The present invention describes methods of diagnosing Alzheimer's disease in a subject comprising determining in a sample derived from a body fluid of said subject the amount of antibodies capable of binding to a pGlu Aβ peptide and comparing the amount of said antibodies to a reference amount; wherein the amount of said antibodies compared to the reference amount is indicative of Alzheimer's disease. The invention also relates to methods of diagnosing mild cognitive impairment (MCI) in a subject, as well as to methods of predicting the likelihood that a subject having MCI will develop Alzheimer's disease. The invention also describes a pGlu Aβ peptide for use in medicine and/or veterinary medicine as well as to said pGlu Aβ peptide for use in the diagnosis and/or prognosis of AD and/or MCI. The invention also describes the use of an antibody capable of binding to a pGlu Aβ peptide as a biomarker for Alzheimer's disease, to methods for identifying and/or isolating an antibody capable of binding to a pGlu Aβ peptide and to an antibody capable of binding to a pGlu Aβ. Moreover, the invention also relates to a kit.

Abstract

Figure 1
BIOMARKERS AND METHODS FOR DIAGNOSING ALZHEIMER'S DISEASE AND/OR MILD COGNITIVE IMPAIRMENT

FIELD OF THE INVENTION

The present invention relates to the diagnosis and prognosis of Alzheimer's disease and to biomarkers for Alzheimer's disease. In addition, the present invention relates to the diagnosis and prognosis of mild cognitive impairment and to biomarkers for mild cognitive impairment. More specifically, said biomarkers are antibodies capable of binding to a pGlu Aβ peptide. The invention also relates to pGlu Aβ peptides for use in medicine and/or veterinary medicine and for their use in the diagnosis and/or prognosis of Alzheimer's disease and/or mild cognitive impairment.

BACKGROUND OF THE INVENTION

"Alzheimer's disease" or "AD", as interchangeably used herein, refers to a well-known disease characterized by a progressive dementia that occurs in middle or late life. AD is the most common form of dementia. Abundant evidence suggests that a key event in AD pathogenesis is the conversion of the amyloid β peptide (Aβ, A-beta, or Abeta; in the following "Aβ") from soluble to aggregated forms in the brain. Aβ is generated by proteolytic cleavage of its precursor, the amyloid precursor protein (APP). Aβ is the main protein component of the plaques found in the brains of Alzheimer patients. There are several both N-terminally and C-terminally truncated forms of Aβ. The two major C-terminal variants of Aβ consist of a shorter form ending at Val-40 ("Aβ1-40" or "Aβ40"), and a longer form ending at Ala-42 ("Aβ1-42" or "Aβ42"). Other C-terminally truncated Aβ-peptides include Aβ1-37, Aβ1-38, Aβ1-39 (Blennow. 2004). The first molecular events leading to Alzheimer's disease are supposed to appear 10 to 20 years before cognitive symptoms become apparent in affected individuals. Therefore, identification of biomarkers predictive for AD might allow screening for individuals who may benefit from preventive therapies before cognitive impairment can be observed.
Aβ peptides have been discussed to play a central role in the pathogenesis of AD, and the levels of Aβ42 in the cerebrospinal fluid (“CSF”) is a routine diagnostic procedure with 75-95% sensitivity and specificity. In fact, at present, only CSF Aβ42 levels is a well established Aβ-related AD biomarker with high sensitivity and specificity. Moreover, a longitudinal study showed that CSF Aβ42 decreases within 12 months significantly in AD patients, while Tau and Phospho-Tau did not. Although much more useful for clinical practice, blood based Aβ levels are controversially discussed as a useful biomarker for AD. There seems to be no correlation between CSF and blood Aβ level, as shown by several groups. In addition, studies on plasma Aβ levels have shown contradictory results, including increased Aβ40 and decreased Aβ42 in AD patients compared to healthy controls, no differences or increased Aβ42 in AD patients. The same inconsistency emerged from a series of prospective studies, in which a higher risk for developing AD has been associated both with higher baseline plasma Aβ42 concentration or higher Aβ40 with no effect on Aβ42. Finally, another report linked a high risk for developing AD to a lower Aβ42/40 ratio. The variable observations in these studies may be due to differences in study design, including variations in age and disease severity of included subjects. A longitudinal study showed that plasma Aβ40 and Aβ42 levels measured at age 70 years were not significantly associated with incident AD. All of the studies though, have shown substantial overlap in plasma Aβ40 and Aβ42 levels between patients and controls, thus limiting the diagnostic value of plasma Aβ assessment. Some reports find that CSF and blood levels of Aβ do not correlate, neither in AD patients nor in healthy controls. On the other side, one report suggests that there is a correlation between CSF and plasma levels of Aβ40 and Aβ42 in healthy individuals, whereas no such correlation could be seen for AD or MCI (“mild cognitive impairment”) cases. As reviewed by Kawarabayashi et al., the broad overlap in the plasma Aβ protein levels between patients with Alzheimer’s disease and control individuals indicates that the plasma Aβ level cannot differentiate cases of sporadic Alzheimer's disease from control cases. Although the significance of Aβ for diagnosing Alzheimer's disease is controversial, high plasma concentrations of Aβ40 and low plasma concentrations of Aβ42 are discussed to indicate an increased risk of dementia (Kawarabayashi 2008).

Besides the detection of biochemical AD markers, autoimmune responses to, for example, aggregates of Aβ25-35 have become a current research focus (Gruden 2004, Nath 2003). There seems to be a reduction in the magnitude of lymphocyte stimulation by APP in AD patients, and naturally occurring anti-Aβ antibodies seem to be present in sera and CSF of AD patients and healthy controls (Du 2001). Titers of anti-Aβ42 autoantibodies were reported to be lower in AD patients compared to healthy individuals (Du 2001, Weksler 2002), or higher in AD patients (Nath 2003). Another study reported no correlation between autoantibodies titers and no correlation to plasma Aβ40 or 42 levels. In other
reports, the serum content of autoantibodies to β-amyloid protein Aβ1-42, its neurotoxic fragment Aβ25-35, vasopressin, bradykinin, thrombin, antithrombin III, α2-macroglobulin, and angiotensin II was measured in patients with various forms of Alzheimer's dementias, (Myagkova 2003. Myagkova 2001). However, these studies lack the inclusion of a control group and therefore do not provide evidence of the suitability of said autoantibodies for the diagnosis of AD. Generally, whether autoimmunity plays a direct role in the pathogenesis of AD is a matter of scientific debates.

"Mild cognitive impairment" or "MCF", as interchangeably used herein, is another well-known disease characterized by cognitive impairments beyond that expected for the age and education of an individual. It is considered to be the transitional stage between normal aging and dementia. MCI is frequently seen as a risk factor for AD.

Pyroglutamatic peptides or proteins, i.e. peptides or proteins containing an N-terminal pyroglutamate ("pGlu") residue, are e.g. formed spontaneously by conversion of an N-terminal glutamate residue, pGlu residues may also be formed enzymatically such as by means of the enzyme glutaminyl cyclase ("QC"). This enzyme also catalyzes the conversion of an N-terminal glutamate to a pyroglutamate (in the following: "pGlu"). In Alzheimer's disease (AD), glutaminyl cyclase catalyzes N-terminal pGlu-formation of amyloid beta (Aβ) in vivo. That is, glutamate at the N-terminus of truncated Aβ can be subsequently cyclized into pyroglutamic acid (pGlu) at position 3 or 11 of Aβ, resulting in Aβ3(pGlu)-40/42 and Aβ11(pGlu)-40/42 (Schilling, 2008). Because of their abundance, resistance to proteolysis, rapid aggregation and neurotoxicity, N-terminally truncated and, in particular, pyroglutamate (pGlu)-modified Aβ peptides have been suggested as being important in the initiation of pathological cascades resulting in the development of Alzheimer's disease. According to one hypothesis Aβ 3(pGlu)-42 acts as a seed for Aβ aggregation by self-aggregation and co-aggregation with Aβ 1-40/42. Therefore, Aβ 3(pGlu)-40/42 peptides seem to represent Aβ forms with exceptional potency for disturbing neuronal function (Schilling, 2008). Glutaminyl cyclase (QC) was shown to catalyze the formation of Aβ 3(pGlu)-40/42 after amyloidogenic processing of APP in two different cell lines.

Frequently, AD and MCI are diagnosed on basis of the cognitive state. Generally, it would be desirable to provide for possibilities of diagnosing AD in a subject on basis of a molecular biomarker, e.g. in a sample derived from a body fluid of said subject, rather than on basis of the cognitive state of the patient. Likewise, it would be desirable to provide for possibilities of diagnosing MCI in a subject on basis of a molecular biomarker. e.g. in a sample derived from a body fluid of said subject.

Moreover, the identification of biomarkers in body fluids other than CSF, such as plasma, would be desirable because of the better accessibility of said body fluids and less invasive sampling procedure.
Plasma Aβ levels are commonly quantified with enzyme-linked immunoabsorbent assays (ELISA) using antibodies against a variety of Aβ epitopes. However, quantification of Aβ in blood is technically difficult and limited by the fact that most of it is bound to blood components, including lipids, serum albumin, α2-macroglobulin, immunoglobulins, apolipoprotein J, transthyretin, apoferritin, as well as complement components C1q and C3. In light of the above, it would be desirable to provide for biomarkers for AD, particularly biomarkers other than Aβ peptides, as well as for biomarkers for MCI. Accordingly, one object of the present invention is to provide for biomarkers for AD in human body fluids, particularly for other biomarkers than Aβ peptides. Another object of the present invention is to provide for biomarkers for MCI. Still another object of the present invention is to provide for biomarkers in human body fluids that are easily accessible, such as body fluids other than CSF. Still another object of the present invention is to provide for simple, quick and/or cost-effective methods for the diagnosis of AD and such methods for the diagnosis of MCI.

Having performed detailed studies on the levels of anti-Aβ antibodies, particularly circulating autoantibodies of the class M immunoglobulins (IgM), in patients with Alzheimer’s disease (AD), healthy controls (HC) and patients with mild cognitive impairment (MCI), the present inventors have surprisingly found new biomarkers for AD and MCI as well as methods for diagnosing Alzheimer’s disease, methods for diagnosing MCI and methods for predicting the likelihood that a subject having MCI will develop AD as disclosed herein.

SUMMARY OF THE INVENTION

The present invention relates to a method of diagnosing Alzheimer’s disease in a subject, comprising determining in a sample derived from a body fluid of said subject the amount of antibodies capable of binding to a pGlu Aβ peptide; and comparing the amount of said antibodies to a reference amount; wherein the amount of said antibodies compared to the reference amount is indicative that the subject is affected by AD. In addition, the invention relates to embodiments of such methods as defined in the claims.

The invention also relates to methods of diagnosing mild cognitive impairment (MCI) in a subject as defined in the claims, as well as to methods of predicting the likelihood that a subject having mild MCI will develop AD as defined in the claims.

The invention also relates to a pGlu Aβ peptide for use in medicine and/or veterinary medicine as well as to a pGlu Aβ peptide for use in the diagnosis and/or prognosis of AD and/or MCI, and particularly to a pGlu Aβ peptide as defined in the claims.

The invention also relates to the use of an antibody capable of binding to a pGlu Aβ peptide as a biomarker for AD and/or MCI, and particularly to the uses as defined in the
claims. Moreover, the invention relates to methods for identifying and/or isolating an antibody capable of binding to a pGlu Aβ peptide as defined in the claims. The invention also provides for IgM antibodies capable of binding to a pGlu Aβ peptide as defined in the claims. In addition, the invention relates to kits as defined in the claim.

DETAILED DESCRIPTION OF THE INVENTION

The inventors have surprisingly found biomarkers for AD and MCI. methods for diagnosing AD, methods for diagnosing MCI and methods of predicting the likelihood that a subject having mild MCI will develop AD.

Accordingly, in a first aspect, the present invention relates to a method of diagnosing Alzheimer’s disease (AD) in a subject, comprising:

(a) Determining, in a sample derived from a body fluid of said subject, the amount of antibodies capable of binding to a pGlu Aβ peptide; and

(b) comparing the amount of said antibodies to a reference amount:

wherein the amount of said antibodies compared to the reference amount is indicative that the subject is affected by AD.

A “subject” as used herein is a non-human animal or a human. Preferably, the subject is a mammal such as a mouse, a rat, a guinea pig, a cow, a sheep, a pig, a horse, a primate or a human. More preferably, the subject is a human.

A “sample” as used herein refers to any type of sample suitable for determining the concentration of antibodies contained therein.

As used herein, the term “a sample derived from a body fluid” includes the possibility that the sample is directly obtained from a body fluid and is used in the method without the involvement of any processing steps. Said term also includes the possibility that the sample is obtained from the body fluid and subsequently is subjected to one or more processing steps. Suitable processing steps depend on the type of body fluid used and are - in each case - well known to a person skilled in the art. Processing steps include but are not limited to steps of: dilution, concentration, fractionation, purifications, precipitation, heating, cooling, and centrifugation. Accordingly, the components of the sample and the concentrations of the components of the sample may be equal to or different from that of the body fluid. In one embodiment, the concentration of said antibodies in the sample is equal to the concentration of said antibodies in the body fluid. According to another embodiment, the concentration of said antibodies in the sample is increased compared to the concentration of said antibodies in the body fluid. Preferably, the increase in concentration is obtainable by one or more fractionation or purification steps. Said steps include but are not limited to immunoprecipitation, immunoelectrophoresis, gel
electrophoresis, and chromatography such as affinity chromatography and gel filtration chromatography. In a preferred embodiment, the sample has been subjected to gel filtration HPLC. In any case, a person skilled in the art will chose the processing steps such that the amount of said antibodies can still be determined after processing.

A "body fluid" of a subject may be any appropriate body fluid. Different types of body fluids are well known in the art. According to the invention, the body fluid is preferably a body fluid known to comprise antibodies, more preferably antibodies of the immunoglobulin M type (IgM antibodies).

As used herein, by "antibody" is meant a protein of the immunoglobulin family comprising Fe and Fab fragments, which protein is capable of combining, interacting or otherwise associating with an antigen, preferably by an antigen binding fragment, more preferably by the antigen determining region. An "antibody" may be a polyclonal or a monoclonal antibody. Typically, an antibody has a dissociation constant, \( K_d \) of about \( 10^7 \) to about \( 10^1 \) M. "Antibodies" as used herein either refers to a plurality of antibody molecules. Antibodies may be monoclonal and/or polyclonal antibodies. "Polyclonal antibodies" are antibodies derived from different B cell clones. They are a mixture of immunoglobulin molecules against a specific antigen, each recognizing a different epitope. "Monoclonal antibodies" are antibodies derived from a specific B cell clone. They are essentially identical antibody molecules against a specific antigen, each recognizing the same epitope.

The antibodies may be of any subtype such as IgA, IgD, IgE, IgG, IgM, IgY, IgW. According to the invention, preferably, the antibodies are IgM antibodies.

An "antigen" refers to a substance that is capable of interacting with the antibody via an antigen recognition site. An antigen comprises one or more epitopes. An "epitope" is well-known in the art as a particular region of the antigen molecule which region is recognized by an antibody.

A "pGlu" or "pE" as used herein means "pyroglutamate" and, particularly, an N-terminal amino acid residue of a peptide or protein (cf. the formula below; the free binding depicted on the bottom of the left-hand side connects to the "-NH-" group of the adjacent residue in the peptide or protein, respectively). In vivo, a pyroglutamate residue (pGlu) may for example be generated via the conversion of an N-terminal glutamate ("Glu" or "E") or glutamine ("Gln" or "Q") residue by the enzyme glutaminyl cyclase ("QC").

![Pyroglutamate Residue](image)

An "Aβ peptide" or "Abeta peptide" as referred to herein is a peptide comprising at least four consecutive amino acids of Aβ. An Aβ peptide may be aggregated or non-aggregated. Aβ is well known within the art as a peptide generated by proteolytic cleavage of its precursor, the amyloid precursor protein (APP). Full-length Aβ typically comprises about
42 to 43 amino acids, but also longer versions of Aβ, such as an Aβ of 49 amino acids
(Aβ49). have been described. Aβ1-42 has the following sequence:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVVGVIAY

Residue 43, which may optionally be present is "V". Moreover, several both N-terminally
and C-terminally truncated forms of Aβ are known within the art. Exemplary truncated
forms of Aβ include but are not limited to Aβ1-37, Aβ1-38, Aβ1-39, Aβ1-40 and Aβ1-41.

A "pGlu Aβ peptide" as used herein refers to any Aβ peptide comprising a pGlu residue as
an N-terminal residue. A pGlu Aβ peptide may be aggregated or non-aggregated. Said N-
terminal pGlu may occur at any position of Aβ, at which, originally, a Glu or Gln residue is
present, and preferably at positions 3 or 11, resulting in pGlu Aβ 3-x or pGlu Aβ 11-y, x
and y denoting the C-terminus of the pGlu Aβ peptide.

In addition, as used herein, a "derivative of a pGlu Aβ peptide" is a pGlu Aβ peptide
having one or more amino acid additions, substitutions or deletions compared to the

10 corresponding pGlu Aβ peptide, or may be a covalently modified pGlu Aβ peptide. Said
derivatives have a sequence homology of at least 80%, preferably at least 85%, preferably
at least 90%, preferably at least 95%, preferably at least 99% to the corresponding pGlu Aβ
peptide. According to the present invention, a derivative of a pGlu Aβ peptide comprises at
least one epitope of its corresponding pGlu Aβ peptide such as pGlu Aβ 3-x or pGlu Aβ
11-y. Preferably, the derivative comprises all epitopes of its corresponding pGlu Aβ
peptide such as pGlu Aβ 3-x or pGlu Aβ 11-y. Thus, the derivative is preferably capable of
being bound by the antibodies capable of binding to a pGlu Aβ peptide.

Preferably, the pGlu Aβ peptide is any of the following; a) pGlu Aβ 3-x, wherein x denotes
the C-terminus of the peptide, particularly wherein is selected from the group consisting of
the integers from 5 to 43, b) pGlu Aβ 11-y, wherein y denotes the C-terminus of the
peptide, particularly wherein y is selected from the group consisting of the integers from
13 to 43, c) a derivative of a pGlu Aβ 3-x according to a), or a pGlu Aβ 11-y according to
b). Preferably, the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y, x and y denoting the
C-terminus of the pGlu Aβ peptide, or a derivative thereof, particularly wherein x is
selected from the group consisting of the integers from 5 to 43 and y is selected from the
group consisting of the integers from 13 to 43, particularly wherein x is any one of 7, 37,
38, 39, 40, 41, 42, 43 and y is any one of 15, 37, 38, 39, 40, 41, 42, 43. According to one
embodiment preferably, x is 35 or smaller. Preferably, x is 25 or smaller. Preferably, x is
15 or smaller. Preferably, x is 10 or smaller. More preferably, x is 7. Accordingly, the pGlu
Aβ peptide, preferably, is pEFRHD (SEQ ID NO: 2). Preferably, y is 35 or smaller.
Preferably, y is 25 or smaller. More preferably, y is 15.

As used herein, an antibody is said to be "capable of binding to a pGlu Aβ peptide" if it
binds to said pGlu Aβ peptide with a dissociation constant, Kd, of at least 10^-6 M.
preferably at least 10⁻⁷ M, preferably at least 10⁻⁸ M, preferably at least 10⁻⁹ M, preferably at least 10⁻¹⁰ M, preferably at least 10⁻¹¹ M.

An antibody is said to be "not capable of binding to a pGlu Aβ peptide" if it does not bind to said pGlu Aβ peptide with a dissociation constant referred to above.

Likewise, an antibody is said to be "not capable of binding to a corresponding non-pGlu Aβ peptide" if it binds to said corresponding non-pGlu Aβ peptide with a substantially lower affinity. For example, said antibody does not bind to said corresponding non-pGlu Aβ peptide with a dissociation constant referred to above. Preferably, the Kd of an antibody not capable of binding to a corresponding non-pGlu Aβ peptide is 50% or less, preferably 5, 4, 3, 2, 1% or less, preferably 0.1% or less, preferably 0.01% or less of the Kd for its binding to said pGlu Aβ peptide. A skilled person can readily determine, whether such antibody is not capable of binding to a corresponding non-pGlu Aβ peptide e.g. by means of an ELISA. Generally, in preferred embodiments of the invention, an antibody capable of binding to a pGlu Aβ peptide is an antibody not capable of binding to a corresponding non-pGlu Aβ peptide. In a particular embodiment of the invention, the antibody capable of binding to a pGlu Aβ peptide is not the antibody produced by the hybridoma cell line deposited under accession number of FERM BP-7995 at International Patent Organism Depository in Japan.

A "reference amount" as used herein refers to a control amount of antibodies capable of binding to a pGlu Aβ peptide, with which the amount of antibodies capable of binding to a pGlu Aβ peptide in the sample to be tested determined in a method of diagnosing AD according to the invention is compared. The reference amount is, for example, determined using a sample derived from a corresponding body fluid of a healthy subject or of a subject having AD. The reference amount may also be determined using a sample of the same subject derived from a corresponding body fluid that has been obtained at an earlier point in time, particularly at a point in time at which the subject was not affected by AD or MCI as determined by any suitable method according to the prior art, or at a point in time in which the body fluid of the subject has first or previously, respectively, been examined. It is understood that, after official validation of the developed methodology for the claimed diagnosis and/or prognosis of AD or MCI, a standard guide value could be defined, which might replace the described preferred control amount of antibodies.

By a "corresponding body fluid" is meant the same type of body fluid of another or the same subject, respectively. For example, where the type of body fluid is blood, a corresponding body fluid is blood, as well. Preferably, the reference amount is determined using a corresponding sample of a corresponding body fluid. By a "corresponding sample" is meant that the sample has been subjected to the same processing steps as the sample derived from a body fluid of the subject in order to optimize the comparability of the results.
As used herein in connection with a reference amount, a subject is said to be a "subject having AD" when the subject has been diagnosed with any suitable method for diagnosing AD known in the art, such as the ones disclosed by McKhann et al. (McKhann 1984, specifically incorporated herein by reference). Otherwise, a suitable method for diagnosing AD may be based on the measurement of e.g. Aβ42, tau or phospho-tau in CSF. Alternatively, a subject is said to be a subject "having AD", when the subject has been diagnosed with a method according to the first aspect of the invention.

A result obtained according to a method of the invention being "indicative that the subject is affected by AD" means that the corresponding subject has any form of AD such as mild, moderate or advanced AD. If desirable, the outcome can be double-checked by any method for diagnosing AD known in the art, such as the ones referred to above.

According to a preferred embodiment of the first aspect:

(i) where the reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject, a significantly decreased amount of said antibodies compared to the reference amount is indicative that the subject is affected by AD; and/or

(ii) where the reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, an essentially same or decreased amount of said antibodies compared to the reference amount is indicative that the subject is affected by AD.

Moreover,

(iii) where the reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject an essentially same or increased amount of said antibodies compared to the reference amount is indicative that the subject is not affected by AD; and/or

(iv) where the reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, a significantly increased amount of said antibodies compared to the reference amount is indicative that the subject is not affected by AD.

According to the first aspect of the invention, a "significantly decreased" amount compared to the reference amount is preferably an amount that is decreased by at least 15%, preferably at least 20%, preferably at least 25%, preferably at least 30%, preferably at least 35%, and preferably at least 40% of the reference amount. Alternatively, the decrease is preferably in the range of 20-80%, preferably in the range of 25-40%, preferably in the range of 28-35%, preferably in the range of 28-30% compared to the reference amount. In addition, a "significantly increased" amount compared to the
reference amount is preferably an amount that is increased by at least 15%, preferably at least 20%, preferably at least 25%, preferably at least 30%, preferably at least 35%, and preferably at least 40% of the reference amount. Alternatively, the increase is preferably in the range of 20-80%, preferably in the range of 25-40%, preferably in the range of 28-35%, preferably in the range of 28-30% compared to the reference amount.

In addition, an "essentially same" amount compared to the reference amount is preferably an amount that is in the range of 95-105%, preferably in the range of 96-104%, preferably in the range of 97-103%, preferably in the range of 98-102% compared to the reference amount. In addition, an "increased" amount compared to the reference amount is preferably an amount that is increased by more than 5%, preferably more than 10%, compared to the reference amount. In addition, a "decreased" amount compared to the reference amount is preferably an amount that is decreased by more than 5%, preferably more than 10%, compared to the reference amount.

In a second aspect, the present invention relates to a method of diagnosing mild cognitive impairment in a subject, comprising:

(a) determining, in a sample derived from a body fluid of said subject, the amount of antibodies capable of binding to a pGlu Aβ peptide; and
(b) comparing the amount of said antibodies to at least one reference amount;

wherein the amount of said antibodies compared to said at least one reference amount is indicative that the subject is affected by MCI.

According to a preferred embodiment of the second aspect:

(i) where a first reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject and a second reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, a decreased amount of said antibodies compared to the first reference amount and an increased amount of said antibodies compared to the second reference amount is indicative that the subject is affected by MCI; and/or

(ii) where the reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject, an essentially same or increased amount of said antibodies compared to the reference amount is indicative that the subject is not affected by MCI; and/or

(iii) where the reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, an essentially same or decreased amount of said antibodies compared to the reference amount is indicative that the subject is not affected by MCI.

A result obtained according to a method of the invention is being "indicative that the subject is affected by MCI" means that the corresponding subject has MCI. Optionally, the
diagnosis of MCI may be confirmed by any of the known methods of diagnosing MCI such as the one disclosed by Winblad et al. (Winblad 2004, which is specifically incorporated herein by reference). If desirable, the outcome may be double-checked by any method for diagnosing AD known in the art, such as the ones referred to above, in order to confirm that the subject does not have AD.

According to the second aspect of the invention, an "essentially same" amount compared to the reference amount is preferably an amount that is in the range of 95-105%, preferably in the range of 96-104%, preferably in the range of 97-103%, preferably in the range of 98-102% compared to the reference amount. In addition, an "increased" amount compared to the reference amount is preferably an amount that is increased by more than 5%, preferably more than 10%, compared to the reference amount. In addition, a "decreased" amount compared to the reference amount is preferably an amount that is decreased by more than 5%, preferably more than 10%, compared to the reference amount.

In a third aspect, the present invention relates to a method of predicting the likelihood that a subject having mild cognitive impairment (MCI) will develop Alzheimer’s disease (AD), the method comprising

(a) determining, in a sample derived from a body fluid of said subject, the amount of antibodies capable of binding to a pGlu Aβ peptide; and

(b) comparing the amount of said antibodies to at least one reference amount;

wherein the amount of said antibodies compared to said at least one reference amount allows predicting that the subject is likely to develop AD.

According to the third aspect of the invention, a subject is said to be a "subject having MCI" e.g. when the MCI criteria ("Petersen criteria") disclosed by Winblad et al. (Winblad 2004, which is specifically incorporated herein by reference) are fulfilled: (i) the person is neither normal nor demented; (ii) there is evidence of cognitive deterioration shown by either objectively measured decline over time and/or subjective report of decline by self and/or informant in conjunction with objective cognitive deficits; and (iii) activities of daily living are preserved and complex instrumental functions are either intact or minimally impaired. Alternatively, a subject is said to be a subject "having MCI" when the subject has been diagnosed with a method according to the second aspect of the invention.

As used herein, a subject is "likely to develop AD" if the probability that the subject will be affected by AD in the future, is greater than the statistical probability, preferably greater than 30%, preferably greater than 50%, preferably greater than 70%, preferably greater than 90%. In order to increase this probability, the subject may be further monitored, e.g. in accordance with the methods disclosed hereinbelow, to obtain an improved prediction.
According to one preferred embodiment of the third aspect, a first reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject and a second reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, and wherein:

(i) where the difference of the amount of said antibodies to the first reference amount is smaller than the difference of the amount of said antibodies to the second reference amount, the subject is likely not to develop AD; and/or

(ii) where the difference of the amount of said antibodies to the second reference amount is smaller than the difference of the amount of said antibodies to the first reference amount, the subject is likely to develop AD.

According to another preferred embodiment of the third aspect, the method comprises the steps of

(a') determining, in a first sample derived from a body fluid of the subject, the amount of said antibodies to obtain a first amount of said antibodies;

(b') comparing the first amount of said antibodies to at least one reference amount;

(a'') determining, in a second sample derived from said body fluid of the subject, the amount of said antibodies to obtain a second amount of said antibodies; and

(c) comparing the first amount of said antibodies to the second amount of said antibodies;

wherein the body fluid referred to in step (a) has been obtained from the subject 6-12 months before the body fluid referred to in step (a'');

wherein the at least one reference amount is a first reference amount determined using a sample derived from a corresponding body fluid of a healthy subject and a second reference amount determined using a sample derived from a corresponding body fluid of a subject having AD, and wherein:

(i) where the difference of the first amount of said antibodies to the first reference amount is smaller than the difference of the first amount of said antibodies to the second reference amount, and the second amount of said antibodies is decreased compared to the first amount of said antibodies, the subject is likely to develop AD; and/or

(ii) where the difference of the first amount of said antibodies to the first reference amount is smaller than the difference of the first amount of said antibodies to the second reference amount, and the second amount of said antibodies is essentially the same as the first amount of said antibodies, the subject is likely not to develop AD; and/or

(iii) where the difference of the first amount of said antibodies to the second reference amount is smaller than the difference of the first amount of said antibodies to the first
reference amount, and the second amount of said antibodies is decreased compared
to the first amount of said antibodies, the subject is likely to develop AD; and/or
(iv) where the difference of the first amount of said antibodies to the second reference
amount is smaller than the difference of the first amount of said antibodies to the first
reference amount, and the second amount of said antibodies is essentially the same as
the first amount of said antibodies, the subject is likely not to develop AD.
According to this aspect, the samples referred to in steps (a') and (a'"") may be derived from the body fluids at any suitable time, the amounts referred to in steps (a"") and (a'"")
may be determined at any suitable time, and the amounts of antibodies referred to in steps
(b"") and (c) may be compared at any suitable time, as long as the body fluid referred to in
step (a) has been obtained from the subject 6-12 months before the body fluid referred to in
step (a""). For example, the samples may be derived from the body fluids at the same time
and the amounts of antibodies may subsequently, or after an intermediate storage period,
be determined and compared.
According to another preferred embodiment of the third aspect, the method comprises the
steps of
(o) determining, in a sample derived from a body fluid of the subject, the amount of
antibodies capable of binding to a pGlu Aβ peptide to obtain a reference amount;
(a) determining, in a sample derived from a body fluid of the subject, the amount of
antibodies capable of binding to a pGlu Aβ peptide; and
(b"") comparing the amount of said antibodies determined in step (a) to said reference
amount;
wherein the body fluid referred to in step (o) has been obtained from the subject 6-12
months before the body fluid referred to in step (a),
wherein:
(i) where the amount of said antibodies determined in step (a) is decreased compared to
said reference amount, the subject is likely to develop AD; and/or
(ii) where the amount of said antibodies determined in step (a) is essentially the same as
said reference amount, the subject is likely not to develop AD; and/or
(iii) where the amount of said antibodies determined in step (a) is increased compared to
said reference amount, the subject is likely not to develop AD.
According to this aspect, the samples referred to in steps (o) and (a) may be derived from
the body fluids at any suitable time, the amounts referred to in steps (o) and (a) may be
determined at any suitable time, and the amount of antibodies referred to in step (b"") may
be compared at any suitable time, as long as the body fluid referred to in step (o) has been
obtained from the subject 6-12 months before the body fluid referred to in step (a). For
example, the samples may be derived from the body fluids at the same time and the
amounts of antibodies may subsequently, or after an intermediate storage period, be determined and compared.

According to the third aspect of the invention, an "essentially same" amount compared to another amount, such as the reference amount, is preferably an amount that is in the range of 95-105%, preferably in the range of 96-104%, preferably in the range of 97-103%, preferably in the range of 98-102% compared to the other amount, such as the reference amount. In addition, an "increased" amount compared to another amount, such as the reference amount, is preferably an amount that is increased by more than 5%. preferably more than 10%, compared to the other amount, such as the reference amount. In addition, a "decreased" amount compared to another amount, such as the reference amount, is preferably an amount that is decreased by more than 5%. preferably more than 10%, compared to the other amount, such as the reference amount. In addition, by a "difference" as used herein is the meant the difference of the bigger value to the smaller value, expressed as an absolute value. A difference is said to be "smaller" than another difference, if the absolute value of the one difference is smaller than that of the other difference. That is, for example where the first reference amount is 2000 AU ("arbitrary units") and the second reference amount is 1000 AU and the amount of said antibodies is 1800 AU, the difference to the first reference amount is 200 AU, the difference to the second reference amount is 800 AU and, hence, the difference to the first reference amount is smaller than that to the second reference amount. According to the third aspect of the invention, the time period of 6-12 months is preferably from 6-7 months, preferably from 7-8 months, preferably from 8-9 months, preferably from 9-10 months, preferably from 10-11 months, preferably from 11-12 months.

Generally, according to the present invention, the reference amount may be determined using a sample derived from a corresponding body fluid of a healthy subject or a pool of samples derived from corresponding body fluids of a plurality of healthy subjects, of a subject having AD or of pool of samples derived from corresponding body fluids of a plurality of subject having AD. Alternatively, it may be determined using a sample derived from the same subject, wherein the blood fluid, from which the reference amount is derived has been obtained from the subject at an earlier point in time, particularly at a point in time at which the subject was not affected by AD or MCI as determined by any suitable method according to the prior art. or at a point in time in which the body fluid of the subject has first or previously, respectively, been examined. It is understood that, after official validation of the developed methodology for the claimed diagnosis and/or prognosis of AD or MCI, a standard guide value could be defined, which might replace the described preferred control amount of antibodies. As used herein, a "pool of samples" may be at least two. preferably at least five, preferably at least ten, preferably at least fifteen
samples, for example at least one hundred samples, preferably at least one thousand samples. Preferably, the pool of samples is less than ten thousand samples, preferably less than one hundred thousand samples. In a preferred embodiment of the invention, the reference amount is determined using a pool of samples derived from corresponding body fluids of a plurality of healthy subjects or a plurality of subjects having Alzheimer's disease.

The amount of antibodies capable of binding to a pGlu Aβ peptide may be determined by any suitable method known in the art such as by an immunoassay or by chromatography. An "immunoassay", as used herein, relates to an assay involving an immunoglobulin such as an antibody, particularly to an assay allowing the determination of the amount of an immunoglobulin, such as an antibody, e.g. by taking advantage of the specific binding of an antibody to its antigen. "Chromatography" or "chromatographic method" refers to a family of laboratory techniques for the separation of mixtures involving passing a mixture contained in a mobile phase through a stationary phase. As used herein, "chromatography" may be any type of chromatography known to a person skilled in the art. In the context of the determination of the amount of antibodies, the chromatographic method, e.g. affinity chromatography, allows quantification of a certain antibody or antibody species. Preferably, the amount of said antibodies is determined by a method selected from the group consisting of ELISA, immunofluorescence, radioimmunoassay, magnetic immunoassay, Western blot, dot blot, immunodiffusion, agglutination, nephelometry, immunoelectrophoresis, affinity chromatography, and HPLC, more preferably by ELISA. An exemplary method of determining the amount of said antibodies by ELISA is described in the Examples hereinbelow.

According to preferred embodiments of the invention:

a) the amount of said antibodies is quantified in each of the samples by using a calibration curve and/ or

b) the amount of said antibodies and the reference amount or reference amounts in each sample are normalized to an internal reference standard in the sample.

A calibration curve as used herein refers to a means well known in the art that e.g. allows relative or absolute quantification of the amount of antibodies in a sample. Said curve is e.g. obtained by measuring the signal intensity in a given assay for a number of different dilutions of a reference antibody. The measured points a calibration curve is obtained by suitable curve fitting (regression analysis) of the measured points. Based on said curve, the relative or absolute concentration of e.g. the corresponding antibodies contained in a sample may be calculated or otherwise obtained from the measured signal intensity.

As used herein, such "reference antibody" is preferably an antibody capable of binding to said pGlu Aβ peptide. Preferably, the pGlu Aβ peptide is as further defined hereinabove.
Preferably, said pGlu Aβ peptide is pEFRHD (SEQ ID NO: 1). Preferably, the reference antibody is obtainable from a sample derived from a body fluid of a subject, wherein the concentration of the reference antibody in said sample is increased compared to the concentration of the reference antibody in said body fluid. Preferably, the increase in concentration is obtainable by one or more fractionation or purification steps selected from the group consisting of immunoprecipitation, immunoelectrophoresis, gel electrophoresis, chromatography such as affinity chromatography and gel filtration chromatography, particularly wherein the sample has been subjected to gel filtration HPLC.

As used herein, an 'internal reference standard' is a reference standard based on a protein contained in each sample that may optionally be utilized to ensure or improve the comparability of the samples by normalization of the amounts of said antibodies and the reference amount(s) to said internal reference standard. Preferably, said internal reference standard is selected from the amount of antibodies binding to a reference peptide or reference protein, and the amount of a reference peptide or reference protein, particularly wherein said internal reference standard is expressed in essentially the same amount in healthy subjects, subjects having MCI and subjects having AD. The internal reference standard may be an antibody such as an IgM autoantibody binding to a protein such as albumins, globulins, fibrinogen, etc. or may also be a corresponding non-pGlu Aβ peptide, a non-pGlu Aβ peptide or full-length non-Glu Aβ. As stated herein, the present inventors have found that e.g. the titer of IgM autoantibodies against AβI-42 was not significantly different between AD patients and healthy controls, hence allowing a use as an internal reference standard. The internal reference standard may also be a protein selected from albumins, globulins, fibrinogen, transferrin, coeruloplasmin, ferritin, homocystein or may also be a lipid selected from cholesterol, LDL-cholesterol, HDL-cholesterol, triglycerides.

Other suitable reference standards are known to a person skilled in the art.

A "non-pGlu Aβ peptide" as used herein refers to an Aβ peptide not containing a pGlu residue. A "corresponding non-pGlu Aβ peptide" as used herein refers to an Aβ peptide corresponding to the pGlu Aβ peptide, but differing in the N-terminal residue, which is not a pGlu residue, but may be a Glu or Gln residue.

According to the inventions, preferably, the antibody and/or antibodies are autologous antibodies. More preferably the antibodies are autologous IgM antibodies. An "autologous antibody" as used herein refers to an antibody which is autologous with regard to a subject, if the antibody is produced in vivo by the immune system of said subject.

According to the inventions, preferably, the body fluid is a human body fluid selected from blood, blood plasma, blood serum, cerebrospinal fluid, lymph, urine, saliva, tears, semen, breast milk. Preferably, the body fluid is a body fluid that is easily obtained from a subject. Preferably, the body fluid is a body fluid other than cerebrospinal fluid. More preferably, the body fluid is blood or blood plasma.
The method according to the first aspect of the invention may be used for monitoring the disease status or progression of AD in a patient, monitoring the effectiveness of a therapy of AD in a patient, or screening one or more subjects for subjects having AD. The method according to the second or third aspect of the invention for monitoring the disease status or progression of MCI in a patient, monitoring the effectiveness of a therapy of MCI in a patient, or screening one or more subjects for subjects having MCI. The disease status or progression of AD or MCI may be monitored by performing the method of diagnosing AD or MCI according to the invention at two or more points in time separated by intervals of at least 3 months, preferably from 3 months to 15 years, preferably from 6 to 12 months. In the art, this assay format is frequently referred to as "longitudinal study". Similarly, the effectiveness of a therapy of AD or MCI in a patient may be monitored by performing the method of diagnosing AD according to the invention at two or more points in time separated by intervals of at least 3 months, preferably from 3 months to 15 years, preferably from 6 to 12 months, wherein a therapy is considered effective, in case that the amount of antibodies capable of binding to a pGlu Aβ compared to a reference amount increases over time. In addition, by applying the method of diagnosing AD according to the invention in a plurality of subjects, said subjects may be screened for subjects having Alzheimer's disease.

In a fourth aspect, the invention relates to a pGlu Aβ peptide for use in medicine and/or veterinary medicine. In another aspect, the invention relates to a pGlu Aβ peptide for use in the diagnosis and/or prognosis of Alzheimer's disease and/or mild cognitive impairment. According to the latter two aspects of the invention, the pGlu Aβ peptide or derivative thereof, preferably, is not selected from the group consisting of pGlu Aβ3-42, pGlu Aβ3-40 and a modified pGlu Aβ3-7, said modified pGlu Aβ3-7 having a Cys residue attached to its C-terminal end. Otherwise, and generally according to the present invention, the pGlu Aβ peptide, the derivative thereof, and the preferred embodiments of said peptide and derivative are as defined above. Examples of suitable uses are uses in a diagnostic method for AD and/or MCI. Moreover, said pGlu Aβ peptides may be used in a method for diagnosing AD or MCI as described herein, or in any other method for diagnosing AD and/or MCI. Preferably, each of the latter uses involves the detection and or determination of the amount of an antibody or antibodies capable of binding to a pGlu Aβ peptide. It is understood that based on the teachings herein, many such uses will be apparent to a person skilled in the art.

In a fifth aspect, the invention relates to the use of an autologous antibody capable of binding to a pGlu Aβ peptide as a biomarker for Alzheimer’s disease (AD) or mild cognitive impairment (MCI) in a subject. Preferably, the antibody is an IgM antibody. The
use of the fifth aspect of the invention is preferably an "ex vivo" use and particularly an "in vitro" use. Generally, a "biomarker" is a substance used as an indicator of a biologic state, i.e. an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. In the context of the present invention, a "biomarker for Alzheimer's disease" is a substance, preferably a protein, more preferably an antibody, suitable as an indicator of AD, more particularly of the presence or absence of AD. In the context of the present invention, a "biomarker for mild cognitive impairment" is a substance, preferably a protein, more preferably an antibody, suitable as an indicator of MCI. more particularly of the presence or absence of MCI. It is understood that based on the teachings herein and on his general knowledge about biomarkers, many particular uses according to the fifth aspect of the invention will be apparent to a person skilled in the art.

In another aspect, the invention relates to a kit comprising a pGlu Aβ peptide. Preferably, the pGlu Aβ peptide and its preferred embodiments are as defined above. Generally, "kits" are well known within the art. According to the invention, optionally, the kit comprises an instruction manual containing operating instructions for the kit. Preferably, the kit is for diagnosing AD or MCI in a subject and/or for predicting the likelihood that a subject having MCI will develop AD. Accordingly, in a preferred embodiment, said instruction manual contains operating instructions for the use of the kit in accordance with the methods of the invention described above. Preferably, the kit additionally comprises at least one reference sample and/or a reference antibody, particularly wherein said reference antibody as well as its preferred embodiments are as defined above. As used herein, said "reference sample" is a sample derived from a corresponding body fluid that allows the determination of a reference amount referred to in the methods of the invention. That is, said reference sample may be derived from a body fluid of a healthy subject or may be a pool of samples derived from body fluids of a plurality of healthy subjects. In addition, said reference sample may be derived from a body fluid of a subject having AD or may be a pool of samples derived from body fluids of a plurality of subject having AD.

In yet another aspect, the invention relates to a method for identifying and/or isolating an antibody capable of binding to a pGlu Aβ peptide, the method comprising the steps of:
(a) providing a sample derived from a body fluid of a subject or from an in vitro cell culture, said sample containing said antibody,
(b) optionally - bringing the sample into contact with a pGlu Aβ peptide, and
(c) identifying and/or isolating the antibody.
Preferably, the pGlu Aβ peptide and its preferred embodiments are as defined above.
In one embodiment of this method, said antibody is identified and/or isolated by means of affinity chromatography, such as affinity chromatography HPLC. In another embodiment
of this method, said antibody is identified and/or isolated by using a culture of cells capable of expressing an antibody that is capable of binding to a pGlu Aβ peptide. Said culture may comprise a plurality of cells comprising a plurality of antibodies. In this case, a cell capable of expressing an antibody capable of binding to a pGlu Aβ peptide may be selected according to any suitable method known to a person skilled in the art. e.g. by utilizing the affinity of said antibody to said pGlu Aβ peptide. Moreover, various approaches using recombinant DNA technology have been employed since the late 1980s. In one exemplary approach, the DNA that encodes the binding portion of monoclonal mouse antibodies is taken and merged with human antibody-producing DNA. Then, mammalian cell cultures are used to express this DNA and produce these half-mouse and half-human antibodies. Depending on how big a part of the mouse antibody is used, the antibodies are designated as chimeric antibodies or humanized antibodies. Another approach involves mice genetically engineered to produce more human-like antibodies. Any of these approaches may be used. In addition, methods for identifying and/or isolating a particular antibody capable of binding to a particular peptide are very well known to a person skilled in the art and any such method may be employed.

In still another aspect, the invention relates to an antibody capable of binding to a pGlu Aβ peptide, wherein the antibody is not capable of binding to a corresponding non-pGlu Aβ peptide, particularly wherein the antibody is an IgM antibody. A "non- pGlu Aβ peptide" is as defined above. Preferably, the antibody is obtainable by a method for identifying and/or isolating an antibody capable of binding to a pGlu Aβ peptide as defined above. Preferably, the pGlu Aβ peptide and its preferred embodiments are as defined above. In a preferred embodiment the antibody is capable of binding to pEFRHD.

**SHORT DESCRIPTION OF THE FIGURES**

Figure 1. A. Gel-filtration HPLC of pooled plasma samples. Proteins are separated according to their molecular weight, with the larger proteins eluting first. Total protein concentration of each fraction was measured at 280 nm (grey line). The characteristic peaks were as follows: empty volume with a MW >500 kDa (larger proteins and aggregates). IgGs with a MW of 150 kDa, and the albumin peak at 66 kDa. A direct ELISA performed on each fraction to measure the quantity of anti-pGlu Aβ IgM (squares) confirms that IgMs elute in the empty volume fractions. B. Western blot against IgM heavy-chain in the empty volume fraction.

Figure 2. A. Sandwich ELISA: 96-well titer plates are coated with different Aβ fragments (grey). After incubation with plasma samples, the wells are washed and incubated with an
anti-IgM heavy chain antibody conjugated with HRP (Blue). Signal of bound molecules is therefore only produced by complexes IgMs (red) recognizing that specific fragment. B. Calibration curve obtained by serial dilutions of the IgM fraction. The IgMs against pGlu Aβ were obtained from the plasma by means of immobilized pEFRHD peptides. Bound human IgMs were then quantified by means of a secondary antibody.

Figure 3. A. Levels of anti-pGlu Aβ IgM in the different diagnostic groups. B. The comparison of the mean values shows a significant reduction in AD group compared to HC. In addition, there was a trend for anti-pGlu Aβ IgM reduction in MCI versus HC C. ROC curve analysis shows a discrete Area Under the Curve (AUC) of 0.68 in discriminating AD and HC samples. By setting sensitivity at a value higher than 80% corresponding specificity was 60%. D. While the anti-pGlu Aβ IgM levels correlated significantly with MMSE scores in MCI patients (rho = 0.58, df = 13, p = 0.022), no correlation was observed in the group of AD patients.

EXEMPLIFYING SECTION

The following examples are meant to further illustrate, but not limit, the invention. The examples comprise technical features and it will be appreciated that the invention relates also to combinations of technical features presented in this exemplifying section.

1. **Data analysis**

   All biomarker values were expressed as means +/- standard deviation. Differences between the groups were determined by ANOVA followed by Student Rests. The diagnostic powers were determined by ROC curve analysis. Chi-square test on independence was used to analyze, if the gender distribution was significantly different between the three groups and if there is a difference between the two patient groups in relation to the number of family members with dementia. AU analyses were performed using GraphPad Prism (GraphPad software, Inc., La Jolla, CA, USA and SPSS 16 for Windows). Significance level was α = 0.05. All tests were two-tailed.

2. **Patients and samples**

   Plasma samples (stored at -70°C) from 30 out-patients with mild to moderate AD. 15 patients with mild cognitive impairment (MCI) and 30 healthy controls (HC) were analyzed. The patients were recruited at the Memory Clinic at the Department of Geriatrics. Uppsala University Hospital. All AD patients were diagnosed according to NINCDS criteria (McKhann 1984, specifically incorporated herein by reference). The MCI
patients met the Petersen criteria (Winblad. 2004. specifically incorporated herein by reference) for MCI.

There were no significant age differences between the three groups (Alzheimer patients (AD). MCI and healthy controls) (F = 0.3, df = 2, 72: p = 0.74). Patients with MCI diagnosis were not significantly older at onset of the disease than AD patients (F = 2.3, df = 1. 42; p = 0.13). As expected, the calculated mean MMSE score was higher in MCI compared to AD patients (F = 44.3. df = 1. 40: p < 0.0005). Gender distribution was not significantly different between the three groups (Chi² = 1.2, df = 2; p = 0.55) and the proportion of family members with dementia did not differ significantly between the two patients groups (Chi² = 0.8. df = 1; p = 0.38) (Table 1).

Table 1. Demographic and clinical data of the studied groups

<table>
<thead>
<tr>
<th>Gender (male; female)</th>
<th>AD</th>
<th>MCI</th>
<th>HC</th>
<th>Chi²</th>
<th>df</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m:12; f: 18</td>
<td>m: 6, f 7</td>
<td>m:11; f 19</td>
<td>1.2</td>
<td>2</td>
<td>0.55</td>
</tr>
<tr>
<td>Dementia in family</td>
<td>yes:16; no: 12</td>
<td>yes: 6; no: 8</td>
<td></td>
<td>0.8</td>
<td>1</td>
<td>0.38</td>
</tr>
<tr>
<td>Age (years)</td>
<td>n</td>
<td>m ± sd</td>
<td>n</td>
<td>m ± sd</td>
<td>F</td>
<td>df</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>72.3 ± 6.7</td>
<td>15</td>
<td>73.7 ± 4.3</td>
<td>0.3</td>
<td>2, 72</td>
</tr>
<tr>
<td>Age at onset (years)</td>
<td>30</td>
<td>67.9 ± 7.9</td>
<td>14</td>
<td>71.4 ± 4.9</td>
<td>2.3</td>
<td>1, 42</td>
</tr>
<tr>
<td>MMSE (at screening)</td>
<td>27</td>
<td>17.3 ± 6.1</td>
<td>15</td>
<td>27.9 ± 1.3</td>
<td>44.3</td>
<td>1, 40</td>
</tr>
</tbody>
</table>

AD = Alzheimer’s disease, MCI = mild cognitive impaired individuals, HC = healthy controls, m = mean, sd = standard deviation, Chi² = Chi-square statistic, F = F-statistic, df = degrees of freedom, p = probability, m = male, f = female.

3. Gel filtration

Plasma samples from 5 AD patients and 5 healthy controls showing high reactivity to pGlu Aβ-IgM immune complexes in a preliminary ELISA assay using pEFRHD as the antigen were grouped and analyzed by gel filtration HPLC. Five hundred microliters of pooled samples were analyzed using a gel-filtration column Superdex 200 10/300 GL (GE Healthcare, Freiburg, Germany) on an ACTA Basic system (GE Healthcare, Freiburg, Germany). The elution was carried out in PBS at a flow rate of 1 ml per minute, and sample absorbance was monitored at 280 nm. Fractions were collected every 30 seconds, immunoreactivity was tested by ELISA using pEFRHD as the antigen and the fractions corresponding to the highest immunoreactivity were pooled and used as reference standard. Before running the samples, a calibration run was carried out according to the manufacturer’s instructions.

Plasma samples from the above 10 individuals were pooled and analyzed by gel filtration. Fractions collected from the column were tested for the presence of anti-pGlu Aβ IgM by
ELISA assays using pEFRHD. A strong immunoreactivity against anti-pGlu Aβ IgM was observed in the fractions eluting at high molecular weight (> 500 kDa) (Figure 1A). The fractions contained components migrating at the expected molecular weight for reduced IgM that were stained by using an anti-human IgM antibody (Figure 1B).

4. Western Blot

Gel-filtration fractions corresponding to 280 nm absorbance peaks were separated on a 4-12% variogel (Anamed, Groß-Bieberau, Germany) and then transferred to a nitrocellulose membrane. The membrane was heated in PBS in a microwave for 5 minutes and then blocked in 10% non-fat dry milk in TBS containing 0.05% Tween (TBS-T) for 1 h at room temperature. Blots were then incubated with peroxidase-conjugated goat anti-human IgM (Sigma-Aldrich) at 1:1000 in TBS-T for 2 hours or, alternatively, with a primary antibody directed against Aβ residues 4-10 (WO-2, The Genetics Company, Switzerland) overnight at 4°C and secondary peroxidase-conjugated antibodies for 2 h at room temperature.

Protein bands were revealed using ehemiluminescence solution and peroxide hydrogen as substrates on a Curix60 developing machine (Agfa-Gevaert N.V., Mortsel, Belgium).

5. ELISA assay to detect anti-Aβ IgM

96-well ELISA plates were coated with 50 microliters per well of the following antigens: 1 μg/mL of a synthetic fragment of human Aβ starting at position 3 with a modified pyroglutamate and ending at position 7 (pEFRHD) (SEQ ID NO: 2); 1 μg/mL of synthetic full length human AβI-42 (SEQ ID NO: 1); 0.1 μg/mL of synthetic human Aβ38-42 (N-GVVIA-C) (SEQ ID NO: 3); 0.1 μg/mL of synthetic human Aβ37-40 (N-GGVV-C) (SEQ ID NO: 4) (all peptides from Synaptic Systems GmbH, Goettingen, Germany) in PBS at 4°C overnight and then blocked for 2 hours with 3% BSA in PBS. After blocking, 50 μL of fractions, serially diluted reference standards or samples in PBS-B-T were incubated for 1.5 h at 37°C. The autologous IgM antibodies were revealed using peroxidase conjugated anti-human IgM (Sigma-Aldrich Chemie GmbH, Munich, Germany) at a dilution of 1:20000 in PBS-B-T and developed with TMB (Pierce manufacturing, Appleton, IL, USA) and Hydrogen peroxide as the substrate. The amount of IgM in the plasma was expressed in arbitrary units/mL (AU/mL) by using Gel-filtration purified anti-pGlu Aβ IgM reference standards to design a calibration curve; concentration was determined by interpolation of samples absorbance on the calibration curve. The samples with values above 32 AU/mL (matching the upper limit of the calibration curve) were further diluted and re-measured.

The assay was standardized and its analytical performance evaluated: coefficient of variation (CV) inter- and intra-assay was less than 10%. Range of linearity of the assay was between 0.9 and 32 AU/mL.
To assess the relevance of these findings, plasma samples from 75 patients with AD, MCI, and from healthy controls were analyzed for the presence of natural IgM autoantibodies against different Aβ epitopes. Serial dilutions of the gel filtration-purified IgM fractions have been used to draw a reference calibration curve (Figure 2). By interpolation with the standard curve, anti-pGlu Aβ IgM concentration in the plasma samples has been expressed in arbitrary units per mL (AU/mL). The titer of IgM autoantibodies against Aβ1-42, Aβ37-40 and Aβ38-42 was not significantly different between AD patients and HC (data not shown). The mean level of anti-pGlu Aβ-IgM was significantly decreased in AD patients as compared to healthy controls (3-group ANOVA: F = 3.2. df = 2, 72. p = 0.045: AD vs. HC: -29%, p = 0.021). In addition, there was a trend for anti-pGlu Aβ-IgM reduction in MCI patients versus healthy controls (-28%, p = 0.071), while there was no significant difference between the two patient groups (AD vs. MCI: -2%. p = 0.92) (Figure 3, Table 2).

Table 2. Comparison of anti-pGlu Aβ IgM between the diagnostic group s

<table>
<thead>
<tr>
<th></th>
<th>AD</th>
<th>MCI</th>
<th>HC</th>
<th>ANOVA 3 group comparison</th>
<th>Subgroup comparison</th>
<th>AD vs. HC</th>
<th>MCI vs. HC</th>
<th>MCI vs. AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>m ± sd</td>
<td>n</td>
<td>m ± sd</td>
<td>n</td>
<td>m ± sd</td>
<td>F</td>
<td>df</td>
<td>p</td>
</tr>
<tr>
<td>anti-pGlu-Aβ IgM</td>
<td>50 1708 ± 838</td>
<td>15 1741 ± 648</td>
<td>30 2408 ± 1532</td>
<td>3.2 2.72 0.045</td>
<td>0.021 0.071 0.92</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AD = Alzheimer disease, MCI = mild cognitive impairment, HC = healthy controls, ANOVA = analysis of variance, m = mean, sd = standard deviation, F = F-statistic, df = degrees of freedom, p = probability

ROC curve analysis of the anti-pGlu Aβ IgM showed that specificity was 60% when sensitivity was set to 80%, and sensitivity was 32% when specificity was set to 80% comparing AD patients with HC, with an area under the curve (AUC) of 0.68 (Figure 3C, Table 3).

Table 3. Comparison of specificity, sensitivity. Positive Predictive Value (PPV = true positive (TP)/TP+false positive). Negative Predictive Value (NPV = true negative (TN)/TN+false negative) of anti-pGlu Aβ IgM assay when setting alternatively sensitivity and specificity to 80%. according to ROC curve analysis.

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Cut-off (AU/mL)</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-pGlu-Aβ IgM</td>
<td>&lt;2016</td>
<td>80% (24/30)</td>
<td>60% (12/30)</td>
<td>67%</td>
<td>75%</td>
</tr>
<tr>
<td></td>
<td>&lt;1277</td>
<td>33% (10/30)</td>
<td>80% (8/30)</td>
<td>63%</td>
<td>55%</td>
</tr>
</tbody>
</table>
Interestingly, in the group of AD patients there was no significant correlation between anti-pGlu Aβ IgM and cognitive decline analyzed by MMSE (rho = -0.1, df = 25, p = 0.65). These two variables correlated however significantly positive in MCI patients (rho = 0.58, df = 13, p = 0.022: Figure 3D).

6. Discussion

In the present study plasma samples from 30 AD patients, 15 MCI patients, and 30 age- and sex-matched HC have been analyzed to determine the levels of circulating Aβ-IgM. After gel-filtration and size fractionation, Aβ was found to be associated with the IgM fractions using an ELISA. The present inventors observed that autoantibodies against pGlu Aβ (N-terminal truncated Aβ starting at position three with pyroglutamate) were significantly decreased in AD patients as compared to healthy controls. In addition, in the group of MCI patients there was a significant positive correlation between anti-pGlu Aβ IgM and cognitive status. None of the other IgM autoantibody levels against Aβ showed any significant difference between the groups.

These results indicate that circulating anti-pGlu Aβ IgMs result from autoimmune-mediated processes involved in AD. In any case, autoimmunity driven homeostasis of plasma protein levels is a natural process and its understanding may be useful in both diagnosing and treating AD. One study group showed that active immunization against aggregated Aβ42 resulted in a slower cognitive decline in a dose-dependent fashion. Patients generating a higher titer of anti-Aβ antibodies performed better in cognitive test than patients who did not respond to the immunization protocol. The increase in antibody titer did not correspond to a decrease in Aβ blood levels however. Significant changes were observed in serum and CSF of patients with AD after treatment with intravenous Aβ IgGs.

The levels of Aβ autoantibodies of the IgG class for monitoring the effect of passive or active immunization in AD have already been evaluated by recent studies. It is speculative to discuss a possible role of Aβ-IgM levels as a predictive indicator of immunotherapy efficacy. A possible hypothesis includes that they serve as a “buffering system” to keep free potential toxic endogenous peptides and proteins under homeostatic control and lead to their clearance (Gruden 2007). Previous studies have shown the presence of anti-Aβ autoantibodies (Xu 1997) and circulating IC of Aβ and IgGs of different classes (Gruden 2004). APP transgenic mice injected intravenously with monoclonal anti-Aβ antibody showed a rapid and massive increase of CNS-derived Aβ in the plasma suggesting that antibodies may participate in Aβ clearance from the brain. However, whether this immune activity is defensive or toxic is still a matter of debate (cf. e.g., Nath 2003).
LITERATURE


Claims

1. A method of diagnosing Alzheimer’s disease (AD) in a subject, comprising:
   (a) determining, in a sample derived from a body fluid of said subject, the amount
       of antibodies capable of binding to a pGlu Aβ peptide; and
   (b) comparing the amount of said antibodies to a reference amount;
   wherein the amount of said antibodies compared to the reference amount is
   indicative that the subject is affected by AD.

2. The method of claim 1, wherein
   (i) where the reference amount is determined using a sample derived from a
       corresponding body fluid of a healthy subject, a significantly decreased amount
       of said antibodies compared to the reference amount is indicative that the
       subject is affected by AD; and/ or
   (ii) where the reference amount is determined using a sample derived from a
       corresponding body fluid of a subject having AD, an essentially same or
       decreased amount of said antibodies compared to the reference amount is
       indicative that the subject is affected by AD.

3. A method of diagnosing mild cognitive impairment (MCI) in a subject, comprising:
   (a) determining, in a sample derived from a body fluid of said subject, the amount
       of antibodies capable of binding to a pGlu Aβ peptide; and
   (b) comparing the amount of said antibodies to at least one reference amount:
   wherein the amount of said antibodies compared to said at least one reference
   amount is indicative that the subject is affected by MCI.

4. The method of claim 3, wherein
   (i) where a first reference amount is determined using a sample derived from a
       corresponding body fluid of a healthy subject and a second reference amount is
       determined using a sample derived from a corresponding body fluid of a
       subject having AD, a decreased amount of said antibodies compared to the first
       reference amount and an increased amount of said antibodies compared to the
       second reference amount is indicative that the subject is affected by MCI.
5. A method of predicting the likelihood that a subject having mild cognitive impairment (MCI) will develop Alzheimer's disease (AD), the method comprising
(a) determining, in a sample derived from a body fluid of said subject, the amount of antibodies capable of binding to a pGlu Aβ peptide; and
(b) comparing the amount of said antibodies to at least one reference amount:
wherein the amount of said antibodies compared to said at least one reference amount allows predicting that the subject is likely to develop AD.

6. The method of claim 5, wherein a first reference amount is determined using a sample derived from a corresponding body fluid of a healthy subject and a second reference amount is determined using a sample derived from a corresponding body fluid of a subject having AD, and wherein:
(i) where the difference of the amount of said antibodies to the first reference amount is smaller than the difference of the amount of said antibodies to the second reference amount, the subject is likely not to develop AD; and/or
(ii) where the difference of the amount of said antibodies to the second reference amount is smaller than the difference of the amount of said antibodies to the first reference amount, the subject is likely to develop AD.

7. The method of claim 5, the method comprising the steps of
(a’) determining, in a first sample derived from a body fluid of the subject the amount of said antibodies to obtain a first amount of said antibodies:
(b’) comparing the first amount of said antibodies to at least one reference amount;
(a’”) determining, in a second sample derived from said body fluid of the subject the amount of said antibodies to obtain a second amount of said antibodies; and
(c) comparing the first amount of said antibodies to the second amount of said antibodies:
wherein the body fluid referred to in step (a’) has been obtained from the subject 6-12 months before the body fluid referred to in step (a’”);
wherein the at least one reference amount is a first reference amount determined using a sample derived from a corresponding body fluid of a healthy subject and a second reference amount determined using a sample derived from a corresponding body fluid of a subject having AD, and wherein:
(i) where the difference of the first amount of said antibodies to the first reference amount is smaller than the difference of the first amount of said antibodies to the second reference amount, and the second amount of said antibodies is decreased compared to the first amount of said antibodies, the subject is likely to develop AD; and/or
(ii) where the difference of the first amount of said antibodies to the first reference amount is smaller than the difference of the first amount of said antibodies to the second reference amount, and the second amount of said antibodies is essentially the same as the first amount of said antibodies, the subject is likely not to develop AD: and/or

(iii) where the difference of the first amount of said antibodies to the second reference amount is smaller than the difference of the first amount of said antibodies to the first reference amount, and the second amount of said antibodies is decreased compared to the first amount of said antibodies, the subject is likely to develop AD; and/or

(iv) where the difference of the first amount of said antibodies to the second reference amount is smaller than the difference of the first amount of said antibodies to the first reference amount, and the second amount of said antibodies is essentially the same as the first amount of said antibodies, the subject is likely not to develop AD,

8. The method of claim 5, the method comprising the steps of

(0) determining, in a sample derived from a body fluid of the subject, the amount of antibodies capable of binding to a pGlu Aβ peptide to obtain a reference amount:

(a) determining, in a sample derived from a body fluid of the subject, the amount of antibodies capable of binding to a pGlu Aβ peptide; and

(b") comparing the amount of said antibodies determined in step (a) to said reference amount;

wherein the body fluid referred to in step (a) has been obtained from the subject 6-12 months before the body fluid referred to in step (a), wherein:

(i) where the amount of said antibodies determined in step (a) is decreased compared to said reference amount, the subject is likely to develop AD; and/or

(ii) where the amount of said antibodies determined in step (a) is essentially the same as said reference amount, the subject is likely not to develop AD; and/or

(iii) where the amount of said antibodies determined in step (a) is increased compared to said reference amount, the subject is likely not to develop AD.

9. The method of any of claims 1-7, wherein the reference amount is determined using a pool of samples derived from corresponding body fluids of a plurality of healthy subjects or a plurality of subjects having Alzheimer's disease.
10. The method of any of the preceding claims, wherein the amount of said antibodies is determined by any one of ELISA, immunofluorescence, radioimmunoassay, magnetic immunoassay, Western blot, dot blot, immunodiffusion, agglutination, nephelometry, immunoelectrophoresis, affinity chromatography, and HPLC, particularly by ELISA.

11. The method of any of the preceding claims, wherein
a) the amount of said antibodies is quantified in each of the samples by using a calibration curve,
particularly wherein said calibration curve is obtained by using a reference antibody capable of binding to said pGlu Aβ peptide: and/or
b) the amount of said antibodies and the reference amount or reference amounts in each sample are normalized to an internal reference standard in the sample.

12. The method of claim 11, wherein the reference antibody is obtainable from a sample derived from a body fluid of a subject, wherein the concentration of the reference antibody in said sample is increased compared to the concentration of the reference antibody in said body fluid.

13. The method of claim 12, wherein the increase in concentration is obtainable by one or more fractionation or purification steps selected from the group consisting of immunoprecipitation, immunoelectrophoresis, gel electrophoresis, chromatography such as affinity chromatography and gel filtration chromatography, particularly wherein the sample has been subjected to gel filtration HPLC.

14. The method of any of the preceding claims, wherein the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y, x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof, particularly wherein x is selected from the group consisting of the integers from 5 to 43 and y is selected from the group consisting of the integers from 13 to 43, particularly wherein x is any one of 7, 37, 38, 39, 40, 41, 42, 43 and y is any one of 15, 37, 38, 39, 40, 41, 42, 43.

15. The method of any of the preceding claims, wherein the antibodies are autologous antibodies, particularly wherein the antibodies are autologous IgM antibodies.
16. The method of any of the preceding claims, wherein the body fluid is a human body fluid selected from blood, blood plasma, blood serum, cerebrospinal fluid, lymph, urine, saliva, tears, semen, breast milk, particularly wherein the body fluid is blood or blood plasma.

17. The method of claim 1 or 2, optionally as further defined according to any one of claims 9 to 16, for monitoring the disease status or progression of AD in a patient, monitoring the effectiveness of a therapy of AD in a patient, or screening one or more subjects for subjects having AD.

18. The method of any one of claims 3 to 8, optionally as further defined according to any one of claims 9 to 16 for monitoring the disease status or progression of MCI in a patient, monitoring the effectiveness of a therapy of MCI in a patient, or screening one or more subjects for subjects having MCI.

19. A pGlu Aβ peptide for use in medicine and/or veterinary medicine.

20. A pů lu Aβ peptide for use in the diagnosis and/or prognosis of Alzheimer's disease and/or mild cognitive impairment.

21. The pGlu Aβ peptide according to claim 18 or 19, wherein the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y. x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof, particularly wherein x is selected from the group consisting of the integers from 5 to 43 and y is selected from the group consisting of the integers from 13 to 43, particularly wherein x is any one of 7, 37, 38, 39, 40, 41, 42, 43 and y is any one of 15, 37, 38, 39, 40, 41, 42, 43.

22. Use of an autologous antibody capable of binding to a pGlu Aβ peptide as a biomarker for Alzheimer's disease (AD) or mild cognitive impairment (MCI) in a subject, particularly wherein said antibody is an IgM antibody.

23. The use according to claim 22, wherein the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y, x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof, particularly wherein x is selected from the group consisting of the integers from 5 to 43 and y is selected from the group consisting of the integers from 13 to 43,
particularly wherein x is any one of 7, 37, 38, 39, 40, 41, 42, 43 and y is any one of 15, 37, 38, 39, 40, 41, 42, 43.


25. The kit according to claim 24, wherein the kit additionally comprises at least one reference sample and/or a reference antibody.

26. The kit according to claim 24 or 25, wherein the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y. x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof.

27. A method for identifying and/or isolating an antibody capable of binding to a pGlu Aβ peptide, the method comprising the steps of:
   (a) providing a sample derived from a body fluid of a subject or from an in vitro cell culture, said sample containing said antibody.
   (b) - optionally - bringing the sample into contact with a pGlu Aβ peptide, and
   (c) identifying and/or isolating the antibody.

28. The method according to claim 27, wherein the pGlu Aβ peptide is pGlu Aβ 3-x or pGlu Aβ 11-y. x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof.

29. An antibody capable of binding to a pGlu Aβ peptide, wherein the antibody is not capable of binding to a corresponding non-pGlu Aβ peptide, particularly wherein the antibody is an IgM antibody.
30. The antibody according to claim 29, wherein the antibody is obtainable by a method according to claim 27 or 28.

31. The antibody according to claim 29 or 30, wherein the pGlu Aβ peptide is pGlu Aβ
3-x or pGlu Aβ 11-y, x and y denoting the C-terminus of the pGlu Aβ peptide, or a derivative thereof,
particularly wherein x is selected from the group consisting of the integers from 5 to 43 and y is selected from the group consisting of the integers from 13 to 43,
particularly wherein x is any one of 7, 37, 38, 39, 40, 41, 42, 43 and y is any one of 15, 37, 38, 39, 40, 41, 42, 43.
Figure 1
Figure 2
Figure 3
Figure 3 (continued)
INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2010/056255

A. CLASSIFICATION OF SUBJECT MATTER

INV. G01N33/68
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)

GOIN

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

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

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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D. Further documents are listed in the continuation of Box C

See patent family annex

Date of the actual completion of the international search
15 July 2010

Date of mailing of the international search report
06/08/2010

Name and mailing address of the ISA
European Patent Office P B 581 B Patentlaan 2 NL-2280 HV Rijswijk
Tel (+31-70) 340-2040
Fax (+31-70) 340-3016

Authorized officer
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<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No</th>
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</table>
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of
   a. (means)
      - [X] on paper
      - [X] in electronic form
   b. (time)
      - [X] in the international application as filed
      - [X] together with the international application in electronic form
      - [ ] subsequently to this Authority for the purpose of search

2. In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments