



- (51) **International Patent Classification:**
A61K 39/00 (2006.01) A61K 39/395 (2006.01)
C07K 16/18 (2006.01)
- (21) **International Application Number:**
PCT/EP2018/052239
- (22) **International Filing Date:**
30 January 2018 (30.01.2018)
- (25) **Filing Language:** English
- (26) **Publication Language:** English
- (30) **Priority Data:**
17305110.3 31 January 2017 (31.01.2017) EP
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(81) **Designated States** (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(54) **Title:** NEURONAL CELL PROTECTIVE EFFECT OF ANTIBODIES SPECIFIC FOR THE PROTOFIBRILLAR FORM OF THE BETA-AMYLOID PEPTIDE

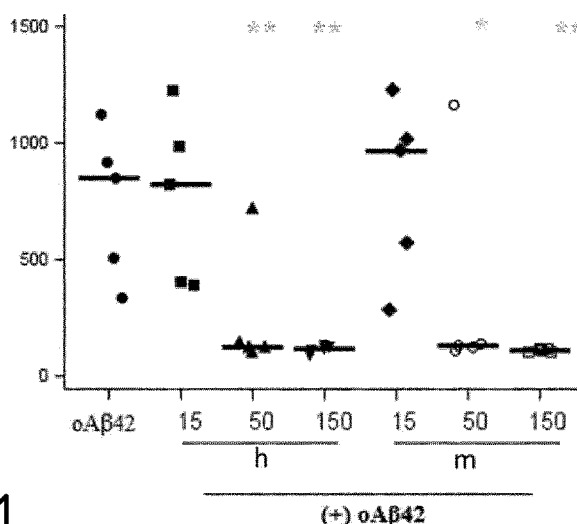


FIG.1

(57) **Abstract:** The present invention relates to an antibody specific for the protofibrillar form of the Aβ peptide for use in the prevention and/or the treatment of a neurodegenerative disease involving Aβ peptide aggregation, in particular for the prevention of Alzheimer's disease and/or the treatment of an early stage of symptomatic Alzheimer's disease. The antibody may thus be used before the appearance of memory loss and impaired cognitive functions or at the earliest stages of the disease. Said antibody has indeed a neuronal cell protective effect against Aβ peptide-induced neurotoxicity.



Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

NEURONAL CELL PROTECTIVE EFFECT OF ANTIBODIES SPECIFIC FOR THE PROTOFIBRILLAR FORM OF THE BETA-AMYLOID PEPTIDE

5 Field of the invention

The present invention relates to the prevention and/or treatment of Alzheimer's disease.

Background

10 Alzheimer's disease is a complex multifactorial neurodegenerative disorder, which particularly results in loss of memory and impairment of cognitive functions. Neuropathological findings of Alzheimer's disease are characterized by the accumulation of extracellular deposits of beta-amyloid (A β) peptide in senile plaques and the intracellular aggregation of tau protein in neurofibrillary tangles. Alzheimer's disease (AD) progression is linked to an increased density of fibrillar A β plaques.

15 Document WO2010/130946 discloses humanized antibodies specific for the protofibrillar form of A β peptide and their use for the treatment of diseases associated with neurodegenerative disorders, such as Alzheimer's disease. The protofibrillar form of A β peptide disclosed in document WO2010/130946 corresponds to oligomers having a molecular weight greater than 200 kDa. Said humanized antibodies are characterized by a
20 greatly improved affinity for the protofibrillar form of peptide A β by comparison to their affinity to the other forms of this peptide, which results in their specific binding to senile plaques and not to diffuse deposits.

Senile plaques are mainly composed of insoluble filamentous aggregates having a high molecular weight, the so-called fibrils, and of protofibrillar forms that have been
25 shown to have a molecular weight greater than 200 kDa. On the contrary, diffuse deposits comprise no or little fibrils and protofibrillar forms of A β peptide. It is believed that diffuse plaques are precursors to senile plaques. Insoluble fibrils are indeed formed by the progressive aggregation of soluble A β peptides, which results in fibrillar forms that are initially soluble, i.e. protofibrillar forms, before to be converted into insoluble forms.

30 Soluble oligomers of A β peptide, including dimers, trimers and larger oligomers, are suggested to cause synaptic and cognitive dysfunction in the early stages of Alzheimer's disease. These soluble oligomers may be the major synaptotoxic A β species leading to neuronal dysfunction.

35 Alzheimer's disease has been recently reconceptualized as a progressive sequence of pathophysiological changes that includes asymptomatic, symptomatic prodementia (mild cognitive impairment (MCI) due to AD or prodromal AD) and dementia phases. In

the asymptomatic phase, biomarkers are used to establish the presence of AD pathology in subjects with no or very subtle overt symptoms.

There is still a need to provide solutions for the prevention of Alzheimer's disease, more particularly by intervention(s) initiated in cognitively unimpaired at-risk individuals to prevent the clinical onset of Alzheimer's disease and/or for the treatment of the earliest stages of the disease.

Description of the invention

The Inventors have surprisingly found that antibodies specific for the protofibrillar form of the A β peptide, in particular specific for oligomers having a molecular weight greater than 200 kDa, have a neuronal cell protective effect against a preparation comprising several SDS-stable forms of A β peptide, which forms include small oligomers, large oligomers and aggregates having a molecular weight of less than 200 kDa.

Whereas these antibodies specific for the protofibrillar form of the A β peptide were previously disclosed as specifically binding to senile plaques and not to diffuse deposits, it was unexpected that these antibodies can prevent the neurotoxic effects of a A β preparation comprising several forms derived from synthetic A β 42.

The Inventors have indeed shown that the humanized antibody Ab1 (consisting of two heavy chains of sequence SEQ ID NO: 2 and two light chains of sequence SEQ ID NO: 4) significantly inhibits the neurotoxic effects induced by a preparation comprising several SDS-stable forms of A β 42 peptide (referred to as "oA β 42") in mouse primary neuronal cell cultures. Said humanized antibody indeed inhibits in a concentration-dependent manner both oA β 42-induced increase in capsase-3/7 enzymatic activity and oA β 42-induced decrease in neurite network.

The antibodies specific for the protofibrillar form of the A β peptide are thus particularly useful for the prevention of a disease involving A β peptide aggregation, in particular for the prevention of Alzheimer's disease, more particularly to prevent the clinical onset of Alzheimer's disease in a subject at risk of developing symptomatic Alzheimer's disease. These antibodies indeed allow preventing memory loss and impairment of cognitive functions. More particularly, they allow preventing memory loss and impairment of cognitive functions, which result from toxic A β oligomers and not from senile plaques.

The present invention thus relates to an antibody specific for the protofibrillar form of the A β peptide for use in the prevention of Alzheimer's disease and/or in the treatment of an early stage of symptomatic Alzheimer's disease.

The present invention also relates to a method for preventing Alzheimer's disease and/or treating an early stage of symptomatic Alzheimer's disease in a subject in need thereof, wherein said method comprises administering to said subject an effective amount of an antibody specific for the protofibrillar form of the A β peptide.

5 The present invention particularly relates to an antibody specific for the protofibrillar form of the A β peptide for use in the prevention of Alzheimer's disease in a subject (i) who does not suffer from memory loss and/or from impaired cognitive function and/or (ii) who does not have senile plaques, does not have neurodegeneration and/or does not have synapse dysfunction.

10 The antibody for use according to the invention preferably prevents, at least partially, A β -induced cytotoxicity in neuronal cells, in particular prevents or reduces A β -induced cytotoxicity in neuronal cells.

The antibody for use according to the invention preferably prevents at least partially, A β -induced apoptosis of neuronal cells and/or prevents, at least partially, A β -
15 induced neurite network decrease.

The antibody is preferably a humanized antibody.

In one preferred embodiment, the antibody comprises (i) the CDRs of sequence SEQ ID NO: 10, 12, 14, 16, 18 and 20, (ii) the CDRs of sequence SEQ ID NO: 10, 12, 14, 32, 18 and 20 or (iii) the CDRs of sequence SEQ ID NO: 10, 12, 30, 32, 18 and 20.

20 The antibody preferably comprises the CDRs encoded by (i) the nucleotide sequences SEQ ID NO: 9, 11, 13, 15, 17 and 19, (ii) the nucleotide sequences SEQ ID NO: 9, 11, 13, 31, 17 and 19, (iii) the nucleotide sequences SEQ ID NO: 9, 11, 29, 31, 17 and 19 or (iv) by nucleotide sequences differing by respectively 1, 2, 3, 4 or 5 nucleotides from these sequences of (i), (ii) or (iii).

25 In one preferred embodiment, the variable part of the heavy chain of the antibody is encoded by a sequence having at least 80% identity with sequence SEQ ID NO: 5 or SEQ ID NO: 27 and/or (ii) the variable part of the light chain of the antibody is encoded by a sequence having at least 80% identity with sequence SEQ ID NO: 7 or SEQ ID NO: 23.

30 In one preferred embodiment, the variable part of the heavy chain of the antibody comprises a sequence having at least 80% identity with sequence SEQ ID NO: 6 or SEQ ID NO: 28 and/or the variable part of the light chain of the antibody comprises a sequence having at least 80% identity with sequence SEQ ID NO: 8 or SEQ ID NO: 24.

35 The antibody preferably comprises (i) a heavy chain encoded by a sequence having at least 80% identity with the nucleotide sequence SEQ ID NO: 1 or SEQ ID NO: 25 and/or (ii) a light chain encoded by a sequence having at least 80% identity with the nucleotide sequence SEQ ID NO: 3 or SEQ ID NO: 21.

The antibody preferably comprises (i) a heavy chain having at least 80% identity with the polypeptide sequence SEQ ID NO: 2 or SEQ ID NO: 26 and/or (ii) a light chain having at least 80% identity with the polypeptide sequence SEQ ID NO: 4 or SEQ ID NO: 22.

5 The antibody preferably comprises sequences encoded by the nucleotide sequences (i) SEQ ID NO: 5 and 7, (ii) the nucleotide sequences SEQ ID NO: 5 and 23 or (iii) the nucleotide sequences SEQ ID NO: 27 and 23.

The antibody is preferably characterized in that its sequence comprises (i) the polypeptide sequences SEQ ID NO: 6 and 8, (ii) the polypeptide sequences
10 SEQ ID NO: 6 and 24 or (iii) the polypeptide sequences SEQ ID NO: 28 and 24.

The antibody preferably comprises the sequences encoded by (i) the nucleotide sequences SEQ ID NO: 1 and 3, (ii) the nucleotide sequences SEQ ID NO: 1 and 21 or (iii) the nucleotide sequences SEQ ID NO: 25 and 21.

The antibody preferably comprises (i) the polypeptide sequences SEQ ID NO: 2
15 and 4, (ii) the polypeptide sequences SEQ ID NO: 2 and 22 or (iii) the polypeptide sequences SEQ ID NO: 26 and 22.

A β peptide and aggregated forms thereof

By the expression "A β peptide" or "amyloid beta peptide" or "Abeta peptide", it is
20 herein meant a peptide of 36 to 43 amino acids resulting from sequential cleavage of the amyloid precursor protein (APP) by beta secretase and gamma secretase.

The A β 42 peptide is the more hydrophobic and fibrillogenic peptide and constitutes the main species deposited in the brain.

A β peptides can be found as monomers or can aggregate to form soluble oligomers
25 or insoluble fibrils.

A soluble oligomer is for example a dimer, trimer, tetramer, larger oligomer or a protofibrillar form of A β peptide.

By the expression "protofibrillar form", it is herein meant an oligomeric form of A β peptides, which is soluble *in vitro*, which can be isolated as an entity of molecular weight
30 greater than 200 kDa, 300 kDa, 400 kDa or 500 kDa and which can fix agents such as thioflavin-S or Congo Red.

By the expression "senile plaque", it is herein meant a plaque composed of an amyloid core (fixing thioflavin S or Congo Red) surrounded by dystrophic neurites and a reaction of glial cells. *In vivo*, senile plaques mainly comprise insoluble fibrils and
35 protofibrillar forms having a molecular weight greater than 200 kDa. Senile plaques are found in particular in patients with Alzheimer's disease.

By the expression “diffuse amyloid deposit” our “diffuse deposit”, it is herein meant amorphous A β substructure that do not fix thioflavin S or Congo Red and comprise no or little fibrils and protofibrillar forms. Diffuse amyloid deposits are far more numerous, but are not associated with the disease.

5

Subject to be treated

A subject in need to be treated for the prevention and/or the treatment of Alzheimer's disease according to the invention may be a human being or a non-human mammal, preferably a human being.

10

When the subject is a human being, the terms “individual” or “patient” may also be used.

Said human being may be of any age, for example an infant, child, adolescent, adult, elderly people.

A non-human mammal is preferably a mouse, rat, cat, dog, rabbit or primate.

15

The subject may be a subject at risk to develop symptomatic Alzheimer's disease, in particular for the prevention of Alzheimer's disease, or a subject at an early stage of symptomatic Alzheimer's disease, in particular for the treatment of symptomatic Alzheimer's disease.

20

A subject at risk to develop symptomatic Alzheimer's disease does not suffer from memory loss and does not suffer from impairment of cognitive functions. In other words, a subject at risk to develop symptomatic Alzheimer's disease has normal cognition or optionally subtle cognitive decline.

25

Memory loss may be assessed by any method well-known by the skilled person, such as but not limited to, the Free and Cued Selective Reminding Test, the Rey Auditory Verbal Learning Test and/or the California Verbal Learning Test.

30

Impairment of cognitive functions may be assessed by any method well-known by the skilled person, such as but not limited to, the Mini-Mental State Examination, the Alzheimer's Disease Assessment Scale -Cognitive Subscale, the Neuropsychological Test Battery and/or the Repeatable Battery for the Assessment of Neuropsychological Status.

A subject at risk to develop symptomatic Alzheimer's disease may be a pre-Alzheimer subject or a subject with asymptomatic Alzheimer's disease.

A pre-Alzheimer subject does not have any biomarker associated with a risk of developing symptomatic Alzheimer's disease.

A subject with asymptomatic Alzheimer's disease has at least one biomarker and/or at least one genetic factor associated with a risk of developing symptomatic Alzheimer's disease.

Such biomarkers associated with a risk of developing symptomatic Alzheimer's disease include biomarkers of A β peptide accumulation in the brain (for example, abnormal tracer retention on amyloid PET imaging and/or low levels of A β peptide in cerebrospinal fluid (CSF)) and/or biomarkers of neuronal degeneration or injury (for example elevated levels of tau protein in CSF and/or decreased brain fluorodeoxyglucose uptake on PET imaging and/or brain atrophy on structural magnetic resonance).

Such genetic factors associated with a risk of developing symptomatic Alzheimer's disease include, but are not limited to,

- for autosomal dominant Alzheimer's disease, mutation(s) in APP, PSEN1 and/or PSEN2 genes;
- for sporadic Alzheimer's disease, mutation(s) of gene(s) involved in lipid metabolism (APOE4, CLU and/or ABCA7), in the immune system (CLU, CR1, ABCA7, CD33, EPHA1 and/or the MS4A gene cluster), and/or in synaptic functioning (PICALM, CD33, CD2AP, EPHA1 and/or BIN1).

Memory loss and impairment of cognitive functions result, at least partially, from synapse dysfunction and/or neurodegeneration.

Thus, a subject at risk to develop symptomatic Alzheimer's disease does not have synapse dysfunction and/or neurodegeneration or has a level of synapse dysfunction and/or neurodegeneration not translating into symptoms.

By the expression "synapse dysfunction", it is herein meant abnormal alterations in the number, structure and/or organization of synapses, in particular in neurodegenerative conditions, leading to impairment in the functional activity of synapses. Synapse dysfunction includes mechanisms of synapse loss.

Synapse dysfunction may be assessed by any method well-known by the skilled person, such as for example assessment of functional brain connectivity using resting state- or task-functional MRI (*Magnetic Resonance Imaging*) (see for example Knight et al. 2016, *Magnetic Resonance Imaging to Detect Early Molecular and Cellular Changes in Alzheimer's Disease, Front. Aging Neurosci. Vol. 8, article 139*) and/or measurement of levels of synaptic protein(s) (for example, neurogranin) in CSF.

Synapse dysfunction, as observed *in vivo*, for example corresponds *in vitro* to impaired neurite network.

By the expression “impaired neurite network”, it is herein meant a decreased neurite network, i.e. decrease in the size of neurite length.

Neurite network may be assessed by any method well-known by the skilled person, such as immunocytochemistry followed by cell imaging.

5 By the expression “neurodegeneration”, it is herein meant loss of neuronal cells and/or decrease in neuronal function or activity.

10 Neurodegeneration may be assessed by any method well-known by the skilled person, such as for example elevated levels of tau protein in CSF and/or decreased brain fluorodeoxyglucose uptake on PET imaging and/or brain atrophy on structural magnetic resonance.

Neurodegeneration, as observed *in vivo*, for example corresponds *in vitro* to abnormal neuronal cell apoptosis.

Abnormal neuronal cell apoptosis means an increased neuronal cell apoptosis by comparison to a control healthy cell.

15 Neuronal cell apoptosis may be assessed by any method well-known by the skilled person, such as measurement of enzymatic activity of caspase 3/7.

In one preferred embodiment of the invention, a subject at risk to develop symptomatic Alzheimer’s disease is:

- 20
- a subject with normal cognition having at least one family member that is suffering from or is at risk to suffer from symptomatic Alzheimer’s disease, more preferably from an autosomal dominant form of Alzheimer’s disease, and/or
 - a subject with normal cognition diagnosed as carrying at least one mutation involved in an autosomal dominant form of Alzheimer’s disease, and/or
 - 25 - a subject with normal cognition or with subtle cognitive decline, at risk of developing symptomatic Alzheimer’s disease on the basis of biomarker(s) and/or genetic factor(s) associated with a risk of developing symptomatic Alzheimer’s disease, this subject being for example at risk of developing a sporadic form of Alzheimer’s disease.

30 Said biomarker(s) and/or genetic factor(s) associated with a risk of developing symptomatic Alzheimer’s disease are as defined above.

By the expression “family member”, it is preferably meant a member of previous generations (for example, parent, grand-parent or great-grand parent), a brother, a sister or optionally a child or grandchild. A family member is generally a parent or sibling.

35 By the expression “autosomal dominant form of Alzheimer’s disease”, it is herein meant an Alzheimer’s disease resulting from at least one genetic mutation.

Autosomal dominant forms of Alzheimer's disease are well-known by the skilled person. Autosomal dominant forms of Alzheimer's disease for example include at least one mutation in at least one gene selected in the group consisting of a gene encoding Amyloid Beta Precursor Protein (APP), a gene encoding Presenilin 1 (PSEN1), and a gene encoding Presenilin 2 (PSEN2).

Non-limitative examples of such mutations are described by Cacace et al. (2016, Alzheimer's & Dementia vol.12, pp 733-748).

A subject having developed symptomatic Alzheimer's disease, i.e. suffering from symptomatic Alzheimer's disease, is preferably at an early stage of symptomatic Alzheimer's disease.

By "early stage of symptomatic Alzheimer's disease", it is herein meant a stage wherein memory loss and/or impairment of cognitive functions begin.

An example of early stage of symptomatic Alzheimer's disease is symptomatic predementia.

Symptomatic predementia for example includes mild cognitive impairment (MCI) due to Alzheimer's disease or prodromal Alzheimer's disease. Characteristics of MCI due to Alzheimer's disease and of prodromal Alzheimer's disease may be found in Albert et al., 2011, Alzheimer's & Dementia, 7, 270-279 and Dubois et al., 2016, Alzheimer's & Dementia, 12, 292-323, respectively.

A later stage of symptomatic Alzheimer's disease is dementia due to Alzheimer's disease, preferably mild dementia due to Alzheimer's disease. Characteristics of dementia due to Alzheimer's disease may be found in McKhann, 2011, Alzheimer's & Dementia, 7, 263-269.

In one preferred embodiment of the invention, the subject does not suffer from symptomatic Alzheimer's disease.

In a preferred embodiment of the invention, the subject does not have senile plaques.

The absence of senile plaque may for example be assessed by amyloid PET (*Positron Emission Tomography*) imaging of the brain.

Prevention of Alzheimer's disease or treatment of an early stage of symptomatic Alzheimer's disease

By the expression "prevention of Alzheimer's disease", it is herein meant to prevent, at least partially, (in particular, prevent, reduce and/or delay) the memory loss

and/or impairment of cognitive function in a subject to be treated, preferably in a subject at risk to develop symptomatic Alzheimer's disease, in particular by preventing, at least partially, A β -induced cytotoxicity in neuronal cells (in particular by preventing, delaying and/or reducing A β -induced cytotoxicity in neuronal cells).

5 Desirable effects of the prevention of Alzheimer's disease include:

- preventing, at least partially, neurodegeneration, in particular preventing, delaying and/or reducing neurodegeneration, and/or
- preventing, at least partially, synaptic dysfunction, for example preventing, delaying and/or reducing synaptic dysfunction, in particular preventing, at least
- 10 partially, the loss of synapse (in particular preventing, delaying and/or reducing the loss of synapse).

By the expression "treatment of an early stage of symptomatic Alzheimer's disease", it is herein meant to treat, at least partially, (in particular, stop, slow down and/or reduce)

15 the memory loss and/or impairment of cognitive function in a subject to be treated, preferably in a subject suffering from an early stage of symptomatic Alzheimer's disease, for example a subject suffering from symptomatic predementia, in particular by preventing, at least partially, A β -induced cytotoxicity in neuronal cells (in particular by stopping, slowing down and/or reducing A β -induced cytotoxicity in neuronal cells).

20 Desirable effects of treatment of an early stage of symptomatic Alzheimer's disease include:

- stopping or slowing down neurodegeneration and/or
- stopping or slowing down the development of synaptic dysfunction, in particular stopping or slowing down the loss of synapse.

25

The expressions "memory loss", "impairment of cognitive function" and "subject to be treated" are as defined above.

Neurodegeneration, synapse dysfunction and synapse loss are as defined above and may be assessed as described above.

30 Memory loss and impairment of cognitive functions to be prevented and/or treated according to the invention result from toxic A β oligomers, and not from senile plaques.

Antibody

35 Naturally occurring antibodies typically comprise a tetramer. Each such tetramer is typically composed of two identical pairs of polypeptide chains, each pair having one full-length "light" chain (typically having a molecular weight of about 25 kDa) and one full-

length "heavy" chain (typically having a molecular weight of about 50-70 kDa). The terms "heavy chain" and "light chain" as used herein refer to any immunoglobulin polypeptide having sufficient variable domain sequence to confer specificity for a target antigen. The amino-terminal portion of each light and heavy chain typically includes a variable domain of about 100 to 110 or more amino acids that typically is responsible for antigen recognition. The carboxy-terminal portion of each chain typically defines a constant domain responsible for effector function. Thus, in a naturally occurring antibody, a full-length heavy chain immunoglobulin polypeptide includes a variable domain (VH) and three constant domains (CH1, CH2, and CH3), wherein the VH domain is at the amino-terminus of the polypeptide and the CH3 domain is at the carboxyl-terminus, and a full-length light chain immunoglobulin polypeptide includes a variable domain (VL) and a constant domain (CL), wherein the VL domain is at the amino-terminus of the polypeptide and the CL domain is at the carboxyl-terminus.

Human light chains are typically classified as kappa and lambda light chains, and human heavy chains are typically classified as mu, delta, gamma, alpha, or epsilon, and define the antibody's isotype as IgM, IgD, IgG, IgA, and IgE, respectively. IgG has several subclasses, including, but not limited to, IgG1, IgG2, IgG3, and IgG4. IgM has subclasses including, but not limited to, IgM1 and IgM2. IgA is similarly subdivided into subclasses including, but not limited to, IgA1 and IgA2. Within full-length light and heavy chains, the variable and constant domains typically are joined by a "J" region of about 12 or more amino acids, with the heavy chain also including a "D" region of about 10 more amino acids. See, e.g., FUNDAMENTAL IMMUNOLOGY (Paul, W., ed., Raven Press, 2nd ed., 1989). The variable regions of each light/heavy chain pair typically form an antigen binding site. The variable domains of naturally occurring antibodies typically exhibit the same general structure of relatively conserved framework regions (FR) joined by three hypervariable regions, also called complementarity determining regions or CDRs. The CDRs from the two chains of each pair typically are aligned by the framework regions, which may enable binding to a specific epitope. From the amino-terminus to the carboxyl-terminus, both light and heavy chain variable domains typically comprise the domains FR1, CDR1, FR2, CDR2, FR3, CDR3 and FR4.

The term "CDR set" refers to a group of three CDRs that occur in a single variable region capable of binding the antigen. The exact boundaries of these CDRs have been defined differently according to different systems. The system described by Kabat (Kabat et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST (National Institutes of Health, Bethesda, Md. (1987) and (1991)) not only provides an unambiguous residue numbering system applicable to any variable region of an antibody, but also

provides precise residue boundaries defining the three CDRs. These CDRs may be referred to as Kabat CDRs. Chothia and coworkers (Chothia and Lesk, 1987, J. Mol. Biol. 196: 901—17; Chothia et al., 1989, Nature 342: 877—83) found that certain sub-portions within Kabat CDRs adopt nearly identical peptide backbone conformations, despite having great diversity at the level of amino acid sequence. These sub-portions were designated as L1, L2, and L3 or H1, H2, and H3 where the "L" and the "H" designates the light chain and the heavy chain regions, respectively. These regions may be referred to as Chothia CDRs, which have boundaries that overlap with Kabat CDRs. Other boundaries defining CDRs overlapping with the Kabat CDRs have been described by Padlan, 1995, FASEB J. 9: 133- 39; MacCallum, 1996, J. Mol. Biol. 262(5): 732-45; and Lefranc, 2003, Dev. Comp. Immunol. 27: 55-77. Still other CDR boundary definitions may not strictly follow one of the herein systems, but will nonetheless overlap with the Kabat CDRs, although they may be shortened or lengthened in light of prediction or experimental findings that particular residues or groups of residues or even entire CDRs do not significantly impact antigen binding. The methods used herein may utilize CDRs defined according to any of these systems, although certain embodiments use Kabat or Chothia defined CDRs. Identification of predicted CDRs using the amino acid sequence is well known in the field, such as in Martin, A.C. "Protein sequence and structure analysis of antibody variable domains," In Antibody Engineering, Vol. 2. Kontermann R., Dubel S., eds. Springer-Verlag, Berlin, p. 33-51 (2010). The amino acid sequence of the heavy and/or light chain variable domain may be also inspected to identify the sequences of the CDRs by other conventional methods, e.g., by comparison to known amino acid sequences of other heavy and light chain variable regions to determine the regions of sequence hypervariability. The numbered sequences may be aligned by eye, or by employing an alignment program such as one of the CLUSTAL suite of programs, as described in Thompson, 1994, Nucleic Acids Res. 22: 4673-80. Molecular models are conventionally used to correctly delineate framework and CDR regions and thus correct the sequence-based assignments.

The term "antibody" as used herein encompasses an antibody as defined above and any functional fragment of antibody. Preferably, the functional fragment of an antibody is a fragment of type Fv, scFv (*single chain Fv*), Fab (*Fragment antigen binding*), F(ab')₂, Fab', scFv-Fc, diabody, multispecific antibody (notably bispecific), synthetic polypeptide containing the sequence of one or more CDRs, which generally possesses the same specificity of fixation as the antibody from which they are derived. Functional fragments of an antibody can be obtained by methods such as digestion by enzymes, such as pepsin

or papain and/or by cleavage of the disulphide bridges by chemical reduction. Nanobodies also come under the definition of an antibody.

The term "Fc" as used herein refers to a molecule comprising the sequence of a non-antigen-binding fragment resulting from digestion of an antibody or produced by other means, whether in monomeric or multimeric form, and can contain the hinge region. The original immunoglobulin source of the native Fc is preferably of human origin and can be any of the immunoglobulins, although IgG1 and IgG2 are preferred. Fc molecules are made up of monomeric polypeptides that can be linked into dimeric or multimeric forms by covalent (i.e., disulfide bonds) and non-covalent association. The number of intermolecular disulfide bonds between monomeric subunits of native Fc molecules ranges from 1 to 4 depending on class (e.g., IgG, IgA, and IgE) or subclass (e.g., IgG1, IgG2, IgG3, IgA1, and IgGA2). One example of a Fc is a disulfide-bonded dimer resulting from papain digestion of an IgG. The term "Fc" as used herein is generic to the monomeric, dimeric, and multimeric forms.

A F(ab) fragment typically includes one light chain and the VH and CH1 domains of one heavy chain, wherein the VH-CH1 heavy chain portion of the F(ab) fragment cannot form a disulfide bond with another heavy chain polypeptide. As used herein, a F(ab) fragment can also include one light chain containing two variable domains separated by an amino acid linker and one heavy chain containing two variable domains separated by an amino acid linker and a CH1 domain.

A F(ab') fragment typically includes one light chain and a portion of one heavy chain that contains more of the constant region (between the CH1 and CH2 domains), such that an interchain disulfide bond can be formed between two heavy chains to form a F(ab')₂ molecule.

"Epitope" means the site of the antigen to which the antibody binds. If the antigen is a polymer, such as a protein or a polysaccharide, the epitope can be formed by contiguous or non-contiguous residues. Here the epitope is conformational, i.e. related to the three-dimensional structure of the protofibrillar A- β peptide.

The term "VH" as used herein refers to the variable region of a heavy chain of an antibody, including the heavy chain of a fragment Fv, scFv, dsFv, Fab, Fab' or F(ab)'.

The term "VL" as used herein refers to the variable region of a light chain of an antibody, including the light chain of a fragment Fv, scFv, dsFv, Fab, Fab' or F(ab)'.

"Humanized antibody" means an antibody that contains mainly human immunoglobulin sequences. This term generally refers to a non-human immunoglobulin that has been modified by incorporating human sequences or residues found in human sequences.

In general, humanized antibodies comprise one or typically two variable domains in which all or part of the CDR regions correspond to parts derived from the non-human parent sequence and in which all or part of the FR regions are derived from a human immunoglobulin sequence. The humanized antibody can then comprise at least one
5 portion of a constant region of immunoglobulin (Fc), in particular that of the selected reference human immunoglobulin.

A humanized antibody is preferably the least immunogenic as possible in a human. For that purpose, it is possible that one or two amino acids of one or more CDRs are modified by an amino acid that is less immunogenic for the human host, without
10 substantially reducing the binding specificity of the antibody to the protofibrillar form of the A β peptide. Furthermore, the residues of the framework regions do not need to be human and it is possible that they are not modified, as they do not contribute to the immunogenic potential of the antibody.

Several methods of humanization are known by a person skilled in the art for
15 modifying a non-human parent antibody to an antibody that is less immunogenic in humans. Complete identity of the sequences with a human antibody is not essential. In fact complete sequence identity is not necessarily a predictive indicator of reduced immunogenicity and modification of a limited number of residues can lead to humanized antibodies presenting a very attenuated immunogenic potential in humans (Molecular
20 Immunology (2007) 44, 1986-1998).

Some methods are for example the inclusion of CDRs (grafting) (EPO 0 239 400; WO 91/09967; and U.S. Pat. Nos. 5,530,101 and 5,585,089), the resurfacing (EPO 0 592
106; EPO 0 519 596; Padlan, 1991, Molec Imm 28(4/5): 489-498; Studnicka et al., 1994, Prot Eng 7(6):805-814; and Roguska et al., 1994, PNAS 91:969-973) or chain mixing
25 (U.S. Pat. No. 5,565,332).

The humanized antibody may have variable parts that are modified according to the technology explained in international application WO 2009/032661.

This technique notably uses dynamic molecular simulation based on three-dimensional models of antibodies, said models being constructed by homology.

The antibody may have diminished effector functions, such as immunoglobulins
30 bearing mutations of the Fc domain reducing its affinity for the receptors of the immune system or such as nanobodies.

“Effector functions” means any fixation of the Fc domain of the antibody to receptors or proteins inducing immune responses. Decreasing these effector functions makes it
35 possible to reduce adverse effects such as the induction of microhaemorrhages (Racke et al. J Neurosci 2005, 25:629).

Affinity can be measured by any technique known by a person skilled in the art. It is advantageously measured by the Biostat Speed technique developed on the basis of the algorithms described by Ratkovsk D A and Reedy T J (Bio-metrics, 1986, 42, 575-82).

5 In order to express the heavy chain(s) and/or light chain(s) of an antibody, the polynucleotides coding for said chain(s) may be inserted in expression vector(s). The expression vector can be a plasmid, YAC, cosmid, retrovirus, episome derived from EBV, or any vector that a person skilled in the art may judge suitable for expression of said chain(s).

10 The vector can be used for transforming cells, advantageously derived from one cell line. Said cell line is even more advantageously derived from a mammal. It is advantageously the CHO line or a line derived from this line, or the HEK293 line or a line derived from this line.

The transformation of the cells can be carried out by any method well-known by a person skilled in the art for introducing polynucleotides into a host cell. Said method can be transformation by means of dextran, precipitation by calcium phosphate, transfection by means of polybrene, protoplast fusion, electroporation, encapsulation of the polynucleotides in liposomes, biolistic injection and direct micro-injection of DNA into the nucleus.

20

Sequence identity

By "an amino acid or nucleic acid sequence at least x% identical to a reference sequence", it is intended that the sequence of the subject peptide or nucleic acid is identical to the reference sequence or differ from the reference sequence by up to 100-x amino acid or nucleotide alterations per each 100 amino acids or nucleotides of the reference sequence. In other words, to obtain a polypeptide or nucleic acid having a sequence at least x% identical to a reference sequence, up to 100-x% of the amino acids or nucleotides in the subject sequence may be inserted, deleted or substituted with another amino acid or nucleotide.

30 A polypeptide having at least 80%, 85%, 90%, 95% or 99% identity with a reference sequence may comprise mutation(s), such as deletion(s), insertion(s) and/or substitution(s) compared to the reference sequence. In case of substitutions, the substitution preferably corresponds to a conservative substitution as indicated in the Table 1 below and/or to a substitution for a less immunogenic amino acid, more particularly for a human host.

35

A nucleic acid sequence "at least 80%, 85%, 90%, 95% or 99% identical" to a reference sequence may comprise mutation(s), such as deletion(s), insertion(s) and/or substitution(s) compared to the reference sequence. In case of substitution, the substitution preferably corresponds to a silent substitution or a substitution leading to a conservative substitution in the translated amino acid sequence or to a less immunogenic amino acid, by comparison to the reference sequence, for example as indicated in the Table 1 above.

Table 1

| Conservative substitutions | Type of Amino Acid |
|--|--|
| Ala, Val, Leu, Ile, Met, Pro, Phe, Trp | Amino acids with aliphatic hydrophobic side chains |
| Ser, Tyr, Asn, Gln, Cys | Amino acids with uncharged but polar side chains |
| Asp, Glu | Amino acids with acidic side chains |
| Lys, Arg, His | Amino acids with basic side chains |
| Gly | Neutral side chain |

In a preferred embodiment, the nucleic sequence at least 80%, 85%, 90%, 95% or 99% identical to a reference sequence only differs from the reference sequence by silent substitution(s) and/or substitution(s) leading to a conservative amino-acid substitution and/or to a less immunogenic amino acid.

Methods for comparing the identity of two or more sequences are well known in the art. For instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1, for example the programs BESTFIT and GAP, may be used to determine the % identity between two sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman and finds the best single region of similarity between two sequences. Other programs for determining identity between sequences are also known in the art, for instance the Needle program, which is based on the Needleman and Wunsch algorithm, described in Needleman and Wunsch (1970) J. Mol Biol. 48:443-453, with for example the following parameters for polypeptide sequence comparison: comparison matrix: BLOSUM62, gap open penalty: 10 and gap extend penalty: 0.5, end gap penalty: false, end gap open penalty = 10, end gap extend penalty = 0.5; and the following parameters for polynucleotide sequence comparison: comparison matrix: DNAFULL; gap open penalty = 10, gap extend penalty = 0.5, end gap penalty: false, end gap open penalty = 10, end gap extend penalty = 0.5.

Antibody specific for the protofibrillar form of the A β peptide

The antibody for use according to the invention is specific for the protofibrillar form of the A β peptide.

5 The antibody thus specifically binds to the protofibrillar form of the A β peptide, i.e. to a high molecular weight peptide, which is as defined above. The antibody particularly binds to the A β peptide having a molecular weight greater than 200, 300, 400 or 500 kDa.

10 The antibody preferably binds specifically to the protofibrillar form of the A β peptide but not to the other proteins of amyloid structure (for example IAPP, Islet Amyloid Polypeptide).

By "specific binding", it is herein meant a difference by a factor of at least about 10, 20, 30, 40, 50, or 100 between the strength of binding to the protofibrillar form of the A β peptide relative to the binding to the other forms of this peptide.

15 The antibody preferably has an affinity for the protofibrillar form of peptide A β at least 100 times greater, more preferably at least 150 times greater, still more preferably at least 170 times greater than its affinity for the other forms of this peptide. The affinity may be the EC50 measured by ELISA.

The antibody is thus characterized by its ability to bind to A β peptides aggregated into protofibrills.

20 The antibody for use according to the invention also prevents, at least partially, A β -induced apoptosis of neuronal cells and/or prevents, at least partially, A β -induced neurite network decrease, in particular *in vitro*.

25 The expression "the antibody prevents, at least partially, A β -induced apoptosis of neuronal cells" particularly means that said antibody prevents or reduces A β -induced apoptosis of neuronal cells *in vitro*, in particular by comparison to A β -induced apoptosis of neuronal cells in the absence of said antibody.

30 The expression "the antibody prevents, at least partially, A β -induced neurite network decrease" particularly means that the antibody prevents or reduces A β -induced neurite network decrease *in vitro*, in particular by comparison to A β -induced neurite network decrease in the absence of said antibody.

Neuronal cell apoptosis and neurite network may be assessed as described above or in the Examples.

The antibody is preferably a humanized antibody.

The humanized antibody preferably has reduced effector functions, making it possible to limit adverse effects such as the development of microhemorrhages and/or vasogenic edemas.

5 A preferred antibody for use according to the invention no longer possesses effector functions.

Advantageously, the antibody may be an immunoglobulin G4 whose Fc domain has undergone mutations reducing the production of half-molecules.

Advantageously, the antibody may be an immunoglobulin G4 whose Fc domain has undergone mutations reducing the effector activity.

10 The antibody may be extensively sialylated. In one embodiment, the antibody is not extensively sialylated.

By the expression "extensively sialylated", it is herein meant that at least 80 %, preferably at least 85 %, more preferably at least 90 %, even more preferably at least 95%, still most preferably at least 97% or most preferably at least 99% of the N-glycans carried by the Fc domain of the antibody comprise two sialic acid residues by oligosaccharide chain.

15 An extensively sialylated antibody is for example obtained by expressing the antibody in a cell line expressing β -galactosyltransferase and a sialyltransferase activity from a nucleic sequence encoding a heavy chain bearing a mutation in the Fc domain, such as at position D265, F243 and/or V264 (corresponding to position D260, F238 and/or V259 in sequences SEQ ID NO: 1 or SEQ ID NO: 25).

The mutation in the Fc domain is for example a substitution of the amino acid by an amino acid selected from the group consisting of alanine, glycine, leucine and lysine.

20 The mutation is for example D260L, D260K, D260A, F238A or V259A by reference to sequences SEQ ID NO: 1 or SEQ ID NO: 25.

30 The antibody preferably comprises at least one CDR encoded by a nucleotide sequence having a sequence identical to one of the sequences SEQ ID NO: 9, 11, 13, 15, 17 and 19, or by sequences differing respectively by 1, 2, 3, 4 or 5 nucleotides from these sequences.

The antibody preferably comprises at least one CDR having a sequence identical to one of the sequences SEQ ID NO: 10, 12, 14, 16, 18 and 20.

35 The antibody preferably comprises at least one CDR whose sequence differs by one to two amino acids relative to one of the sequences SEQ ID NO: 10, 12, 14, 16, 18, 20 and 32, inasmuch as the antibody maintains its binding specificity.

A preferred antibody comprises the CDRs of sequence SEQ ID NO: 10, 12, 14, 16, 18 and 20, the CDRs of sequence SEQ ID NO: 10, 12, 14, 32, 18 and 20, or the CDRs of sequence SEQ ID NO: 10, 12, 30, 32, 18 and 20.

5 The antibody for example comprises the CDRs encoded by (i) the nucleotide sequences SEQ ID NO: 9, 11, 13, 15, 17 and 19, (ii) the nucleotide sequences SEQ ID NO: 9, 11, 13, 31, 17 and 19, (iii) the nucleotide sequences SEQ ID NO: 9, 11, 29, 31, 17 and 19 or (iv) by sequences differing respectively by 0, 1, 2, 3, 4 or 5 nucleotides from these sequences of (i), (ii) or (iii).

In one preferred embodiment, the antibody is a humanized antibody:

10 - comprising the CDRs of sequence SEQ ID NO: 10, 12, 14, 16, 18 and 20, the CDRs of sequence SEQ ID NO: 10, 12, 14, 32, 18 and 20 or the CDRs of sequence SEQ ID NO: 10, 12, 30, 32, 18 and 20, and

15 - having an affinity for the protofibrillar form of the A β peptide being at least 100 times greater than its affinity for the other forms of this peptide, the affinity being preferably the EC50 measured by ELISA.

In one advantageous embodiment, the antibody comprises or consists of (i) a variable part of its heavy chain (VH) encoded by a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the sequence SEQ ID NO: 5 or the sequence SEQ ID NO: 27 and/or (ii) a variable part of its light chain (VL) encoded by a sequence having at least
20 80%, 85%, 90%, 95% or 99% identity with the sequence SEQ ID NO: 7 or the sequence SEQ ID NO: 23.

In one advantageous embodiment, the antibody comprises or consists of (i) a variable part of its heavy chain (VH) comprising a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the sequence SEQ ID NO: 6 or the sequence SEQ ID NO:
25 28 and/or (ii) a variable part of its light chain (VL) comprising a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the sequence SEQ ID NO: 8 or the sequence SEQ ID NO: 24.

In one even more advantageous embodiment, the humanized antibody comprises or consists of (i) a heavy chain comprising a variable part (VH) encoded by the nucleotide
30 sequences SEQ ID NO: 5 or SEQ ID NO 27 and/or (ii) a light chain comprising a variable part (VL) encoded by the nucleotide sequences SEQ ID NO 7 or SEQ ID NO 23.

In one even more advantageous embodiment, the humanized antibody comprises or consists of (i) a heavy chain comprising a variable part (VH) of polypeptide sequence SEQ
35 ID NO: 6 or SEQ ID NO: 28 and/or (ii) a light chain comprising a variable part (VL) of polypeptide sequence SEQ ID NO 8 or SEQ ID NO 24.

In one advantageous embodiment, the humanized antibody comprises or consists of the polypeptide sequences encoded by (i) the nucleotide sequences SEQ ID NO: 5 and 7, (ii) the nucleotide sequences SEQ ID NO: 5 and 23 or (iii) the nucleotide sequences SEQ ID NO: 27 and 23.

5 In one advantageous embodiment, the humanized antibody comprises or consists of (i) the polypeptide sequences SEQ ID NO: 6 and 8, (ii) the polypeptide sequences SEQ ID NO: 6 and 24, or (iii) the polypeptide sequences SEQ ID NO: 28 and 24.

10 The antibody may comprise or consists of (i) a heavy chain encoded by a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the nucleotide sequence SEQ ID NO: 1 or SEQ ID NO: 25, and/or (ii) a light chain encoded by a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the nucleotide sequence SEQ ID NO: 3 or SEQ ID NO: 21.

15 The antibody may comprise or consists of (i) a heavy chain having at least 80%, 85%, 90%, 95% or 99% identity with the polypeptide sequence SEQ ID NO: 2 or with the polypeptide sequence SEQ ID NO: 26, and/or (ii) a light chain comprising a sequence having at least 80%, 85%, 90%, 95% or 99% identity with the polypeptide sequence SEQ ID NO: 4 or SEQ ID NO: 22.

20 The humanized antibody for example comprises or consists of the sequences encoded by the nucleotide sequences SEQ ID NO: 1 and 3, the nucleotide sequences SEQ ID NO: 1 and 21, or the nucleotide sequences SEQ ID NO: 25 and 21.

The humanized antibody for example comprises or consists of the polypeptide sequences SEQ ID NO: 2 and 4, the polypeptide sequences SEQ ID NO: 2 and 22 or the polypeptide sequences SEQ ID NO: 26 and 22.

25 The humanized antibody, for example antibody Ab1 (VH1 + VL1), Ab2 (VH1 + VL2) or Ab3 (VH2+VL2) may be produced by transient expression in a mammalian line, for example line HEK293 designated FreeStyle 293-F.

30 The cDNAs coding for the humanized variable domain of the light chain and heavy chain (for example, VL1 or VL2; and VH1 or VH2) are fused with the cDNAs coding for the human constant regions, for example Ckappa and IgG4, respectively. The sequence of the constant region IgG4 may be the sequence of the variant having the substitutions S241P and L248E in Kabat's nomenclature, for a significant reduction in the production of half-molecules (*see for example Angla et al., 1993, Mol. Immunol., 30: 105-108*) and the effector functions (*see for example WO 97/09351*).

35 The nucleic acid sequences coding for the heavy chain (for example VH1+CH1 or VH2 + CH2) and for the light chain (for example VL1 + CL1 or VL2 + CL2), respectively,

may be cloned independently in two expression vectors. The humanized antibody is then produced by transient expression in a mammalian cell, after co-transfection with these two expression vectors. The antibodies are then preferably purified by affinity chromatography, for example on a column of MabSelect gel (Amersham), preferably formulated in a buffer, for example a PBS buffer, and optionally submitted to sterile filtration (0.2 μm).

Pharmaceutical composition

The antibody specific for the protofibrillar form of the A β peptide for use according to the invention is preferably provided in a pharmaceutical composition.

The term "pharmaceutical composition" as used herein refers to a composition capable of inducing a desired therapeutic effect, when properly administered to a subject.

The pharmaceutical composition comprises an antibody specific for the protofibrillar form of the A β peptide as defined above and at least one pharmaceutically acceptable carrier.

The expression "pharmaceutically acceptable carrier" or "physiologically acceptable carrier" as used herein refers to one or more formulation materials suitable for accomplishing or enhancing the delivery of an antibody.

The pharmaceutical composition preferably comprises at least one pharmaceutically acceptable vehicle for an injectable formulation. Such vehicles are well known by the skilled person and include sterile, isotonic saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride, and/or mixtures of said salts), or dry compositions, notably lyophilized, which, by adding sterilized water or physiological serum as appropriate, permit injectable salutes to be constituted.

As an example, a pharmaceutical composition comprises (1) a Dulbecco phosphate buffer (pH \sim 7.4), optionally containing 1mg/ml to 25 mg/ml of human serum albumin, (2) 0.9% w/v of sodium chloride (NaCl), and (3) 5% (w/v) of dextrose. It can also comprise an antioxidant such as tryptamine and a stabilizer such as Tween 20.

The pharmaceutical composition preferably comprises an effective amount of the antibody specific for the protofibrillar form of the A β peptide and at least one pharmaceutically acceptable carrier.

The terms "effective amount" and "therapeutically effective amount" when used in reference to a pharmaceutical composition comprising an antibody specific for the protofibrillar form of the A β peptide refer to an amount or dosage sufficient to produce a desired therapeutic result. More specifically, a therapeutically effective amount is an amount of antibody sufficient to prevent, at least partially, for some period of time, one or

more of the clinically defined pathological processes associated with the condition being treated. The effective amount may vary depending on the specific antibody that is being used, and also depends on a variety of factors and conditions related to the subject being treated and the severity of the disorder. For example, if the antibody is to be administered *in vivo*, factors such as the age, weight, and health of the patient as well as dose response curves and toxicity data obtained in preclinical animal work would be among those factors considered. The determination of an effective amount or therapeutically effective amount of a given pharmaceutical composition is well within the ability of those skilled in the art.

The pharmaceutical composition may be appropriate for administration by the parenteral, intranasal, intravenous, intramuscular, subcutaneous, intra-cerebral and/or intraocular route.

Antibody specific for the protofibrillar form of the A β peptide for use in the prevention of Alzheimer's disease and/or in the treatment of an early stage of symptomatic Alzheimer's disease

The present invention particularly relates to an antibody specific for the protofibrillar form of the A β peptide as defined above, for use in the prevention of Alzheimer's disease, and/or in the treatment of an early stage of symptomatic Alzheimer's disease, in particular in a subject in need thereof.

The present invention also relates to a method for preventing Alzheimer's disease and/or treating an early stage of symptomatic Alzheimer's disease in a subject in need thereof, wherein said method comprises administering to said subject an effective amount of an antibody specific for the protofibrillar form of the A β peptide as defined above.

The expressions "prevention of Alzheimer's disease", "treatment of an early stage of symptomatic Alzheimer's disease" and the "antibody specific for the protofibrillar form of the A β peptide" are particularly as defined above.

Said antibody may be provided in the form of a pharmaceutical composition, as defined above.

The subject in need thereof is as defined above in the section "*Subject to be treated*".

The subject in need for the prevention of Alzheimer's disease is preferably a subject at risk to develop asymptomatic Alzheimer's disease, for example a pre-Alzheimer subject or a subject with asymptomatic Alzheimer's disease.

The subject in need for the treatment of an early stage of symptomatic Alzheimer's disease preferably has symptomatic predementia.

The antibody is preferably used or administered by the parenteral, intranasal, intravenous, intramuscular, subcutaneous, intra-cerebral and/or intraocular route.

The doses of antibody depend on the desired effect, the duration of the treatment and the route of administration used. Generally the doctor will determine the appropriate dosage in relation to the stage of the disease, the subject's age and weight, or any other subject-related factor that has to be taken into account.

The antibody is preferably used or administered in a quantity comprised from 5 mg to 4500 mg of antibody per administration, more preferably from 100 mg to 1200 mg of antibody per administration, still more preferably from 100 mg to 450 mg of antibody per administration, particularly for an adult.

The present invention will be further illustrated in view of the following examples and figures.

All references cited herein, including journal articles or abstracts, published or unpublished patent application, issued patents or any other references, are entirely incorporated by reference herein, including all data, tables, figures and text presented in the cited references.

Brief description of the figures

Figure 1: Inhibition of oA β 42-induced increase in caspase-3/7 enzymatic activity by humanized antibody Ab1 (h) and its murine counterpart (m) in mouse primary neuronal cultures. Ordinate: caspase 3/7 enzymatic activity (% of control). Abscissa: concentration in μ g/ml. Points represent the mean by experiment and by treatment of data expressed as percentage of control group. Bars correspond to median values. * p<0.05; ** p <0.01: two-tailed Dunnett's test versus oA β 42.

Figure 2: Inhibition of oA β 42-induced decrease in neurite network by humanized antibody Ab1 (h) and its murine antibody counterpart (m) in mouse primary neuronal cultures. Ordinate: neurite network (% of control). Abscissa: concentration in μ g/ml. Points represent the mean by experiment and by treatment of data expressed as percentage of control group. x correspond to mean values. * p<0.01; ** p <0.001: two-tailed Dunnett's test versus oA β 42.

Figure 3: Comparison of the effect of humanized antibody Ab1 (h) (150 μ g/mL) and its murine counterpart (m) (150 μ g/mL) to that of corresponding isotype control hIgG4 (150 μ g/mL) and mIgG1 (150 μ g/mL) respectively on caspase-3/7 enzymatic activity in oA β 42-treated mouse primary neuronal cultures. ** p <0.001 versus IgG4. *** p <0.001 versus IgG1. p: correspond to the p-values of a contrast analysis with Bonferroni-Holm

adjustment for multiplicity after a one-way analysis on rank transformed data in order to compare 7 groups to IgG corresponding groups: Ab1 and its murine counterpart at each concentration to hIgG4 and mIgG1 respectively at the same concentration in absence or presence of oA β 42. Only the results for the highest tested concentration in presence of oA β 42 are represented (Ab1 at 150 μ g/mL + oA β 42 versus IgG4 and its murine antibody at 150 μ g/mL + oA β 42 versus IgG1). Points represent the mean by experiment and by treatment of data expressed as percentage of control group. Bars correspond to median values.

Figure 4: Comparison of the effect of humanized antibody Ab1 (h) (150 μ g/mL) and its murine counterpart (m) (150 μ g/mL) to that corresponding isotype control hIgG4 (150 μ g/mL) and mIgG1(150 μ g/mL) respectively on neurite network in oA β 42-treated mouse primary neuronal cultures. ** p <0.001 versus IgG4 *** p <0.001 versus IgG1. p: correspond to the p-values of a contrast analysis with Bonferroni-Holm adjustment for multiplicity after a one-way analysis on rank transformed data in order to compare 7 groups to IgG: Ab1 and its murine counterpart at each concentration to hIgG4 and mIgG1 respectively at the same concentration in absence or presence of oA β 42. Only the results for the highest tested concentration in presence of oA β 42 are represented (Ab1 at 150 μ g/mL + oA β 42 versus IgG4 at 150 μ g/mL and murine antibody at 150 μ g/mL + oA β 42 versus IgG1 at 150 μ g/mL). Points represent the mean by experiment and by treatment of data expressed as percentage of control group. x correspond to mean values.

Figure 5: Western Blot of aggregated A β -42 (oAb42; 5 μ M) preparation using anti-A β 4G8 mAb. MW: Molecular Weight Marker.

Brief description of the sequences

The table 2 below summarizes the sequences of the humanized antibodies Ab1, Ab2 and Ab3 specific for the protofibrillar form of the A β peptide.

The number (1 or 2) following "VH", "VL", "CH" or "CL" refers herein to a sequence variant, and not for example to the conventional designation of the three constant regions CH1, CH2 and CH3.

Table 2

| | Nucleotide sequences | Protein sequences |
|-----------------------------------|-----------------------|-----------------------|
| Antibody Ab1 VH1VL1 | | |
| VH ₁ + CH ₁ | SEQ ID NO: 1 | SEQ ID NO: 2 |
| VL ₁ + CL ₁ | SEQ ID NO: 3 | SEQ ID NO: 4 |
| VH ₁ | SEQ ID NO: 5 | SEQ ID NO: 6 |
| VL ₁ | SEQ ID NO: 7 | SEQ ID NO: 8 |
| CDR 1, 2, 3 of VH ₁ | SEQ ID NO: 9, 11, 13 | SEQ ID NO: 10, 12, 14 |
| CDR 1, 2, 3 of VL ₁ | SEQ ID NO: 15, 17, 19 | SEQ ID NO: 16, 18, 20 |
| Antibody Ab2 VH1 VL2 | | |
| VH ₁ + CH ₁ | SEQ ID NO: 1 | SEQ ID NO: 2 |
| VL ₂ + CL ₂ | SEQ ID NO: 21 | SEQ ID NO: 22 |
| VH ₁ | SEQ ID NO: 5 | SEQ ID NO: 6 |
| VL ₂ | SEQ ID NO: 23 | SEQ ID NO: 24 |
| CDR 1, 2, 3 of VH ₁ | SEQ ID NO: 9, 11, 13 | SEQ ID NO: 10, 12, 14 |
| CDR 1, 2, 3 of VL ₂ | SEQ ID NO: 31, 17, 19 | SEQ ID NO: 32, 18, 20 |
| Antibody Ab3 VH2 VL2 | | |
| VH ₂ + CH ₂ | SEQ ID NO: 25 | SEQ ID NO: 26 |
| VL ₂ + CL ₂ | SEQ ID NO: 21 | SEQ ID NO: 22 |
| VH ₂ | SEQ ID NO: 27 | SEQ ID NO: 28 |
| VL ₂ | SEQ ID NO: 23 | SEQ ID NO: 24 |
| CDR 1, 2, 3 of VH ₂ | SEQ ID NO: 9, 11, 29 | SEQ ID NO: 10, 12, 30 |
| CDR 1, 2, 3 of VL ₂ | SEQ ID NO: 31, 17, 19 | SEQ ID NO: 32, 18, 20 |

EXAMPLES

5 **EXAMPLE 1:** Neuronal cytoprotective effect of an antibody specific for the
 protofibrillar form of the A β peptide *in vitro*

Material and methods(i) Animals

10 Cerebral cortex from 16-day old mouse embryos (OF1, Charles River Laboratories,
 France) were used for the preparation of primary neuronal cultures.

(ii) Compounds

Test compounds: humanized antibody Ab1 (50 mg/mL) and its murine counterpart (30 mg/mL) were synthesized by the Global Biotherapeutics Department of Sanofi R&D, Vitry-sur-Seine, France.

5 Control isotypes: mouse IgG1 (antiDM4_hyb_CAA162_IgG1) (7.28 mg/mL) and human IgG4 (antiDM4_hyb_CAA162_IgG4) (4.97 mg/mL) were synthesized by the Global Biotherapeutics Department of Sanofi R&D, Vitry-sur-Seine, France.

10 On the day of experiment, the concentrated working solution of all antibodies and control isotypes was prepared as a 10x solution, by diluting each stock solution to 1.5 mg/mL in Dulbecco's Modified Eagle Medium (DMEM). Lower tested concentrations were prepared by diluting the concentrated working solution in DMEM. Final concentrations of tested antibody within the culture wells were 150, 50 or 15 μ g/mL, ie, corresponding to 1, 0.33 or 0.1 μ M respectively.

15 Beta Amyloid (1-42): human (A β 42), reference 641-15 (California Peptide Research Inc, USA), was prepared as a stock solution at 1 mM in hexafluoroisopropanol, Sigma H8508 (HFIP). The resulting solution was aliquoted in microcentrifuge tubes (VWR 20170-293), then the HFIP was allowed to evaporate in the Sorbonne hood, and the resulting clear peptide film was dried under vacuum in a SpeedVac and stored dessicated at -20°C under vacuum.

20 On the day of experiment, the concentrated working solution was prepared from frozen HFIP-treated aliquots, resuspended at 5 mM in Dimethyl Sulfoxide (DMSO) (Sigma D2650, batch RNBB9702) by pipette mixing then followed by incubation in a ultrasonic bath for 10 minutes (Branson). A β 42 oligomers (oA β 42) were obtained by diluting the 5 mM A β 42 working solution to 100 μ M in ice-cold cell culture medium (phenol red-free Ham's F12, Pan Biotech P04-14559), vortexing 30 seconds, and incubating 1 hour at room temperature. This type of preparation has been reported to contain A β 42 aggregates containing mainly high-molecular weight A β 42 oligomers. This preparation of A β 42 was added to the culture medium at a final concentration of 5 μ M.

(iii) Primary neuronal cultures

30 Primary neuronal cultures were prepared from brain of 16 days-old mouse (OF1) embryos by dissecting and then dissociating in 0.25% trypsin cerebral cortices. After centrifugation at 120 g at room temperature, the resulting cell pellet resuspended in 10 mL of culture medium at a cell density of 4x10⁵ cells/mL was plated in poly-D-lysine-coated wells of 96-well culture microplate (Greiner, 655946, Germany) in 100 μ L of culture DMEM supplemented with N2 and B27.

35

After 6 days *in vitro*, the culture medium was removed and replaced by new one without B27, and the neurons were pretreated with anti-A β antibodies for 1 hour. Then the neurons were incubated with oA β 42 (5 μ M) for 48 hours. Control application of drug-free medium but supplemented with the corresponding DMSO concentration (ie, 0.1%, same as for oA β 42 treatment) was run in parallel to application of test substance.

The effect of tested compounds was evaluated using the two following readouts: caspase-3/7 enzymatic activity and neurite network.

(iv) Quantification of caspase-3/7 enzymatic activity

Following experimental treatment, 50 μ L of medium was eliminated from the wells, and 50 μ L of a caspase-Glo 3/7 Assay kit solution (Promega Corporation USA, G7790) were mixed to each well and then incubated for 4 hours at room temperature. After incubation the fluorescence of each sample was quantified using a Spectramax Gemini plate reader (XS, s/n XSO 2810, Molecular Devices, SoftMax PRO3.1 software) using 499 nm as the excitation wavelength and 521 nm as the emission wavelength. The measurement of fluorescence intensity is proportional to caspase-3/7 enzymatic activity.

(v) Quantification of neurite network

Fixation of cells

Following experimental treatment, 100 μ L of a 4% formaldehyde solution (Merck, 100496) was added to each well and then incubated for 10 minutes at room temperature. The medium was then removed and replaced by 100 μ L of a solution of 4% formaldehyde solution and allowed to incubate for further 10 minutes at room temperature. The medium was then removed and the wells were washed four times with 100 μ L of Dulbecco's Phosphate Buffered saline (DPBS) (Gibco, 14190). The microplates were stored at 4°C in DPBS, before staining.

Immunocytochemistry

Immunocytofluorescence procedure was applied on fixed cells in wells of culture microplates. Briefly, cell membranes were permeabilized for 30 minutes in DPBS/10% fetal bovine serum (FBS)-0.2% Triton X-100 at room temperature. Permeabilized cells were incubated overnight at 4°C with a primary antibody recognizing the neuronal protein marker MAP2 (microtubule-associated protein 2), here anti-MAP2 rabbit polyclonal antibody (Millipore AB5622, batch 2049208, 1/1 000 dilution) and then, after a washing step in PBS+0.1% Tween followed by an incubation with a secondary antibody corresponding to Alexa Fluor 594 coupled-anti-rabbit antibody (Molecular probes, A11012, batch 47098A, 1/1 000 dilution) for 1 hour at room temperature. After an additional washing step, fixed cells were then incubated 15 minutes with Hoechst 33342 (Molecular probes H3570) to stain for nuclei (0.2 μ g/mL) and finally rinsed with DPBS before analysis

by imaging. The measurement of neuronal associated extensions, ie, neurites was based on the MAP-2 positive immunofluorescence positive signal.

Quantitative image analysis

Analysis of neurite network was performed on MAP-2 immunostained and Hoechst stained cells from the 96-well culture microplates using high content image analysis. Automated fluorescent image acquisition of 5 fields per well at two color excitation wavelengths was performed on the In Cell Analyzer 2200 imaging system (GE Healthcare, Cardiff, UK) equipped with a 20x high numerical aperture magnification and a 16 bit sCMOS camera in a solid-state light excitation.

The quantitative image analysis process was performed on a computer-based workstation using IN Cell Developer Toolbox software (GE Healthcare). After segmentation of nuclei (based on Hoechst staining) and neurites (based on detected MAP2 immuno-reactivity) the neurite area was measured after defining an "object mask" on each cell followed by a postprocessing step on each field acquired and automatically edited by the In Cell Developer Toolbox software in Microsoft Excel spreadsheets. The measurement of neurite area (expressed as μm^2) was used as neurite network index.

The data used for the analysis are generally from five independent experiments.

(vi) Statistical analysis

For each parameter, the data were expressed as the percentage of the control group. Then, the mean by experiment for each treatment group was calculated for the statistical analyses. Statistical analyses were performed using SAS 9.2 software. A probability less than 5% ($p < 0.05$) was considered as significant.

Results

The aggregated A β 42 preparation used in the experiments for inducing neurotoxicity comprises several SDS-stable species including small and large oligomers (*see Figure 1*).

In mouse primary neuronal cultures, it was shown that oA β 42 induces a toxic effect through a marked increase in caspase 3/7 enzymatic activity (+ 648.6 %) ($p = 0.0105$).

A significant inhibition of oA β 42-induced toxicity is observed after co-treatment with humanized antibody Ab1 at 50 and 150 $\mu\text{g}/\text{mL}$ corresponding to a protective effect of 97% and 98% ($p = 0.0062$ and $p < 0.0001$ versus oA β 42, respectively) (*see Figure 1*).

A significant inhibition of oA β 42-induced toxicity is observed after co-treatment with the murine antibody at 50 and 150 $\mu\text{g}/\text{mL}$ corresponding to a protective effect of 96% and 98% ($p = 0.0352$ and $p < 0.0001$ versus oA β 42, respectively) (*see Figure 1*).

In the absence of oA β 42, no significant differences is observed between control and humanized antibody Ab1 at 50 and 150 $\mu\text{g}/\text{mL}$ ($p = 0.5309$ and $p = 0.4207$ respectively). No

significant differences is observed between control and murine antibody when tested alone at concentration of 15 and 50 $\mu\text{g}/\text{mL}$ ($p=0.9279$ and $p=0.1811$ respectively) (Table 3). A statistical significant increase in caspase-3/7 enzymatic activity is observed with the murine antibody at the highest concentration of 150 $\mu\text{g}/\text{mL}$ versus control (+ 15%; $p=0.0352$).

It was also shown that oA β 42 induces a toxic effect in mouse primary neuronal cultures through a marked decrease in neurite network in mouse primary neuronal cultures (-47 % versus control; $p=0.0009$).

A significant inhibition of oA β 42-induced decrease in neurite network is observed after co-treatment with oA β 42 and humanized antibody Ab1 at 50 and 150 $\mu\text{g}/\text{mL}$ corresponding to a neuroprotective effect of 84% and 111% respectively ($p < 0.0001$ for both concentrations versus oA β 42) (*see Figure 2*).

An apparent inhibition of oA β 42-induced decrease in neurite network is observed after co-treatment with oA β 42 and the murine antibody at 50 and 150 $\mu\text{g}/\text{mL}$ ($p=0.0023$ and $p<0.0001$ respectively, versus oA β 42) (*see Figure 2*).

In the absence of oA β 42, no treatment effect in the level of neurite network is observed ($p=0.3580$). In the presence of oA β 42, significant differences are observed between Ab1 and isotype control hlgG4 tested at 150 $\mu\text{g}/\text{mL}$ for both readouts analyzed, ie, caspase 3/7 enzymatic activity and neurite network ($p=0.0007$ and $p<0.0001$ respectively) (*see Figures 3 and 4*).

Significant differences are also observed between the murine antibody and isotype control mlgG1 at 150 $\mu\text{g}/\text{mL}$ in the presence of oA β 42 for both readouts analyzed, ie, caspase 3/7 enzymatic activity and neurite network ($p<0.0001$ for both parameters) and at 50 $\mu\text{g}/\text{mL}$ in the presence of oA β 42 for neurite network ($p=0.0026$) (*See Figures 3 and 4*).

Conclusion

In the assay conditions, the oA β 42 preparation is neurotoxic for mouse primary neuronal cultures, as observed by a significant and marked increase in caspase 3/7 enzymatic activity and, in parallel, by a significant decrease in neurite network.

In the assay conditions, co-treatments of oA β 42 with humanized antibody Ab1 or its murine counterpart significantly inhibit the neurotoxic effects induced by oA β 42 on mouse primary neuronal cultures, as demonstrated by the concentration-dependent inhibition of oA β 42-induced increase in caspase-3/7 enzymatic activity and of oA β 42-induced decrease in neurite network.

An antibody directed against the protofibrillar form of the A β peptide, such as Ab1, has surprisingly a neuro-protective activity against oA β 42-induced neurotoxicity *in vitro* and is thus useful as therapeutics for amyloid-induced pathology at the earliest stages of the disease and for the prevention of the disease.

5

EXAMPLE 2: Neuronal cytoprotective effect of antibody Ab1 versus other antibodies

Material and methods

Primary cultures of cortical neurons were dissected out from 16-day-old mouse embryos (OF1) and plated in wells of poly-D-lysine coated 96-well culture plates (Greiner) in a Dulbecco's Modified Eagle Medium (DMEM) supplemented with N2 and B27 (40,000 cells/well/100 μ l).

After 6 days *in vitro*, all the medium was removed and replaced by new one without B27, and the neurons were pre-incubated with anti-A β antibodies for 1 hour. The neurons were then incubated with oA β 42 preparation (obtained as in Example 1), during 48 hours. Control application of drug-free medium but supplemented with the corresponding DMSO concentration (same as oA β 42) were run in parallel to applications of test substance.

The test compounds were Ab1, Ab4 (consisting of two heavy chains of sequence SEQ ID NO: 33 and two light chains of sequence SEQ ID NO: 34) and Ab5 (consisting of two heavy chains of sequence SEQ ID NO: 35 and two light chains of sequence SEQ ID NO: 36). The control antibodies were hIgG4 and hIgG1.

The concentrations of antibody were 50 μ g/mL or 150 μ g/mL.

Caspase 3/7 activity and neuritic network inhibition were assessed as in Example 1.

Results

As regard to the two other test antibodies, Ab4 specifically binds to a conformational epitope on A β fibrils and Ab5 to A β small and large Abeta assemblies.

It was first checked that, for both parameters, Caspase 3/7 and Neuritic network, a significant difference was observed between the control without oA β 42 and the oA β 42 group and that no significant treatment effect of the test antibodies was observed in the absence of oA β 42.

As previously shown in Example 1, significant differences were observed for antibody Ab1 + oA β 42 at the tested concentrations compared to oA β 42, for both caspase 3/7 and neuritic network parameters. However, significant differences compared to oA β 42 were observed only for the neuritic network as regard to Ab4 at concentrations of 50 and 150 μ g/mL and Ab5 at concentration 50 and 150 μ g/mL.

35

CLAIMS

- 5 1. An antibody specific for the protofibrillar form of the A β peptide for use in the prevention of Alzheimer's disease and/or in the treatment of an early stage of symptomatic Alzheimer's disease.
- 10 2. The antibody for use according to claim 1, in the prevention of Alzheimer's disease in a subject who does not suffer from memory loss and/or from impaired cognitive function.
- 15 3. The antibody for use according to claim 1 or 2, in the prevention of Alzheimer's disease in a subject who does not have senile plaques, does not have neurodegeneration and/or does not have synapse dysfunction.
- 20 4. The antibody for use according to any one of claims 1 to 3, wherein said antibody prevents, at least partially, A β -induced cytotoxicity in neuronal cells.
- 25 5. The antibody for use according to claim 4, wherein said antibody prevents, at least partially, A β -induced apoptosis of neuronal cells and/or prevents, at least partially, A β -induced neurite network decrease.
- 30 6. The antibody for use according to any one of claims 1 to 5, wherein said antibody is a humanized antibody.
- 35 7. The antibody for use according to any one of claims 1 to 6, wherein said antibody comprises (i) the CDRs of sequence SEQ ID NO: 10, 12, 14, 16, 18 and 20, (ii) the CDRs of sequence SEQ ID NO: 10, 12, 14, 32, 18 and 20 or (iii) the CDRs of sequence SEQ ID NO: 10, 12, 30, 32, 18 and 20.
8. The antibody for use according to any one of claims 1 to 7, wherein said antibody comprises the CDRs encoded by (i) the nucleotide sequences SEQ ID NO: 9, 11, 13, 15, 17 and 19, (ii) the nucleotide sequences SEQ ID NO: 9, 11, 13, 31, 17 and 19, (iii) the nucleotide sequences SEQ ID NO: 9, 11, 29, 31, 17 and 19 or (iv) by nucleotide sequences differing by respectively 1, 2, 3, 4 or 5 nucleotides from these sequences of (i), (ii) or (iii).

- 5 **9.** The antibody for use according to any one of claims 1 to 8, wherein (i) the variable part of its heavy chain is encoded by a sequence having at least 80% identity with sequence SEQ ID NO: 5 or SEQ ID NO: 27 and/or (ii) the variable part of its light chain is encoded by a sequence having at least 80% identity with sequence SEQ ID NO: 7 or SEQ ID NO: 23.
- 10 **10.** The antibody for use according to any one of claims 1 to 9, wherein (i) the variable part of its heavy chain comprises a sequence having at least 80% identity with sequence SEQ ID NO: 6 or SEQ ID NO: 28 and/or (ii) the variable part of its light chain comprises a sequence having at least 80% identity with sequence SEQ ID NO: 8 or SEQ ID NO: 24.
- 15 **11.** The antibody for use according to any one of claims 1 to 10, wherein said antibody comprises (i) a heavy chain encoded by a sequence having at least 80% identity with the nucleotide sequence SEQ ID NO: 1 or SEQ ID NO: 25 and/or (ii) a light chain encoded by a sequence having at least 80% identity with the nucleotide sequence SEQ ID NO: 3 or SEQ ID NO: 21.
- 20 **12.** The antibody for use according to any one of claims 1 to 11, wherein said antibody comprises (i) a heavy chain having at least 80% identity with the polypeptide sequence SEQ ID NO: 2 or SEQ ID NO: 26 and/or (ii) a light chain having at least 80% identity with the polypeptide sequence SEQ ID NO: 4 or SEQ ID NO: 22.
- 25 **13.** The antibody for use according to any one of claims 1 to 12, wherein said antibody comprises sequences encoded by the nucleotide sequences (i) SEQ ID NO: 5 and 7, (ii) the nucleotide sequences SEQ ID NO: 5 and 23 or (iii) the nucleotide sequences SEQ ID NO: 27 and 23.
- 30 **14.** The antibody for use according to any one of claims 1 to 13, wherein its sequence comprises (i) the polypeptide sequences SEQ ID NO: 6 and 8, (ii) the polypeptide sequences SEQ ID NO: 6 and 24 or (iii) the polypeptide sequences SEQ ID NO: 28 and 24.
- 35 **15.** The antibody for use according to any one of claims 1 to 14, wherein said antibody comprises the sequences encoded by (i) the nucleotide sequences SEQ ID NO: 1 and 3, (ii) the nucleotide sequences SEQ ID NO: 1 and 21 or (iii) the nucleotide sequences SEQ ID NO: 25 and 21.

16. The antibody for use according to any one of claims 1 to 15, wherein said antibody comprises (i) the polypeptide sequences SEQ ID NO: 2 and 4, (ii) the polypeptide sequences SEQ ID NO: 2 and 22 or (iii) the polypeptide sequences SEQ ID NO: 26 and 22.

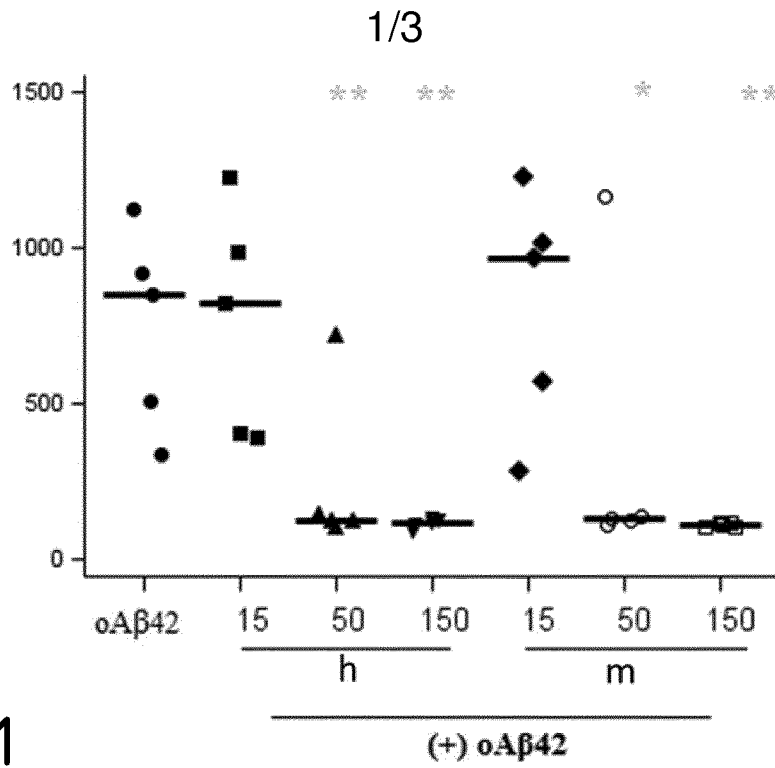


FIG.1

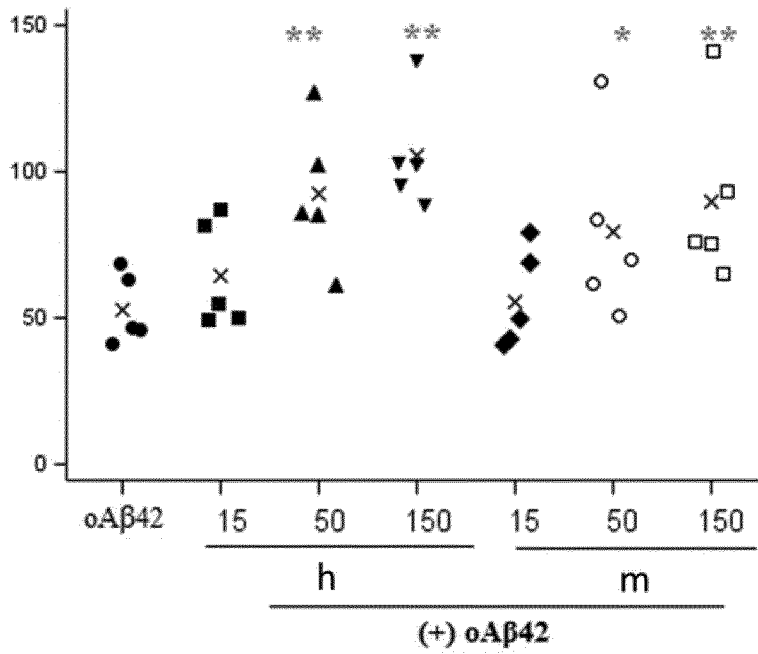


FIG.2

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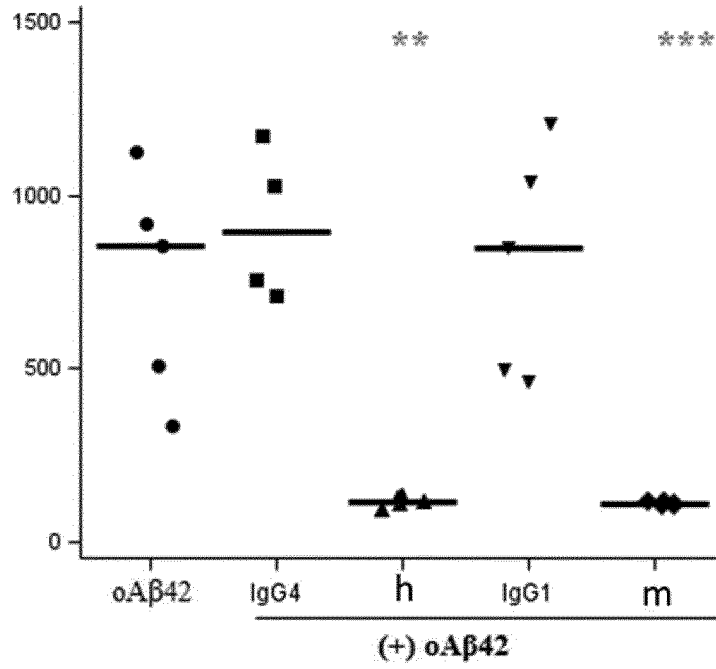


FIG.3

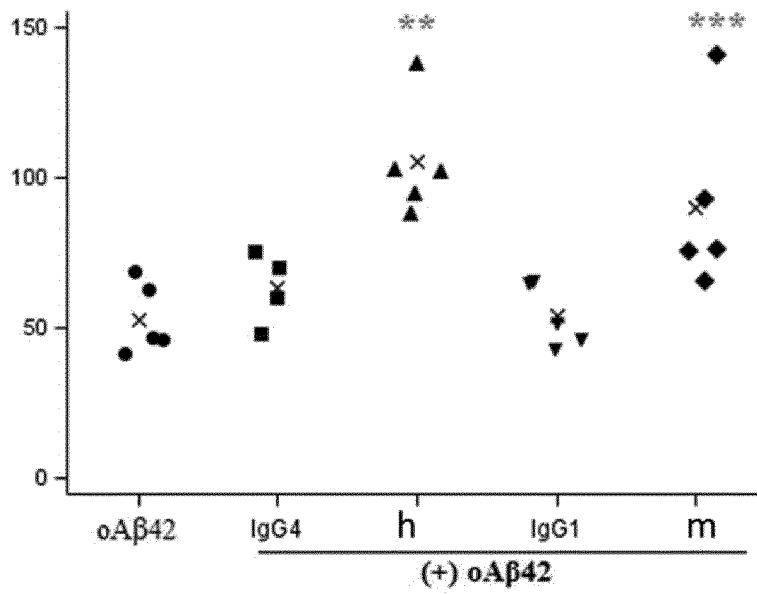


FIG.4

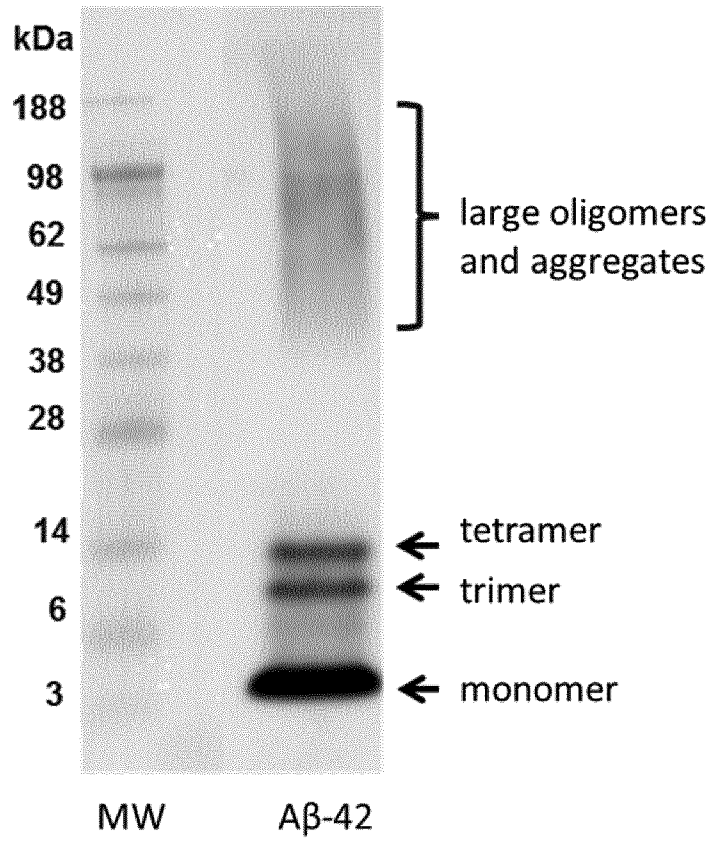


FIG.5

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2018/052239

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/00 C07K16/18 A61K39/395
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)
A61K C07K
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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| "A" document defining the general state of the art which is not considered to be of particular relevance | "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 |
| "E" earlier application or patent but published on or after the international filing date | "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 |
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| "O" document referring to an oral disclosure, use, exhibition or other means | "&" document member of the same patent family |
| "P" document published prior to the international filing date but later than the priority date claimed | |

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| Date of the actual completion of the international search 12 March 2018 | Date of mailing of the international search report 26/03/2018 |
| Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016 | Authorized officer Fleitmann, J |

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