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Method for the Diagnosis or Prediagnosis of a Beta-Amyloidopathy

The accumulation of proteins or protein fragments (peptides) in the brain is a significant feature of age-dependent neurodegenerative diseases. In Alzheimer's disease (Alzheimer disease, AD) and cerebral β-amyloid angiopathy (CAA), the aggregation of β-amyloid peptides(Aβ) is pathogenic, the underlying mechanism being unknown. Aβ proteostasis, i.e. the equilibrium of production and degradation/removal by means of receptors or proteases, is disturbed in AD and CAA. However, little notice was previously given to the removal of the Aβ peptides by cellular transporters (ABC transporters). Cirrito et al. were able to show that the ABCB1 transporter (Pgp) is involved in the transport of Aß across the blood-brain barrier and that an ablation of Pgp on the blood-brain barrier in a murine model of Alzheimer's disease intensifies the Aβ deposition (Cirrito et al. J. Clin. Invest. 2005, 115(11), 3285-3290). In Parkinson's disease (Morbus Parkinson) the protein α-synuclein, which inter alia regulates dopamine secretion in the substantia nigra, accumulates. In α-synucleinopathy Morbus Parkinson it is known that ABC transporters play a decisive part in the transport (Kortekaas et al., Ann Neurol 2005, 57, 176-179). Here, several subfamilies A-G exist, which can transport various substrates in either direction (metabolites, medicaments, peptides, proteins, ions) and are even capable of mutually replacing one another in the transport function (e.g. ABCB1 and ABCC1, Tao et al. Cancer Chemotherapy and Pharmacology, 64, 5, 961-969).

By means of various genetically modified murine models, it was possible to show that the ABC transporter (common structural element of the ABC transporters is an ATP-binding cassette and a transport pore) ABCC1, is an important protein/peptide transporter, in particular Aβ transporter, which has exceptional functional effects on cerebral protein accumulation. ABCC1 is also an important α-synuclein transporter.

Below, the studies on transporter activity are presented on Aβ transport by way of example.

For the determination of the ABCC1 activity *in vivo*, the ABCB1, the ABCG2 or the ABCC1 transporter respectively was removed by genetic engineering in APP-expressing, transgenic mice (knockout mice).

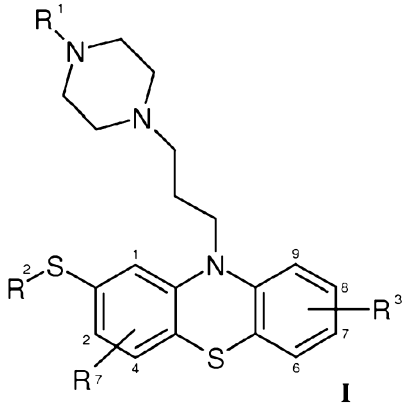
Thereby it was found that:

i) the quantity of Aβ in the mice which lacked the ABCC1 transporter was increased 12-fold,

ii) loss of the ABCB1 transporter leads only to a 3-fold increase and

iii) loss of ABCG2 has no Aβ-accumulating effect.

Substances which influence the ABCC1 transporter in a suitable manner so as to be able to treat neurodegenerative diseases, in particular β-amyloidopathies or α-synucleopathies, are 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines according to the general formula I,



I

wherein the residues

R^1 and R^2 are the same or different and each mutually independently are C_1-C_6 alkyl groups, which mutually independently optionally have a further substituent selected from alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group and halogen atom, wherein the respective alkyl groups optionally have at least one further halogen atom and the residue

R^3 is located at one of the positions 6-9 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl groups optionally bear at least one further halogen atom, or is an NR^4R^5 or OR^6 group, wherein R^4, R^5 and

R^6 are the same or different and are each mutually independently selected from hydrogen and C_1-C_3 alkyl group and the residue

R^7 is located at one of the positions 1, 2 or 4 of the phenothiazine ring system and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl groups optionally bear at least one further halogen atom, or is an NR^8R^9 or OR^{10} group, wherein R^8, R^9 and R^{10} are the same or different and are each mutually independently selected from hydrogen and C_1-C_3 alkyl group.

Both with α-synucleinopathies and also with β-amyloidopathies, there is a need for possible ways for recognizing or diagnosing or pre-diagnosing these.

The purpose of the invention was therefore the provision of a method with which the β-amyloidopathy Alzheimer's disease can be diagnosed or prediagnosed. This problem is solved with a method according to claim 1. Preferred embodiments follow from the dependent claims. In other words, the problem is solved by a method for the diagnosis or prediagnosis of the β-amyloidopathy Alzheimer's disease or for ascertaining the risk of a test subject suffering from this disease, wherein the test subject already partakes of substances which are transported across the cerebral ABCC1 transporter, consisting of the following steps:

a) determining the amount of the ingested substance in body-fluid samples from the test subject at a particular time point;

b) repeating the step a) determination at at least one further, later time point;

c) comparing the amounts ascertained in step a) and b) with amounts which have been defined at the same time points as characteristic of test subjects who exhibited no clinical signs of the β-amyloidopathy Alzheimer's disease at the time of sampling, wherein the substances which are transported across the cerebral ABCC1 transporter are selected from antibiotics, virostatics/antiviral medicaments, antiallergics/antihistamines, cardiovascular medicaments, antidepressants, antihyperuricaemics, cytostatics, vitamins/vitamin analogues, anti-inflammatories, antiepileptics, hormones/hormone derivatives, leukotrienes, fluorescent samples, GSH-,

sulfate- or glucuronide-coupled metabolites of natural substances (endogenously produced), toxins or medicaments selected from the group consisting of 2,4-dinitrophenyl-SG, bimane-SG, N-ethylmaleimide-SG, doxorubicin-SG, thiotepa-SG, cyclophosphamide-SG, melphalan-SG, chlorambucil-SG, ethacrynic acid-SG, metolachlor-SG, atrazine-SG, sulforaphane-SG, aflatoxin B1 epoxide-SG, 4-nitroquinoline 1-oxide-SG, As(SG)3, etoposide-Gluc, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)-3β-O-Gluc, SN-38-gluc, 4-methylumbelliferyl-β-d-gluc, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)-benzothiazole sulfate (E3040S)-Gluc, leukotriene C4, prostaglandin A2-SG, 15-deoxy-Δ12,14-prostaglandin J2-SG, hydroxynonenal-SG, 17β-oestradiol-17-β-d-gluc, glucuronosylbilirubin, bis-lucuronosylbilirubin, hyodeoxycholate-6-α-Gluc, oestrone 3-sulfate, dehydroepiandrosterone sulfate, sulfatolithocholate.

That the test subject already partakes of a substance which is transported across the cerebral ABCC1 transporter means that this substance does not have to be administered first. Rather, it is already present in the body of the test subject, for example because of a medicinal treatment of another disease. The body fluid samples from the test subject which are tested are preferably samples of blood plasma, blood serum and/or spinal fluid. This indirect analysis of the transporter activity of the ABCC1 transporter can be used for the diagnosis/prediagnosis of Alzheimer's disease. In test subjects who are already partaking of ABCC1 transportable substances by other routes, the active substance profile in body fluids, preferably blood plasma, serum and/or spinal fluid, can be tested. A time-dependent measurement, for test subjects in whom a decreased ABCC1 transporter activity compared to healthy test subjects is present, shows a retarded or displaced substance concentration curve (concentration c plotted against time 3), i.e. the maximum of the curve appears at a different time.

When a displaced curved compared to the healthy case is found, this is an indication of an altered ABCC1 transport activity. This means than substances such as Aβ are

then also more poorly transported and is thus an indication of a corresponding disease.

Both the murine model and also the pharmacological influencing of the ABCC1 show that this is an important cellular transmembrane transporter for the Aβ protein and imply that the blood-brain barrier and the choroid plexus occupy a key position for Aβ excretion from the brain. It could be shown that selective pharmacological activation of the ABCC1 transporter significantly decreases the cerebral loading with Aβ and thus is therapeutically usable for the treatment of diseases with impaired brain proteostasis. Furthermore, the analysis of the transporter activity of the ABCC1 transporter can be used as described above for the indirect or direct diagnosis/prediagnosis of a corresponding disease. The direct analysis would be possible via the administration of substances which are transported across the ABCC1 transporter, and their determination. The direct analysis has already been explained above.

Changes in export mechanisms which are connected with ABC transporter can substantially influence the temporal aggregation profile of Aβ and other brain proteins. Consequently, influencing the function of the ABCC1 transporter has a favourable effect on the risk of suffering from neurodegenerative diseases, in particular Alzheimer. In this sense, "treatment of neurodegenerative diseases" includes the prophylaxis and also the treatment of already existing diseases.

The role of the ABC transporters in Aβ excretion was first studied in that it was demonstrated that ABCC1 is capable of transporting Aβ. For this, *in vitro* transwell assays were used with endothelial cells (endothelial cell transwell assay, ECTA) from primary, cultured, capillary endothelial cells from mouse brains (cell culture preparation):

Primary cultures of endothelial cells from cerebral capillaries of ABCB1-deficient, ABCC1-deficient (knock out) mice and of control mice (C57BI/6, FVB/N) were used in order to study the Aβ-specific transport activity. The transport of Aβ from the abluminal (brain) into the luminal (blood) compartment is impaired in ABCB1-

deficient and ABCC1-deficient endothelia. The average Aβ transport rate during the first six hours after administration of Aβ peptides (Aβ42) was 2.2pg/min for the control cells. In contrast to this, the ABCC1-deficient cells reached only half the transport capacity (1.0pg/min). In the ABCB1-deficient cells, the Aβ was almost absent (0.3pg/min). Further studies on capillary endothelia and cells from the choroid plexus showed that the transporter ABCB1 is strongly expressed in the cerebral capillary endothelia, whereas the endothelial ABCC1 expression in cerebral capillaries is less.

On the basis of newly generated ABC transporter-deficient Alzheimer murine models, the relative significance of members of the ABC transporter family was then studied *in vivo*. The genetically modified mice respectively have a deficiency (knock out) of specific ABC transporters ABCG2, ABCB1 or ABCC1.

The Aß immunohistochemistry of brain sections showed:

i) significant increases in the cortical number and the size of Aβ-positive plaques in ABCC1-deficient mice compared to control mice (see Fig. 1 and 2a-c).

ii) ABCB1-deficient mice showed a smaller increase in the number and size of Aβ plaques than ABCC1-deficient mice.

iii) Between control mice and ABCG2-deficient mice, no significant difference could be observed (Fig. 2a-c).

For the determination of the amount of buffer-soluble Aβ (mostly monomers and smaller oligomers) and guanidine-soluble Aβ (mostly fibrillar or aggregated material), enzyme-coupled immunoabsorption tests (enzyme-linked immuno-absorbent assays, ELISAs) for Aß were used.

In agreement with the morphological results from the immunohistochemistry, the ABCC1-deficient mice showed a significant increase for aggregated Aβ in comparison to the control mice at all measurement time points. The cerebral loading with Aβ was the strongest at an age of 25 weeks. At this time point, the Aβ values (Aβ42) were 12 times higher than in the control mice. Buffer-soluble Aβ also

increased with age, but after 25 weeks, at the time point of the highest plaque loading, the values for the soluble Aβ in the ABCC1-deficient group fell markedly.

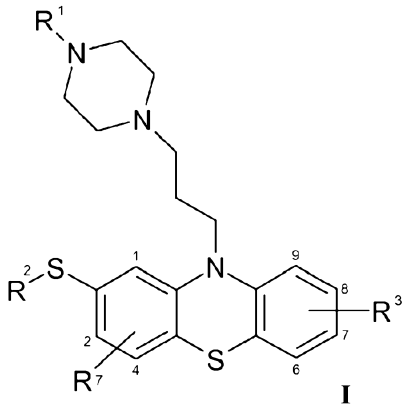
Further studies were performed, which provided further evidence for the connection between the sometimes lacking removal by ABCC1 and the aggregation of Aβ.

The transport kinetics of ABC transporters depend inter alia on specific protein/peptide characteristics such as the specific charge. The Dutch-type variant of the amyloid precursor proteins (Dutch mutant, APP_{dt}), which introduces an additional negative charge close to the α-secretase cleavage site of APP and thus leads to a severe cerebral amyloid angiopathy (CAA), influences the elimination of the $A\beta_{dt}$ across the blood-brain barrier. The Western blot analyses of cerebral capillaries and choroid plexus (CP) from control mice showed strong expression of ABCB1 in cerebral capillary endothelia (BC) and of ABCC1 in the CP (Fig. 3d). Since ABC transporters play an important part in elimination of Aβ, it was assumed that ABC transporter-deficient (at the blood-brain barrier and at the blood choroid plexus barrier) APP_{dt} transgenic mice display intensified accumulation of $A\beta_{dt}$ in meningeal vessels. The severity of the CAA in the ABC-deficient APP_{dt} mice was quantified at the age of 24 months. In agreement with the assumption, at least 51% of the vessels were severely impaired (>75% of the vessel wall laden with Aβ) in the ABCC1-deficient animals as against 23% in the controls (Fig. 3c).

On the basis of these results, it was investigated how far the content of soluble Aß in the brain could be decreased/influenced by active substance-mediated activation of ABC transporters. Mice with amyloid deposits were treated for 30 days with the anti-emetic thiethylperazine (Torecan®, 2-(ethylthio)-10-[3-(4-methylpiperazin-1-yl)propyl]-10*H*-phenothiazine). Twice daily, 3mg/kg body weight were administered intramuscularly. The prophylactic treatment began already before the mice developed senile plaques. ELISA measurements on the treated animals showed a reduction in the amount of Aβ of at least 31% in the treated mice in comparison to animals treated with vehicle (vehicle = water) (Fig. 3e). The results are reproduced graphically in Fig. 3.

The ability to remove Aβ was found to be a key factor in regulation of the intracerebral accumulation of Aβ.

Thiethylperazine (Torecan®) was found to be a particularly efficient activator of the ABCC1 transporter. Further derivatives starting from the same basic structure also showed good results in activation of the ABCC1 transporter. The relevant derivatives are shown in the general formula I



I

wherein the residues

R^1 and R^2 are the same or different and are each mutually independently C_1-C_6 alkyl groups, which mutually independently optionally have a further substituent selected from alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio, alkylthio group and halogen atom, wherein the respective alkyl groups optionally bear at least one further halogen atom and the residue

R^3 is located at one of the positions 6-9 of the phenothiazine ring system, preferably at position 6, 7 or 8, and is a hydrogen atom or an alkyl, aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl groups optionally bear at least one further halogen atom, or is an NR^4R^5 or OR^6 group, wherein R^4, R^5 and R^6 are the same or different and are each mutually independently selected from hydrogen and C_1-C_3 alkyl group and the residue

R^7 is located at one of the positions 1, 2 or 4 of the phenothiazine ring system, preferably at position 2 or 4, and is a hydrogen atom or an alkyl,

aryl, acyl (preferably acetyl), amino, nitro, sulfonyl, hydroxyl, alkoxy, aryloxy, arylthio or alkylthio group or a halogen atom, wherein the respective alkyl groups optionally bear at least one further halogen atom, or is an NR^8R^9 or OR^{10} group, wherein R^8, R^9 and R^{10} are the same or different and are each mutually independently selected from hydrogen and C_1-C_3 alkyl group.

These derivatives are correspondingly well suited for the treatment of neurodegenerative diseases, in particular of β-amyloidopathies or α-synucleinopathies, wherein as mentioned above treatment includes both the prophylaxis and also the treatment of already existing diseases. The halogen atom/the halogen atoms are preferably selected from fluorine and chlorine. The acyl groups (-(C=O)-R) of the residues $R^{1,2,3,7}$ are preferably acetyl groups (-$C(=O)CH_3$). Preferably the residues R^1 and R^2 are the same or different and each mutually independently is a C_1-C_6 alkyl group or a C_1-C_6 alkyl group (preferably C_1 alkyl), substituted with an acetyl group, and the residues R^3 and R^7 hydrogen or an acetyl group. In one preferred embodiment the residues R^1 and R^2 are the same or different and each mutually independently a C_1-C_3 alkyl group. Furthermore, it is preferred that the residues R^3 and R^7 are hydrogen. It is particularly preferred if the residue R^1 is a methyl group, the residue R^2 an ethyl group and the residues R^3 and R^7 are hydrogen (thiethylperazine, Torecan®). In the use for the treatment of neurodegenerative diseases it has been found advantageous to add to the 2-(R^2-thio)-10-[3-(4-R^1-piperazin-1-yl)propyl]-10*H*-phenothiazines further active substances, preferably 1-benzhydrylpiperazine, most preferably 1-benzhydryl-4-cinnamyl-piperazine (Cinnarizin).

Various neurodegenerative diseases are diagnosable by means of the indirect analysis described above, wherein the present invention relates to the diagnosis of Alzheimer's disease (AD). Possible, but not covered by the present invention, is the case that the neurodegenerative disease is an α-synucleinopathy, in particular Parkinson's disease (PD). Both diseases, i.e. β-amyloidopathy and α-synucleinopathies, are characterized by cerebral protein deposits, which can be diagnosed on the basis of the activity of the ABCC1 transporter.

Other diseases, also diagnosable on the basis of the ABCC1 transporter activity, are mentioned below. Thus a further diagnosable disease, not however covered by the present invention, is Lewy body dementia (LBD). This is also characterized by cerebral protein aggregation, i.e. is an α-synucleinopathy like Parkinson's.

Another diagnosable neurogenerative disease, not however covered by the present invention, is Huntington's disease (HD). Another diagnosable neurodegenerative disease, not however covered by the present invention, is a prion disease, in particular Creutzfeld-Jacob disease (CJD) or fatal familial insomnia (FFI). Another diagnosable neurodegenerative disease, not however covered by the present invention, is a tauopathy, in particular cortico-basal degeneration (CBD), Steel-Richardson-Olszewski syndrome (PSP, progressive supranuclear palsy - progressive supranuclear gaze paresis) or Pick's disease (PiD). Another diagnosable neurodegenerative disease, not however covered by the present invention, is a frontotemporal degeneration (FTLD), in particular ubiquitin-positive degeneration, TDP43-positive degeneration or degenerations negative for ubiquitin and TDP43. Another diagnosable neurodegenerative disease, not however covered by the present invention, is amyotrophic lateral sclerosis (ALS). Another diagnosable neurodegenerative disease, not however covered by the present invention, is a spinocerebellar ataxia (SCA) or spastic para-paresis (SPG). Another diagnosable neurodegenerative/neuroimmunological disease, not however covered by the present invention, is multiple sclerosis (MS) or an MS-related syndrome, in particular ADEM or Devic syndrome.

Description of the Diagrams

Fig.1a
shows that the cortical density of neuritic plaques is increased by ca. 75% in ABCC1-deficient mice (ABCC1ko);

Fig.1b,c

shows that the average plaque size is increased (+34%) because of the greater number of plaques (+63%) with a size of more than 700µm^2 and a lower frequency of smaller plaques (-24%). Error bar, standard error (n≥3);

Fig.1d

shows that the IHC staining in ABCG2-deficient (ABCG2ko), ABCB1-deficient (ABCB1ko), ABCC1-deficient (ABCC1ko) mice and in control mice shows a higher area density of Aβ in ABCC1-deficient animals. Typical plaques of the same size are shown in the enlargement, scaling bars represent 500µm (overall view) and 50µm (enlargement) (*p<0.05);

Fig.2a

shows that the plaque density in the cortex (covering) and the size is increased in specific ABC transporter-knockout mice. In particular, ABCC1-deficient (ABCC1ko) mice show an increased Aß-amyloid loading (light grey bars, each outside right in the individual groupings), w = week on the x-axis;

Fig.2b

shows that the total plaque size in ABCC1-deficient (ABCC1ko) and ABCB1-deficient (ABCB1ko) mice at the age of 25 weeks is increased, w = week on the x-axis;

Fig.2c

shows that the total increase in the plaque size is associated with the appearance of fewer small plaques and more larger plaques (>700 µm^2), whereas the number of medium-sized plaques remains at the same value, error bar, standard error (n≥5), *p<0.05;

Fig.3

shows that the deficiency of ABCC1 promotes the accumulation of Aβ and $A\beta_{dt}$ and that the activation of ABCC1 (by administration of Torecan) decreases the Aß values; wherein

Fig.3a

shows that at an age of 25 weeks ABCC1 deficiency leads to a marked increase (ca. 12-fold) in insoluble Aβ; and

Fig.3b

shows that the quantity of buffer-soluble Aβ42 at an age of 25 weeks is markedly reduced in comparison to 22 weeks (-56%). This is probably due to deposition in insoluble depots. At the same age, the area covered by Aβ deposits, which is measured in the immunohistochemistry, is increased by 83% (error bar, standard error $n \geq 5$, $p < 0.05$);

Fig.3c

shows that 53% of the blood vessels are severely affected by CAA (>75% of the vessel walls exhibit Aβ). This relates to ABCC1-deficient mice (ABCC1ko) in comparison to 23% in the controls (n=3);

Fig.3d

shows that the expression of ABCC1 is predominantly to be seen in the choroid plexus (CP), whereas ABCB1 is mainly expressed in the capillaries of the brain (BP);

Fig.3 e

shows that the activation of ABCC1 by thiethylperazine (Torecan) decreases the Aβ values in mice (-28%), error bar, standard error (n=4, *$p < 0.05$).

Examples

Animals

APP transgenic mice (APP, APP_{dt}) were sourced from The Jackson Laboratory (Bar Harbor, USA) and Tübingen University (Tübingen, Germany). The NEP-deficient mice were sourced from the Riken Brain Research Institute (Saitama, Japan). ABCG2-, ABCB1-, and ABCC1-deficient mice were sourced from Taconic-Farms (Denmark). All transgenic and knockout mouse lines were interbred for at least 9 generations in the genetic FVB background. The mice were kept in 12h/12h light/darkness cycle at 23°C with free access to food and water.

Methods

Tissue Preparation

For tissue preparation, the mice were killed by cervical dislocation and transcardially perfused with PBS (phosphate-buffered physiological saline). The brain was removed and one hemisphere stored in buffered 4% paraformaldehyde

for paraffin embedding and immunohistochemistry. The other hemisphere was shock frozen in liquid nitrogen and stored at -80°C for biochemical analyses.

ELISA

ELISA kits (TH40HS, TK42HS) from The Genetics Company (TGC, Schlieren, Switzerland) were used for the quantification of Aβ. Brain hemispheres were homogenized using PreCellys24 (12 s, 6,500 rpm). After addition of carbonate buffer (pH 8.0) the homogenates were mixed using PreCellys (5 s, 5,000 rpm) and centrifuged for 90 mins at 4°C and 24,000 g, in order to separate insoluble from soluble Aβ species. The remaining supernatant (buffer-soluble fraction) was mixed with 8M guanidine hydrochloride in a ratio of 1:1.6. For the extraction of the aggregated Aβ species, the pellet was dissolved in 8 volumes of 5M guanidine hydrochloride, shaken for 3 hrs at room temperature and centrifuged at 24,000 g for 20 min at 4°C. The remaining supernatant was the guanidine-soluble fraction (GuaHCl). Protein contents of all samples were measured three times, for which a Nanodrop1000 spectrophotometer was used (ThermoFisher Scientific, Wilmington, USA). The ELISAs were performed according to the manufacturer's instructions using suitable dilutions.

Western Blots

For the Western blots, tissue homogenates were prepared. The total protein concentrations of the extracts were determined using a BCA assay (Pierce, part of Thermo Fisher Scientific, Rockford, USA). After the electrophoresis of 10 µg total protein per track, the proteins were blotted on PVDF membranes. After blockade in 5% dried milk in TBST buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween20) for 1h at room temperature, the blots were tested either for ABCB1 (1:500, D-11, Santa Cruz), ABCC1 (1:200, Alexis Bio) or β-actin (1:20,000, Sigma) overnight at 4°C. As detection antibodies, anti-mouse-HRP, anti-rat-HRP or anti-hare-HRP were used. For the visualization, an Amersham ECL Plus detection kit and a RoperCoolSnap HQ^2 camera were used.

Immunohistochemistry (IHC)

Formalin-fixed brains were embedded in paraffin and cut into 4µm thick sections. After removal of the paraffin, the sections were further treated with a Bond-Max™ Autostainer (Menarini/Leica, Germany). Immunostaining was initiated after blockade of endogenous peroxidase (5 min) and epitope recovery (epitope retrieval) for 5 mins with 95% formic acid (for antibody 6F3D, Dako, Germany) and 70% formic acid (for antibody 4G8, Millipore, Germany). Primary antibodies were routinely incubated at room temperature for 30 mins with the following dilutions: 6F3D (1:100), 4G8 (1:500). Primary antibodies were detected with the BondMax™ Bond Polymer Refine detection kit and according to the standard protocol DAB R30. The sections were fully digitized with a resolution of 230nm using a MiraxDesk/MiraxMidi scanner and then automatically analysed using the AxioVision software package (Zeiss, Germany).

Assessment of the Severity of the CAA

Brain sections from APP_{dt} mice were stained with 4G8 antibody. At least two non-consecutive sections were tested blind for CAA of the cerebral membrane vessels. All cerebral membrane vessels were counted manually and the severity of the CAA was categorized as follows:

Category I: not affected

Category II: ≤25% of the periphery stained positive

Category III: ≤50% of the periphery stained positive

Category IV: ≤75% of the periphery stained positive

Category V: ≤100% of the periphery stained positive

The average number of vessels for each category was calculated relative to the total number of vessels found.

Endothelial Cell Transwell Assay (ECTA)

Endothelial cells of mouse brain capillaries were prepared as described in Coisne *et al.* (Coisne, C. et al. Mouse syngenic *in vitro* blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Laboratory Investigation; 85, 734-746 (2005)). At least 3-4 week-old mice were decapitated and the brains removed. After dissection of the brain stem, the white matter and the

cerebral membrane, the tissue was homogenized in two volumes of washing buffer B (WBB) (Hanks buffered salt solution (HBBS), 10 mM HEPES, 0.1% BSA) using a 15 ml glass douncer (Wheaton Industries, Millville, NJ; USA). One volume of 30% dextran solution was added to the homogenate. This was centrifuged twice at 3,000 g and 4°C. The pellet, which contained the vessels, was resuspended in WBB and large vessels were broken up manually by harsh pipetting of the solution. Vacuum filtration through 60 µm membranes (SEFAR, Switzerland) was used to separate large vessels from the capillaries. After combined treatment with collagenase/dispase (HBSS, 10 mM HEPES, 0.15 µg/ml TCLK, 10 µg/ml DNAse-I, 1 mg/ml collagenase/dispase (Roche), single cell suspension was achieved by further harsh pipetting of the solution. Endothelial cells were introduced into Matrigel-coated transwell inserts (0.4 µm pores, Greiner Bio-One, Germany) with a density of 120,000 cells per insert and allowed to grow on a supporting glial culture.

Sulfur yellow was used for the determination of the paracellular flow during the assay. The culture medium of the abluminal compartment was replaced with a solution which contained 10 ng Aß42 (1.6 nM end concentration). Next, samples were taken from the luminal compartment after 2h, 6h and 24h and the Aß content was determined by ELISA (TK42-highsense, TGC, Switzerland). The transport rate was determined as described by Coisne *et al.* (Coisne, C. et al. Mouse syngenic *in vitro* blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Laboratory Investigation; 85, 734-746 (2005)).

ELISA Statistics

The Lilliefors godness-of-fit test (alpha=0.05) was applied to the ELISA data and to the log transformed ELISA data in order to distinguish between the assumption of normal distributed sample date and the assumption of log-normal distributed sample data. In spite of the small sample size, for both data sets the null hypothesis (H_0) was rejected for 5 out of 44 samples. In agreement with the observation of predominantly positive shift (skew) and strictly positive sample data, the assumption of normal distributed data was discarded. Average values and confidence intervals were calculated on the assumption of an underlying log-

normal distribution. The Wilcoxon rank-sum test was used in order to compare the ELISA data of the different mouse strains for each time point.

PATENTKRAV

1. Fremgangsmåde til diagnosticering eller prædiagnosticering af β-amyloidopatien Alzheimers sygdom, der er associeret med en cerebral proteinaflejring og en reduceret aktivitet af den cerebrale ABCC1-transportør, eller til klarlægning af risikoen hos en testperson, der lider af en sådan sygdom, hvor testpersonen allerede tager stoffer, som transporteres over den cerebrale ABCC1-transportør, hvilken fremgangsmåde består af følgende trin:

a) bestemmelse af mængden af den indtagne stof i legmesvæskeprøver fra personen der testes på et bestemt tidspunkt;

b) gentagelse af bestemmelsestrinnet a) på mindst ét yderligere, senere tidspunkt;

c) sammenligning af de i trin a) og b) klarlagte mængder med de mængder, der er blevet defineret på de samme tidspunkter som karakteristiske for personer der testes, som ikke udviste kliniske symptomer på β-amyloidopatien Alzheimers sygdom på prøveudtagningstidspunktet, hvor de stoffer, der transporterer over den cerebrale ABCC1-transportør, er valgt blandt antibiotika, virostatika/antivirale medikamenter, antiallergika/antihistaminer, kardiovaskulære medikamenter, antidepressiva, antihyperurikæmi-midler, cytostatika, vitaminer/vitaminanaloger, antiinflammatoriske midler, antiepileptika, hormoner/hormonderivater, leukotriener, fluorescerende prøver, GSH-, sulfat- eller glucuronidkoblede metabolitter af naturlige stoffer (endogent producerede), toksiner eller medikamenter valgt fra gruppen bestående af 2,4-dinitrophenyl-SG, biman-SG, N-ethylmaleimid-SG, doxorubicin-SG, thiotepa-SG, cyclophosphamid-SG, melphalan-SG, chlorambucil-SG, ethacrynsyre-SG, metolachlor-SG, atrazin-SG, sulforaphan-SG, aflatoxin B1-epoxid-SG, 4-nitroquinolin 1-oxid-SG, As(SG)3, etoposid-Gluc, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL)-3β-O-Gluc, SN-38-gluc, 4-methylumbelliferyl-β-D-gluc, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)-benzothiazolsulfat (E3040S)-Gluc, leukotrien C4, prostaglandin A2-SG, 15-deoxy-Δ12,14-prostaglandin J2-SG, hydroxynonenal-SG, 17β-estradiol-17-β-D-gluc, glucuronosylbilirubin, bis-lucuronosyl-bilirubin, hyodeoxycholat-6-α-Gluc, estron-3-sulfat, dehydroepiandrosteronsulfat, sulfatolithocholat.

2. Fremgangsmåde til diagnosticering eller prædiagnosticering af β-amyloidopatien Alzheimers sygdom eller til klarlægning af risikoen hos en testperson, der lider af en sådan sygdom, ifølge krav 1, **kendetegnet ved, at** legmesvæskeprøverne fra testpersonen er prøver af blodplasma, blodserum og/eller spinalvæske.

Fig.1a

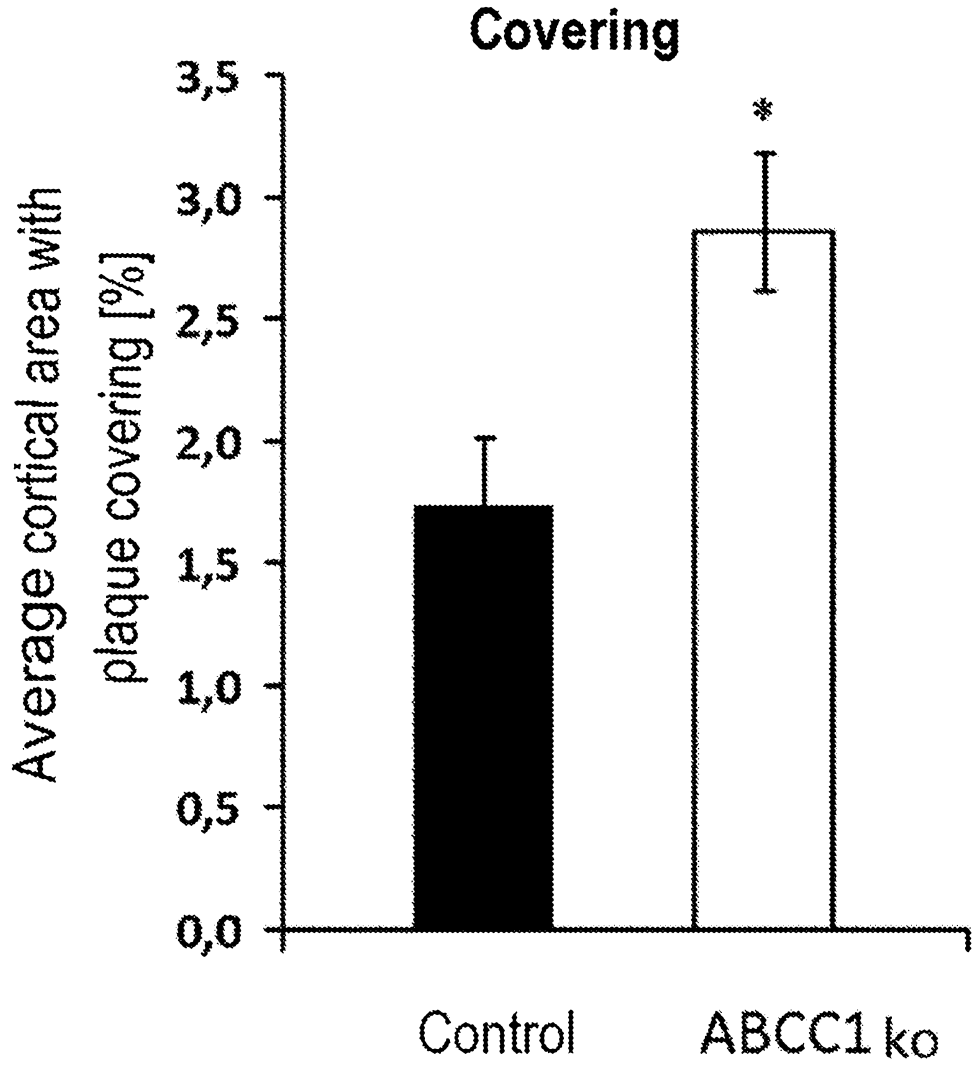

Covering
Average cortical area with plaque covering [%]
3,5
3,0
2,5
2,0
1,5
1,0
0,5
0,0
*
Control
ABCC1 ko

Fig.1b

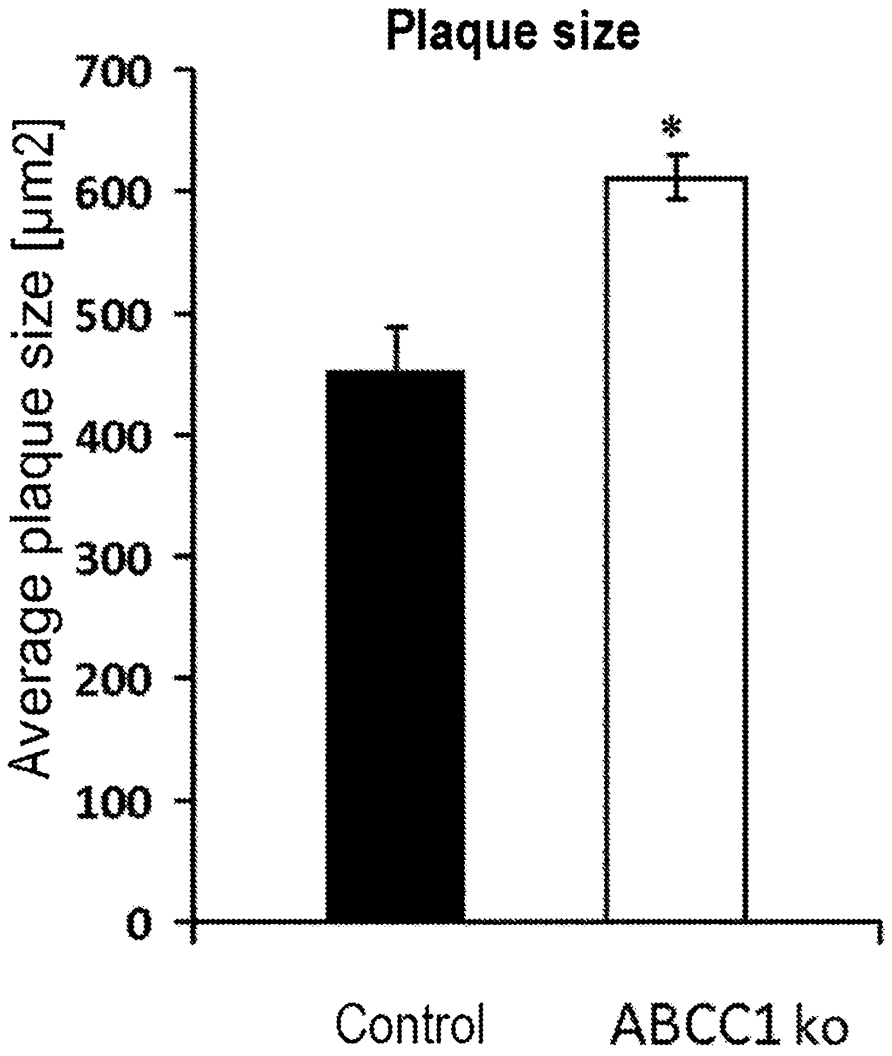

Plaque size
Average plaque size [µm2]
700
600
500
400
300
200
100
0
*
Control
ABCC1 ko

Fig.1c

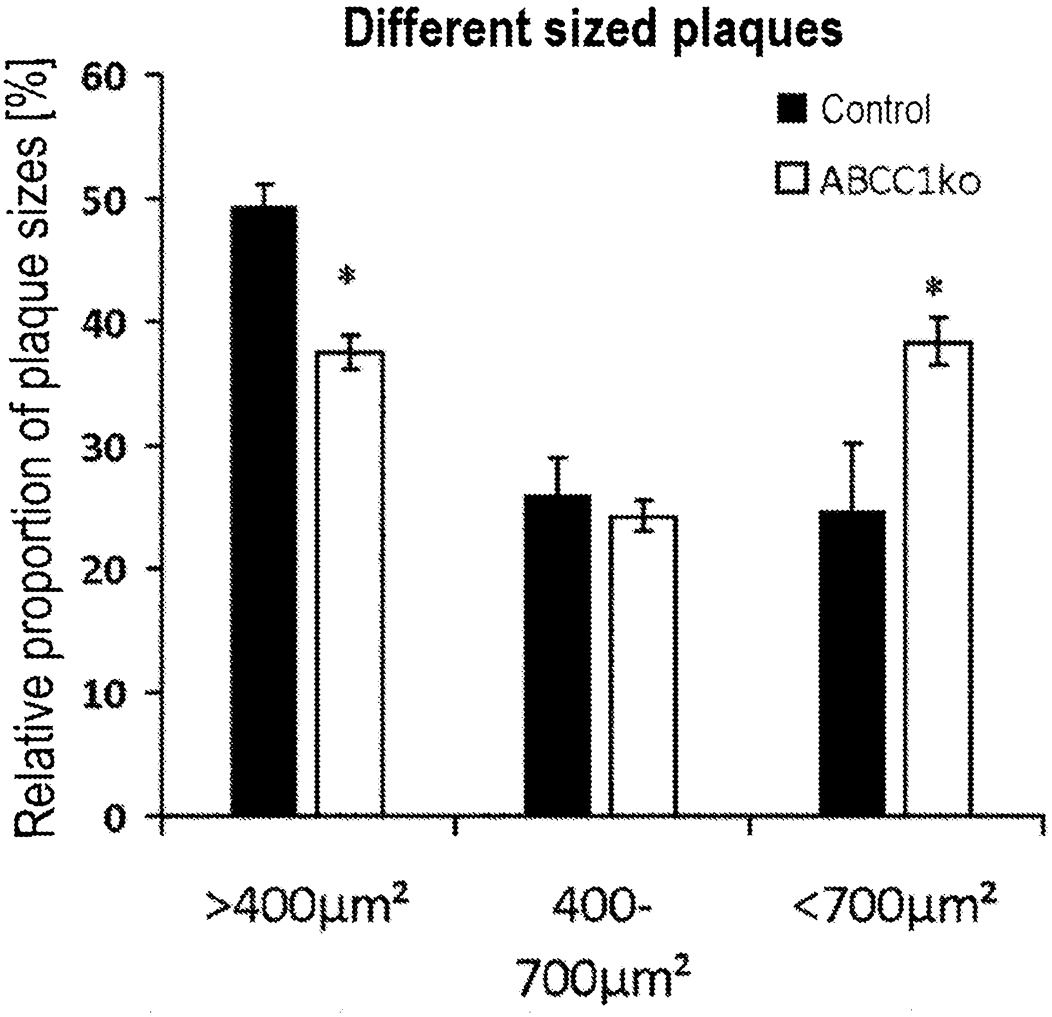

Different sized plaques
Control
ABCC1ko
Relative proportion of plaque sizes [%]
60
50
40
30
20
10
0
*
*
>400µm²
400-
700µm²
<700µm²

Fig.1d

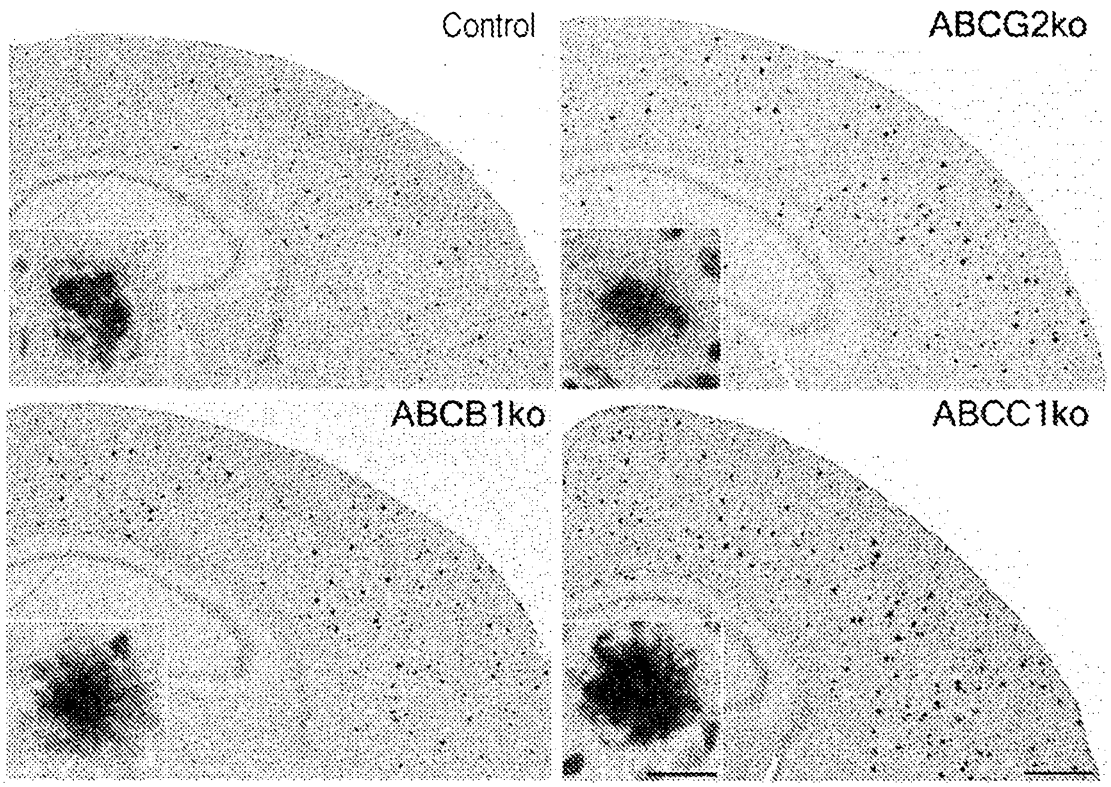


Fig.2a

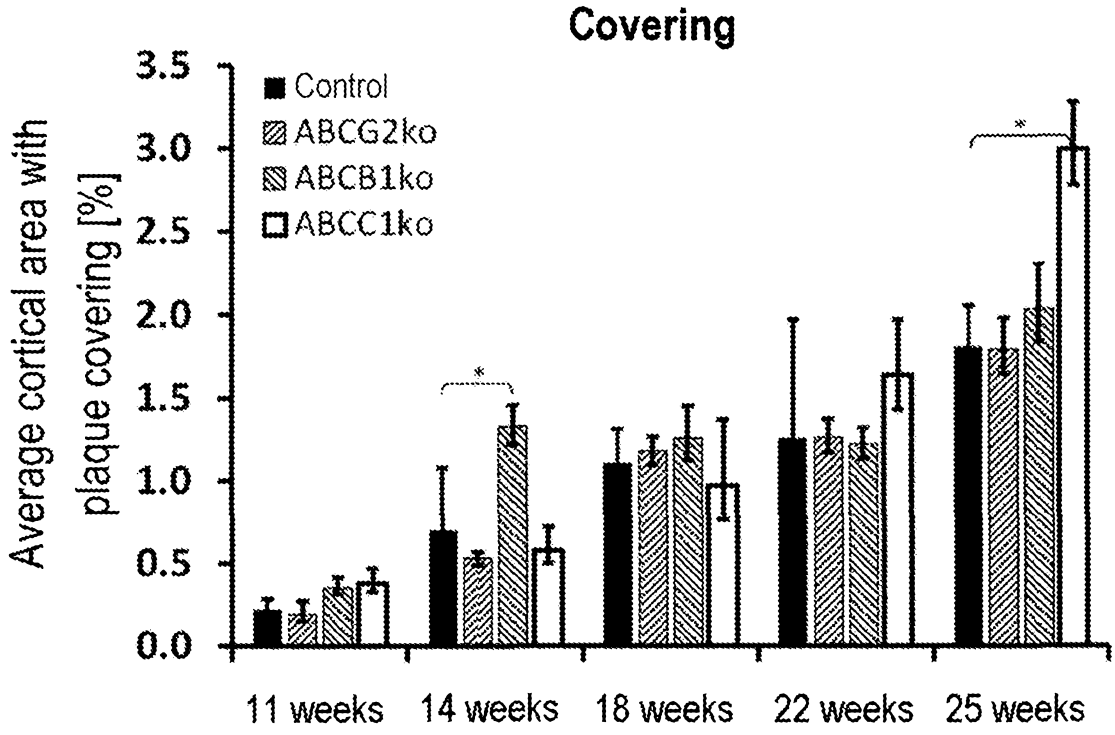

Covering
Average cortical area with plaque covering [%]
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
ABCG2ko
ABCB1ko
ABCC1ko
*
*
11 weeks
14 weeks
18 weeks
22 weeks
25 weeks

Fig.2b

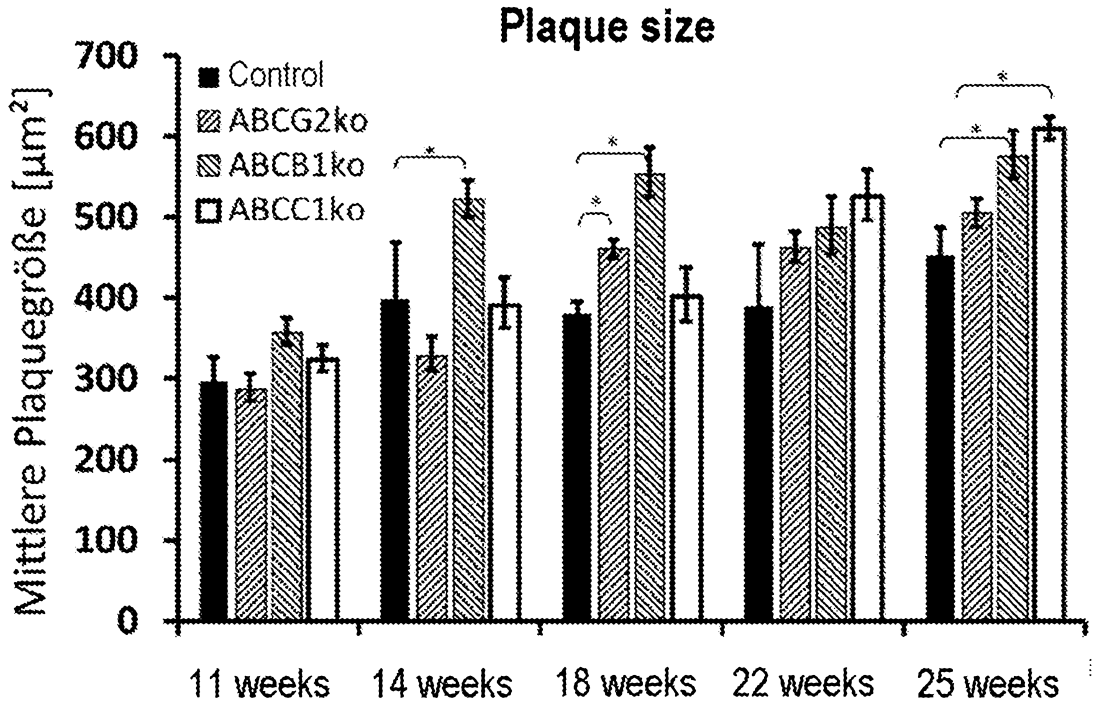

Plaque size
Mittlere Plaquegröße [µm²]
700
600
500
400
300
200
100
0
Control
ABCG2ko
ABCB1ko
ABCC1ko
*
11 weeks
14 weeks
18 weeks
22 weeks
25 weeks

Fig.2c

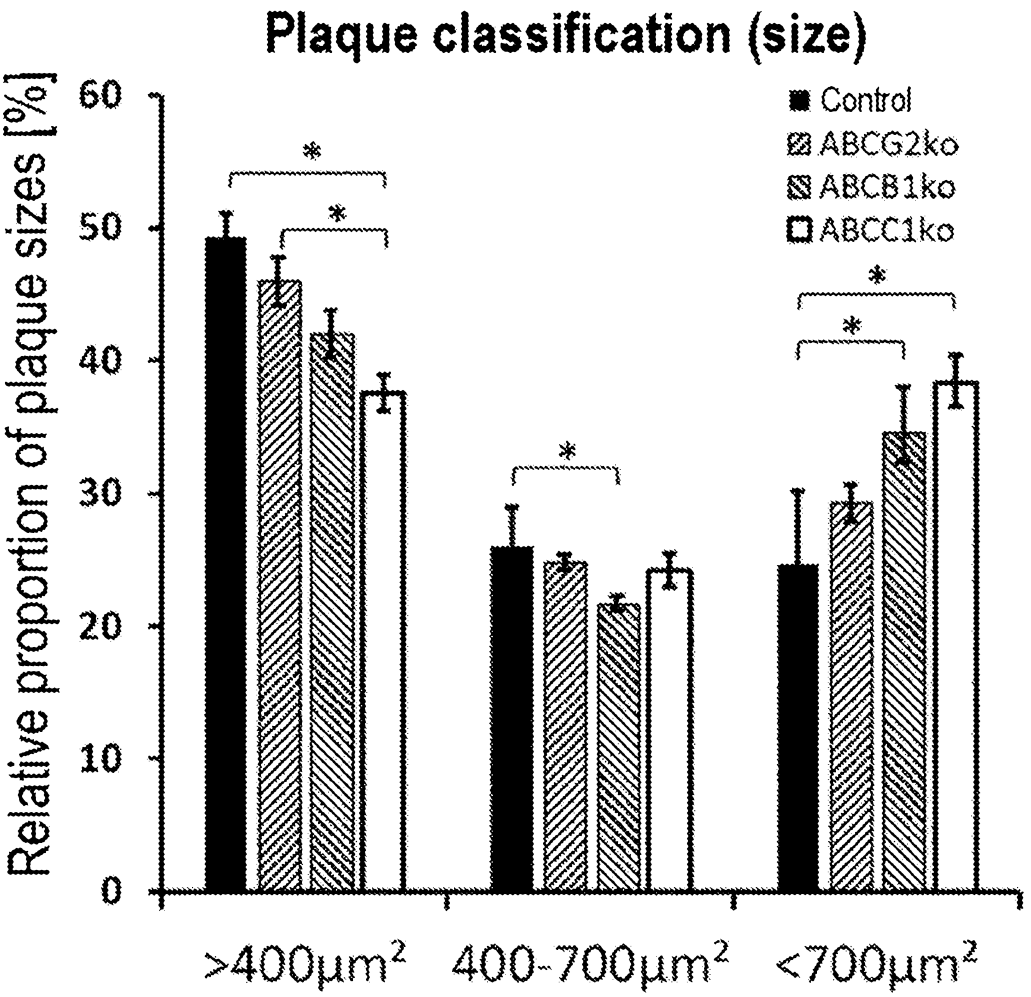

Plaque classification (size)
Relative proportion of plaque sizes [%]
60
50
40
30
20
10
0
Control
ABCG2ko
ABCB1ko
ABCC1ko
*
*
*
*
*
>400µm²
400-700µm²
<700µm²

Fig.3a

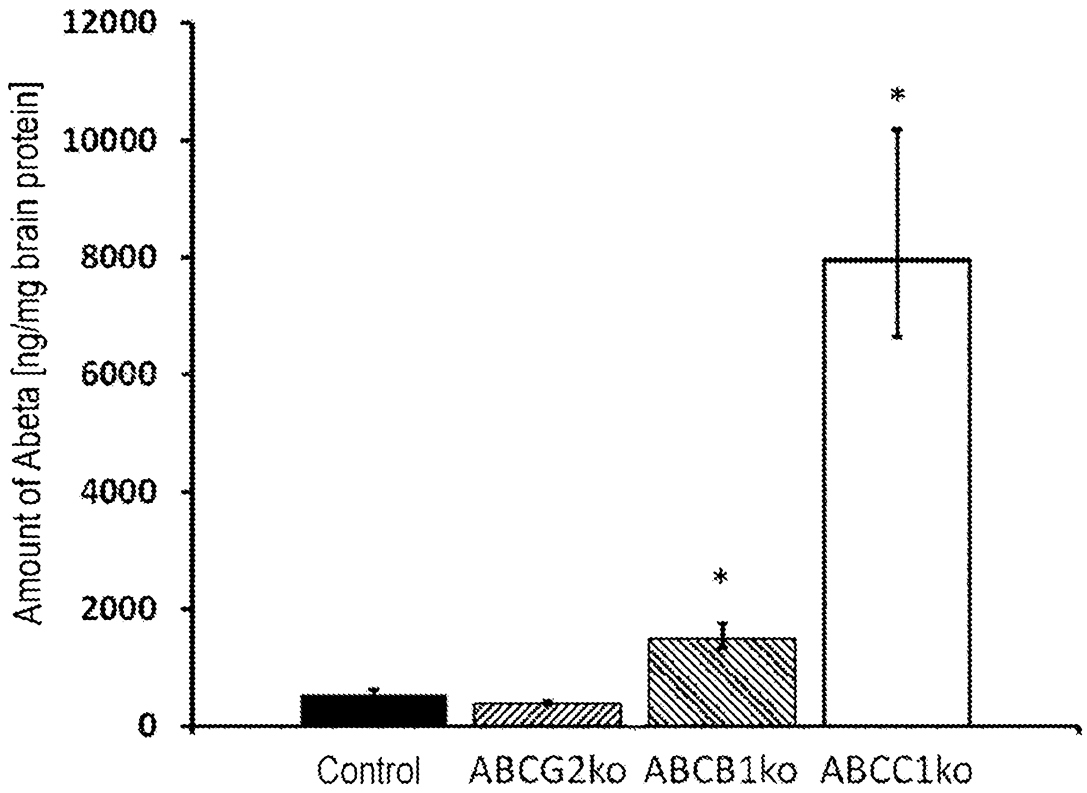

12000
10000
8000
6000
4000
2000
0
Amount of Abeta [ng/mg brain protein]
*
*
Control
ABCG2ko
ABCB1ko
ABCC1ko

Fig.3b

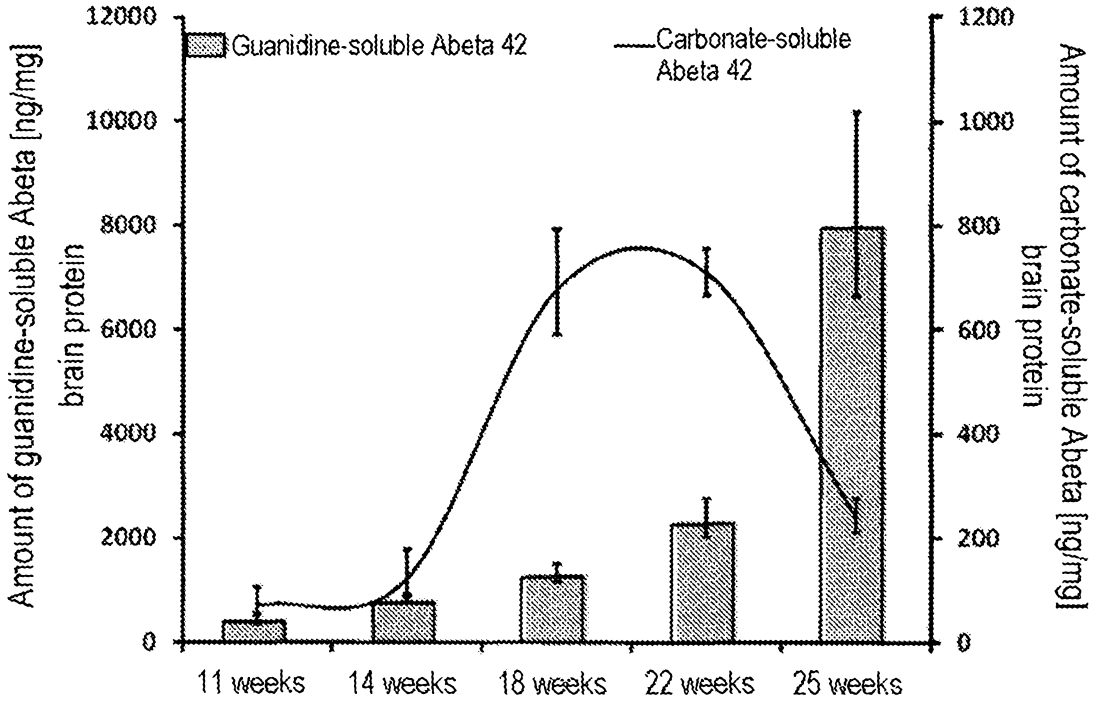

Amount of guanidine-soluble Abeta [ng/mg] brain protein
Amount of carbonate-soluble Abeta [ng/mg] brain protein
Guanidine-soluble Abeta 42
Carbonate-soluble Abeta 42
12000
10000
8000
6000
4000
2000
0
1200
1000
800
600
400
200
0
11 weeks
14 weeks
18 weeks
22 weeks
25 weeks

Fig.3c

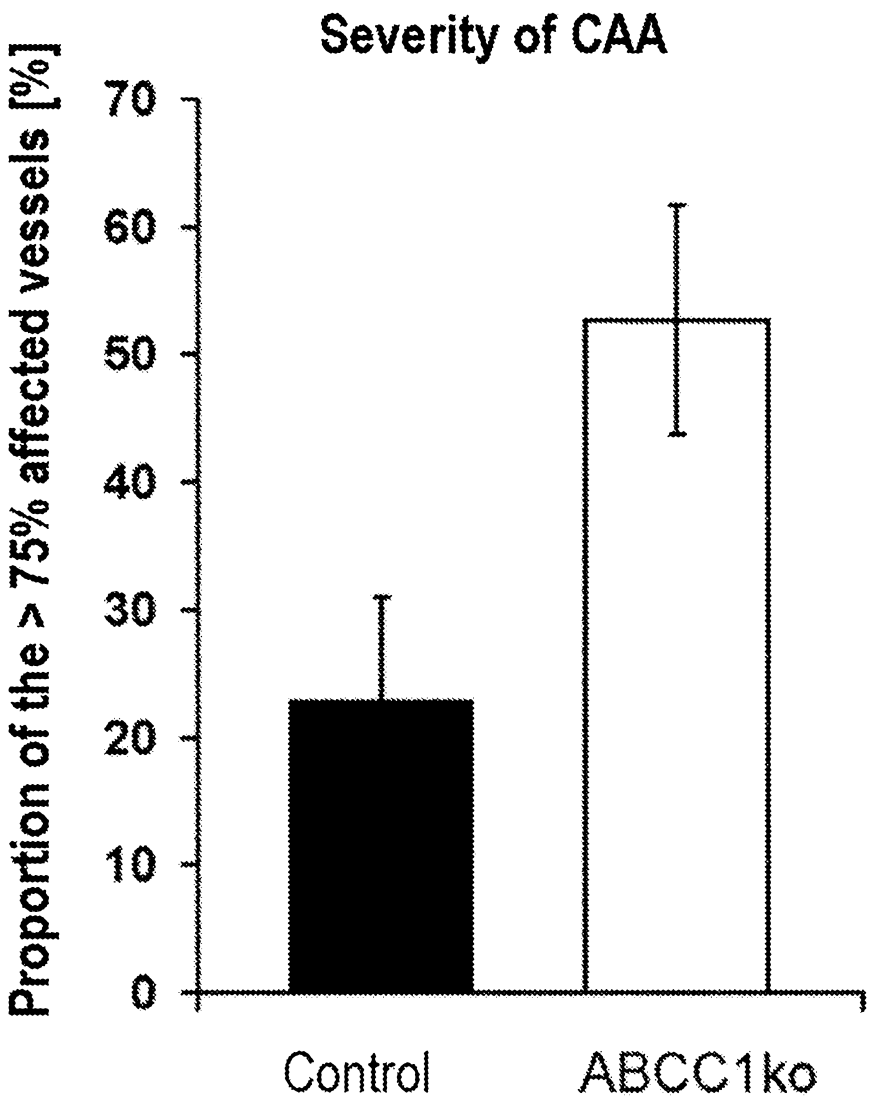

Severity of CAA
Proportion of the > 75% affected vessels [%]
70
60
50
40
30
20
10
0
Control
ABCC1ko

Fig.3d

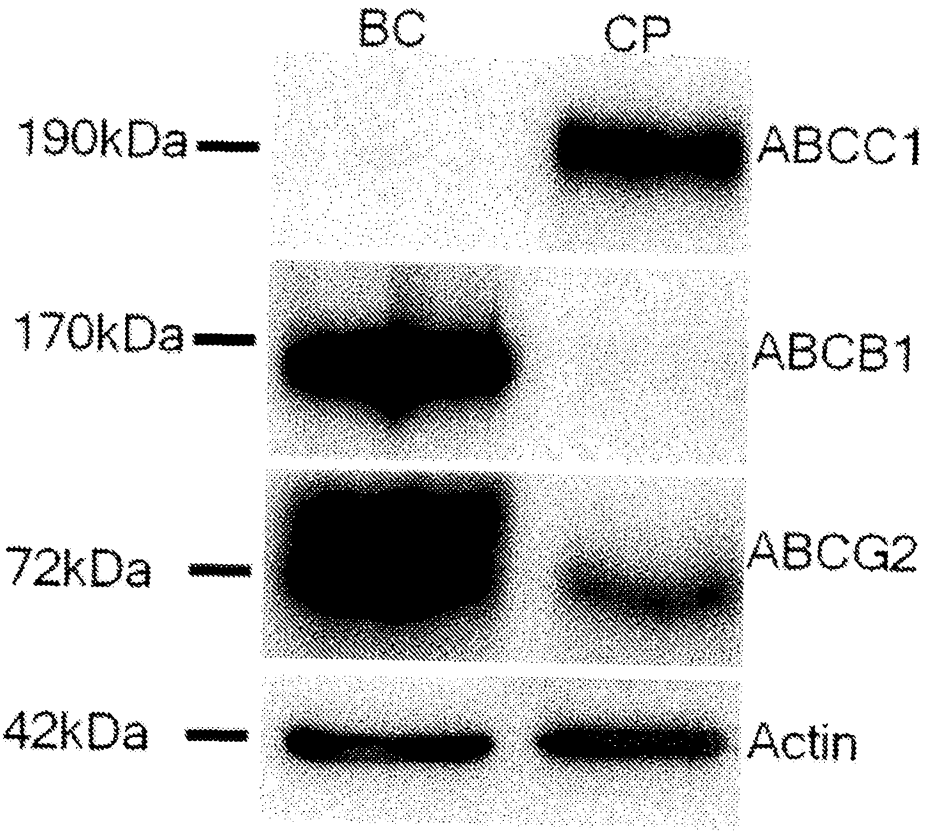
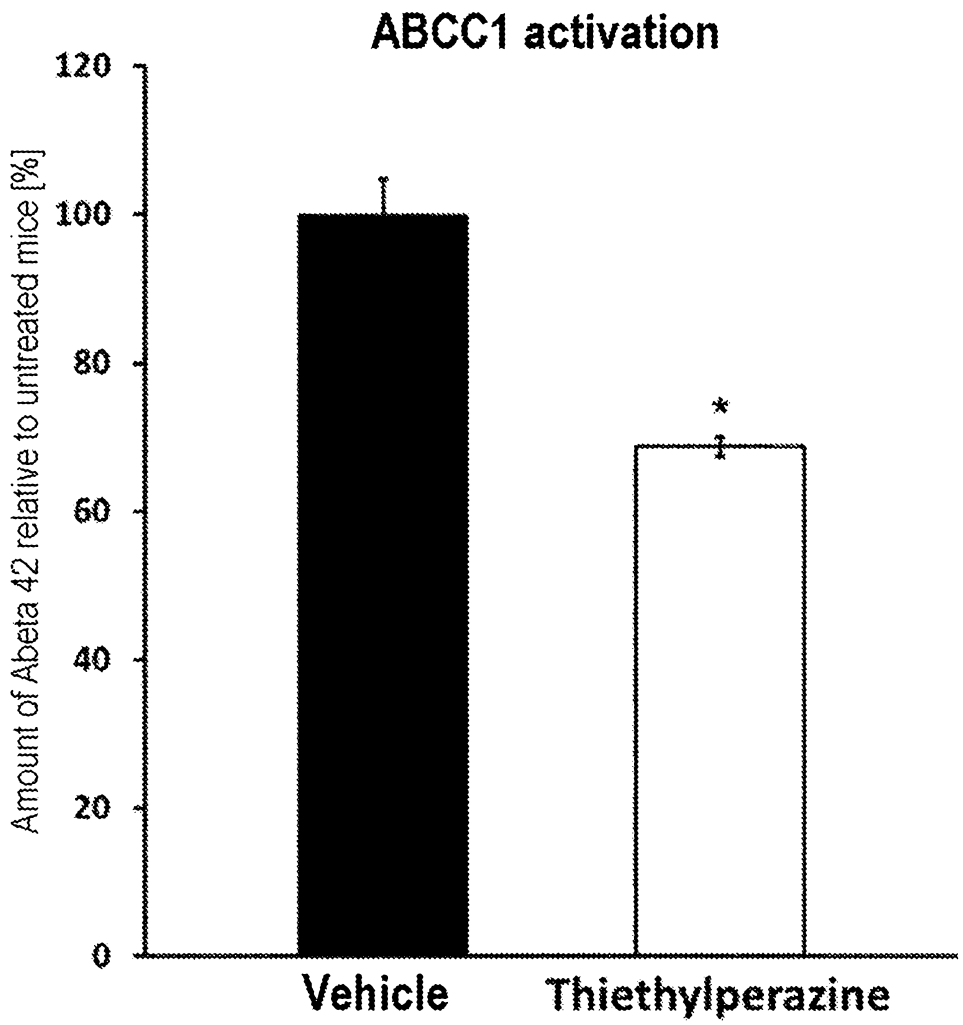


Fig.3e


ABCC1 activation
Amount of Abeta 42 relative to untreated mice [%]
120
100
80
60
40
20
0
*
Vehicle
Thiethylperazine