitored that support patient stratification (identify patients who benefit the most vs. identify patients who are at high risk to develop side effects) and increase patient´s safety. Longitudinal measurements of the three-dimensional structures and the exact quantification thereof allow also for the first time, to establish different stages of the disease. Up to now, besides the evaluation of the biomarkers and the subsequent A/T/N classification the stages i) early ii) mild, iii) moderate or iv) severe are based on cognitive performance alone. Cognitive performance is a result of the underlying pathological processes and toxic accumulation, and formation of toxic oligomeric aggregates should also be taken into account when staging an individual. With the invention, longitudinal staging according to pathological processes on a cellular or molecular level can be performed that is not only timely measured but also much more precise than the information about a cognitive performance.

The aim of the invention is therefore to classify patients with regard to amyloid pathology (A status) and tau pathology (T status) and to describe the presence of neurodegeneration (N status). The total amount of each of the proteins relevant for the respective category A and or T and or N are to be i) quantified after normalisation and ii) a quantification of the different three-dimensional quaternary structures (monomers, oligomers) of each of the proteins is done.

On the basis of these examinations, besides an early, minimal invasive, precise ATN classification that can be made that supports the diagnosis of AD or SNAP or supports the

exclusion of a corresponding pathology, a highly precise and individual and disease specific quantification of the stage of the disease can be done according to the quantities of different three-dimensional structures of each protein.

In order to fully understand how much of the relevant proteins and how much of the respective quaternary structures can be found in the nasal secretion (=protein specific biomarker signature) for each protein and all categories (A/T/N), highly pre-classified reference cohorts (A+ vs A-, T+ vs T-, N+ vs N-) were preselected on the basis of the actual gold standard for the ATN classification following the NIA-AA guidelines- CSF measurements and amyloid-PET as well as MRI studies in combination with clinical examinations done by professionals with more than 15 years of experience. Especially in cases where, for example, the CSF results were ambiguous or unclear (e.g. results that were +/- 10% around the cut-off defined for the machine and the parameter), it was important for the exact classification of these reference cohorts to also include the clinical expertise of the above-mentioned experts for the determination of the final ATN classification. In the appended Examples the inventors analyzed as a proof of evidence of the claimed method nasal secretion samples of patients that were also classified via CSF analysis according to the A/T/N classification. In these pre-classified reference cohorts, nasal secretion samples were measured according to the method described herein. For each patient within the different cohorts and for each of the proteins of interest after normalization i) the total content of each analyte was determined ii) and the respective three-dimensional quaternary substructures of each analyte (e.g. monomers and oligomers or bigger n-mers) were separated according to size or molecular weight. They were then exactly quantified individually resulting in protein and disease state-specific and size or molecular weight-specific bands that can be analyzed separately or in relation towards each other or in relation to other protein- and disease-specific individual bands. This was repeated in the respective ATN cohorts (A+, A-, T+, T-, N+, N-) for each individual protein until a statistically significant number of measurements had been made for subsequent analysis. The protein- and disease state- specific bands or ratios of the bands to each other (intra-protein band analysis) or among each other (inter-protein band analysis) were identified for each of the proteins individually in each of the respective cohort. This allowed to establish cut-offs to distinguish a pathological change from a non-pathological change based on the method

described herein that is equivalent to the CSF- or PET- or clinically based ATN classification system. These so developed cut-offs can be to classify the measurement results in terms of the presence or absence of amyloid (A)-and tau- pathology (T) or signs of neurodegeneration (N) and aid in the diagnosis of AD or SNAP as well as support the exclusion of AD and SNAP. This can replace the highly invasive procedures (e.g. CSF) and radioactive diagnostic procedures (e.g. PET scans).

As shown in **Figure 3**, the 56 kDa oligomer band of Aβ40 shows an increased signal in patients without AD (corresponding to a A- classification) in comparison to patients with clear AD (classified as A+) in nasal secretion samples.

Figure 6 shows that the total level of detected Aβ42 is decreased in nasal secretion samples of an AD patient (classified as A+), relative to the total level of an A- patient. Figure 6 also shows that AD patients show altered band ratios between the 56 and 32 kDa oligomers compared to A- patients.

Figure 8 shows higher detected levels of tTau, seen at approximately 55 kDa in patients with clear signs for neurodegeneration (classified as N+) than in patients without signs for neurodegeneration (classified as N-).

Figure 9 shows that the total level of detected pTau is decreased in patients with clear Tau pathology (T+) compared to patients without Tau pathology (T-).

Furthermore, **Figure 16** shows the total intensity of Aβ42 as detected in nasal secretion samples (corresponding to the total level of Aβ42) in patients that were classified as A+ or A- according to CSF analysis. From the comparison a cut-off may be determined which may be 11. When in nasal secretion samples of a subject/patient an Aβ42 intensity of below 11 is measured said subject may be classified as A+.

Figures 17 to 20 show additional potential cut-offs for the relevant analytes that allow A/T/N classification in nasal secretion samples. Thus, it is plausibly laid down in the Examples that A/T/N classification via a minimally invasive method using nasal secretion samples is possible.

Figures 22 and 23 show that between A+ and A- can be discriminated using protein specific biomarker signatures.

Figures 24 and 25 show that classification specific scores obtained by multiple logistic regression correlate well with the observed A status determined via CSF.

Figures 26 and 27 show that using different protein-specific biomarker signatures as input for multiple logistic regression results in excellent negative and positive predictive power.

The present invention, inter alia, allows for:

- Minimally invasive diagnosis (which can be applied when minimal symptoms/prodromal symptoms are apparent or even in an at-risk population (e.g. 1° relatives without any symptoms), avoiding the risks related to a lumbar puncture or radioactive tracer (PET Scan);
- Simple and inexpensive diagnosis in order to reduce the number of unreported cases (estimations of unreported cases are at 50%, as current diagnostics are highly complex and are therefore only carried out in specialized centers and because of the invasiveness they are no longer carried out in the case of very pronounced symptoms and very advanced stages of the disease and old age, nor can they be applied repeatedly over the course of the disease);
- Early diagnosis;
- Differentiation of A+ and A- in subjects;
- Mapping of the progression of the disease via the determination of oligomerization levels and highly specific patterns of disease relevant biomarker (protein-specific biomarker signature or ATN biomarker signature);
- Therapy monitoring;
- Longitudinal profiling through repetitive testing of the same patient at different time points (comparing the initial individual protein specific biomarker signature(s) of the patient or the classification specific score at a certain point in time of the same person with the protein specific biomarker signature(s) or the classification specific score of the same person at a different point in time); and
- Patient stratification: by classifying the patient sample, specific therapeutic approaches can be selected that are particularly suitable in this individual case, and patients can be selected who are particularly well suited to be included in special study settings in order to benefit from particularly new therapies or other approaches under investigation.

DETAILED DESCRIPTION OF THE INVENTION

In the following the invention is described in more detail.

In particular, the invention relates to the following items:

1. An *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification (classification according to "A", the value of a beta amyloid biomarker; "T," the value of a Tau biomarker; and "N," biomarkers of neurodegeneration or neuronal injury) in nasal secretion samples obtained from said subject.

2. The method of item 1 comprising the following steps (a) to (e):
   a) determining in a nasal secretion sample obtained from the subject
      i) the protein-specific biomarker signature(s) of beta amyloid,
      ii) the protein-specific biomarker signature(s) of phosphorylated Tau, preferably pTau-181 and/or pTau-231, and/or
      iii) the protein-specific biomarker signature(s) of total Tau;
   b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;
   c) comparing the classification specific scores determined in b) to predetermined classification specific scores;
   d) classifying the subject as
      i) A+ or A- according to the comparison result of the classification specific score for A determined in c),
      ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or
      iii) N+ or N- according to the comparison result of the classification specific score for n determined in c); and
   e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

3. The method of item 2, wherein the protein-specific biomarker signature(s) of a) comprise the whole protein level of beta amyloid, phosphorylated Tau and/or total Tau and/or the protein level of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and Total tau.

4. The method item 3, wherein the protein levels are determined via a technique comprising separation of the quaternary structures and/or isoforms according to size, molecular weight or charge.

5. The method of item 4, wherein the technique is an electrophoretic technique.

6. The method of any of the preceding items, wherein the quaternary structures and/or isoforms are separated between about 2 kDa and about 440 kDa.

7. The method of any of the preceding items, wherein the isoform of beta amyloid is Aβ40 and/or Aβ42.

8. The method of any of the preceding items, wherein the quaternary structures of beta amyloid have a molecular weight of about 4 kDa, about 8 kDa, about 12 kDa, about 16 kDa, about 19 kDa, about 24 kDa, about 32 kDa, about 40 kDa, about 44 kDa, about 48 kDa, about 52 kDa, about 56 kDa, about 60 kDa to 72 kDa, about 84 kDa to 120 kDa and/or more than about 140 kDa

9. The method of any one of the preceding items, wherein the quaternary structures of phosphorylated Tau have a molecular weight of about 30 kDa, about 38 kDa, about 55 kDa to 62 kDa, about 96 kDa to 106 kDa, and about 140 to 160 kDa.

10. The method of any one of the preceding items, wherein the quaternary structures of total Tau have a molecular weight of about 30 kDa, about 38 kDa, about 48 kDa, about

55 kDa to 62 kDa, about 96 kDa to 106 kDa, about 140 kDa to 160 kDa and more than 160 kDa.

11. The method of any of the preceding items, wherein determination of the classification specific score comprises

i) the whole protein level of beta amyloid, phosphorylated Tau and/or total Tau;

ii) the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau;

iii) the ratio of whole protein levels of beta amyloid, phosphorylated Tau and/or total Tau;

iv) the ratio of whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau;

v) the ratio of the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau to the whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau; and/or

vi) the ratio of protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of different quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau.

12. The method of any one of the preceding items, wherein determination of the classification specific score comprises application to the protein-specific biomarker signature(s) logistic regression fits, neural networks, ensemble learning, dimensional-reduction techniques and/or Bayesian classifiers.

13. The method of any one of the preceding items, wherein step c) is comparing the classification specific scores to a predetermined cut-off (preferably obtained from the reference cohorts).

14. The method of any one of the preceding items, wherein the nasal secretion sample is obtained from one or both olfactory cleft(s) of the subject.

15. The method of any one of the preceding items, wherein the nasal secretion sample is obtained by

a) placing an absorption material (AM) in the olfactory cleft(s);

b) incubating the AM in the olfactory cleft(s);

c) recovering the AM; and

d) isolating the nasal secretion sample from the AM.

16. The method of any one of the preceding items, wherein the AM is placed in the vicinity of the olfactory cleft.

17. The method of any one of the preceding items, wherein the AM consists of a material selected from the group of synthetic material (e.g. PVA) and/or organic material (cotton).

18. The method of any one of the preceding items, wherein the AM is incubated in the olfactory cleft for about 1 to 60 min.

19. The method of any one of the preceding items, wherein the AM is incubated in the olfactory cleft for 20 min.

20. The method of any of the preceding items, wherein the nasal secretion sample is obtained using nosecollect®.

21. The method of any one of the preceding items, wherein the classification A+/T-/N-, A+/T+/N-, A+/T-/N+ and A+/T+/N+ is indicative for Alzheimer's disease.

22. The method of any one of the preceding items, wherein the classification A-/T+/N-, A-/T-/N+ and A-/T+/N+ is indicative for a suspected non-Alzheimer pathophysiology (SNAP).

23. The method of any one of the preceding items, wherein the classification A-/T-/N- is indicative that the subject does not have Alzheimer's disease.

24. The method of any one of the preceding items, said method further comprising the measurement of further physiological parameters and/or further markers of the neurodegenerative disease.

25. The method of any one of the preceding items, said method further comprising the selection of a treatment of the neurodegenerative disease.

26. The method of any of the preceding items, wherein the protein-specific biomarker signature(s) is/are used to monitor a therapeutic response.

27. A kit comprising the components required for the method of the preceding items.

As mentioned above the present invention provides methods that allow diagnosis of neurodegenerative diseases such as AD and SNAP in nasal secretion samples. Specifically, the described methods allow A/T/N classification in nasal secretion samples. A/T/N classification is a classification system well-known in the art which categorizes individuals based on biomarker evidence of pathology, comprising classification according to "A", the value of a beta amyloid biomarker; "T", the value of a Tau biomarker; and/or "N" biomarkers of neurodegeneration or neuronal injury. Accordingly, the present invention relates to a method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject. More specifically, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject. The skilled person understands that the term

"A/T/N classification in nasal secretion samples" refers to the biomarker classification of an individual person/subject in either + or – based on the A/T/N system determined in a nasal secretion sample obtained from the individual person/subject. An illustration of diagnostic categories based on the ATN system is reported in **Figure 21**.

"*In vitro*" as used herein means that the method steps of the herein described methods are not performed in the nose of the subject but are performed outside of the body of the subject. The terms "subject", "patient" and "individual" may be used synonymously herein and refers to an organism (preferably a human) that is diagnosed with the herein described methods and may be treated according to the diagnosis and/or is selected e.g. for a study according to the diagnosis.

"Diagnosing" is used herein in the broadest sense and refers to the process of determining a disease or condition.

"Predicting" is used herein in the broadest sense and may mean that it can be predicted whether a subject e.g. will in the future be developing a amyloid pathology or describe or predict the overall progression rate as either slow, mid or fast and predict if a subject will benefit from a certain therapeutic treatment regime or could specifically being enrolled in a study.

Preferably, a neurodegenerative disease is diagnosed or predicted herein. In the context of the present invention, the A/T/N classification may be used for the diagnosis and/or prediction of AD, SNAP or the exclusion of AD. Preferably, the neurodegenerative disease is AD or SNAP. All these aspects depend on the protein specific biomarker signature(s) or the classification specific score(s) of a subject/patient.

"A/T/N classification" is a globally accepted system to diagnose AD and initiate appropriate therapeutic consequences (Jack (2016), Neurology, 87(5), 539-547; Grontvedt (2020), J. Alzheimers Dis. 74, 829-837). The A/T/N classification system relates to a biomarker classification, wherein "A" refers to the value of an Aβ biomarker (e.g. Aβ42), "T" refers the value of a Tau pathology biomarker (e.g. pTau) and "N" refers a quantitative or topographic biomarker of neurodegeneration. According to the A/T/N classification the subject is classified in three binary categories A, T and N. That is, according to the A/T/N classification the subject is classified as A+ or A-, T+ or T- and N+ or N-. Herein below it is explained how the subject is

classified as A+ or A-, T+ or T- and N+ or N-. It is pointed out that for some applications it may be sufficient that the subject is only classified in one of the three categories. For example, the subject may be diagnosed with AD when the subject was classified only according to the A category and was classified as A+. In general, the subject suffers from AD when said subject is classified as A+. An illustration of diagnostic categories based on the ATN system is reported in **Figure 21**

The skilled person is well aware how nasal secretion samples can be obtained from a subject. Nasal secretion samples can be obtained by swabbing, brushing, for example by swabbing or brushing the anterior nasal cavity, preferably swabbing or brushing the olfactory cleft mucosa, or nasal lavage/rinsing or biopsy of the olfactory mucosa. However, these techniques have certain disadvantages. Suction may result in loss of sample material, nasal lavage/rinsing may result in an uncontrollable loss of sample material into the nasopharynx and uncontrollable dilution and due to the fact that the actual area of interest, namely the olfactory cleft, only makes up a small part of the nose, washing out the whole nose is yielding in a further dilution of the proteins of interest. Brushing, swapping and smearing is invasive and the brush must be guided. Since the olfactory cleft is in sagittal alignment, without general anesthesia or local anesthesia one cannot reach the olfactory cleft manually, blood admixtures can alternate measurements, as there are organs that produce Aβ42, for example, and therefore measurement can be falsified. Biopsy is invasive and since the area of interest is up to 23 cm^2, a small biopsy of e.g. 1 mm^2 is not displaying the whole pathology in its entirety. Accordingly, the collection of the nasal secretion sample by absorption is preferred. In context of the present invention it is preferred that the nasal secretion samples are obtained from the olfactory cleft, in particular from the olfactory mucosa. The nasal secretion samples may be obtained from one or both olfactory clefts, preferably both olfactory clefts.

No human has a completely symmetrical nose. Accordingly, the olfactory clefts and the olfactory mucosa in both nostrils may have a different size. Accordingly, it is preferred that the nasal secretion samples obtained from both olfactory clefts of a subject are pooled at some point during analysis. Accordingly, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising

A/T/N classification in nasal secretion samples obtained from both olfactory clefts of said subject. Furthermore, the invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from the olfactory mucosa of both olfactory clefts of said subject.

It is preferred that an absorption material is placed in the olfactory cleft preferably on the olfactory mucosa and incubated for a certain amount of time in the olfactory cleft or on the olfactory mucosa. After incubation the absorption material is recovered and the nasal secretion sample is isolated/obtained from the absorption material.

Accordingly, it is envisaged that in the herein described methods the nasal secretion sample is obtained by

a) placing an absorption material in the olfactory cleft(s);
b) incubating the absorption material in the olfactory cleft(s);
c) recovering the absorption material; and
d) isolating the nasal secretion sample from the absorption material.

It is also envisaged that the absorption material is placed in the vicinity of the olfactory cleft.

The absorption material used in the herein described methods is not particularly limited and the skilled person is readily capable of choosing suitable absorption material. Non limiting examples of absorption material used in context of the described methods may be synthetic material such as polyvinyl alcohol (PVA) or organic material such as cotton or a mixture.

It is preferred that the absorption material is in form of a sponge.

It is envisaged that the absorption material is incubated in the olfactory cleft for about 1 to 60 min, such as about 1 min, about 2 min, about 3 min, about 4 min, about 5 min, about 6 min, about 7 min, about 8 min, about 9 min, about 10 min, about 11 min, about 12 min, about 13 min, about 14 min, about 15 min, about 16 min, about 17 min, about 18 min, about 19 min, about 20 min, about 21 min, about 22 min, about 23 min, about 24 min, about 25 min, about

26 min, about 27 min, about 28 min, about 29 min, about 30 min, about 31 min, about 32 min, about 33 min, about 34 min, about 35 min, about 36 min, about 37 min, about 38 min, about 39 min, about 40 min, about 41 min, about 42 min, about 43 min, about 44 min, about 45 min, about 46 min, about 47 min, about 48 min, about 49 min, about 50 min, about 51 min, about 52 min, about 53 min, about 54 min, about 55 min, about 56 min, about 57 min, about 58 min, about 59 min or about 60 min and all values in between such as about 14.7 min. It is preferred in context of the herein described methods that the absorption material is incubated in the olfactory cleft for 20 min.

Accordingly, it is envisaged that in the herein described methods the nasal secretion samples is obtained by

a) placing an absorption material in the olfactory cleft(s);
b) incubating the absorption material in the olfactory cleft(s) for about 1 to 60 min, preferably 20 min;
c) recovering the absorption material; and
d) isolating the nasal secretion sample from the absorption material.

It is envisaged that the nasal secretion sample is obtained using a the proprietarily developed absorbing material (AM) and a medical device to facilitate insertion of the AM at correct position (nosecollect®) as described in WO 2022/101311.

The operator collecting nasal secretion may be usually a psychiatrist or neurologist but may also be a different physician or trained medical personnel. It is preferred that the entire pathology is covered in both nostrils by collecting the sample from the whole olfactory mucosa area of about 23 cm^2. It is preferred that the absorption material (e.g. in form of a sponge) is inserted in the sagittal orientation to cover the whole olfactory cleft in its entirety and that it expands in the caudal direction and not in coronal (laterally) when inserted in the olfactory cleft.

For collection of nasal secretion samples, the absorption material (e.g. polyvinyl alcohol (PVA)) may be either applied with nosecollect® (as described in e.g. WO 2022/101311) or manually into the olfactory cleft on both sides of a subject's nose by trained personnel. In the case of

manual insertion, a Hartmann Nasal Speculum (13 cm, Karl Storz SE & Co. KG, 400500) as well as a Jansen Bayonet Nasal Forceps (16.5 cm, Karl Storz SE & Co. KG, 426516) may be used. A thread attached to the absorption material and hanging out of the nostril may be carefully fixed to the subject's cheek by an adhesive to prevent accidental displacement. The absorption material may be left in place for 20 to 30 minutes (or any other time mentioned herein, preferably 20 min). In case of dry mucosa, the subject may perform physical activity (walking around, climbing stairs), eating or drinking or the nasal mucosa might previously be irrigated with e.g. saline solution to stimulate nasal secretion.

In the following it is exemplarily disclosed how the nasal samples could be obtained and processed:

After incubation in the nose, the fixation of the threads may be loosened on subject's cheek and the absorption material, which is saturated with nasal secretion, may be removed from both sides of the nose by pulling the threads. If the absorption material of one or both nostrils of a test person is completely bloody, it may be collected in separate tubes (Eppendorf 50 ml Protein LoBind Tube, Eppendorf, 0030122240) by the collecting personnel. If the absorption material of both sides is clean or only small blood spots are visible, the saturated absorption material of the second side (including the thread) may be combined with the absorption material from the first side into the same prelabeled tube (Eppendorf 50 ml Protein LoBind Tube, Eppendorf, 0030122240). The tubes with the removed absorption material may be collected upright in a suitable plastic box.

The box with the sample tubes may be held at room temperature (RT) for a maximum of 10 to 20 minutes, preferably 10 minutes. If several samples are taken and it is not guaranteed that no sample is at RT for more than 10 to 20 minutes, preferably 10 minutes, the box with the first samples may be immediately stored at -80°C and new samples are successively added there. A temperature logger (Testo 184 T4, Testo SE & Co. KGaA, 05721844) may be added to the samples to guarantee compliance with the cold chain.

The box, containing the collected nasal secretion samples may be stored at -80°C with the lid facing up until shipment to the analytical laboratory. Storage in a -80°C freezer is preferred, however other conditions like dry ice are also possible. In this case it should be assured that

the complete box is surrounded by dry ice to guarantee equivalent temperature conditions all over the box.

The box containing the collected samples and the temperature logger may be transferred into a Styrofoam box with the lid facing up. The whole Styrofoam box may be filled with dry ice. The sample box should be completely surrounded by the dry ice. Samples can then be shipped from e.g. the clinical site (overnight) to the analytical lab by e.g. a commercial logistics partner. The styrofoam box containing the nasal secretion samples that were collected as described herein may be opened at an analytical lab (preferably shortly after delivery). It may be checked if the collected samples are in good condition. This means, that they were frozen at -80°C during the whole shipment (checked by the status of the remaining dry ice and the report of the included temperature logger) and the integrity of the tubes is fine (unbroken, sealed). The samples may then be stored at the analytical lab in specific racks or boxes in the -80°C freezer until preprocessing. On the day of elution, the samples may be removed from the -80°C freezer and thawed for 30 minutes at RT.

The thawed specimens may be visually inspected for signs of blood under a class II safety cabinet. Absorption material with blood spots may not be eluted together with bloodless absorption material. Therefore, blood spots may be cut out of the absorption material using a disinfected pair of scissors (microscopy scissors, curved, pointed/pointed, VWR, 233-1454) and a forceps (straight, blunt, VWR, 232-2116) before the absorption material from the two sides of the nose (i.e., two nostrils) of one subject are eluted together. The threads may be cut from the PVAs using a disinfected pair of scissors.

For elution, Pierce Centrifuge Columns (10 ml, Thermo Fisher Scientific, PIER89898) may be prepared by removing the silica membrane. The absorption material may be placed into these prepared columns, which may be put back into the original sample tube of the corresponding subject to avoid unnecessary loss of proteins. If the blood spots cannot be removed, the absorption material of the two nostrils may be placed in separate centrifugation columns and tubes, respectively.

The tubes with the centrifugation column containing the absorption material may be centrifuged for 5 minutes at 4566 rcf at room temperature (RT). After that, the centrifuge column and the absorption material may be discarded. The complete volume of the eluted nasal secretion may be transferred from the original Eppendorf 50 ml Protein LoBind tube,

that was used for centrifugation, into a pre-cooled Eppendorf 1.5 ml Protein LoBind tube (Eppendorf, 0030108116) and kept on ice.

The transferred eluates may be centrifuged for 10 minutes at 17000 rcf at RT in a suitable benchtop centrifuge to pellet the solid components for purification. After centrifugation, the tubes may be placed on ice under a class II safety cabinet and the supernatant may be transferred into a new pre-cooled Eppendorf 1.5 ml Protein LoBind tube without pipetting solid or viscous portions. The volume of the transferred eluate may be estimated.

If blood stains could not be removed from the absorption material before centrifugation, the absorption material of the two nose holes from one subject may be eluted separately. In this case, the two eluates from one subject continue to be processed separately. The eluate with the lowest value on the color scale may be analyzed alone or the eluate from both may be mixed for analysis.

The eluates may be evaluated based on the available color scale (1-5) (**Figure 1**). If a sample is rated color scale 5, the sample may not be further processed.

Several working aliquots may be prepared in pre-cooled Eppendorf 1.5 ml Protein LoBind tubes under a class II safety cabinet. Samples and aliquots may be kept on ice during preparation. They may be stored at -80°C for long term storage.

As mentioned above the present invention provides a method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject. It is envisaged that (a) protein-specific biomarker signature(s) for the key analytes is (are) determined in the herein described methods. The term "protein specific biomarker signature(s)" means in the context of the present invention the sum of information/parameters/values obtained from the herein described measurements for a biomarker/analyte/protein and may include the total level of biomarker/analyte/protein, a separation of the biomarker/analyte/protein by molecular weight based on its quaternary structures/three dimensional structures (e.g., monomers and different oligomers) and the level/amount/quantity of the different quaternary structures (e.g., the level/amount/quantity of monomers, the level/amount/quantity of oligomers).

The term "classification specific score" means in the context of the present invention information/parameters/values that is obtained from information/parameters/values of

protein specific biomarker signature(s), e.g. by using mathematical computation. It is envisaged that for each one of the categories A, T, N a classification specific score is determined. By comparing this classification specific score for each one of the categories A, T, N to reference classification specific scores for each category A, T, N (preferably predetermined reference classification specific scores) (see section 4) a correlation to globally accepted A, T, N classification can be done. For example, correlation can be done to predetermined CSF-based A, T, N classification cohorts. Other examples for predetermined reference classification specific scores may be obtained from A, T, N classification cohorts based on the results of PET or by clinical expertise combining different parameters following the NIA -AA framework for ATN classification and the SOPs for the respective measurements. According to the ATN system, each individual is rated for the amount of (i) Aβ42 (in CSF Aβ42 or by amount of amyloid in positron emission tomography-computed tomography (PET-CT): "A"), (ii) hyperphosphorylated Tau (pTau181 in CSF or detection of Tau in Tau PET-CT: "T"), and (iii) neurodegeneration (atrophy on structural magnetic resonance imaging (MRI), fluordeoxyglucose (FDG), PET-CT, or CSF total tau (tTau): "N"). According to the A/T/N classification the subject is classified in three binary categories A, T and N. That is, according to the A/T/N classification the subject is classified as A+ or A-, T+ or T- and N+ or N-. In general, the patient suffers from AD when said patient is classified as A+.

This enables following the NIA-AA framework without the need to perform a highly invasive lumbar puncture or radioactive tracer imaging procedures by using the methods of the prior art. However, as also explained elsewhere herein it is also envisaged that, in some cases, only for one or only for two of the categories A, T, N a classification specific score is determined for the diagnosis and/or prediction.

"Protein-specific biomarker for an analyte" and "protein-specific biomarker of an analyte" may be used synonymously herein.

Preferably the key analytes are (different isoforms of) beta Amyloid (e.g. beta Amyloid 40 (Aβ40) and beta Amyloid 42 (Aβ42) but also pan-Amyloid beta) phosphorylated Tau (pTau; e.g. pTau181 or pTau217) and total Tau (tTau). It is important to note that for each one, i.e. each isoform of beta Amyloid, pTau and tTau more than one protein-specific biomarker signature may be determined in context of the herein described methods. As explained herein

beta Amyloid exists in the organism in different isoforms (e.g. Aβ40 and Aβ42). As also explained herein these different isoforms in turn can form different quaternary structures (oligomers). Accordingly, there is a total level of a certain isoform (i.e. the sum of all quaternary structures of said isoform) and the level of a specific quaternary structures of said isoform. For example, **Figure 3** shows that for Aβ40 e.g. the quaternary structures with a molecular weight of 56 kDa and 97 kDa exist. Said quaternary structure are present in different levels (as evident from different chemiluminescent signals in **Figure 3**).

For example when it is explained herein that the protein-specific biomarker signature(s) for beta Amyloid is (are) determined that means that (a) protein-specific biomarker signature(s) for Aβ40 and/or Aβ42 may be determined. However, in addition a protein-specific biomarker signature for total beta Amyloid (see explanations what total beta Amyloid means herein below) may be determined. Thus, it is envisaged that a protein-specific biomarker signature for total beta Amyloid, a protein-specific biomarker signature for Aβ40 and a protein-specific biomarker signature for Aβ42 is determined. All three protein-specific biomarker signatures may then be used to determine a classification specific score. A protein-specific biomarker signature may comprise different values/parameters (as e.g. exemplified in the Examples). For example, a protein-specific biomarker signature for Aβ42 may comprise as values/parameters the total level of Aβ42 and the levels of all detectable quaternary structures of Aβ42. However, if deemed sufficient for subsequent calculation of the classification specific score the protein-specific biomarker signature for Aβ42 may only comprise as values/parameters the total level of Aβ42 and the levels of one or several specific quaternary structure(s). What is explained here for beta Amyloid is of course also envisaged for (all isoforms of) pTau and tTau. Out of pTau, pTau-181 is preferred but other isoforms might also be preferred, e.g. pTau231 or pTau217.

The term "analyte" as used herein refers to the proteins that may be associated with neurodegenerative diseases and are analyzed in context of the herein described methods for A/T/N classification.

The term "biomarker" as used herein refers to the proteins that may be associated with neurodegenerative diseases and are analyzed in context of the herein described methods for A/T/N classification. In context of the present invention the term "analyte" or "biomarker"

may refer to the overall e.g. beta Amyloid for which one or several protein-specific biomarker signature(s) is (are) determined. In other words, "analyte" or "biomarker" may refer to the sum of several "analytes" or "biomarkers". That means when it is herein referred to that an "analyte" or "biomarker" is analyzed/tested this may mean that one or several protein-specific biomarker signature(s) is (are) determined. Exemplarily when it is herein referred to that the "analyte" or "biomarker" beta Amyloid is analyzed/tested the protein-specific biomarker signatures for e.g. Aβ40 and Aβ42 may be determined. But it may also refer only to one isoform of beta Amyloid (e.g. Aβ42) for which a protein-specific biomarker signature is determined.

The term "analyte" may be used synonymously with "protein" or "biomarker". It is evident for the skilled person that depending on the context "protein" may refer to an analyte or biomarker that is analyzed in the herein described methods. However, "protein" can also refer to e.g. all proteins in a nasal secretion sample (i.e. all proteins in the nasal secretion sample including proteins that are not known to be associated with neurodegenerative diseases).

Accordingly, it is also evident for the skilled person that e.g. the term "(whole) protein level" may refer to e.g. the level of all proteins in a given sample. However, the term "(whole) protein level of beta Amyloid" refers to the protein level of beta Amyloid. The term “(whole) protein level of Aβ42” refers to protein level of beta Amyloid isoform Aβ42. The same applies for each of the other isoforms of the proteins of interest.

It is possible in the herein described methods that beta Amyloid is used for A classification, phosphorylated Tau for T classification and total Tau for N classification. However, it is pointed out that all analytes may be used for all three classifications, such that e.g. parameters of protein-specific biomarker signatures of total Tau are used to determine T classification.

The analytes are analyzed by techniques described herein (e.g. using electrophoretic techniques using different capillary sizes (low, mid, high molecular size)). Each measurement generates (a) signature(s) that is (are) specific to the respective protein and also specific for the individual disease state. The signature(s) is (are) basically made up of a large number of values or parameters. These values or parameters include the total level of analyte, a

separation of the analyte by molecular weight into different quaternary structures (e.g. monomers and oligomers) and the level of the different quaternary structures (e.g. the level of monomers, the level of oligomers). The sum of the information results in (a) biomarker specific signature(s) (referred to in here as protein-specific biomarker signature) for each of the analytes. The protein-specific biomarker signature(s) for e.g. beta amyloid is (are) also referred to herein as beta amyloid specific biomarker signature. Based on a mathematical computation of these protein-specific biomarker signatures (see e.g. Examples section 3. and 5.; e.g. by the ratio of total level of Aβ42 to the total level of Aβ40 in combination with a ratio of the level of specific quaternary structures e.g. of 34 kDa to 56 kDa) a classification specific score for A and or T and or N can be generated. By comparing this classification specific score for each one of the categories A, T, N to the reference classification specific scores (e.g., predefined reference classification specific scores) for each one of the categories A, T, N (see e.g. Examples section 4) a correlation with the world-wide established A/T/N classification following NIA-AA framework can be performed. From said classification the disease can be diagnosed or predicted.

Accordingly, it is envisaged that the herein described methods comprise the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject
   i) the protein-specific biomarker signature(s) of beta amyloid, and/or
   ii) the protein-specific biomarker signature(s) of phosphorylated Tau, and/or
   iii) the protein-specific biomarker signature(s) of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as
   i) A+ or A- according to the comparison result of the classification specific score for A determined in c), and/or
   ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or

iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

Accordingly, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject, wherein the method comprises the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject

i) the protein-specific biomarker signature(s) of beta amyloid, and/or

ii) the protein-specific biomarker signature(s) of phosphorylated Tau, and/or

iii) the protein-specific biomarker signature(s) of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as

i) A+ or A- according to the comparison result of the classification specific score for A determined in c), and/or

ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or

iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

It is envisaged that in the herein described methods for the protein-specific biomarker signature(s) of beta amyloid, Aβ40 and/or Aβ42 are analyzed. In other words, it is envisaged that the isoforms of beta amyloid are Aβ40 and/or Aβ42 and total Aß. It is also envisaged that in the herein described methods for the protein-specific biomarker signature(s) of beta

amyloid, Aβ40, Aβ42 and/or total beta amyloid are analyzed. Total beta amyloid is also referred to pan beta amyloid or pan Aβ. Total beta amyloid may mean that all isoforms are analyzed and the corresponding information provided as the protein-specific biomarker signature for total beta amyloid (also called total beta amyloid specific biomarker signature). This may be done in that an antibody is used that does not distinguish between different isoform of beta amyloid (e.g. as described in section 3.2.3 of the Examples) but detects all isoform e.g. by binding to all isoforms. However, it is pointed out that for total beta amyloid as used herein may also simply mean that not a single isoform but several isoforms are detected that do not necessarily represent all beta amyloid molecules in the sample. For example, it is envisaged that e.g. an antibody detects beta amyloid peptides 1 to 41, 1 to 42 (Aβ42) and 1 to 43 but not beta amyloid peptides 1 to 38, 1 to 39 and 1 to 40 (Aβ40). Accordingly, the resulting protein-specific biomarker signature would comprise information about beta amyloid peptides 1 to 41, 1 to 42 and 1 to 43. This may also be referred to as a total beta amyloid specific biomarker weight signature although it does not necessarily represent all beta amyloid molecules in the sample.

Accordingly, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject, wherein the method comprises the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject
   i) the protein-specific biomarker signature(s) of Aβ40, Aβ42 and/or total beta amyloid,
   ii) the protein-specific biomarker signature(s) of phosphorylated Tau, and/or
   iii) the protein-specific biomarker signature(s) of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as

i) A+ or A- according to the comparison result of the classification specific score for A determined in c),

ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or

iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

As mentioned above the protein-specific biomarker signature(s) may comprise a large number of parameters. These parameters may include the total amount (total level) of an analyte, a separation of the analyte by molecular weight into different quaternary structures (e.g. monomers and oligomers) and the levels within the different quaternary structures (e.g. the level of monomers, the level of oligomers). The terms "level", "amount", "protein level" and "protein amount" may be used synonymously herein. The terms "total level", "total amount", "total protein level", "total protein amount", "whole protein level" and "whole protein amount" may be used synonymously herein. These terms may refer to a concentration or may be denoted as a concentration such as mg/ml or µM. However, they may also be denoted as arbitrary units. For example, it is envisaged herein that the analytes/proteins/biomarker of interest are measured via immunodetection. The analyte may be first contacted with a primary antibody specific for said analyte and after association of said primary antibody with said analyte a secondary antibody may be added specific for the primary antibody. The secondary antibody may be labeled with a moiety generating a detectable signal such as a fluorophore or a molecule generating chemiluminescence. The fluorescence or chemiluminescence may be detected and may be proportional to the amount of the analyte in the sample. The fluorescence or chemiluminescence may be denoted in arbitrary units.

It is also envisaged that the analytes of interest in the sample are separated e.g. via molecular weight prior to association with a primary antibody. In this case the detectable signal may be proportional to e.g. the different quaternary structures of the analyte in the sample.

The term "quaternary structure" is used herein in the broadest sense. A protein/polypeptide may have a primary structure, secondary structure, tertiary structure and quaternary structure. The quaternary structure of a protein is the association of several protein chains or polypeptide chains into a (closely packed) arrangement. For example, two proteins/polypeptides/oligopeptides may form a dimer and three proteins/polypeptides/oligopeptides may form a trimer. It is pointed out that when herein it is referred to quaternary structure also monomers (i.e. single proteins or polypeptide chains) are included. Thus, quaternary structures as used herein refer to monomers and oligomers comprising two up to several thousand proteins/polypeptides. Quaternary structures may be distinguished by the number of protein chains or polypeptide chains comprised in the quaternary structure or by molecular weight (e.g. kDa) of the quaternary structure (e.g. as determined by running behavior in e.g. an electrophoretic technique). When it is referred to the Aβ42 56 kDa quaternary structure it is referred to a quaternary structure/oligomer that e.g. shows a molecular weight of about 56 kDa for example as determined via an electrophoretic technique. However, it is evident for the skilled person that said quaternary structure can also be referred to as 56 kDa Aβ42 quaternary structure, 56 kDa Aβ42 oligomer or Aβ42 56 kDa oligomer. Depending on the technique, device, buffer etc. the observed molecular weight in kDa may vary. It is envisaged that the observed molecular weight may vary up to 10 %. Accordingly, the term "about" as used herein may mean that depicted value may vary +/- 10 %.

It is also envisaged that in the herein described methods the protein-specific biomarker signature(s) comprise(s) the protein level of isoforms of beta amyloid, phosphorylated Tau (pTau) and total Tau (tTau).

Isoforms of beta amyloid may have any length of the beta amyloid peptide 1 to 38, 1 to 39, 1 to 40, 1 to 41, 1 to 42, 1 to 43, or any N-terminally truncated isoforms x to 38, x to 39, x to 40, x to 41, x to 42, x to 43, wherein "x" is representative for any of the N-terminally truncated position, e.g. any position between amino acids positions 1 to 30, preferably 1 to 10, most preferably positions 2, 3 or 4.

Isoforms of pTau are any phosphorylated version of Tau, including but not limited to pTau181, pTau202, pTau205, pTau217 pTau231, pTau199, pTau18, pTau396 and/or pTau422, preferably pTau181 and/or pTau231. Isoforms of tTau include variants created by alternative splicing or proteolytic processing (fragments), this includes but is not limited to alternative splice forms 2N4R, 2N3R, 1N4R, 1N3R, 0N4R, 0N3R and/or fragments 1 to 314, 187 to 441, 1 to 255, 1 to 368, 151 to 421, 45 to 230, 243 to 441. Accordingly, in the herein described methods the protein-specific biomarker signature(s) of a) may comprise the whole protein level of beta amyloid, pTau and/or tTau and/or the protein level of quaternary structures and/or isoforms of beta amyloid, pTau and tTau.

Accordingly, it is envisaged that the herein described methods comprise the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject
   i) the protein-specific biomarker signature(s) of beta amyloid comprising the whole protein level of beta amyloid and/or the protein level of quaternary structures and/or isoforms of beta amyloid, and/or
   ii) the protein-specific biomarker signature(s) of phosphorylated Tau comprising the whole protein level of phosphorylated Tau and/or the protein level of quaternary structures and/or isoforms of phosphorylated Tau, and/or
   iii) the protein-specific biomarker signature(s) of the whole protein level of total Tau and/or the protein level of quaternary structures and/or isoforms of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as
   i) A+ or A- according to the comparison result of the classification specific score for A determined in c), and/ or
   ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or

iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

Accordingly, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject, wherein the method comprises the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject

i) the protein-specific biomarker signature(s) of beta amyloid comprising the whole protein level of beta amyloid and/or the protein level of quaternary structures and/or isoforms of beta amyloid, and/or

ii) the protein-specific biomarker signature(s) of phosphorylated Tau comprising the whole protein level of phosphorylated Tau and/or the protein level of quaternary structures and/or isoforms of phosphorylated Tau, and/or

iii) the protein-specific biomarker signature(s) of the whole protein level of total Tau and/or the protein level of quaternary structures and/or isoforms of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as

i) A+ or A- according to the comparison result of the classification specific score for A determined in c), and/or

ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or

iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

The protein-specific biomarker signature(s) of beta Amyloid may comprise the whole protein level of total amyloid beta and/or the protein level of quaternary structures of total amyloid beta and/or the whole protein level of isoforms of amyloid beta and/or the protein level of quaternary structures of isoforms of amyloid beta.
The protein-specific biomarker signature(s) of tTau may comprise the whole protein level of total tTau and/or the protein level of quaternary structures of total tTau and/or the whole protein level of isoforms of tTau and/or the protein level of quaternary structures of isoforms of tTau.
The protein-specific biomarker signature(s) of pTau may comprise the whole protein level of total pTau and/or the protein level of quaternary structures of total pTau and/or the whole protein level of isoforms of pTau and/or the protein level of quaternary structures of isoforms of pTau.

As mentioned, the herein described methods may comprise that the protein levels are determined via a technique comprising separation of the quaternary structures and/or isoforms according to size, molecular weight or charge. For example, any form of gel electrophoresis, isoelectric focusing, size exclusion chromatography, or gel filtration chromatography.

It is also envisaged that the herein described methods comprise an electrophoretic technique. Accordingly, it is envisaged that the protein levels are determined via an electrophoretic technique. Accordingly, it is envisaged that the protein levels are determined via an electrophoretic technique comprising separation of the quaternary structures and/or isoforms.

Accordingly, the present invention relates to an *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal

secretion samples obtained from said subject, wherein the method comprises the following steps (a) to (e):

a) determining in a nasal secretion sample obtained from the subject
   i) the protein-specific biomarker signature(s) of beta amyloid comprising the whole protein level of beta amyloid and/or the protein level of quaternary structures and/or isoforms of beta amyloid, and/or
   ii) the protein-specific biomarker signature(s) of phosphorylated Tau comprising the whole protein level of phosphorylated Tau and/or the protein level of quaternary structures and/or isoforms of phosphorylated Tau, and/or
   iii) the protein-specific biomarker signature(s) of the whole protein level of total Tau and/or the protein level of quaternary structures and/or isoforms of total Tau;

b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;

c) comparing the classification specific scores determined in b) to predetermined classification specific scores;

d) classifying the subject as
   i) A+ or A- according to the comparison result of the classification specific score for A determined in c), and/or
   ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or
   iii) N+ or N- according to the comparison result of the classification specific score for N determined in c); and

e) diagnosing or predicting the neurodegenerative disease based on the classification of d),

wherein the protein levels are determined via an electrophoretic technique.

It is envisaged that the quaternary structures and/or isoforms may be separated between about 2 kDa and about 440 kDa. In other words, the technique comprising separation of the quaternary structures and/or isoforms may separate the quaternary structures and/or isoforms between a molecular weight of between about 2 kDa and about 440 kDa, such as

about 2 to about 40 kDa, about 12 to about 230 kDa or about 66 to about 440 kDa, preferably about 2 to about 40 kDa, about 12 to about 230 kDa.

It is envisaged that a defined total protein content of the nasal secretion sample is applied to the technique comprising separation of the quaternary structures to allow normalization and comparability of different individual samples. The total protein content of the nasal secretion eluate obtained as described herein may be measured by BCA Assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, 10678484). One sample aliquot may be taken from the -80°C freezer and equilibrated to RT on the bench for approximately 30 minutes. The total protein assay should be performed according to the manufacturer's protocol and the concentration may be calculated in mg/ml. These results may be further used for the normalization of the total protein concentration in e.g. gel electrophoresis in following measurements.

To determine a specific biomarker signature a corresponding sample may be run on an automated protein separation and immunodetection system (such as Simple Western™ Jess, BioTechne, 004-650) or on traditional gel electrophoresis and Western Blot. An aliquot of a nasal secretion sample eluate may be thawed at room temperature for 30 minutes. An appropriate amount of volume may taken of the sample so that the final total protein concentration in the assay may be about 0.25 mg/ml, about 0.5 mg/ml, about 0.75 mg/ml, about 1.0 mg/ml, about 1.25 mg/ml, about 1.5 mg/ml, about 1.75 mg/ml or about 2.0 mg/ml. Preferably, the final total protein concentration in the assay is about 1.0 mg/ml.
The sample may be diluted in sample buffer provided by the manufacturer such as BioTechne for the Simple Western™ Jess system. The diluted sample may be loaded into the Simple Western™ Jess system and may be started and run according to the manufacturer's instructions as single plex assay. Capillaries with different gel percentages suitable for low- (2 to 40 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W012), mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004), or high- (66 to 440 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W008) molecular weight separation may be used.

For detection of Aβ40 the following primary antibodies may be used: Human Amyloid beta (aa1-40) Antibody, BioTechne/R&D, MAB96181-100 and/or purified (azide-free) anti-β-Amyloid, 1-40 (11A50-B10), BioLegend, 805409. Both antibodies are specific to the C-terminus of Aβ40. An enzyme-linked secondary antibody (such as Anti-Mouse Detection Module, BioTechne, DM-003) may then be added followed by a chemiluminescent substrate. The Aβ40 biomarker signature may be measured, displaying information on the total level of the analyte and the level of different molecular weight (quaternary structures) of Aβ40 molecules within the same sample. This allows to simultaneously detect mono- and oligomeric Aβ40 molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other. **Figures 2 and 4** illustrate exemplary banding patterns of Aβ40 that may be measured in nasal secretion from patients with different clinical conditions. Each antibody-epitope recognition is represented by a chemiluminescent signal, thus higher signal intensities represent more antibody-epitope recognitions and thus represent higher levels of detected quaternary structures or overall levels of analyte. By calculating the area under the curve (AUC) the amount of antibody-epitope recognition may be measured and, thus, the level of a given quaternary structure or the total level of the protein. A graphical representation of the AUC, calculated from the chemiluminescent signal of the bound antibodies for Aβ40 may be generated. Oligomers of approximately 100 kDa, 56 kDa and 32 kDa, 19 kDa, 16 kDa, 12 kDa, and 8 kDa may be detected, as well as monomers at approximately 4 kDa. The signal of the Aβ40 56 kDa oligomer band may be higher in patients without AD (corresponding to a A-classification) in comparison to patients with clear AD (classified as A+). Without necessarily being bound by scientific theory it is believed that insoluble Aβ42 aggregates in the Alzheimer's patients sequester Aβ40 molecules leading to a decrease of detected Aβ40. The detected healthy monomeric species may be higher in Patients without AD (A-) relative to patients with AD (A+).

For detection of Aβ42 the following primary antibodies may be used: Purified (azide-free) anti-β-Amyloid, 1-42 (12F4), BioLegend, 805501 and/or Anti Amyloid β42(43), Monoclonal Antibody (BC05), Fujifilm Wako, 010-26903. Both antibodies are specific to Aβ42 and may recognize one or several isoforms and oligomeric structures. An enzyme-linked secondary antibody (such as Anti-Mouse Detection Module, BioTechne, DM-003) may then be added

followed by a chemiluminescent substrate. The biomarker signature may be generated, displaying information on the total level of the analyte and the level of different molecular weight of Aβ42 molecules (quaternary structures) within the same sample. This allows to simultaneously detect mono- and oligomeric Aβ42 molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other (**Figure 6**). There may be an overall decrease in the detected total level of Aβ42 (calculated by the total area under the curve for the whole protein-specific biomarker signature) in an AD patient (classified as A+), relative to the total level of an A- individual. Also band ratios between the 56 and 32 kDa oligomers may be altered.

For detection of total Aβ the following primary antibody may be used: primary antibody 6E10 (Purified anti-β-Amyloid, 1-16 Antibody (6E10), BioLegend, 803001) recognizes all forms of Aβ. An enzyme-linked secondary antibody (such as Anti-Mouse Detection Module, BioTechne, DM-003) may then be added followed by a chemiluminescent substrate. The pan Aβ specific biomarker signature may be generated, displaying information on the total level of the analyte, as well as the level of different molecular weight of pan Aβ molecules (quaternary structures) within the same sample. This allows to simultaneously detect mono- and oligomeric Aβ molecules and quantify them separately for each individual molecular weight, in total and in relation to each other (**Figure 7**). The pan-Aβ antibody binds to an epitope that is more N-terminal than the species-specific antibodies described above. This allows for recognition of different quaternary structures compared to the antibodies specific for Aβ40 or Aβ42. It is envisaged that in A+ vs. A- individuals the ratio of the 45 and 32 kDa bands, the total levels, and/or the levels of higher oligomeric structures such as the 183 kDa band are altered. It is further envisaged that these alterations can be used for classification e.g. A+ vs A-.

For detection of tTau the following primary antibodies may be used: Purified anti-Tau, 404-441 Antibody (mouse), BioLegend, 806601 and/ or Tau Monoclonal Antibody (HT7), Biotin (mouse), Thermo Fisher Scientific, MN1000B. Both are specific to tTau and recognize one or several isoforms and oligomeric structures. The enzyme-linked secondary antibody (Anti-Mouse Detection Module, BioTechne, DM-003) may then be added and followed by a

chemiluminescent substrate. The tTau specific biomarker signature may be generated, displaying information on the total level of the analyte, as well as the level of quaternary structures or isoforms of tTau molecules within the same sample. This allows to simultaneously detect mono- and oligomeric tTau molecules and quantify them separately for each individual molecular weight, in total and/ or in relation to each other (**Figure 8**). The detected level of canonical isoform of tTau, seen at approximately 55 kDa may be higher in patients with clear signs for neurodegeneration (classified as N+) than in patients without signs for neurodegeneration (classified as N-). Different other quaternary structures may be measured as well, e.g. total Tau dimers. The ratio of tTau dimers to monomers may also be used to compute the classification specific score for N classification.

For detection of pTau the following primary antibody may be used: Phospho-Tau (Thr181) (D9F4G) Rabbit mAb, Cell Signaling, 12885S is specific to pTau-181 and recognizes one or several isoforms and oligomeric structures. An enzyme-linked secondary antibody (Anti-Rabbit Detection Module BioTechne, DM-001) may be added followed by a chemiluminescent substrate. The pTau specific biomarker signature may be measured, displaying information on the total level of the analyte and the level of quaternary structures with different molecular weight within the same sample. This allows to simultaneously detect mono- and oligomeric pTau-181 molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other (**Figure 9**). The total level of detected pTau may be decreased in patients with clear Tau pathology (T+) compared to patients without Tau pathology (T-). A decreased level of monomers, present at approximately 56 kDa as well as decreased levels of dimers and trimers with apparent molecular of 96 and 139 kDa, respectively, may be seen in patients with clear Tau pathology (T+).

The quaternary structures of beta amyloid may have a molecular weight of about 4 kDa, about 8 kDa, about 12 kDa, about 16 kDa, 19 kDa, about 24 kDa, about 32 kDa, about 40 kDa, about 44 kDa, about 48 kDa, about 52 kDa, about 56 kDa, about 60 to 72 kDa, about 84 to 120 kDa and/or more than about 140 kDa. Thus, it is envisaged that quaternary structures of beta amyloid having the above-mentioned molecular weights are comprised in the protein-specific biomarker signature for beta amyloid.

The quaternary structures of pTau may have a molecular weight of about 30 kDa, about 38 kDa, about 55 to 62 kDa, about 96 to 106 kDa, and about 140 to 160 kDa. Thus, it is envisaged that quaternary structures of pTau having the above mentioned molecular weights are comprised in the protein-specific biomarker signature(s) for phosphorylated Tau.

The quaternary structures of tTau may have a molecular weight of about 30 kDa, about 38 kDa, about 48 kDa, about 55 to 62 kDa, about 96 to 106 kDa, about 140 to 160 kDa and more than 160 kDa. Thus, it is envisaged that quaternary structures of tTau having the above mentioned molecular weights are comprised in the protein-specific biomarker signature(s) for tTau.

It is envisaged that in the herein described methods the classification specific score is compared to a predetermined classification specific score. Based on the comparison result the subject may be classified according to the A/T/N classification. The predetermined classification specific scores may also be referred to as reference classification specific scores. In order to generate a reference for each classification specific score for the categorization A+, A-, T+, T-, N+, N-, nasal secretion samples from patients who also underwent a standard examination known in the art, such as CSF examination, PET scans or clinical expertise, may be collected using the herein described collection, storage, transport, elution and measurement procedure in a separate investigation. The nasal secretion samples of these patients may be classified into the categories A+, A-, T+, T-, N+, N- according to their standard examination (e.g., CSF, PET or clinical diagnosis) results on the basis of standard cut-offs for the respective measurement platform and the assay used following NIA-AA guidelines for A/T/N classification. More specifically, each individual may be rated for the amount of (i) Aβ42 (in CSF Aβ42 or the ratio of Aβ42 to Aβ40, or by amount of amyloid burden detected in positron emission tomography-computed tomography (PET-CT): "A"), (ii) hyperphosphorylated Tau (the amount of pTau181 in CSF or detection of Tau in Tau PET-CT: "T"), and (iii) neurodegeneration (atrophy on structural magnetic resonance imaging (MRI), fluordeoxyglucose (FDG), PET-CT, or CSF total tau (tTau): “N”). According to the A/T/N classification the subject is classified in three binary categories A, T and N. That is, according

to the A/T/N classification the subject is classified as A+ or A-, T+ or T- and N+ or N-. In general, the patient suffers from AD when said patient is classified as A+. Subsequently, protein-specific biomarker signatures may be generated in the nasal secretion for each protein and each classification category individually following the herein described measurement procedure. On the basis of these protein-specific biomarker signatures, reference classification scores may be generated for the categories A+, A-, T+, T-, N+, N-, thus representing the full range of A+ (T+, T-, N+, N-) indicative for a patient with AD pathology, as well as A- (T+, T-, N+, N-) indicative for a patient with SNAP, and A- (T-, N-) indicative that the subject has no AD. These reference classification specific scores may be used as a reference for future classification specific scores. The determined classification specific scores of a nasal secretion sample of a subject can then be compared to said reference classification specific scores to classify the subject as A+, A-, T+, T-, N+, or N-, respectively. In the present invention, it is preferred that the subject or patient used to generate the reference classification specific scores is a is cognitively impaired individual, more preferably reference classification specific score is generated by comparing cognitively impaired individuals which have been classified as having a neurodegenerative disease such as AD (A+) to cognitively impaired individuals classified as non-AD (A-).

To generate the A/T/N classification score for the standard examination analysis (e.g., CSF, PET, clinical diagnosis) from the corresponding subjects for generation of reference classification specific scores, the corresponding samples may be collected at the clinical site. For example, to generate the A/T/N classification score for the CSF analysis from the corresponding subjects for generation of reference classification specific scores, the CSF may be collected at the clinical site by lumbar puncture, preferably in the morning and may be performed between the 3rd to 4th or 4th to 5th lumbar vertebrae. It may be collected in a polypropylene collection tube, discarding the first 20 drops, following international guidelines. Too much empty space in the tube should be avoided. Within the next 4 hours after collection, the CSF may be centrifuged for 10 minutes at approximately 2000 rcf, at room temperature (RT) and transferred into a new polypropylene collection tube. 1500 µl of the transferred CSF may be aliquoted into a separate polypropylene collection tube. CSF samples and aliquots may be stored in a suitable box at -80°C within 4 hours after collection. The box containing the CSF

aliquots and a temperature logger may be transferred into a Styrofoam box with the lid facing up. The whole Styrofoam box may be filled with dry ice. The sample box may be surrounded by the dry ice. Samples may then be shipped (overnight) to the analytical lab e.g. by a commercial logistics partner. The styrofoam box may be opened at the analytical lab shortly after delivery. It may be checked if the collected samples were in good condition. This means, that they should be frozen at -80°C during the whole shipment (checked by the status of the remaining dry ice and the report of the included temperature logger) and the integrity of the tubes should be fine (unbroken, sealed). The samples may then be stored at the analytical lab in specific racks or boxes in the -80°C freezer until preprocessing. The CSF aliquot may be thawed, and measurement may be performed in an ELISA (e.g. Fuji Rebio Lumipulse) according to the manufacturer's protocol. Then, according to the cut-off values described in Table 1, an individual may be assigned an ATN status. Individuals with an A+ status are classified as having AD (**Figure 10**). A/T/N classification may also be performed by imaging. A status is determined by amyloid PET (Minoshima, 2016). T status may be identified by using a Tau tracer (see e.g. as disclosed at https://pi.lilly.com/us/tauvid-uspi.pdf) for Tau PET. Finally, the use of anatomic MR (Mikulis, 2007; https://onlinelibrary.wiley.com/doi/pdf/10.1002/jmri.21041) or FDG PET (Guedj, 2022; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC8803744/) can be used to determine N status.

As described herein nasal secretion samples may be subjected to techniques comprising separation of the quaternary structures and/or isoforms of the relevant analytes/biomarkers according to size, molecular weight or charge. It is envisaged that said techniques produce raw data that are processed further to generate a protein-specific biomarker signature.

After the determination of the different raw data for the biomarkers (Aβ42, Aβ40, pTau, tTau) in a given sample by e.g. a molecular weight separation device, the raw data collected by this device may be processed further. If necessary, the generated data is exported to an easily readable format (such as .csv, .xml, or .xlsx) using software provided by e.g. the manufacturer. For example, the BioTechne Compass software is used to export spectra and plate layout information from run files generated by the BioTechne JESS system. The exported data files may be processed using software referred to as "Run Processing SW" from now on. This

software may be written in Python but could also be written in other programming languages or make use of platforms such as Matlab or Mathematica. The "Run Processing SW" calculates, from the raw data, the values of the individual measurements that constitute the protein-specific biomarker signature, including, but not limited to, the total signal from the analyte (measured as the area under the curve, or AUC), and the signals from individual bands corresponding to specific biomarker/analyte quaternary structures or isoforms. It may also perform further processing, including (but not limited to) evaluating calibrator data to quantify the signal from samples in terms of concentration or absolute amount of protein.
The "Run Processing SW" may generate plots allowing a visual inspection of the results. The "Run Processing SW" may export the calculated values to other formats, including Excel .xlsx. However, the calculated values may also be exported into a database, to .csv files, or to other formats. Results from the same sample from multiple measurements, runs and/or multiple devices may be aggregated by a different software module, referred to as "Sample Processing SW" from now on. The "Sample Processing SW" may use the combination of protein-specific biomarker signatures (i.e., the calculated values of the individual measurements) to calculate classification specific scores that allow to classify the sample according to the dimensions A, T, and N using a classification algorithm developed and calibrated using reference classification scores that may have been determined as described herein. The "Sample Processing SW" may also generate a report in a format like .xlsx that provides an overview of the pertinent measured and calculated values for a sample, as well as the classification specific scores and resulting classification (**Figure 11**).

In the following the determination of a protein-specific biomarker signature is described when a technique is used that comprises separation of the quaternary structures and/or isoforms of the relevant analytes/biomarkers according to molecular weight and immunoassay detection. The run of the corresponding device results, for each sample (corresponding to one patient) and each analyte/biomarker, in a spectrum that gives an intensity (corresponding to the concentration of bound antibodies) as a function of the molecular weight. The "Run Processing SW" may read the spectra generated by the device (exported into a readable format using manufacturer software if necessary). If the device or its manufacturer software provides a way to apply baseline correction to the spectra, the corrected spectra may be used.

Otherwise, the "Run Processing SW" may apply a baseline correction algorithm following one of the common algorithms described in the literature. The "Run Processing SW" may calculate the total intensity in the spectrum by numerically integrating the intensity over the molecular weight in the quantifiable range - i.e., calculating the area under the curve (AUC) with the correct weighting. It may also calculate the contribution to the signal from individual bands or groups of bands by applying a fit algorithm that fits a set of peaks (whose approximate positions and widths are known beforehand and configured in the "Run Processing SW") to the spectrum. The integral over these isolated contributions gives the intensity of the corresponding band (see **Figure 12**).

The result for each sample and run is a set of measurements for one analyte, which is called the protein-specific biomarker signature and which includes, but is not limited to, the total intensity and the intensity from each of the preconfigured bands in the respective molecular weight/size of the analyte. The protein-specific biomarker signature can be visualized in various ways, e.g., as a polar plot (see **Figure 13**).

The biomarker/analyte measurements may be calibrated or not calibrated. It is envisaged herein that the biomarker/analyte measurements may be calibrated for improved quantification of the protein-specific biomarker signature. The skilled person understands that calibration could mean that the samples will be subjected to normalization. Calibrators may be used for two purposes: Firstly, to quantify the detected signal in terms of the total amount of the analyte contained in the sample or in terms of concentration of the analyte in nasal secretion, and secondly to eliminate the influence of external factors such as ambient temperature from the results of runs, by putting the results of the samples in relation to results from calibrators with a well-defined amount of analyte. Runs of the size separation device, or another measurement device, can include one or more calibrators. Each calibrator may contain a known amount of the analyte, with a constant distribution of monomers and/ or oligomers as far as possible. If a set of calibrators is included, the "Run Processing SW" may calculate the total signal from each of the calibrators using the same methods applied to samples. If a single calibrator is used, the software may calibrate amounts of analyte in patient samples by putting their measured intensities in relation to that of the calibrator. If more than

one calibrator is used, the "Run Processing SW" may use the calculated signals and the known amounts to fit a calibration curve (using a 4-parameter logistic function or other appropriate function), which represents a mapping from the total intensity to the amount of analyte. This mapping may be used to calculate the amount of analyte contained in each sample.

Each measurement done on a sample (possibly using different antibodies, different capillary sizes, different ranges of molecular weights, different devices) yields a set of measured and calculated values representing the relative or absolute amounts of a given protein and its quaternary structures, which is referred to as protein-specific biomarker signature. The relative amount may refer to the band intensities (i.e. the area under the curve, which can be calculated by mathematical means and which is a function of the band intensities) and the width of the bands for the herein described biomarkers as determined by via (electrophoretic) techniques described herein.

The sample may be normalized before or during the measurement, e.g. by determining the whole protein amount per ml of the sample and loading a predefined amount of protein or by measuring whole protein within the same run.

The determined band intensities may also be normalized before obtaining the protein-specific biomarker signature. For example, the relative amount may refer to e.g. band intensities from tTau 30 kDa normalized to total Tau levels. The totality of all such measurements for the different proteins can be combined into the ATN biomarker signature of the sample. For each dimension A, T, and N, a scoring algorithm may calculate a number – the classification specific score – from a suitable subset of the values in the protein-specific biomarker signatures; by comparing this classification specific score to e.g. a cutoff, a classification for each dimension can be made (see **Figures 14 and 15**). The measurements that are relevant may be identified from the results of a study that includes a sufficient number of samples from patients with various cognitive impairments (also including patients with only subjective cognitive impairment but without objective cognitive impairment based on typical psychometric measurement means like MMST) with an A/T/N classification based on CSF measurements and/or other diagnostic tools such as PET screening, plasma measurement or clinical diagnosis, as well as age-matched cognitively unimpaired controls. These reference scores are used as a ground truth to display the internationally accepted A/T/N classification system in

nasal secretion samples that normally is based on CSF examination or amyloid PET or by expert consensus.

The results of the same study may also be used to develop a scoring algorithm which maps the relevant subset of the values from the ATN biomarker signature onto a single number for each one of the dimensions A, T, and N (see herein below for more explanation and examples), and to select cutoffs for each dimension that determine whether to classify the sample as "+" or "-", wherein "+" is equivalent to a positive finding of a pathology (e.g. A+ would display a patient with amyloid pathology) and "-" is equivalent to a negative finding, so no pathology being present is the patient sample.

Approaches and tools used in the scoring algorithm to determine the classification specific score and subsequent classification for each category A, T and/or N range from simple to more elaborate (if needed to achieve sufficient specificity). Simple approaches include, but are not limited to:

Application of cut-offs to individual analyte measurements; for example:

A status: a cut-off is applied to the total amount of Aβ42 or total intensity of Aβ42 to determine A status, preferably a cut-off is applied to the relative total amount of Aβ42 or total intensity of Aβ42 and then compared to the previously determined reference cut off to determine A status (**Figure 16**).

N status: a cut-off is applied to the total amount of tTau or total intensity of tTau to determine N status, preferably a cut-off is applied to the relative total amount of tTau or total intensity of tTau and then compared to the previously determined reference cut off for tTau to determine N status (**Figure 17**).

Application of cut-offs to the ratio of total amounts of different analytes; for example:

A status: a threshold is applied to the ratio of the total intensity of Aβ42 to the total intensity of Aβ40 determine A status, preferably a threshold is applied to the relative ratio of the total intensity of Aβ42 to the total intensity of Aβ40 and then compared to previously determined reference cut offs for the ratio of the two relative total amounts to determine A status (**Figure 18**).

Application of cut-offs to the ratio between a band of one analyte to another band of the same analyte, or to the total amount of that same analyte; for example:

A status: a cut-off is applied to the ratio of the sum of the intensities of the Aβ40 bands at 52 and 56 kDa to the intensity of the Aβ40 bands at 62kD to determine A status, preferably a cut-off is applied to the ratio of the sum of the intensities of the Aβ40 bands at 52 and 56 kDa to the intensity of the Aβ40 bands at 62kD and then compared to previously determined cut offs for this ratio to determine A status (**Figure 19**).

Application of cut-offs to the ratio between measurements from one band of one analyte, to the total quantity or specific bands of a different analyte; for example:

T status: a cut-off is applied to the ratio between pTau and tTau 57 kDa bands to determine T status (**Figure 20**).

If application of cut-offs to individual measurements or their ratios does not give satisfactory sensitivity and specificity, multiple measurements may be combined to calculate a classification specific score by methods (mathematical computation) including, but not limited to, the following:

- Logistic regression fits of all total intensities and band-specific intensities, or their ratios, or a subset of them.
- Application of neural networks (including deep learning and support vector machines) to the measurements.
- Ensemble learning: such as the application of dimensional-reduction techniques such as UMAP, t-SNE, or Principal Component Analysis to identify relevant dimensions.
- Bayesian Classifiers.

When it is explained herein that multiple measurements may be combined to calculate a classification specific score this may mean that information/values/parameters of several protein-specific biomarker signatures are combined (e.g. by mathematical computation). That means that to information/values/parameters of several protein-specific biomarker signatures Logistic regression fits, neural networks, Ensemble learning (application of dimensional-reduction techniques such as UMAP, t-SNE, or Principal Component Analysis to identify relevant dimensions) and/ Bayesian Classifiers may be applied.

For classification of e.g. the A status, multiple logistic regression may be used to determine the classification specific scores. The T and N status can be also determined by multiple logistic

regression. In other words, protein-specific biomarker signatures may be inputted into multiple logistic regression. For example, (relative) band intensities from tTau 30 kDa, normalized to total Tau levels, pan Aβ 60-72 kDa, normalized to total pan Aβ levels and/or Aβ42 56 kDa may be inputted into a multiple logistic regression to calculate the classification specific score and the cut-off may be 0.5. This cut off then will be compared to the reference cut off previously determined and related to this cut off, the patient will be determined as either "+" positive for the respective pathology or "-" negative.

In another example, (relative) band intensities (i.e., via specific calculation of the area under the curve) from tTau 30 kDa, normalized to total Tau levels, Aβ42 48 kDa and/or Aβ42 56 kDa may be inputted into a multiple logistic regression and the cut-off may be 0.5. However, it is evident for the skilled person that also other protein-specific biomarker signatures may be inputted into multiple logistic regression. For example, individual molecular weight species levels or total levels from pTau may be used in combination with either Aβ42, Aβ40, pan Aβ and/or tTau. Any combination of individual molecular weight bands or relative amount of bands (e.g. percentage of total analyte) from pTau, Aβ42, Aβ40, pan Aβ and/or tTau may be put into a logistic regression.

The skilled person is well-aware how multiple logistic regression may be applied. For example, Prism software may be used. In the Prism software the default parameters may be used (Prism 9.5.1; user manual: https://www.graphpad.com/guides/prism/latest/user-guide/index.htm).

Different scoring methods may turn out optimal for each of the classification dimensions A, T, and N. For example, N may be decided by a simple threshold on the measured amount of tTau, whereas A may be decided by a combination of ratios between different peaks of Aβ42 and Aβ40.

The methods and parameters to calculate the optimal classification specific score derived from comparison with CSF measurements may be implemented in the "Sample Processing SW". The "Sample Processing SW" may collect the measurement results from all runs that apply to a given sample from one patient, assemble the ATN biomarker signatures, and apply the scoring mechanism. The resulting classification specific scores determine the classification along the A, T, N dimensions.

The "Run Processing SW" and "Sample Processing SW" may be used as command-line tools that process run data and generate sample reports on demand. They may also be incorporated in an automated setup, where new data from runs of automated measurement devices is processed as soon as it becomes available, and sample reports comprising the classification results referring the patient as either A+, A-, T+, T-, N+, N- are generated as soon as the necessary measurements for that sample are complete.

As described herein above certain measured or calculated values of the protein-specific biomarker signature may be used to determine the classification specific score. When it is referred to herein that the determination classification specific score comprises e.g. a protein level this may mean that said protein level is used to determine the classification specific score. It is envisaged that in the herein described methods determination of the classification specific score comprises

i) the whole protein level of beta amyloid, phosphorylated Tau and/or total Tau;

ii) the protein level(s) of quaternary structures and/or isoforms of beta amyloid, pTau and/or tTau;

iii) the ratio of whole protein levels of beta amyloid, pTau and/or tTau;

iv) the ratio of whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of quaternary structures and/or isoforms of beta amyloid, pTau and/or tTau;

v) whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of quaternary structures; and/or

vi) the ratio of protein level(s) of quaternary structures and/or isoforms of beta amyloid, pTau and/or tTau to the protein level(s) of different quaternary structures and/or isoforms of beta amyloid, pTau and/or tTau.

It is evident that when it is referred to a ratio a to b it is also referred to a ratio of b to a (i.e. when it is referred to a/b this also includes b/a). For example, when it is referred to the ratio of a whole protein level to the protein level of a quaternary structures this also includes the ratio of the protein level of a quaternary structure to a whole protein level.

It is evident for the skilled person that when the above described values i) to v) are used for the determination of the classification specific score the corresponding protein-specific

biomarker signature comprises said values or corresponding information that allow calculation of said values.

As mentioned above, more elaborate approaches and tools may be used to determine the classification specific score. That means that more elaborate approaches and tools may be applied to values of protein-specific biomarker signatures. Accordingly, it is envisaged that in the herein described methods determination of the classification specific score comprises application to the protein-specific biomarker signatures logistic regression fits, neural networks, ensemble learning, dimensional-reduction techniques and/or Bayesian classifiers.

As mentioned herein, it is envisaged that the classification specific score is compared to a predetermined classification specific score or a reference classification specific score. The predetermined classification specific score or reference classification specific score may be a cut-off. Thus, it is envisaged that the classification specific score is compared to a predetermined cut-off or reference cut-off. It is described herein how a predetermined classification specific score, a reference classification specific score, a predetermined cut-off or reference cut-off is generated. In order to generate a predetermined classification specific score, a reference classification specific score, a predetermined cut-off or reference cut-off for each classification specific score for the categorization A+, A-, T+, T-, N+, or N-, nasal secretion samples from patients who also underwent CSF examinations may be collected using the herein described collection, storage, transport, elution and measurement procedure in a separate investigation. For example, **Figure 16** shows the total intensity of Aβ42 as detected in nasal secretion samples (corresponding to the total level of Aβ42) in patients that were classified as A+ or A- according to CSF analysis. From the comparison a cut-off may be determined which may be 11. When in nasal secretion samples of a subject an Aβ42 intensity below 11 is measured said subject may be classified as A+. Accordingly, step c) of the herein described methods may be comparing the classification specific scores to a predetermined cut-off.

When all categories of the subject have been classified said subject can be diagnosed.

The classification A+/T-/N-, A+/T+/N-, A+/T-/N+ and A+/T+/N+ is indicative for AD.

The classification A-/T+/N-, A-/T-/N+ and A-/T+/N+ is indicative for a suspected non-Alzheimer pathophysiology (SNAP).

The classification A-/T-/N- is indicative that the subject does not have AD.

It is evident for the skilled person that further physiological parameters and/or further markers of the neurodegenerative disease are measured in the herein described methods. Thus, it is envisaged that the herein described methods may further comprise the measurement of further physiological parameters and/or further markers of the neurodegenerative disease.

Other physiological parameters and/or further markers of the neurodegenerative disease may be included to further streamline the diagnosis and support the physician's decision. Additional parameters could include, but are not limited to, age, cognitive performance measured by psychometrical means (e.g. Mini Mental Status Test (MMST), Montreal-Cognitive-Assessement-Test (MoCa-Test) risk factors like genetic predisposition, sleeping disorders, Neurofilament light (NFL), glial fibrillary acidic protein (GFAP), Alpha-Synuclein, APOE4 status, protein S100b, neurogranin, Strem2, Interleukin, Ubiquitin, Immunoglobulins, imaging parameters like reduced brain volume, vascular pathologies, enlarged cerebrospinal fluid spaces or the general consumption of drugs like smoking and alcohol.

It is envisaged that based on the diagnosis and/or prediction of the herein described methods a treatment for the neurodegenerative disease is selected.

Accordingly, the herein described methods may further comprise the selection of a treatment of the neurodegenerative disease.

Treatments can include, but are not limited to, anti-amyloid based antibody drug therapies like Lecanemab. Lecanemab (Leqembi®) for example is approved in the United States for the

treatment of AD to be initiated in early AD (mild cognitive impairment [MCI] due to AD or mild AD dementia) with confirmed brain amyloid pathology. Treatment subjects should have amyloid pathology as demonstrated by amyloid positron emission tomography (PET) or cerebrospinal fluid (CSF) tests indicative of AD. Reliable and approved nasal secretion-based classification score for A, displaying normal or abnormal biomarker profiles - may become fully validated soon and could be thus considered adequate to identify appropriate subjects for the treatment. Since Lecanemab binds to amyloid oligomers, protofibrils and insoluble fibrils it would be thus highly suitable to be able to detect and quantify all relevant quarternary structures within a subjects´s sample as it could be done accordingly to our biomarker specific signature and classification score. Also the confirmation of efficacy of anti-amyloid drugs like Lecanemab in the clinical setting could be feasible since the change in the rate of decline is relatively subtle but a change in the longitudinally collected specific biomarker signatures could be detected reliably and thus indicate the efficacy of a selected treatment, also supporting dose adaptations and thus reduce side effects.

It is also envisaged that the herein described methods are used to monitor a therapeutic response. Accordingly, the protein-specific biomarker signatures of the herein described methods may be used to monitor a therapeutic response. For example, if a patient is per it's diagnostic result selected as qualifying for e.g. a given anti-amyloid treatment such as Lacanemab, one would expect to initially measure a change in the total levels of Aβ42 and a change in specific corresponding quaternary bands and a subsequently change in the levels of pTau and tTau as well as a change in specific quaternary structures of said proteins. Another possibility could be that a patient's profile does not change but, on the contrary, remains the same over time, which can also be seen as a therapeutic success and can indicate a steady state, i.e. the progression of the disease could be significantly delayed or even stopped.

The invention also provides a kit comprising the components required for the herein described methods. The kit may consist of an easy-to-handle nasal secretion collection tool such as nosecollect® one for each side of the patient's nose, each including a highly specific proprietary absorption material (AM) to safely and consistently collect nasal secretion from the vicinity of the brain-nose interface. The kit may also comprise a suitable transportation

tube, to ensure proper and regulated transport from the patient to the lab without losing or altering the biomarkers of interest. This tool is designed for medical trained personnel and offers the option to collect high quality specimen in daily clinical routine.

The Figures show:

Figure 1: Color Scale to assess blood content of the nasal secretion eluate.

Figure 2: Aβ40: oligomers can be detected at 56 kDa and 100 kDa by Simple Western™ Jess using an Aβ40-specific antibody from R and D.

Figure 3: Aβ40: An example of a classification of CSF-verified A- and A+ individuals by the 56 kDa band. A+ subjects have less of the 56kDa Aβ40 oligomer than A- subjects using an Aβ40-specific antibody from R and D.

Figure 4: Aβ40: Lower molecular weight oligomers and monomers can be detected by Simple Western™ Jess using an Aβ40-specific antibody from R and D. A- subjects have a higher amount of healthy monomeric Aβ-40.

Figure 5: Aβ40: A- subjects have more healthy monomers than A+ as shown in this example plot from two subjects using an Aβ40-specific antibody from R and D.

Figure 6: Aβ42: A+ have decreased total levels of Aβ42 and an altered ratio of oligomeric bands at 56 and 32 kDa as exemplified by one A+ and one A- subject using an Aβ42-specific antibody from Fujifilm Wako.

Figure 7: pan-Aβ antibody recognizes multiple oligomeric Aβ structures in nasal secretion using the 6E10 antibody.

Figure 8: detected tTau levels are increased in N+ relative to N- as shown in this example of an N+ and an N- subject using the Tau specific antibody from Biolegend.

Figure 9: pTau181 levels are decreased in T+ relative to T- as demonstrated in this example of one T+ and one T- patient sample using the pTau specific antibody from Cell Signaling.

Figure 10: ATN classification scheme (figure taken from Jack (2018), Alzheimers. Dement., 14(4), 535-562)

Figure 11: Data flow of a possible embodiment of the system. Other embodiments are possible. A nasal secretion sample is analysed using different capillary systems of Simple Western™ Jess (low, mid, high molecular weight capillary systems) and different analyte specific antibodies. A protein-specific biomarker signature for each analyte is generated. Different values from i) the same protein-specific biomarker signature or ii) from different protein-specific biomarker signatures are further processed by the sample processing software and a classification specific score is generated for each category A, T and/or N. This score is then compared to a reference classification specific score for each category A, T and/or N and then further referred to as either + or - (pathological result or non pathological result) for each category A, T and or N.

Figure 12: The measured intensity (top panel) is approximated by a set of preconfigured bands, which can consist of one or more Gaussian peaks (dotted lines in the lower panels, shown in order of descending area under the curve). Crosses indicate the maxima of each band.

Figure 13: Results of the fitting procedure can be visualized for illustration purposes, e.g., in a polar plot, where each direction represents one of the bands, and the distance from the center represents the area of the peak (i.e., the amount of analyte in that sample)

Figure 14: Illustration of the steps that lead from the experiments to the ATN biomarker signature and to the classification.

Figure 15: Identifying inputs for the classification algorithms: some examples of measurements that show a promising distinction on a small reference sample set.

Figure 16: Example for using the total intensity (Area under the curve) from one analyte as a criterion for a cutoff: the A status of patients was confirmed using CSF analysis. In nasal secretion, the total intensity from Aβ42 was measured. Values suggest a possible cutoff of 11; measurements above the cutoff may be classified as A-.

Figure 17: Example for using the total intensity from one analyte as a criterion for a cutoff: the N status of patients was confirmed using CSF analysis. In nasal secretion, the total intensity from tTau was measured. Values suggest a possible cutoff of 2.7; measurements above the cutoff may be classified as N+.

Figure 18: Example for using a ratio between different analytes as a classification criterion for a cutoff: the diagnosis of A+ was confirmed by pathological CSF. In nasal secretion, the ratio between total intensity for Aβ42 to total intensity for Aβ40 was calculated. Values suggest a cutoff of 0.18; measurements above the cutoff may be classified as A-.

Figure 19: Example for using ratios between bands from the same protein/analyte as a criterion for classification: the A status of patients was confirmed using CSF analysis. In nasal secretion, the ratio between the sum of intensities from Aβ40 bands at 52 and 56 kDa to the intensity from the 62 kDa band was calculated. Values suggest a cutoff of 0.35; measurements above the cutoff may be classified as A+.

Figure 20: Example for using ratios between bands from different proteins as a criterion for classification: the T status of patients was confirmed using CSF analysis. In nasal secretion, the ratio between the total intensity of pTau and 57 kDa band of tTau was calculated. Values suggest a cutoff of 20; values below the cutoff may be classified as T+.

Figure 21: Illustration of diagnostic categories based on ATN system.

Figure 22: Discrimination of A+ and A- by inputting protein-specific biomarker signatures from tTau, total Aβ and Aβ42 into multiple logistic regression results in an AUC of 0.94 (p= 0.0009, n=20). Following run analysis, relative band intensities from tTau 30 kDa, normalized to total Tau levels, pan-Aβ 60-72 kDa, normalized to total pan-Aβ levels and Aβ42 56 kDa were inputted into a multiple logistic regression (n=20). The ROC curve shows the specificity and sensitivity of the assay at a classification specific score cutoff, in this example it is 0.5.

Figure 23: Discrimination of A+ and A- by inputting protein-specific biomarker signatures from tTau and Aβ42 into multiple logistic regression results in an AUC of 0.89 (p= 0.0002, n=30). Following run analysis, relative band intensities from tTau 30 kDa, normalized to total Tau levels, Aβ42 48 kDa and Aβ42 56 kDa were inputted into a multiple logistic regression (n=30).

The ROC curve shows the specificity and sensitivity of the assay at a classification specific score cutoff, in this example it is 0.5.

Figure 24: Classification specific scores of the multiple logistic regression of Figure 22. An observed value of 0 represents an amyloid negative (A-) pathology, an observed value of 1 represents an amyloid positive (A+) pathology, as determined by CSF.

Figure 25: Classification specific scores of the multiple logistic regression of Figure 23. An observed value of 0 represents an amyloid negative (A-) pathology, an observed value of 1 represents an amyloid positive (A+) pathology, as determined by CSF.

Figure 26: Classification specific scores of the multiple logistic regression of Figure 22.
Shown are the parameters of the discrimination when a cut-off of 0.5 is used. An n=20 was used, with three parameters, resulting in a significant p-value of .0009, a negative predictive power of 90.0% and a positive predicitve power of 100%.

Figure 27: Classification specific scores of the multiple logistic regression of Figure 23.
Shown are the parameters of the discrimination when a cut-off of 0.5 is used. An n=30 was used, with three parameters, resulting in a significant p-value of .0002, a negative predictive power of 86.7% and a positive predicitve power of 86.7%.

The following Examples illustrate the invention

1. Collection of nasal secretion

1.1 Application and removal of absorption material for nasal secretion

Samples were collected at four different clinical sites. Therefore, absorption material (AM) polyvinyl alcohol (PVA) was either applied with nosecollect® or manually applied into the olfactory cleft on both sides of a subject's nose (N=2) by trained personnel. In the case of manual insertion, a Hartmann Nasal Speculum (13 cm, Karl Storz SE & Co. KG, 400500) as well as a Jansen Bayonet Nasal Forceps (16.5 cm, Karl Storz SE & Co. KG, 426516) were used. The thread attached to the AM and hanging out of the nostril was carefully fixed to the subject's cheek by an adhesive to prevent accidental displacement. The AM was left in place for 20 to 30 minutes. In case of dry mucosa, the subject performed physical activity (walking around, climbing stairs), eating or drinking to stimulate nasal secretion. After incubation in the nose, the fixation of the threads was loosened on subject's cheek and the AM, which was saturated with nasal secretion, was removed from both sides of the nose by pulling the threads. If one or both AMs of a test person were completely bloody, they were collected in separate tubes (Eppendorf 50 ml Protein LoBind Tube, Eppendorf, 0030122240) by the collecting personnel. If the AM of both sides was clean or only small blood spots were visible, the saturated AM of the second side (including the thread) was combined with the AM from the first side into the same prelabeled tube (Eppendorf 50 ml Protein LoBind Tube, Eppendorf, 0030122240).

1.2 Nasal secretion storage

The tubes with the removed AM were collected upright in a suitable plastic box. The box with the sample tubes was held at room temperature (RT) for a maximum of 10 minutes. If several samples were taken and it was not guaranteed that no sample is at RT for more than 10 minutes, the box with the first samples was immediately stored

at -80°C and new samples were successively added there. A temperature logger (Testo 184 T4, Testo SE & Co. KGaA, 05721844) was added to the samples to guarantee compliance with the cold chain.

The box, containing the collected nasal secretion samples was stored at -80°C with the lid facing up until shipment to the analytical laboratory. Storage in a -80°C freezer was preferred, however other conditions like dry ice were also possible. In this case it was assured that the complete box was surrounded by dry ice to guarantee equivalent temperature conditions all over the box.

1.3 Nasal secretion transport

The box containing the collected samples and the temperature logger was transferred into a Styrofoam box with the lid facing up. The whole Styrofoam box was filled with dry ice. The sample box was completely surrounded by the dry ice. Samples were then shipped over night to the analytical lab by a commercial logistics partner.

2. Sample handling and pre-analytics

2.1 Sample receipt, inspection, and storage

The styrofoam box was opened at the analytical lab shortly after delivery. It was checked if the collected samples were in good condition. This means, that they were frozen at -80°C during the whole shipment (checked by the status of the remaining dry ice and the report of the included temperature logger) and the integrity of the tubes was fine (unbroken, sealed).

The samples were then stored at the analytical lab in specific racks or boxes in the -80°C freezer until preprocessing.

2.2 Elution of nasal secretion

On the day of elution, the samples were removed from the -80°C freezer and thawed for 30 minutes at RT. The thawed specimens were visually inspected for signs of blood under a class II safety cabinet. AM with blood spots was never eluted together with bloodless AM. Therefore, blood spots were cut out of the AM using a disinfected pair of scissors (microscopy scissors, curved, pointed/pointed, VWR, 233-1454) and a forceps (straight, blunt, VWR, 232-2116) before the two AMs from one subject were eluted together. The threads were cut from the PVAs using a disinfected pair of scissors. For elution, Pierce Centrifuge Columns (10 ml, Thermo Fisher Scientifc, PIER89898) were prepared by removing the silica membrane. Both AMs were placed into these prepared columns, which were put back into the original sample tube of the corresponding subject. If the blood spots cannot be removed, the two AMs were placed in separate centrifugation columns and tubes, respectively. The tubes with the centrifugation column containing the AMs were centrifuged for 5 minutes at 4566 rcf at room temperature (RT). After that, the centrifuge column and the AMs were discarded. The complete volume of the eluted nasal secretion was transferred from the original Eppendorf 50 ml Protein LoBind tube, that was used for centrifugation, into a pre-cooled Eppendorf 1.5 ml Protein LoBind tube (Eppendorf, 0030108116) and kept on ice. The transferred eluates were centrifuged for 10 minutes at 17000 rcf at RT in a suitable benchtop centrifuge to pellet the solid components. After centrifugation, the tubes were placed on ice under a class II safety cabinet and the supernatant was transferred into a new pre-cooled Eppendorf 1.5 ml Protein LoBind tube without pipetting solid or viscous portions. The volume of the transferred eluate was estimated. If blood stains could not be removed from the AM before centrifugation, the two AMs from one subject were eluted separately. In this case, the two eluates from one subject continue to be processed separately. The eluates were evaluated based on the available color scale (1-5) (**Figure 1**). If a sample was rated color scale 5, the sample was not further processed.

2.3 Preparation of eluate aliquots

Several working aliquots were prepared in pre-cooled Eppendorf 1.5 ml Protein LoBind tubes under a class II safety cabinet. Samples and aliquots were kept on ice during preparation. They were stored at -80°C for long term storage

3. Analytics of nasal secretion samples

3.1 Measurement of whole protein content

The total protein content of the nasal secretion eluate was measured by BCA Assay (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific, 10678484). One sample aliquot was taken from the -80°C freezer and equilibrated to RT on the bench for approximately 30 minutes. The total protein assay was performed according to the manufacturer's protocol and the concentration was calculated in mg/ml. These results were further used for the normalization of the total protein concentration in gel electrophoresis in the following measurements.

3.2 Determination of protein specific biomarker signature for beta Amyloid and Tau

The following steps describe the determination of the protein specific biomarker signature for the key analytes: beta Amyloid 40 (Aβ40), beta Amyloid 42 (Aβ42), phosphorylated Tau and total Tau. The analysis was carried out using one or two antibodies against each analyte and using different capillary sizes (low, mid, high molecular size). Each measurement generates a signature that is specific to the respective protein and also specific for the individual disease state. The signature is basically made up of a large number of parameters. These parameters include the total level of analyte, a separation of the analyte by molecular weight based on its quaternary structures (e.g., monomers and different oligomers) and the level of the different quaternary structures (e.g., the level of monomers, the level of oligomers). The sum of the information results in a biomarker specific signature for each of the analytes and for each disease state (e.g., A- or A+). Based on a mathematical

computation of these protein specific biomarker signatures (see section 55; e.g. by the ratio of total level of Aβ42 to the total level of Aβ40 in combination with a ratio of the level of specific quaternary structures e.g. of 34 kDa to 56 kDa) a classification specific score for A and or T and or N can be generated. By comparing this classification specific score for each of the categories A, T, N to reference classification specific scores for each category A, T, N (see section 4) a correlation with the established CSF-based A, T, N classification can be done.

3.2.1 Determination of Aβ40 specific biomarker signature

An aliquot of nasal secretion was used to determine the Aβ40 specific biomarker signature. The sample was run on an automated protein separation and immunodetection system (Simple Western™ Jess, BioTechne, 004-650) and on traditional gel electrophoresis and Western Blot (see protocol below). The aliquot was thawed at room temperature for 30 minutes. An appropriate amount of volume was taken of the sample so that the final total protein concentration in the assay was 1.25 mg/ml. The sample was diluted in sample buffer provided by the manufacturer. The diluted sample was loaded into the Simple Western™ Jess system and was started and run according to the manufacturer's instructions as single plex assay. Capillaries with gel percentages suitable for mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004). Primary antibody #1 (Human Amyloid beta (aa1-40) Antibody, BioTechne/R&D, MAB96181-100) was used and is specific to the C-terminus of Aβ40. An enzyme-linked secondary antibody (Anti-Mouse Detection Module, BioTechne, DM-003) is then added followed by a chemiluminescent substrate. The Aβ40 biomarker signature was measured, displaying information on the total level of the analyte and the level of different molecular weight sizes (quaternary structure) of Aβ40 molecules within the same sample. As shown in **Figures 2 and 4**, this allowed to simultaneously detect mono- and oligomeric Aβ40 molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other. **Figures 2 and 4** illustrate exemplary banding patterns of Aβ40 that can be measured in nasal secretion from patients with different clinical conditions. Each lane

represents an individual patient. Along the y-axis the molecular weight is displayed. Larger Aβ40 quaternary structures, e.g. oligomers that have formed from aggregated monomers in the brain are not separated into monomers by SDS and, thus, have higher molecular weight. Each antibody-epitope recognition is represented by a chemiluminescent signal, thus higher signal intensities represent more antibody-epitope recognitions and thus represent higher amount of detected quaternary structures or overall levels of analyte. By calculating the area under the curve (AUC) the amount of antibody-epitope recognition can be measured and, thus, the level of a given quaternary structure or the total level of the protein. A graphical representation of the AUC, calculated from the chemiluminescent signal of the bound antibodies for Aß40 can be seen in **Figures 3 and 6** (for more information see Protein Simple – Jess user manual). Oligomers of approximately 100 kDa, 56 kDa and 32 kDa, and 19 kDa can be detected, as well as monomers at approximately 4 kDa. As can be seen, for example, in **Figure 3** the 56 kDa oligomer band shows a higher signal in patients without AD (corresponding to a A- classification) in comparison to patients with clear AD (classified as A+). Each curve represents a single patient. The relative decrease in the 56 kDa band in patients with AD(A+) could be explained by the accumulation of Aβ40 in insoluble Aβ42 aggregates. It is known that insoluble Aβ42 aggregates in the AD patients sequester Aβ40 molecules leading to a decrease of detected Aβ-40. **Figures 4** shows an increase in the detected monomeric species (see* monomers **Figure 4**) in Patients without AD(A-) relative to patients with AD(A+). **Figure 5** shows the biomarker signature for Aβ40 from a patient with AD (A+) compared to a patient without AD (A-) which also demonstrates that higher levels of Aβ40 monomer at 4 kDa can be detected.

3.2.2 Determination of Aβ42 specific biomarker signature

An aliquot of nasal secretion was used for the determination of the Aβ42 specific biomarker signature. The sample was run on an automated protein separation and immunodetection system (Simple Western™ Jess, BioTechne, 004-650) and on traditional gel electrophoresis and Western Blot. The protocol described below refers to the automated system. The aliquot was thawed at room temperature for 30

minutes. An appropriate amount of volume was taken of the sample so that the final total protein concentration in the assay was 1.25 mg/ml. The sample was diluted in sample buffer provided by the manufacturer. The diluted sample was loaded into the Simple Western™ Jess system and the system was started and run according to the manufacturer's instructions as single plex assay. Capillaries with gel percentages suitable for mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004) molecular weight separation was used. Primary antibody #6 (Anti Amyloid β42(43), Monoclonal Antibody (BC05), Fujifilm Wako, 010-26903) was used and is specific to Aβ42 and may recognize one or several isoforms and oligomeric structures. An enzyme-linked secondary antibody (Anti-Mouse Detection Module, BioTechne, DM-003) is then added followed by a chemiluminescent substrate. The Aβ42 specific biomarker signature is generated, displaying information on the total amount of the analyte and the amount within different molecular weight sizes of Aβ42 molecules (quaternary structures) within the same sample. As shown in **Figure 6**, this allowed to simultaneously detect mono- and oligomeric Aβ42 molecules and quantify them separately for each individual size, in total and/or in relation to each other. **Figure 6** demonstrates two Aβ42 specific biomarker signatures from a patient with AD (A+) in comparison to a patient without AD (A-). The two samples, being run on a mid-molecular weight gel, show that i) the total amount/level of Aβ42 and ii) different (oligomeric) quaternary structures and their corresponding level can be measured. As demonstrated here, there is an overall decrease in the detected total level of Aβ42 (calculated by the total area under the curve for the whole biomarker signature) in an AD patient (classified as A+), relative to the total level of an A- individual. Also the two patients show altered band ratios between the 56 and 32 kDa oligomers.

3.2.3 Determination of the Total Aβ specific biomarker signature

An aliquot of nasal secretion was used for determination of the total Aβ specific biomarker signature. The sample was run on an automated protein separation and immunodetection system (Simple Western™ Jess, BioTechne, 004-650) and on traditional gel electrophoresis and Western Blot. The protocol described below refers to the automated system. The aliquot was thawed at room temperature for 30 minutes. An appropriate amount of volume was taken of the sample so that the final total protein concentration in the assay was 1.25 mg/ml. The sample was diluted in sample buffer provided by the manufacturer. The diluted sample was loaded into the Simple Western™ Jess system and the system was started and run according to the manufacturer's instructions as single plex assay. Capillaries with different gel percentages suitable for mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004) molecular weight separation was used. Primary antibody 6E10 (Purified anti-β-Amyloid, 1-16 Antibody (6E10), BioLegend, 803001) recognizes all forms of Aβ. An enzyme-linked secondary antibody (Anti-Mouse Detection Module, BioTechne, DM-003) is then added followed by a chemiluminescent substrate. The pan Aβ specific biomarker signature is generated, displaying information on the total level of the analyte, as well as the level of different molecular weight sizes of pan Aβ molecules (quaternary structures) within the same sample. As shown in **Figure 7**, this allows to simultaneously detect mono- and oligomeric Aβ molecules and quantify them separately for each individual size, in total and in relation to each other. The pan-Aβ antibodies bind to an epitope that is more N-terminal than the species-specific antibodies described above. This allowed for recognition of different quaternary structures compared to the antibodies used in 3.2.1 and 3.2.2 above. **Figure 7** shows the pan-Aβ specific biomarker signature of a patient without Alzheimer's disease. It is envisaged that in A+ vs A- individuals the ratio of the 45 and 32 kDa bands, the total levels, and/or the levels of higher oligomeric structures such as the 183 kDa band are altered. It is further envisaged that these alterations can be used for classification e.g. A+ vs A-.

3.2.4 Determination of total Tau (tTau) specific biomarker signature

An aliquot of nasal secretion was used for determination of the tTau specific biomarker signature. The sample was run on an automated protein separation and immunodetection system (Simple Western™ Jess, BioTechne, 004-650) and on traditional gel electrophoresis and Western Blot. The protocol described below refers to the automated system. The aliquot was thawed at room temperature for 30 minutes. An appropriate amount of volume was taken of the sample so that the final total protein concentration in the assay was 1.25 mg/ml. The sample was diluted in a sample buffer provided by the manufacturer. The diluted sample was loaded into the Simple Western™ Jess system and the system was started and run according to the manufacturer's instructions as single plex assay. Capillaries with different gel percentages suitable for low- (2 to 40 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W012), mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004), or high- (66 to 440 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W008) molecular weight separation were used. Primary antibody #7 (Purified anti-Tau, 404-441 Antibody (mouse), BioLegend, 806601) was used, and is specific to tTau and recognize one or several isoforms and oligomeric structures. An enzyme-linked secondary antibody (Anti-Mouse Detection Module, BioTechne, DM-003) is then added followed by a chemiluminescent substrate. The tTau specific biomarker signature is generated, displaying information on the total level of the analyte, as well as the level of quaternary structures with different molecular weight within the same sample. As shown in **Figure 8**, this allowed to simultaneously detect mono- and oligomeric tTau molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other. The canonical isoform of tTau, seen at approximately 55 kDa is detected at a higher level in patients with clear signs for neurodegeneration (classified as N+) than in patients without signs for neurodegeneration (classified as N-) (**Figure 8**). Different other quaternary structures can be measured as well, e.g. total Tau dimers. The ratio of tTau dimers to monomers also can be used to compute the classification specific score for N classification (**Figure 8**).

3.2.5 Determination of pTau-181 specific biomarker signature

An aliquot of nasal secretion was used to determine the pTau-181 specific biomarker signature. The sample was run on an automated protein separation and immunodetection system (Simple Western™ Jess, BioTechne, 004-650) and on traditional gel electrophoresis and Western Blot. The protocol described below refers to the automated system. The aliquot was thawed at room temperature for 30 minutes. An appropriate amount of volume was taken of the sample so that the final total protein concentration in the assay was 1.25 mg/ml. The sample was diluted in sample buffer provided by the manufacturer. The diluted sample was loaded into the Simple Western™ Jess system and the system was started and run according to the manufacturer's instructions as single plex assay. Capillaries with different gel percentages suitable for low- (2 to 40 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W012), mid- (12 to 230 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W004), or high- (66 to 440 kDa Separation Module, 8 x 25 Capillary Cartridges, BioTechne, SM-W008) molecular weight separation were used. Primary antibody #8 (Phospho-Tau (Thr181) (D9F4G) Rabbit mAb, Cell Signaling, 12885S) is specific to pTau-181 and recognizes one or several isoforms and oligomeric structures. An enzyme-linked secondary (Anti-Rabbit Detection Module, BioTechne, DM-001) is then added followed by a chemiluminescent substrate. The pTau-181 specific biomarker signature is measured, displaying information on the total level of the analyte and the level of quaternary structures with different molecular weight within the same sample. As shown in **Figure 9**, this allowed us to simultaneously detect mono- and oligomeric pTau-181 molecules and quantify them separately for each individual molecular weight, in total and/or in relation to each other. As shown in **Figure 9**, the total level of pTau-181 can be calculated by the area under the curve (AUC). As shown here, the total level of detected pTau is decreased in patients with clear Tau pathology (T+) compared to patients without Tau pathology (T-). Also shown here, a decreased level of monomers, present at approximately 56 kDa as well as decreased levels of dimers and trimers with apparent

molecular sizes at 96 and 139 kDa, respectively can be seen in patients with clear tau pathology (T+).

4. Generation of ATN reference data

4.1. Generation of reference classification specific scores for A and/or T and/or N according to their CSF results

In order to generate a reference for each classification specific score for the categorization A+, A-, T+, T-, N+, N-, nasal secretion samples from patients who also underwent CSF examinations were collected using the above-described collection, storage, transport, elution and measurement procedure in a separate investigation. The nasal secretion samples of these patients were classified into the categories A+, A-, T+, T-, N+, N- according to their CSF results based on standard cut-offs for the respective measurement platform and the assay used following NIA-AA guidelines for ATN classification. Subsequently, protein-specific biomarker signatures were generated in the nasal secretion for each protein and each classification category individually following the above-described measurement procedure. On the basis of these protein-specific biomarker signatures, reference classification scores were generated for the categories A+, A-, T+, T-, N+, N-, thus representing the full range of A+(T+, T-, N+, N-) indicative for a patient with AD pathology, as well as A-(T+,T-, N+, N-) indicative for a patient with SNAP, and A-(T-, N-) indicative for a cognitive impairment that is not related to AD. These reference classification specific scores will be used as a reference for future classification specific scores.

4.1 CSF collection

To generate the A, T, N classification score for the CSF analysis from the corresponding patients for generation of reference classification specific scores, the CSF was collected at the clinical site by lumbar puncture, preferably in the morning and was performed between the 3rd to 4th or 4th to 5th lumbar vertebrae. It was collected in a

polypropylene collection tube, discarding the first 20 drops, following international guidelines. Too much empty space in the tube was avoided. Within the next 4 hours after collection, the CSF was centrifuged for 10 minutes at approximately 2000 rcf, at room temperature (RT) and transferred into a new polypropylene collection tube. 1500 µl of the transferred CSF were aliquoted into a separate polypropylene collection tube. CSF samples and aliquots were stored in a suitable box at -80°C within 4 hours after collection.

4.2 CSF specimen transport

The box containing the CSF aliquots and a temperature logger was transferred into a Styrofoam box with the lid facing up. The whole Styrofoam box was filled with dry ice. The sample box was completely surrounded by the dry ice. Samples are then shipped over night to the analytical lab by a commercial logistics partner.

4.3 Sample handling and pre-analytics

4.3.1 Sample receipt, inspection, and storage

The styrofoam box was opened at the analytical lab shortly after delivery. It was checked if the collected samples were in good condition. This means, that they were frozen at -80°C during the whole shipment (checked by the status of the remaining dry ice and the report of the included temperature logger) and the integrity of the tubes was fine (unbroken, sealed). The samples were then stored at the analytical lab in specific racks or boxes in the -80°C freezer until preprocessing.

4.3.2 Sample measurement

The CSF aliquot was thawed, and measurement was performed in an ELISA (e.g. Fuji Rebio Lumipulse) according to the manufacturer's protocol. Then, according to the cut-

off values described in Table 1, an individual was assigned an ATN status. Individuals with an A+ status were classified as having Alzheimer's pathology (**Figure 10**).

5. Data Processing

5.1 Overview

After the determination of the different raw data for the biomarkers (Aβ42, Aβ40, pTau-181, tTau) in a given sample by e.g. a size separation device, the raw data collected by this device has to be processed further. If necessary, the generated data is exported to an easily readable format (such as .csv, .xml, or .xlsx) using software provided by that manufacturer. For example, the BioTechne Compass software is used to export spectra and plate layout information from run files generated by the BioTechne JESS system. The exported data files are processed using softwarereferred to as "Run Processing SW" from now on. This software is written in Python but could also be written in other programming languages or make use of platforms such as Matlab or Mathematica. The ""Run Processing SW"" calculates, from the raw data, the values that constitute the protein-specific biomarker signature, including, but not limited to, the total signal from the analyte (measured as the area under the curve, or AUC), and the signals from individual bands corresponding to specific biomarker quaternary structures or isoforms. It also performs further processing, including (but not limited to) evaluating calibrator data to quantify the signal from samples in terms of concentration or absolute amount of protein.

The "Run Processing SW" generates plots allowing a visual inspection of the results. The "Run Processing SW" exports the calculated values to other formats, including Excel .xlsx. (The calculated values may also be exported into a database, to .csv files, or to other formats.) Results from the same sample from multiple measurements, runs and/or multiple devices are aggregated by a different software module, referred to as ""Sample Processing SW"" from now on. The "Sample Processing SW" uses the combination of protein-specific biomarker signatures (i.e., the calculated values of the individual measurements) to calculate classification specific scores that allow to

classify the sample according to the dimensions A, T, and N using a classification algorithm developed and calibrated using reference classification scores that were determined in a separate investigation (see section 4). The "Sample Processing SW" also generates a report in a format like .xlsx that provides an overview of the pertinent measured and calculated values for a sample, as well as the classification specific scores and resulting classification (**Figure 11**).

5.2 Determination of protein-specific biomarker signatures

The run of a molecular weight separation device results, for each sample (corresponding to one patient) and each analyte, in a spectrum that gives an intensity (corresponding to the concentration of bound antibodies) as a function of the molecular weight. The "Run Processing SW" reads the spectra generated by the device (exported into a readable format using manufacturer software if necessary). If the device or its manufacturer software provides a way to apply baseline correction to the spectra, the corrected spectra are used. Otherwise, the "Run Processing SW" applies a baseline correction algorithm following one of the common algorithms described in the literature. The "Run Processing SW" calculates the total intensity in the spectrum by numerically integrating the intensity over the molecular weight in the quantifiable range - i.e., calculating the area under the curve (AUC) with the correct weighting. It also calculates the contribution to the signal from individual bands or groups of bands by applying a fit algorithm that fits a set of peaks (whose approximate positions and widths are known beforehand and configured in the “Run Processing SW”) to the spectrum. The integral over these isolated contributions gives the intensity of the corresponding band (see **Figure 12**).

The result for each sample and run is a set of measurements for one analyte, which we call the protein-specific biomarker signature and which includes, but is not limited to, the total intensity and the intensity from each of the preconfigured bands. The protein-specific biomarker signature can be visualized in various ways, e.g., as a polar plot (see **Figure 13**).

5.3 Calibration of Biomarker Measurements for improved quantification of the protein specific biomarker signature

Calibrators can be used for two purposes: Firstly, to quantify the detected signal in terms of the total amount of the analyte contained in the sample or in terms of concentration of the analyte in nasal secretion, and secondly to eliminate the influence of external factors such as ambient temperature from the results of runs, by putting the results of the samples in relation to results from calibrators with a well-defined amount of analyte. Runs of the size separation or other measurement device, can include one or more calibrators. Each calibrator contains a known amount of the analyte, with a constant distribution of monomers and/ or oligomers as far as possible. If a set of calibrators is included, the "Run Processing Software" calculates the total signal from each of the calibrators using the same methods applied to samples. If a single calibrator is used, the software calibrates amounts of analyte in patient samples by putting their measured intensities in relation to that of the calibrator. If more than one calibrator is used, the SW uses the calculated signals and the known amounts to fit a calibration curve (using a 4-parameter logistic function or other appropriate function), which represents a mapping from the total intensity to the amount of analyte. This mapping is used to calculate the amount of analyte contained in each sample.

5.4 Determination of classification-specific score and ATN classification

Each measurement done on a sample (possibly using different antibodies, different capillary sizes, different ranges of molecular weights, different devices) yields a set of measured and calculated values representing the relative or absolute amounts of a given protein and its quaternary structures, which is referred to as protein-specific biomarker signature. The totality of all such measurements for the different proteins can be combined into the ATN biomarker signature of the sample. For each of the dimension A, T, and N, a scoring algorithm calculates a number – the classification specific score – from a suitable subset of the values of the protein-specific biomarker

signatures. By comparing this classification specific score number to a predefined cutoff, a classification for each dimension can be made (see **Figures 14 and 15**). The measurements that are relevant are identified from the results of a study that includes a sufficient number of samples from patients with various cognitive impairments (also including patients with only subjective cognitive impairment but without objective cognitive impairment based on typical psychometric measurement means like MMST) with an AT(N) classification that is based on the guidelines of the NIA-AA framework. These reference scores are used as a ground truth to calculate e.g. the CSF based ATN classification system in nasal secretion.

The results of the same study are also used to develop a scoring algorithm which maps the relevant subset of the values from the ATN biomarker signature onto a single number for each one of the dimensions A, T, and N (see following section for more explanation and examples), and to select cutoffs for each dimension that determine whether to classify the sample as "+" or "-".

Approaches and tools used in the scoring algorithm to determine the classification specific scores and subsequent classification for each category A, T and/or N range from simple to more elaborate (if needed to achieve sufficient specificity). Simple approaches include, but are not limited to:

Application of cut-offs to individual analyte measurements; for example:

A status: a cut-off is applied to the total amount of Aβ42 or total intensity of Aβ42 to determine A status (**Figure 16**).

N status: a cut-off is applied to the total amount of tTau or total intensity of tTau to determine N status (**Figure 17**).

Application of cut-offs to the ratio of total amounts of different analytes; for example:

A status: a cut-off is applied to the ratio of total intensity Aβ42 to total intensity Aβ40 to determine A status (**Figure 18**).

Application of cut-offs to the ratio between a band of one analyte to another band of the same analyte, or to the total amount of that same analyte; for example:

A status: a cut-off is applied to the ratio between the sum of the intensities of the Aβ40 bands at 52 and 56 kDa, to the intensity of the Aβ40 bands around 62 kDa, to determine A status (**Figure 19**).

Application of cut-offs to the ratio between measurements from one band of one analyte, to the total quantity or specific bands of a different analyte; for example:

T status: a cut-off is applied to the ratio between pTau and tTau 57 kDa bands to determine T status (**Figure 20**).

If application of thresholds to individual measurements or their ratios does not give satisfactory sensitivity and specificity, multiple measurements may be combined to calculate a classification-specific score by methods including, but not limited to, the following:

- Logistic regression fits of all total intensities and band-specific intensities, or their ratios, or a subset of them.
- Application of neural networks (including deep learning and support vector machines) to the measurements.
- Ensemble learning: such as the application of dimensional-reduction techniques such as UMAP, t-SNE, or Principal Component Analysis to identify relevant dimensions.
- Bayesian Classifiers.

Different scoring methods may turn out optimal for each of the classification dimensions A, T, and N. For example, N may be decided by a simple threshold on the measured amount of tTau, whereas A may be decided by a combination of ratios between different peaks of Aβ40 and Aβ42.

5.5 Application of Scoring and Classification

The methods and parameters to calculate the optimal classification-specific scores derived from comparison with CSF measurements are implemented in the "Sample Processing SW". The "Sample Processing SW" collects the measurement results from all runs that apply to a given sample from one patient, assembles the ATN biomarker signatures, and applies the scoring mechanism. The resulting classification-specific scores determine the classification along the AT(N) dimensions.

5.6 Deployment of the Software

The "Run Processing SW" and "Sample Processing SW" can be used as command-line tools that process run data and generate sample reports on demand. They can also be incorporated in an automated setup, where new data from runs of automated measurement devices is processed as soon as it becomes available, and sample reports that show the classification specific scores and the resulting classifications are generated as soon as the necessary measurements for that sample are complete.

6. Discrimination of A+ and A- by protein-specific biomarker signatures

Protein-specific biomarker signatures that were determined as described in Examples 1 to 3 above were inputted into a multiple logistic regression.

6.1 Discrimination of A+ and A- by inputting protein-specific biomarkers signatures from tTau, total Aβ and Aβ42 into a multiple logistic regression

Following run analysis, relative band intensities from individual patients tTau 30 kDa normalized to total Tau levels, pan-Aβ 60-72 kDa normalized to total pan-Aβ levels and Aβ42 56 kDa were inputted into a multiple logistic regression (n=20) using the default settings of Prism software (Prism 9.5.1, user guide: https://www.graphpad.com/guides/prism/latest/user-guide/index.htm). The multiple logistic regression corresponds to the scoring algorithm as depicted in **Figure 14**. The multiple logistic regression results in predicted probabilities (**Figure 24**) that corresponds to the classification specific score. The predicted probability indicates the probability that the outcome is 1 or 0. An outcome of 1 indicated amyloid pathology (A+) and outcome of 0 was designated for amyloid negative (A-). A classification-specific score cutoff of 0.5 was selected (**Figure 24**). **Figure 26** shows the parameters resulting from a cut-off of 0.5. The negative predictive power is 90.91 %. The positive predictive power is 100.0 %.

Figure 22 shows that A+ and A- can be discriminated by protein-specific biomarker signatures; here the values of the protein-specific biomarker signatures of the relative amount of tTau 30 kDa, the relative amount of pan Aβ 60-72 kDa, and Aβ42 56 kDa are used as inputs for a multiple logistic regression and a classification specific score cutoff of 0.5 is applied.

6.2 Discrimination of A+ and A- by inputting protein specific weight signatures from tTau and Aβ42 into a multiple logistic regression

Following run analysis, relative band intensities from tTau 30 kDa, normalized to total Tau levels, Aβ42 48 kDa and Aβ42 56 kDa were inputted into a multiple logistic regression (n=30) using the default settings of Prism software (Prism 9.5.1, user guide: https://www.graphpad.com/guides/prism/latest/user-guide/index.htm). The multiple logistic regression corresponds to the scoring algorithm as depicted in **Figure 14**. The multiple logistic regression results in predicted probabilities (**Figure 25**) that corresponds to the classification specific score. The predicted probability indicates the probability that the outcome is 1 or 0. An outcome of 1 indicated amyloid pathology (A+) and outcome of 0 was designated for amyloid negative (A-). A classification score cutoff of 0.5 was selected (**Figure 25**). **Figure 27** shows the parameters resulting from a cut-off of 0.5. The negative predictive power is 86.67 %. The positive predictive power is 86.67 %.

Figure 23 shows that A+ and A- can be discriminated by protein-specific biomarker signatures; here the protein-specific biomarker signatures of the relative amount of tTau 30 kDa, Aβ42 48 kDa and Aβ42 56 kDa are used as inputs for a multiple logistic regression and a classification specific score cutoff of 0.5 is applied.

Claims

1. An *in vitro* method for diagnosing or predicting a neurodegenerative disease in a subject, said method comprising A/T/N classification in nasal secretion samples obtained from said subject.

2. The method of claim 1 comprising the following steps (a) to (e):
   a) determining in a nasal secretion sample obtained from the subject
      i) the protein-specific biomarker signature(s) of beta amyloid,
      ii) the protein-specific biomarker signature(s) of phosphorylated Tau, and/or
      iii) the protein-specific biomarker signature(s) of total Tau;
   b) determining from the protein-specific biomarker signature(s) determined in a) classification specific scores for A, T and/or N;
   c) comparing the classification specific scores determined in b) to predetermined classification specific scores;
   d) classifying the subject as
      i) A+ or A- according to the comparison result of the classification specific score for A determined in c),
      ii) T+ or T- according to the comparison result of the classification specific score for T determined in c), and/or
      iii) N+ or N- according to the comparison result of the classification specific score for n determined in c); and
   e) diagnosing or predicting the neurodegenerative disease based on the classification of d).

3. The method of claim 2, wherein the protein-specific biomarker signature(s) of a) comprise the whole protein level of beta amyloid, phosphorylated Tau and/or total

Tau and/or the protein level of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and total Tau.

4. The method claim 3, wherein the protein levels are determined via a technique comprising separation of the quaternary structures and/or isoforms according to size, molecular weight or charge.

5. The method of claim 4, wherein the technique is an electrophoretic technique.

6. The method of any of the preceding claims, wherein the quaternary structures and/or isoforms are separated between about 2 kDa and about 440 kDa.

7. The method of any of the preceding claims, wherein the isoform of beta amyloid is Aβ40 and/or Aβ42.

8. The method of any of the preceding claims, wherein the quaternary structures of beta amyloid have a molecular weight of about 4 kDa, about 8 kDa, about 12 kDa, about 16 kDa, about 19 kDa, about 24 kDa, about 32 kDa, about 40 kDa, about 44 kDa, about 48 kDa, about 52 kDa, about 56 kDa, about 60 kDa to 72 kDa, about 84 to 120 kDa and/or more than about 140 kDa.

9. The method of any one of the preceding claims, wherein the quaternary structures of phosphorylated Tau have a molecular weight of about 30 kDa, about 38 kDa, about 55 to 62 kDa, about 96 kDa to 106 kDa, and about 140 to 160 kDa.

10. The method of any one of the preceding claims, wherein the quaternary structures of total Tau have a molecular weight of about 30 kDa, about 38 kDa, about 48 kDa, about 55 kDa to 62 kDa, about 96 kDa to 106, about 140 kDa to 160 kDa and more than 160 kDa.

11. The method of any of the preceding claims, wherein determination of the classification specific score comprises

i) the whole protein level of beta amyloid, phosphorylated Tau and/or total Tau;

ii) the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau;

iii) the ratio of whole protein levels of beta amyloid, phosphorylated Tau and/or total Tau;

iv) the ratio of whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau;

v) the ratio of the protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau to the whole protein level(s) of beta amyloid, phosphorylated Tau and/or total Tau; and/or

vi) the ratio of protein level(s) of quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau to the protein level(s) of different quaternary structures and/or isoforms of beta amyloid, phosphorylated Tau and/or total Tau.

12. The method of any one of the preceding claims, wherein determination of the classification specific score comprises application to the protein-specific biomarker signature(s) logistic regression fits, neural networks, ensemble learning, dimensional-reduction techniques and/or Bayesian classifiers.

13. The method of any one of the preceding claims, wherein step c) is comparing the classification specific scores to a predetermined cut-off.

14. The method of any one of the preceding claims, wherein the nasal secretion sample is obtained from one or both olfactory cleft(s) of the subject.

15. The method of any one of the preceding claims, said method further comprising the selection of a treatment of the neurodegenerative disease.

Figure 1

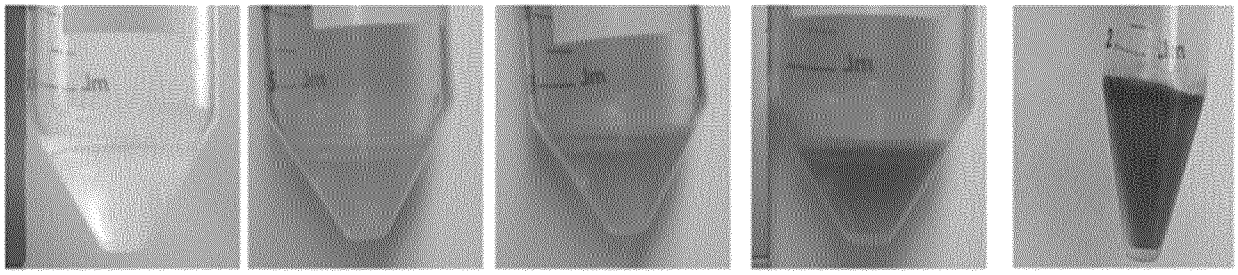

Figure 2

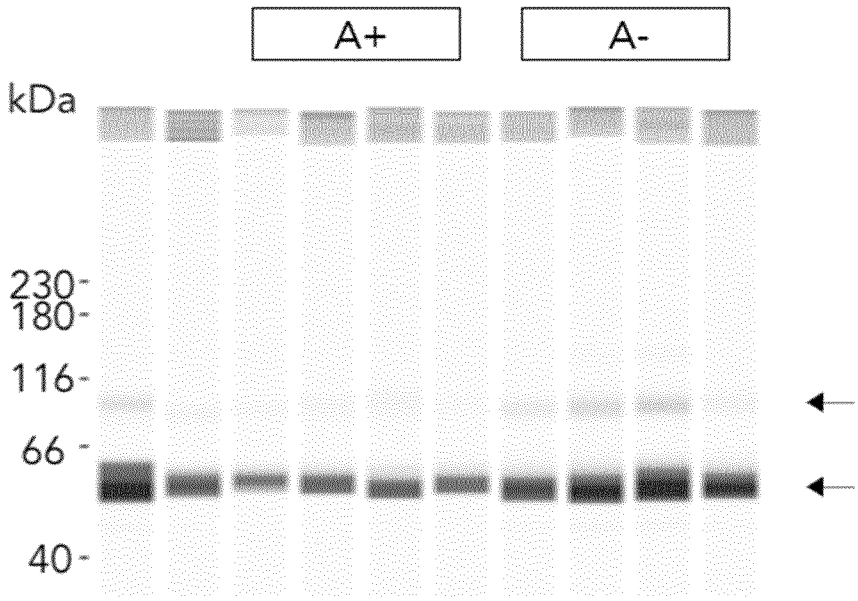

Figure 3

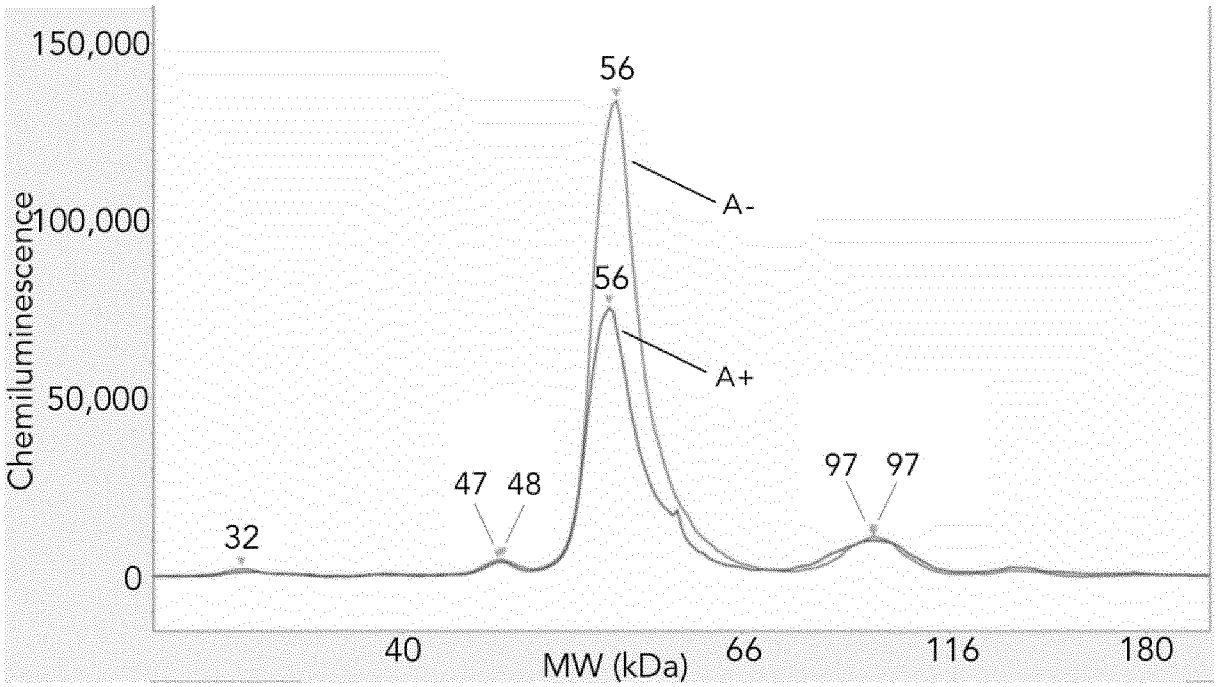

Figure 4

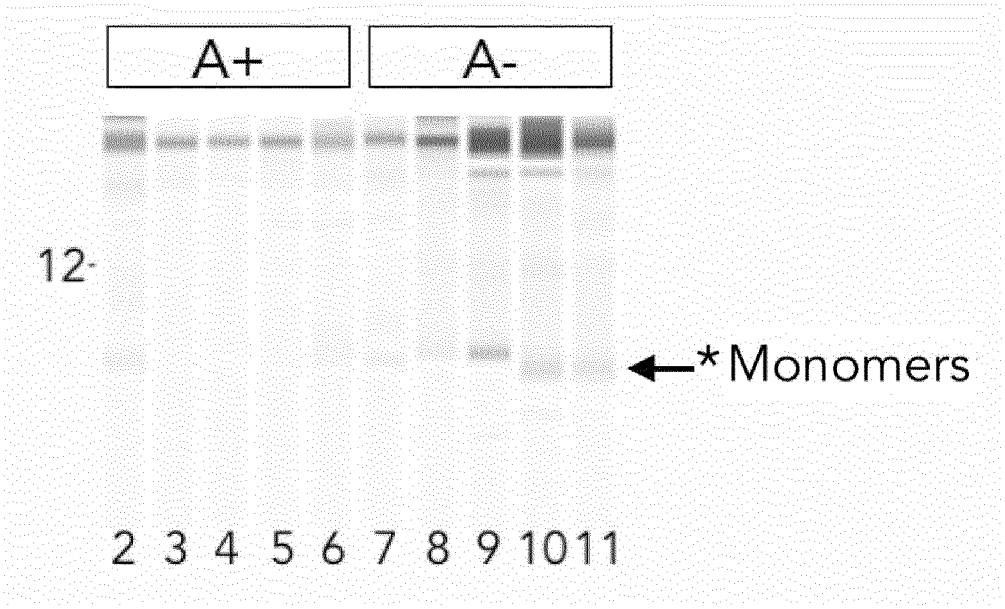

Figure 5

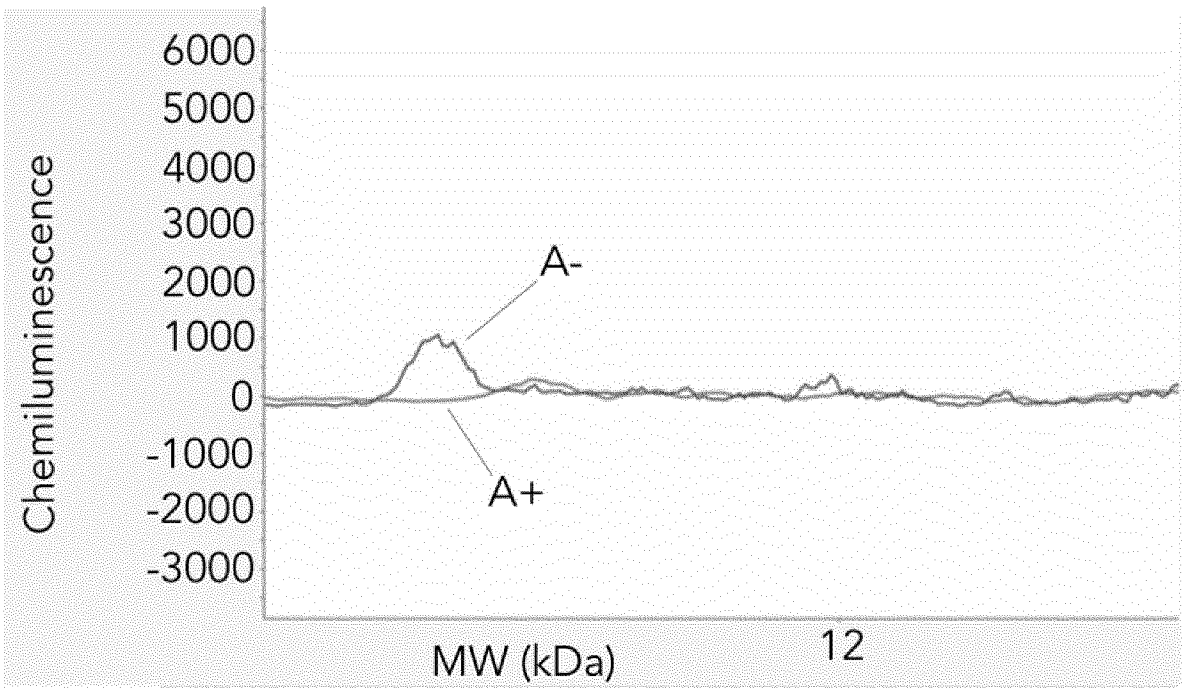
6000
5000
4000
3000
2000
1000
0
-1000
-2000
-3000
Chemiluminescence
A-
A+
MW (kDa)
12

Figure 6

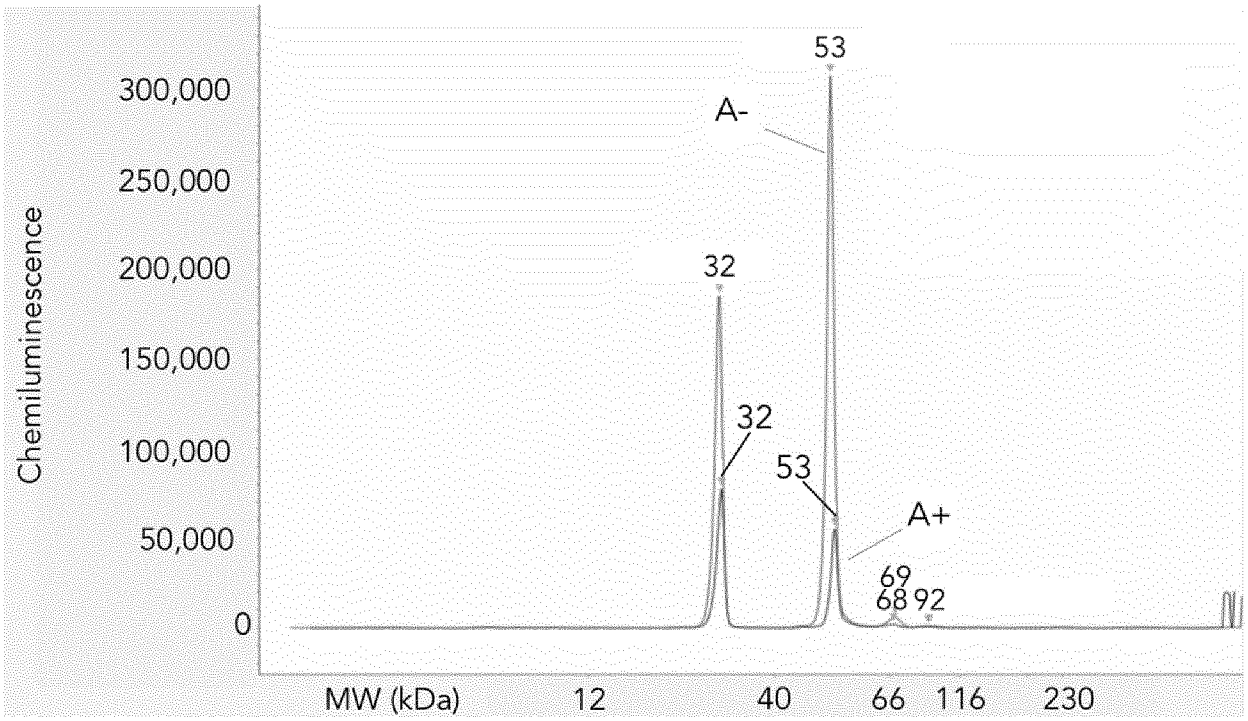
Chemiluminescence
300,000
250,000
200,000
150,000
100,000
50,000
0
MW (kDa)
12
40
66
116
230
53
A-
32
32
53
A+
69
68
92

Figure 7

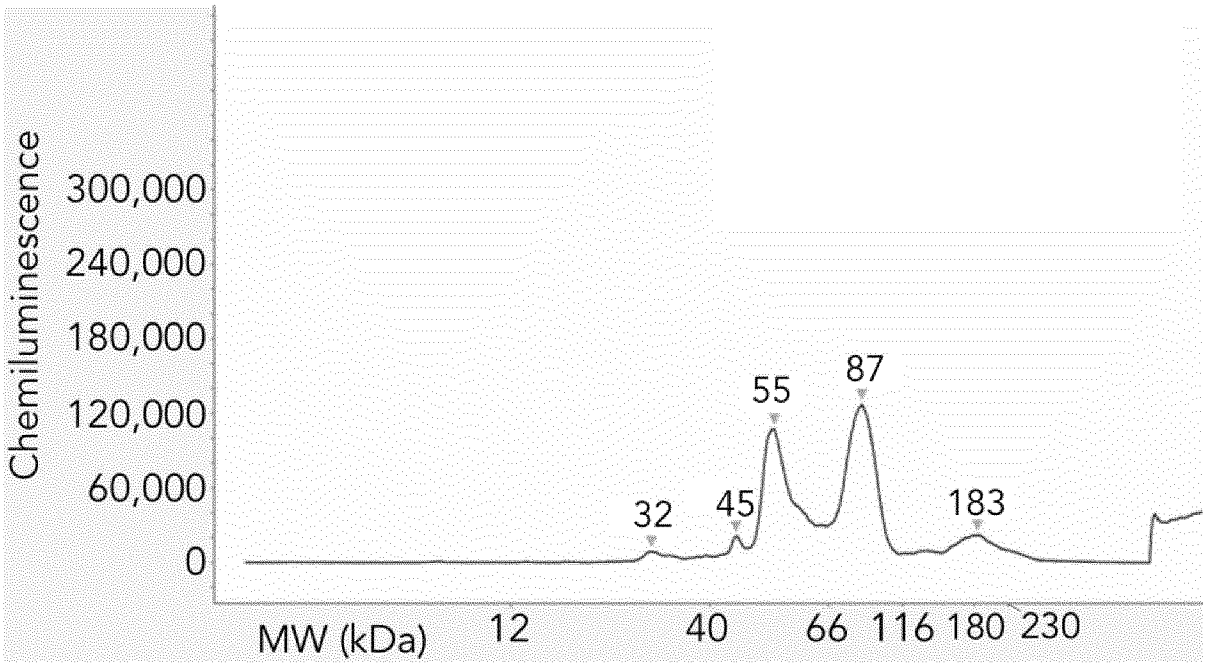
Chemiluminescence
300,000
240,000
180,000
120,000
60,000
0
32
45
55
87
183
MW (kDa)
12
40
66
116
180
230

Figure 8

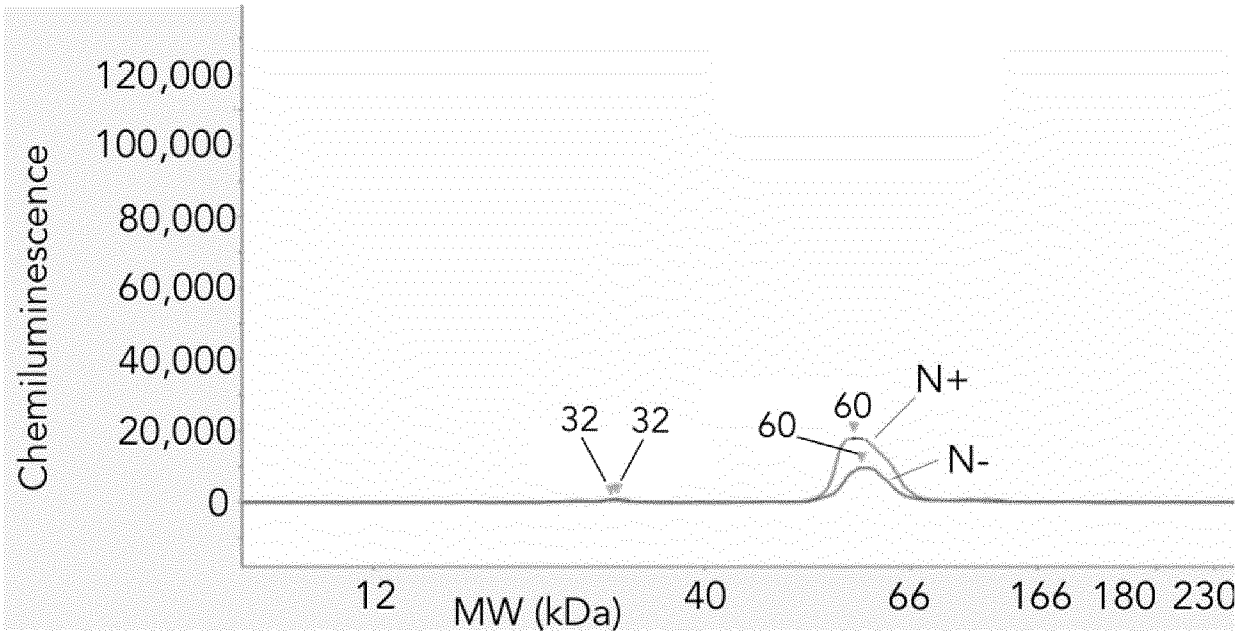

Figure 9

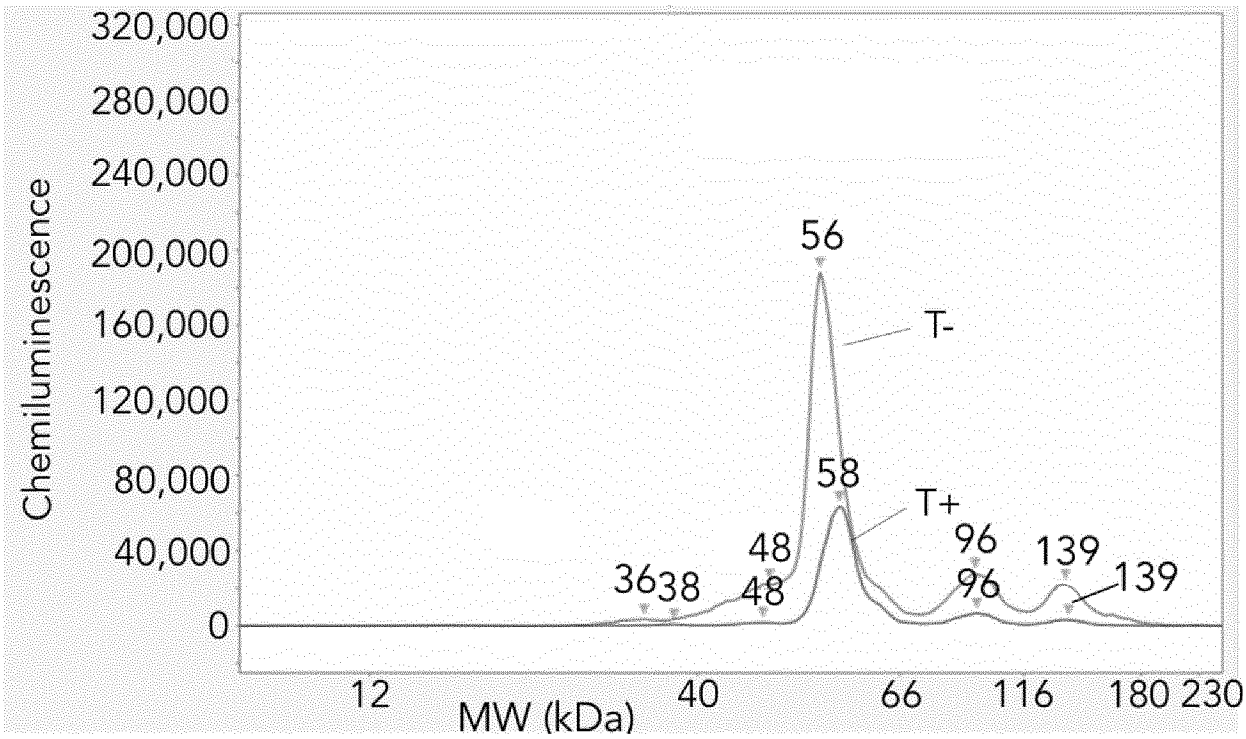

Figure 10

<table>
<tr><th>AT(N) profiles</th><th colspan="2">Biomarker category</th></tr>
<tr><td>A-T-(N)-</td><td colspan="2">Normal AD biomarkers</td></tr>
<tr><td>A+T-(N)-</td><td>Alzheimer's pathologic change</td><td rowspan="4">Alzheimer's continuum</td></tr>
<tr><td>A+T+(N)-</td><td>Alzheimer's disease</td></tr>
<tr><td>A+T+(N)+</td><td>Alzheimer's disease</td></tr>
<tr><td>A+T-(N)+</td><td>Alzheimer's and concomitant suspected non Alzheimer's pathologic change</td></tr>
<tr><td>A-T+(N)-</td><td colspan="2">Non-AD pathologic change</td></tr>
<tr><td>A-T-(N)+</td><td colspan="2">Non-AD pathologic change</td></tr>
<tr><td>A-T+(N)+</td><td colspan="2">Non-AD pathologic change</td></tr>
</table>

[Source: NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease; Clifford R. Jack et al. Alzheimer´s & Dementia 14 (2018) 535 – 562]

Figure 11

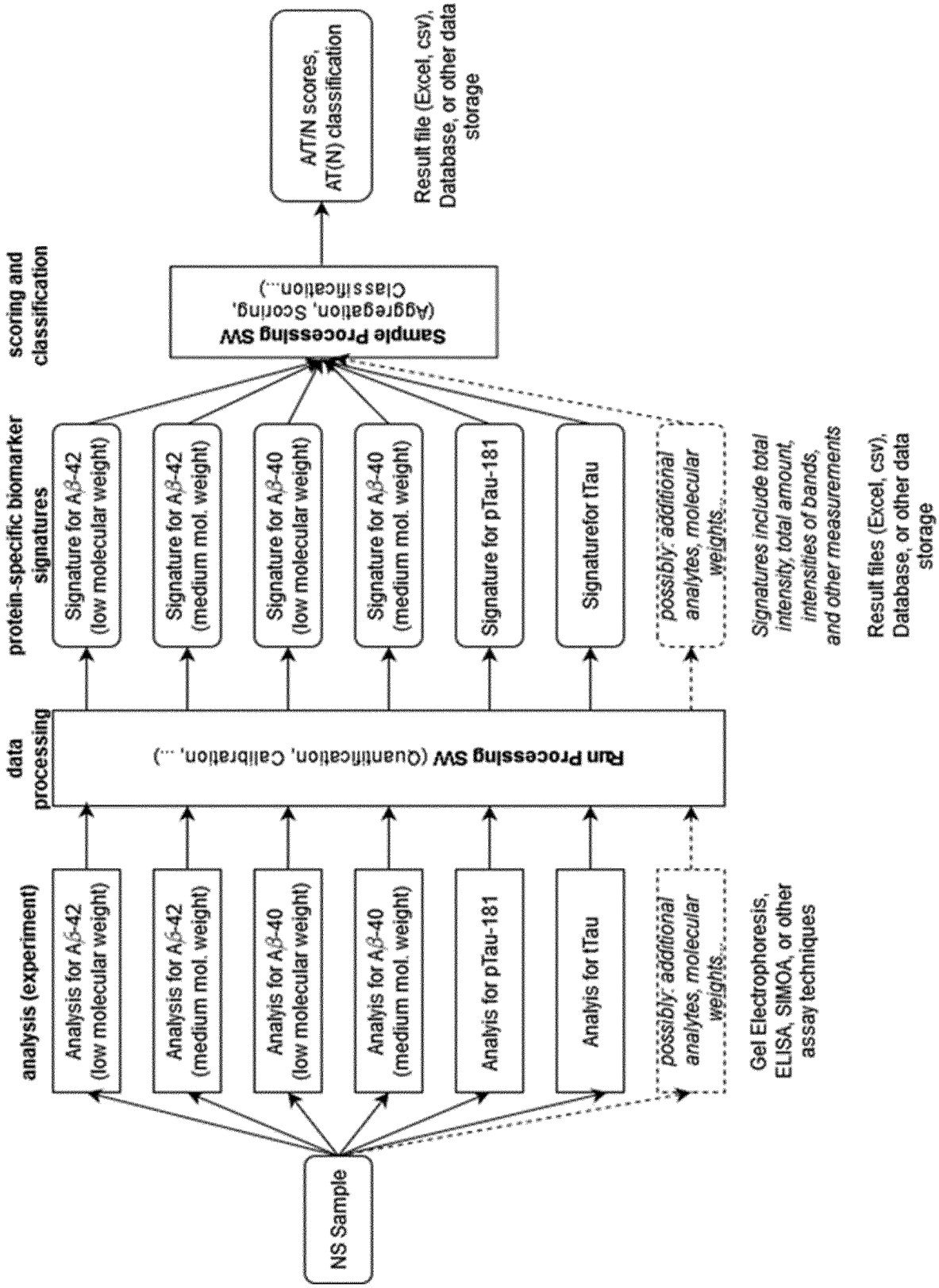
NS Sample
analysis (experiment)
Analysis for Aβ-42 (low molecular weight)
Analysis for Aβ-42 (medium mol. weight)
Analysis for Aβ-40 (low molecular weight)
Analysis for Aβ-40 (medium mol. weight)
Analysis for pTau-181
Analysis for tTau
possibly: additional analytes, molecular weights...
Gel Electrophoresis, ELISA, SIMOA, or other assay techniques
data processing
Run Processing SW (Quantification, Calibration, ...)
protein-specific biomarker signatures
Signature for Aβ-42 (low molecular weight)
Signature for Aβ-42 (medium mol. weight)
Signature for Aβ-40 (low molecular weight)
Signature for Aβ-40 (medium mol. weight)
Signature for pTau-181
Signaturefor tTau
possibly: additional analytes, molecular weights...
Signatures include total intensity, total amount, intensities of bands, and other measurements
Result files (Excel, csv), Database, or other data storage
scoring and classification
Sample Processing SW (Aggregation, Scoring, Classification...)
A/T/N scores, AT(N) classification
Result file (Excel, csv), Database, or other data storage

Figure 12

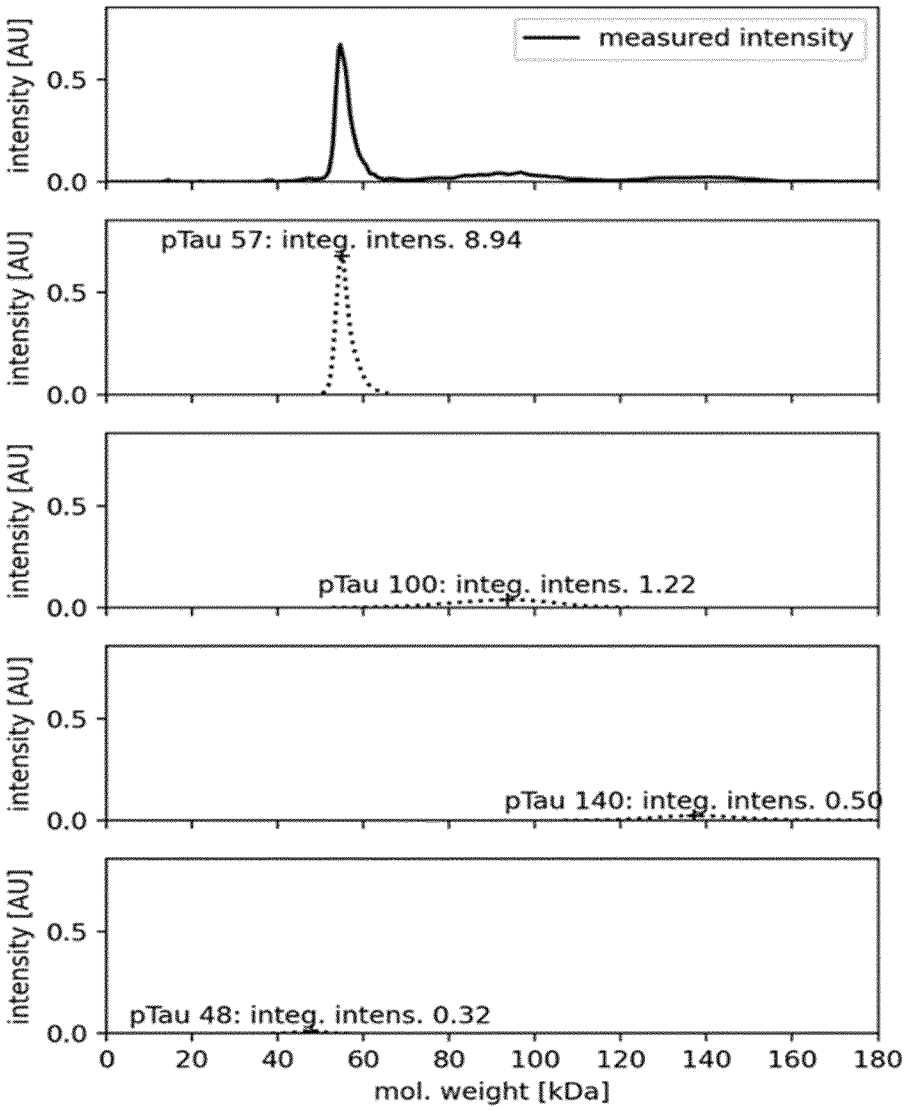
measured intensity
intensity [AU]
0.5
0.0
pTau 57: integ. intens. 8.94
pTau 100: integ. intens. 1.22
pTau 140: integ. intens. 0.50
pTau 48: integ. intens. 0.32
0
20
40
60
80
100
120
140
160
180
mol. weight [kDa]

Figure 13

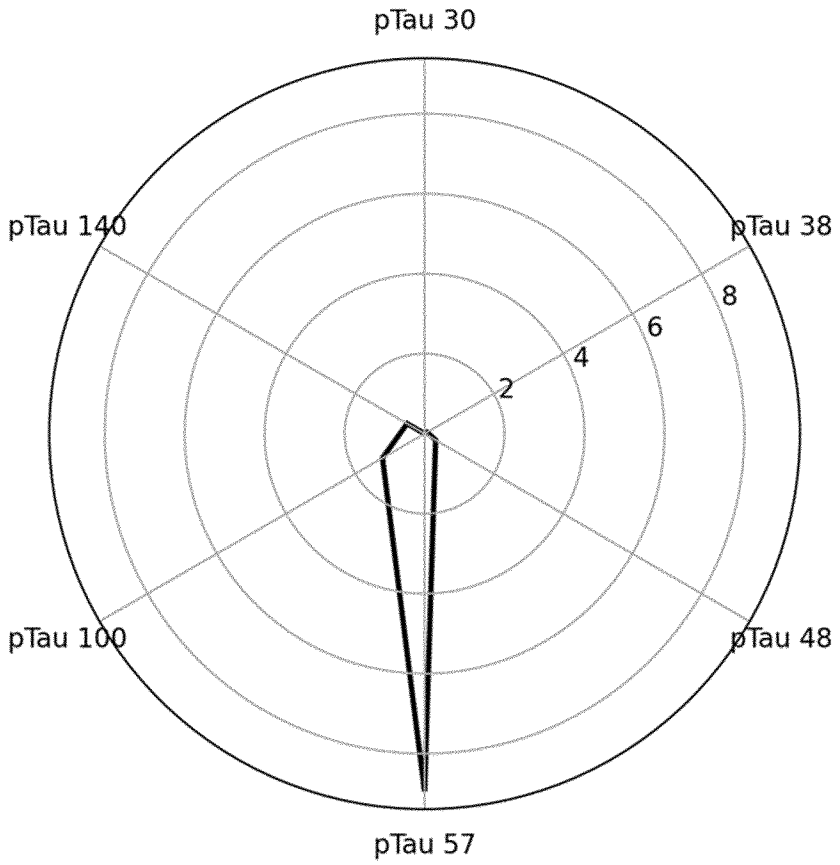

Figure 14

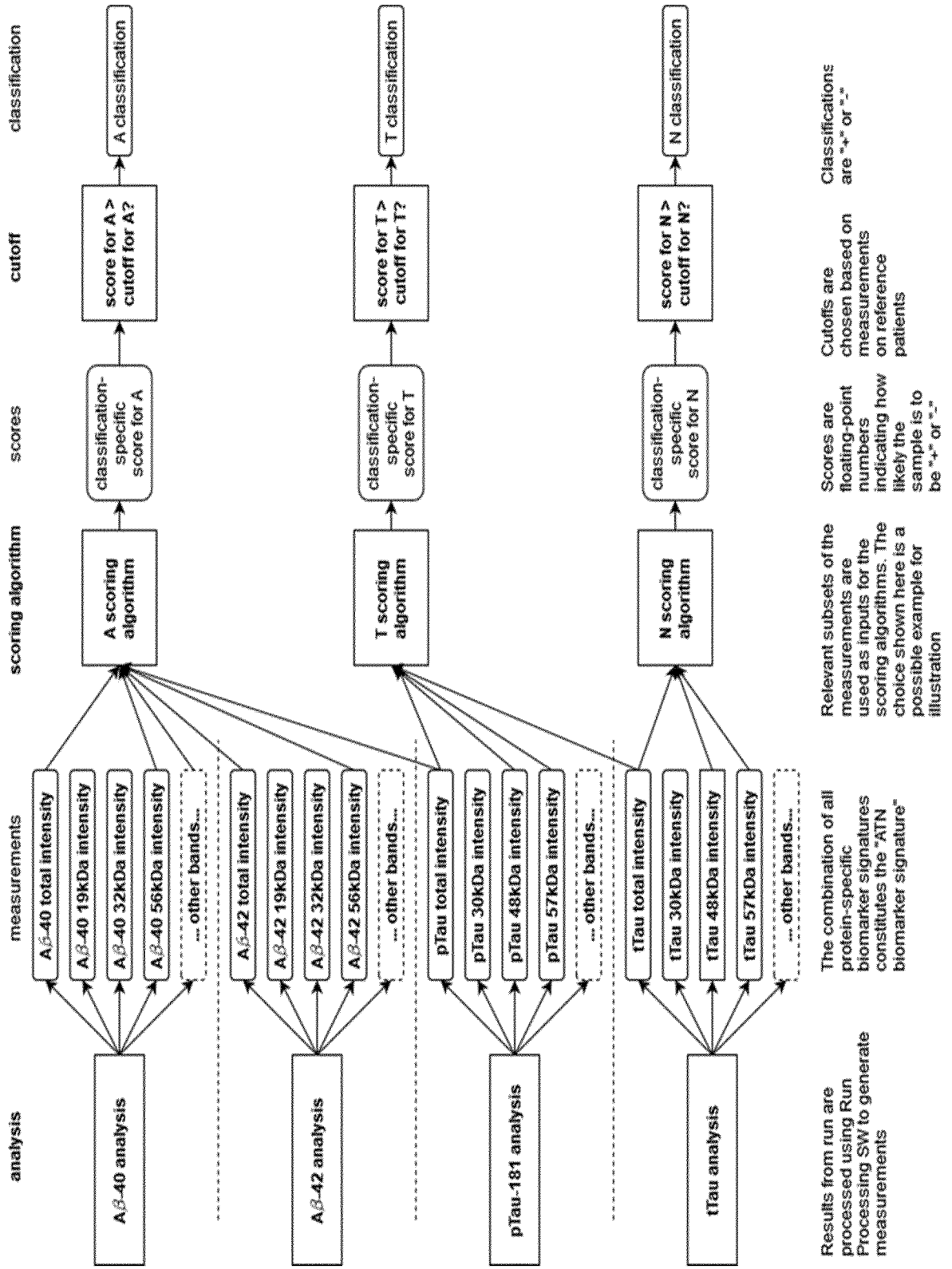
analysis
measurements
scoring algorithm
scores
cutoff
classification
Aβ-40 analysis
Aβ-40 total intensity
Aβ-40 19kDa intensity
Aβ-40 32kDa intensity
Aβ-40 56kDa intensity
... other bands...
A scoring algorithm
classification-specific score for A
score for A > cutoff for A?
A classification
Aβ-42 analysis
Aβ-42 total intensity
Aβ-42 19kDa intensity
Aβ-42 32kDa intensity
Aβ-42 56kDa intensity
... other bands...
T scoring algorithm
classification-specific score for T
score for T > cutoff for T?
T classification
pTau-181 analysis
pTau total intensity
pTau 30kDa intensity
pTau 48kDa intensity
pTau 57kDa intensity
... other bands...
tTau analysis
tTau total intensity
tTau 30kDa intensity
tTau 48kDa intensity
tTau 57kDa intensity
... other bands...
N scoring algorithm
classification-specific score for N
score for N > cutoff for N?
N classification
Results from run are processed using Run Processing SW to generate measurements
The combination of all protein-specific biomarker signatures constitutes the "ATN biomarker signature"
Relevant subsets of the measurements are used as inputs for the scoring algorithms. The choice shown here is a possible example for illustration
Scores are floating-point numbers indicating how likely the sample is to be "+" or "-"
Cutoffs are chosen based on measurements on reference patients
Classifications are "+" or "-"

Figure 15

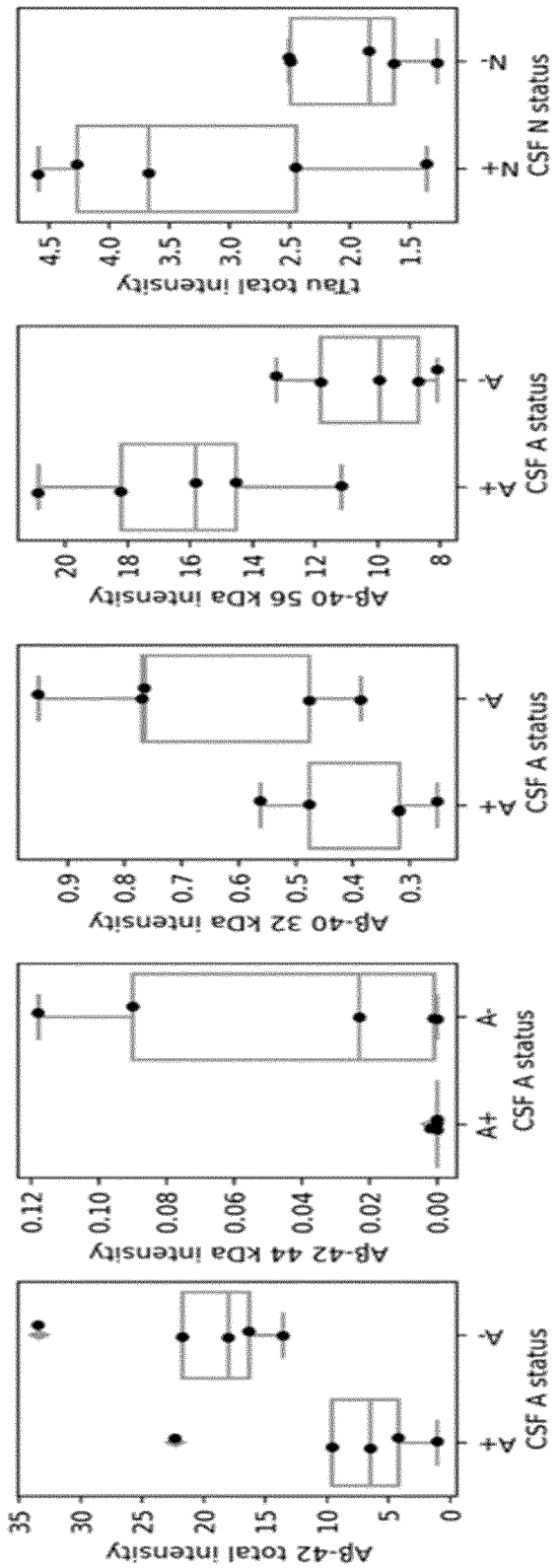
Aβ-42 total intensity
0
5
10
15
20
25
30
35
A+
A-
CSF A status
Aβ-42 44 kDa intensity
0.00
0.02
0.04
0.06
0.08
0.10
0.12
A+
A-
CSF A status
Aβ-40 32 kDa intensity
0.3
0.4
0.5
0.6
0.7
0.8
0.9
A+
A-
CSF A status
Aβ-40 56 kDa intensity
8
10
12
14
16
18
20
A+
A-
CSF A status
tau total intensity
1.5
2.0
2.5
3.0
3.5
4.0
4.5
N+
N-
CSF N status

Figure 16

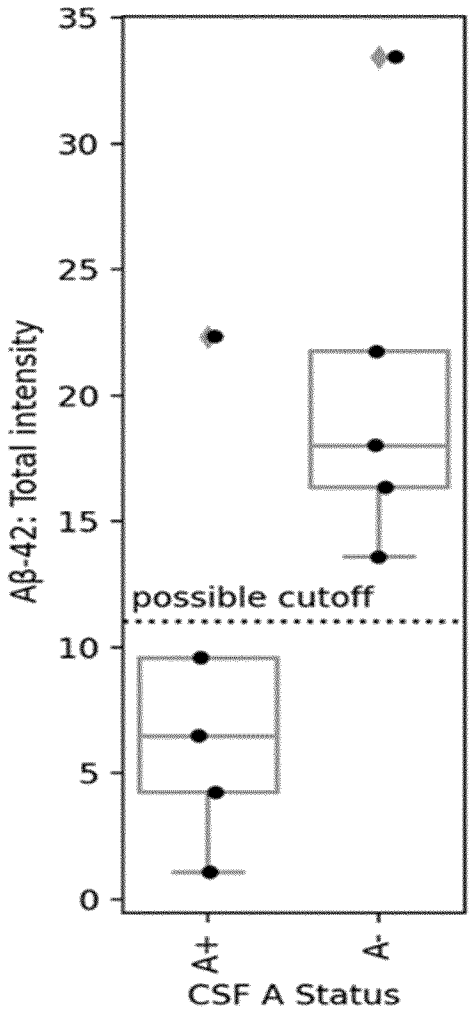
35
30
25
20
15
10
5
0
Aβ-42: Total intensity
possible cutoff
A+
A-
CSF A Status

Figure 17

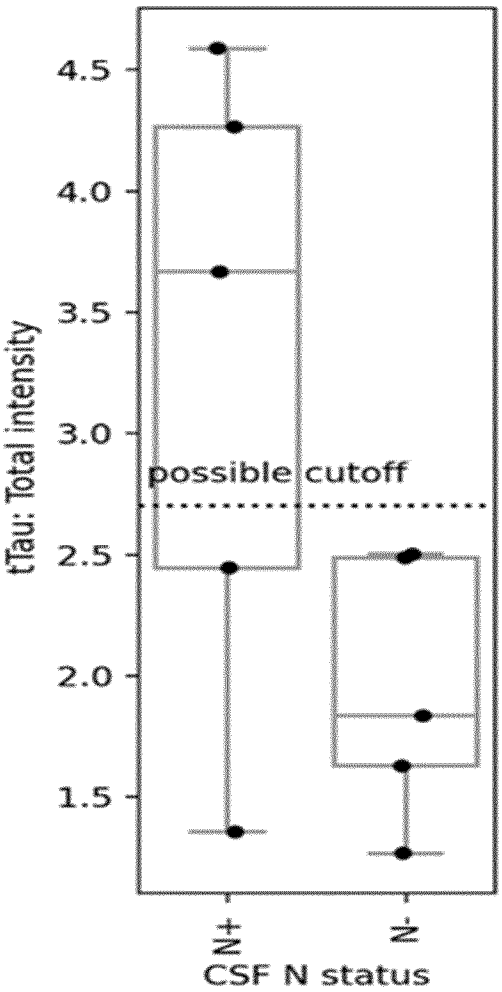
4.5
4.0
3.5
3.0
2.5
2.0
1.5
tTau: Total intensity
possible cutoff
+
N
-
N
CSF N status

Figure 18

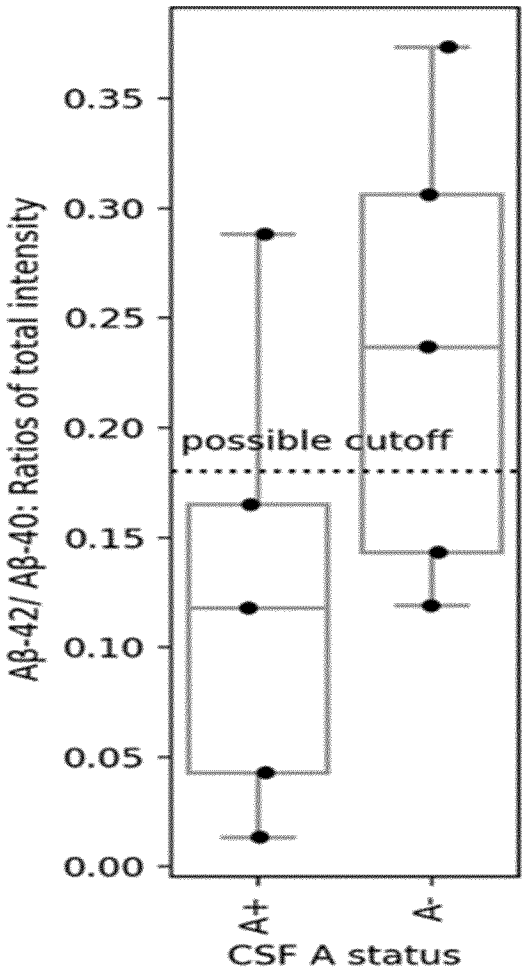
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Aβ-42/ Aβ-40: Ratios of total intensity
possible cutoff
A+
A-
CSF A status

Figure 19

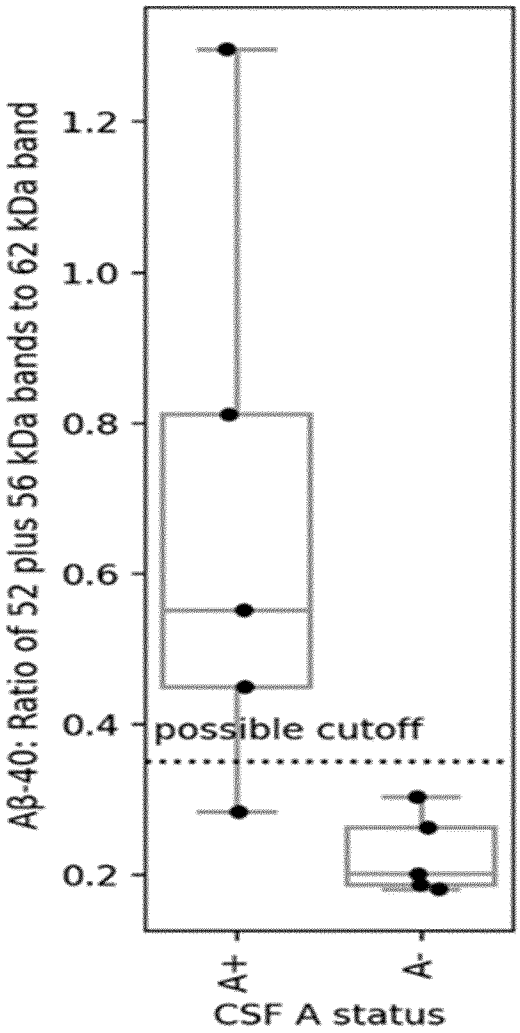
Aβ-40: Ratio of 52 plus 56 kDa bands to 62 kDa band
1.2
1.0
0.8
0.6
0.4
0.2
possible cutoff
A+
A-
CSF A status

Figure 20

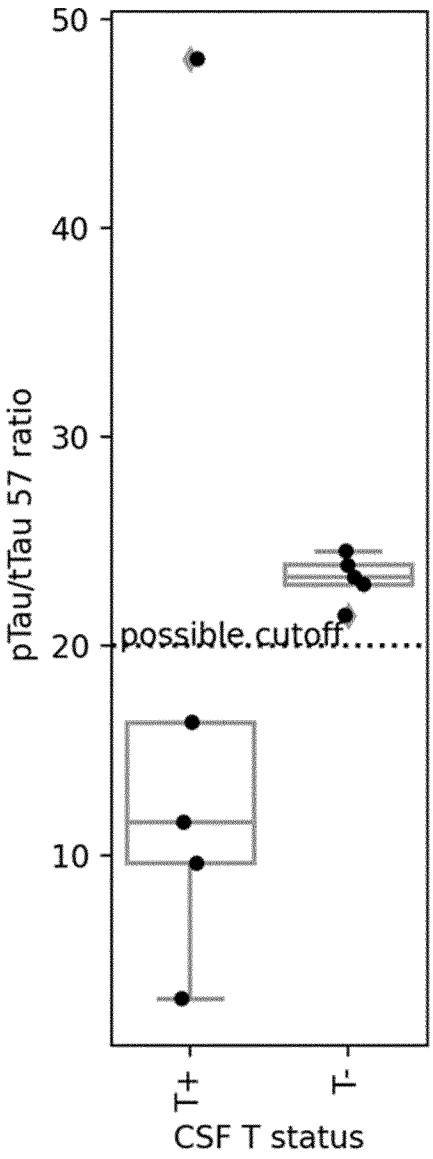
50
40
30
20
10
pTau/tTau 57 ratio
possible cutoff
T+
T-
CSF T status

Figure 21

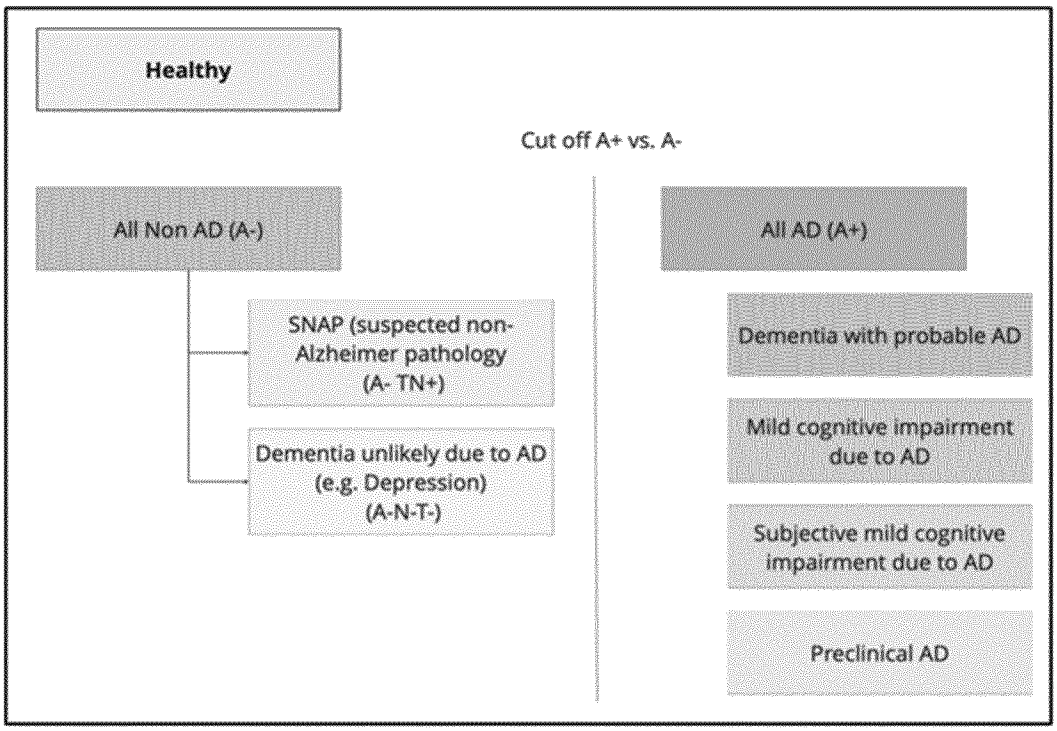
Healthy
Cut off A+ vs. A-
All Non AD (A-)
All AD (A+)
SNAP (suspected non-Alzheimer pathology (A- TN+)
Dementia unlikely due to AD (e.g. Depression) (A-N-T-)
Dementia with probable AD
Mild cognitive impairment due to AD
Subjective mild cognitive impairment due to AD
Preclinical AD

Figure 22

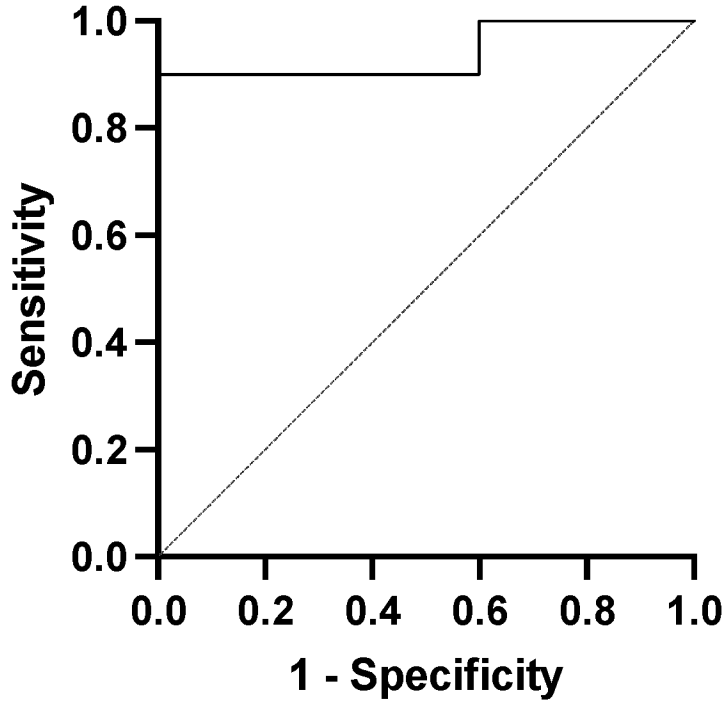

Figure 23

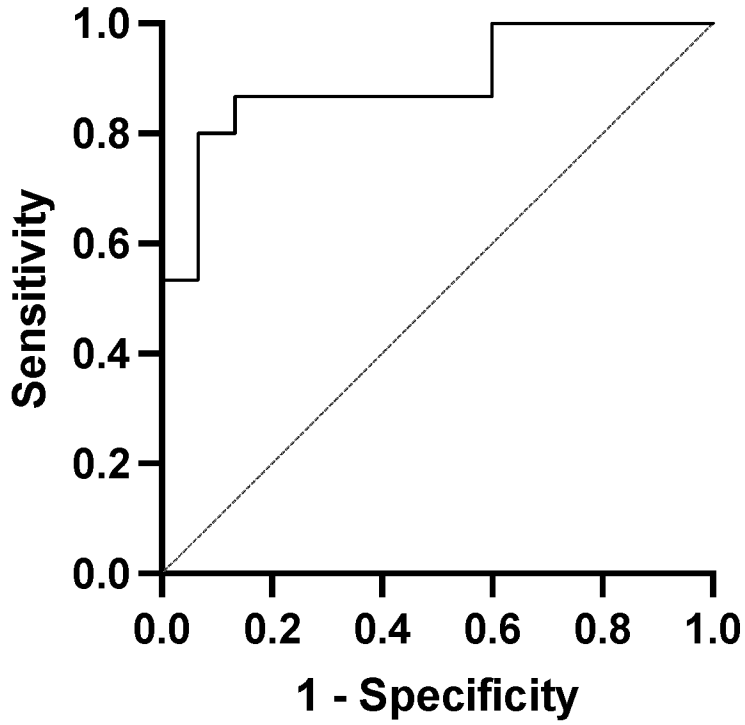

Figure 24

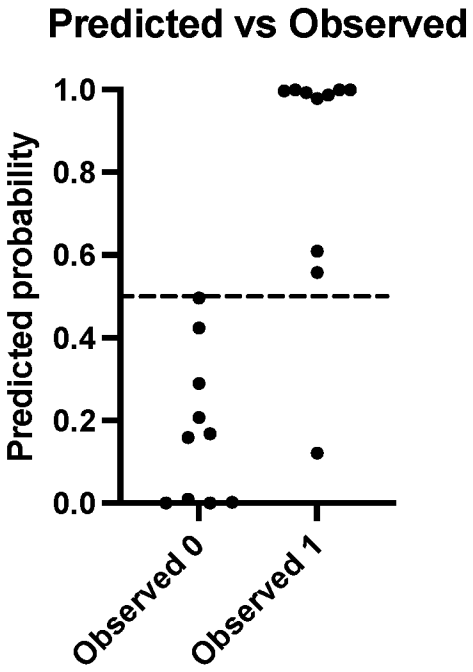

Figure 25

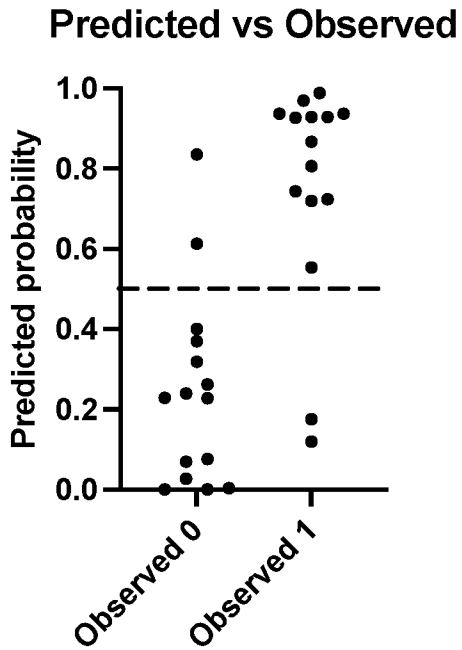

Figure 26

Table Analyzed	Data 1			
Dependent variable	Outcome			
Regression type	Logistic regression			
Model				
Parameter estimates	Variable	Estimate	Standard error	95% CI (profile likelihood)
β0	Intercept	7,029	7,837	-6,195 to 27,89
β1	Tau 30%	-196,5	154,6	-643,0 to -8,924
β2	panAb 60-72%	32,71	24,04	-2,926 to 99,50
β3	ab42 56	-1,742	1,211	-5,363 to -0,2408
Odds ratios	Variable	Estimate	95% CI (profile likelihood)	
β0	Intercept	1129	0,002040 to 1293033766572	
β1	Tau 30%	4,635e-086	5,377e-280 to 0,0001332	
β2	panAb 60-72%	1,601e+014	0,05359 to 1,627e+043	
β3	ab42 56	0,1752	0,004687 to 0,7860	
Model diagnostics	Degrees of Freedom	AICc		
Intercept-only model	19	29,95		
Selected model	16	21,49		
Area under the ROC curve				
Area	0,9400			
Std. Error	0,06079			
95% confidence interval	0,8208 to 1,000			
P value	0,0009			
Classification table	Predicted 0	Predicted 1	Total	% Correctly classified
Observed 0	10	0	10	100,0
Observed 1	1	9	10	90,00
Total	11	9	20	95,00
Negative predictive power (%)	90,91			
Positive predictive power (%)	100,0			
Classification cutoff	0,5			
Pseudo R squared				
Tjur's R squared	0,6489			
Hypothesis tests	Statistic	P value	Null hypothesis	Reject Null Hypothesis?
Hosmer-Lemeshow	4,279	0,8311	Selected model is correct	No
Data summary				
Rows in table	39			
Rows skipped (missing data)	19			
Rows analyzed (#observations)	20			
Number of 1	10			
Number of 0	10			
Number of parameter estimates	4			
#observations/#parameters	5,0			
# of 1/#parameters	2,5			
# of 0/#parameters	2,5			

Figure 27

Table Analyzed	Data 1				
Dependent variable	Outcome				
Regression type	Logistic regression				
Model					
Parameter estimates	Variable	Estimate	Standard error	95% CI (profile likelihood)	
β0	Intercept	7,991	3,025	3,321 to 15,69	
β1	Tau 30%	-144,9	64,11	-307,9 to -49,54	
β2	ab42 56	-0,6836	0,3621	-1,535 to -0,06184	
β3	Ab-42 48	-22,35	14,07	-57,78 to 0,3661	
Odds ratios	Variable	Estimate	95% CI (profile likelihood)		
β0	Intercept	2955	27,70 to 6488196		
β1	Tau 30%	1,231e-063	1,977e-134 to 3,041e-022		
β2	ab42 56	0,5048	0,2155 to 0,9400		
β3	Ab-42 48	1,958e-010	8,060e-026 to 1,442		
Model diagnostics	Degrees of Freedom	AICc			
Intercept-only model	29	43,73			
Selected model	26	32,69			
Area under the ROC curve					
Area	0,8933				
Std. Error	0,06105				
95% confidence interval	0,7737 to 1,000				
P value	0,0002				
Classification table	Predicted 0	Predicted 1	Total	% Correctly classified	
Observed 0	13	2	15	86,67	
Observed 1	2	13	15	86,67	
Total	15	15	30	86,67	
Negative predictive power (%)	86,67				
Positive predictive power (%)	86,67				
Classification cutoff	0,5				
Pseudo R squared					
Tjur's R squared	0,5104				
Hypothesis tests	Statistic	P value	Null hypothesis	Reject Null Hypothesis?	P value summary
Hosmer-Lemeshow	10,43	0,2359	Selected model is correct	No	ns
Data summary					
Rows in table	39				
Rows skipped (missing data)	9				
Rows analyzed (#observations)	30				
Number of 1	15				
Number of 0	15				
Number of parameter estimates	4				
#observations/#parameters	7,5				
# of 1/#parameters	3,8				
# of 0/#parameters	3,8				

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/063001

A. CLASSIFICATION OF SUBJECT MATTER
INV. G01N33/68 C07K14/47
ADD.

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 C07K

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 practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, 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	JUNG DA HAE ET AL: "Non-Invasive Nasal Discharge Fluid and Other Body Fluid Biomarkers in Alzheimer's Disease", PHARMACEUTICS, vol. 14, no. 8, 22 July 2022 (2022-07-22), pages 1-20, XP093071848, DOI: 10.3390/pharmaceutics14081532 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9330777/pdf/pharmaceutics-14-01532.pdf>	1-3
Y	abstract page 3, paragraph 2; table 7	1-3, 11-15
A		4-10

-/--

[x] 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 application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"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	Date of mailing of the international search report
29 May 2024	12/06/2024

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Gundlach, Björn

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	LEE JONGMIN ET AL: "Cerebrospinal Fluid Biomarkers for the Diagnosis and Classification of Alzheimer's Disease Spectrum", JOURNAL OF KOREAN MEDICAL SCIENCES, vol. 35, no. 44, 1 January 2020 (2020-01-01), XP055900886, SEOUL, KR ISSN: 1011-8934, DOI: 10.3346/jkms.2020.35.e361 Retrieved from the Internet: URL:https://jkms.org/DOIx.php?id=10.3346/jkms.2020.35.e361>	1-3, 11-15
A	abstract	4-10

Y	EBENAU JARITH L. ET AL: "ATN classification and clinical progression in subjective cognitive decline : The SCIENCe project", NEUROLOGY, vol. 95, no. 1, 7 July 2020 (2020-07-07), pages e46-e58, XP093081011, US ISSN: 0028-3878, DOI: 10.1212/WNL.0000000000009724 Retrieved from the Internet: URL:https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7371376/pdf/NEUROLOGY2019015339.pdf >	1-3, 11-15
A	abstract	4-10

A	LIU PENG ET AL: "Quaternary Structure Defines a Large Class of Amyloid-[beta] Oligomers Neutralized by Sequestration", CELL REPORTS, vol. 11, no. 11, 1 June 2015 (2015-06-01), pages 1760-1771, XP093080932, US ISSN: 2211-1247, DOI: 10.1016/j.celrep.2015.05.021 abstract	1-15

A	ZGANEC MATJAZ ET AL: "Amino acid substitutions [K16A] and [K28A] distinctly affect amyloid[beta]-protein oligomerization", JOURNAL OF BIOLOGICAL PHYSICS, KLUWER ACADEMIC PUBLISHERS, DORDRECHT, NL, vol. 42, no. 3, 7 May 2016 (2016-05-07), pages 453-476, XP036002796, ISSN: 0092-0606, DOI: 10.1007/S10867-016-9417-4 [retrieved on 2016-05-07] abstract	1-15

	-/--	

1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/063001

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JACK CLIFFORD R ET AL: "NIA-AA Research Framework: Toward a biological definition of Alzheimer's disease", ALZHEIMER'S & DEMENTIA, ELSEVIER, NEW YORK, NY, US, vol. 14, no. 4, 10 April 2018 (2018-04-10) , pages 535-562, XP085378277, ISSN: 1552-5260, DOI: 10.1016/J.JALZ.2018.02.018 cited in the application abstract -----	1-15

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