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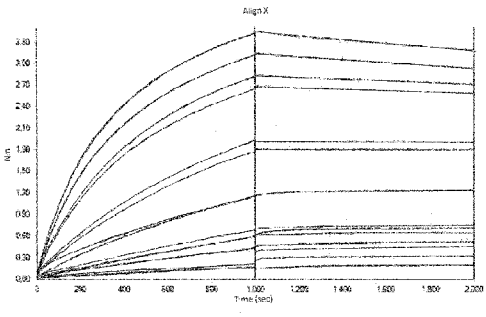
(54) Title: IMMUNOLOGICAL TREATMENT OF CEREBRAL AUTOSOMAL DOMINANT ARTERIOPATHY WITH SUBCORTICAL INFARCTS AND LEUKOENCEPHALOPATHY



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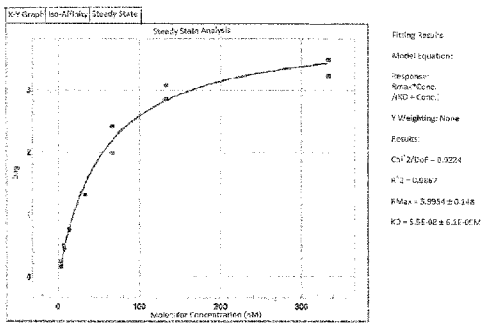

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Figure 1

(57) Abstract: The present invention relates to an anti-Notch 3 antibody therapy useful for treatment of patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. In particular, the invention relates to an anti-Notch3 antibody or a fragment thereof having a 2 fold, 4 fold or 10 fold higher affinity to Notch 3 than to Notch 1 or Notch 2 for use in therapy.

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Immunological treatment of cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

Field of the invention

The present invention relates to an anti-Notch 3 antibody therapy useful for treatment of patients with cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy.

Background of the invention

The Notch gene was first described in 1917 when a strain of the fruit fly Drosophila melanogaster was found to have notched wing blades (Morgan, Am Nat 51:513 (1917)). The gene was cloned some seventy years later and turned out to be a cell surface receptor playing a key role in the development of many different cell types and tissues (Wharton et al., Cell 43:567-581 (1985)). Since then, the gene and its molecular mechanisms have been extensively studied. The generality of the Notch pathway manifests itself at different levels.

The Notch signaling pathway was soon found to be an evolutionarily conserved signaling mechanism from Drosophila to vertebrates and has been found to be involved in many cellular processes, such as differentiation, cell fate decisions, maintenance of stem cells, proliferation, and apoptosis, in various cell types during and after development (See review Artavanis, et al., Science 268:225 (1995)). Knockout mutations were found to be lethal in embryonic mice (Krebs et al. Genes & Dev 14(11):1343-52 (2000). The expression of mutant forms of Notch in developing Xenopus embryos interfere profoundly with normal development (Coffman, et al., Cell 73 (1993)). In humans, there have been several genetic diseases linked to Notch mutations (Artavanis-Tsakonas, et al. Science 284:770-776 (1999)).

Mammals possess four Notch proteins (designated Notch 1 to 4) and five corresponding ligands (Delta- 1, -3, and -4, and Jagged- 1 and -2). The mammalian Notch gene encodes a ~300kd protein that is cleaved during its transport to the cell surface and consequently exists as a heterodimer (NotchECD-

NotchTMIC). The extracellular portion has many epidermal growth factor (EGF)-like repeats followed by three cysteine-rich Notch/Linl2 repeats (LN) (Wharton, et al, Cell 43:567 (1985); Kidd, et al, MoI Cell Biol 6:3431 (1986); Kopczynski, et al, Genes Dev 2:1723 (1988); Yochem, et al, Nature 335:547 (1988)). The amino-terminal EGF-like repeats participate in ligand binding, whereas the C-terminal part of the extracellular portion, including the Lin 12 repeats,prevent signaling in the absence of ligand. The signal induced by ligand binding is transmitted to the nucleus by a process involving proteolytic cleavage of the receptor and nuclear translocation of the intracellular domain (Notch-IC). After entering the nucleus, Notch- IC competes with inhibitory proteins and recruits coactivators, including mastermind-like (MAML) proteins, and acetyltransferases. The Notch-IC complex then binds to a transcription factor RBP-J to convert it from a transcriptional repressor to an activator. The few transcriptional factors identified so far vary in their nature and effects on the cell.

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) causes a type of stroke and dementia whose key features include recurrent subcortical ischaemic events and vascular dementia. Prominent manifestations on neuroimaging include extensive white matter lesions, lacunes and progressive brain atrophy. The disease is caused by highly stereotyped mutations that alter the number of cysteine residues in the extracellular domain of NOTCH3 (Joutel, A et al. 1997. The Lancet 350(9090):1511-5) which is predominantly expressed in vascular smooth muscle cells and pericytes of brain capillaries (Joutel, A et al. 2000. J Clin Invest 105 (5): 597–605). The two hallmark pathologies are extracellular NOTCH3 and GOM deposits, characteristically seen in close vicinity to the plasma membrane of vascular smooth muscle cells in the brain and peripheral vessels as well as of pericytes in the brain capillaries. NOTCH3 deposits are aggregates of the extracellular domain of NOTCH3 (Notch3ECD) and GOM are proteinaceous aggregates, with Notch3ECD being one important constituent (Joutel, A et al . 2000. J Clin Invest 105 (5): 597–605) (Ishiko et al. 2006 Acta Neurophatol 112(3):333-39) (Monet-Leprêtre et al. 2013. Brain: A journal of Neurology 136

(pt 6): 1830-45). Yet, today there are no therapies to prevent or halt the progression of the disease manifestations.

The inventors of the present invention have surprisingly found that anti-Notch 3 antibodies may be used in the treatment of CADASIL patients.

Summary of the invention

The invention relates to notch3 antibodies or fragments thereof. These antibodies are characterised by *inter alia* having a 2 fold, 4 fold or 10 fold higher affinity to Notch 3 than Notch 1 or Notch 2. In particular the antibodies are able to bind the Notch3ECD deposits, and an epitope comprised in amino acids 40 – 1643 of human Notch3 (SEQ ID NO: 3).

In a specific embodiment the CDR regions of the antibody comprises
a heavy chain variable region H-CDR1 comprising SEQ ID NO: 8
a heavy chain variable region H-CDR2 comprising SEQ ID NO: 9
a heavy chain variable region H-CDR3 comprising SEQ ID NO: 10
a light chain variable region L-CDR1 comprising SEQ ID NO:12
a light chain variable region L-CDR2 comprising SEQ ID NO: 13 and
a light chain variable region L-CDR3 comprising SEQ ID NO:14

The invention also relates to a vaccine comprising amino acids 40 – 1643 of human Notch3 (SEQ ID NO: 3) or a fragment thereof or amino acids 657-846 of human Notch3 (SEQ ID NO 7) or a fragment thereof. Such fragments comprise an epitope of Notch3.

Both the antibodies and the vaccines of the invention are useful in therapy and more particularly in treating CADASIL patients.

Brief description of drawings

Figure 1 shows 5E1 binding to human NOTCH3 using OCTET analysis. Kinetic analysis was performed using the Octet RED technology from ForteBio according to the manufactures instruction. Biotinylated NOTCH3 657-848 was immobilized on streptavidin tips at a level of approximately 3 RU each. Subsequent association and dissociation of 5E1 antibody was analyzed at concentrations in the range from 333 nM to 3,3 nM. Data analysis was subsequently done using the ForteBio Data Analysis 7.0 software (Example 3).

Figure 2 shows 5E1 enters the CNS and binds to vascular Notch3ECD deposits in old mice. Old Tg PAC-Notch3R169C mice (10 -12 months old) received a single 10 mg/kg intraperitoneal injection of Alexa 488-conjugated 5E1 (A-I) or 5C9E7 control antibody (J-O) (Example 8). The antibody in vivo binding to Notch3ECD deposits was assessed 3 days after injection on cryosections of brain (A-F; J-L) and kidney (G-I; M-O) immunolabeled with exogenous anti-Notch3ECD-antibody and detected with Alexa 594 secondary antibodies. Shown is the fluorescence emission by the Alexa 488 (injected antibody) (left column), the Alexa 594 (exogenous antibody) (middle column) and the merged images (right column). 5E1 binds to Notch3ECD deposits in brain arteries (A-C), brain capillaries (D-F) and kidney arteries (G-I). Although images displayed on G and M have been acquired using a four times shorter exposure, the kidney artery displays a brighter signal than the brain vessels. (*scale bar* represents 95 μm except in panels D-F where it represents 60 μm).

Figure 2bis shows 5E1 enters the CNS and binds to vascular Notch3ECD deposits in young mice. Young Tg PAC-Notch3R169C mice (2 months old) received a single 10 mg/Kg intraperitoneal injection of unconjugated 5E1 antibody (Example 9). The antibody in vivo target engagement was assessed 3 days after injection on brain sections coimmunolabeled with Alexa 594 conjugated antibody against mouse immunoglobulin (A, detection of 5E1 binding), and Alexa488 conjugated 5E1 (B, detection of total Notch3ECD deposits). The merged image (C) indicates

that the target engagement is almost complete. (*scale bar* represents 76μm). (Example 9).

Figure 3 shows 5E1 treatment does not prevent GOM deposits.

Two months old Tg PAC-Notch3R169C mice were treated for 4 months with weekly intraperitoneal injections of 10 mg/kg of 5E1 (6 mo-5E1, n=5 males) or control IgG1 (6 mo-IgG1, n=5 males) antibodies. A-B Representative electron micrographs of the middle cerebral artery of 6 month old Tg PAC-Notch3R169C mice treated with IgG1 (A) or 5E1 (B). GOM deposits are identified by red arrowheads. C- Diagram showing the number of GOM deposits in 6 month old treated Tg PAC-Notch3R169C mice showing that the 5E1 treatment does not prevent the appearance of GOM deposits. Scale bar is 5 μm. (Examples 10 and 11).

Figure 4 shows that 5E1 chronic treatment protects from cerebrovascular dysfunction.

Two months old Tg PAC-Notch3R169C mice were treated for 4 months with weekly intraperitoneal injections of 10 mg/kg of 5E1 (TgN3R169C-5E1, n=5 males) or control IgG1 (TgN3R169C-IgG1, n=6 males) antibodies and analyzed at 6 months of age for cerebral blood flow (CBF) responses by laser Doppler flowmetry. An additional group of untreated wildtype mice (WT, n= 5 males) aged 6 months was tested in parallel. 5E1 treatment significantly improves attenuation in the increased CBF produced by endothelium vasodilators Acetylcholine, Calcium ionophore A23187 and Bradykinin (A), smooth muscle relaxant adenosine (B) and neural activity (whisker stimulation) (C). (***$p < 0.001$, TgN3R169C-5E1 compared to TgN3R169C-IgG1 or WT mice). Data are expressed as means ± SEM and were analyzed by one-way ANOVA followed by Bonferroni post hoc test. (Examples 10 and 12).

Figure 4 bis shows that 5E1 chronic treatment protects against inward remodeling and reduced myogenic tone of brain arteries.

Two months old TgPAC-Notch3R169C mice were treated for 4 months with weekly intraperitoneal injections of 10 mg/kg of 5E1 (TgN3R169C-5E1, n=7 males) or control IgG1 (TgN3R169C-IgG1, n=7 males) antibodies and analyzed at 6 months of age for passive diameter (inward remodeling) and active diameter (myogenic tone) of pressurized posterior cerebral arteries using an arteriograph system. An additional group of untreated wild-type mice (WT, n= 7 males) aged 6 months was tested in parallel. 5E1 treatment significantly improves reduction in the passive diameter (A) and normalizes the myogenic responses (B). (*$p < 0.05$, ***$p < 0.001$, TgN3R169C-5E1 compared to TgN3R169C-IgG1 mice). Data are expressed as means ± SEM and were analyzed by two-way repeated measures ANOVA followed by Bonferroni post hoc test. (Examples 10 and 13).

Figure 5 shows a schematic presentation of human NOTCH3. Amino acids 1-39 constitute the signal peptide, amino acids 40 – 1643 constitute the extra cellular domain, amino acids 1644 – 1664 constitute the transmembrane domain, and amino acids 1665 – 2321 constitute the Notch 3 intracellular domain. Amino acids 1571-1572 is the cleavage site by furine like protease and amino acids 40-1571 constitutes the Notch3ECD (which abnormally accumulates in CADASIL).

Figure 6 shows determination of antibody plasma kinetics (Example 7). $T_{1/2}$ was determined to be 6-7 days and C_{max} (4 hours) was about 500ug/ml. Quantitation of free plasma mAb level was performed using 5E1 as standard and Notch domain coated plates.

Detailed description of the invention

Definitions

The term "Notch3 "is synonym to the Notch 3 polypeptide and refers to a polypeptide with the amino acids sequence given in SEQ ID NO: 1 (UniProt number Q9UM47), unless another meaning is clear from the context it is given. In SEQ ID NO: 1 amino acids 1-39 constitute the signal peptide (SEQ ID NO: 2), amino acids 40 – 1643 constitute the extra cellular domain (SEQ ID NO: 3),

amino acids 1644 – 1664 constitute the transmembrane domain (SEQ ID NO: 4), and amino acids 1665 – 2321 constitute the Notch 3 intracellular domain (SEQ ID NO: 5). Amino acids 1571-1572 is the cleavage site by furine like protease and amino acids 40-1571 constitutes the Notch3ECD (SEQ ID NO: 6) (which abnormally accumulates in CADASIL).

The term "antibody" refers to an intact immunoglobulin or a functional fragment thereof. The term "isolated antibody" means throughout this specification an immunoglobulin antibody that exists in a physical milieu distinct from that in which it may occur in nature, and differs in chemical formula or sequence from a naturally-occurring protein. The term isolated antibody thus does not include non-isolated antibodies, such as polyclonal antibodies that are naturally occurring, but does include monoclonal antibodies (mAbs) as well as isolated antibody-like polypeptides, chimeric antibodies, humanized antibodies and fragments of isolated antibodies that possess the ability to bind an epitope.

Naturally occurring antibodies typically comprise a tetramer which is usually composed of at least two heavy (H) chains and at least two light (L) chains. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region, usually comprised of three domains (CH1, CH2 ad CH3). Heavy chains can be of any isotype, including IgG (IgG1, IgG2, IgG3 and IgG4 subtypes), IgA (IgA1 and IgA2 subtypes), IgM and IgE. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region (CL). Light chain includes kappa chains and lambda chains. The heavy and light chain variable region is typically responsible for antigen recognition, whilst the heavy and light chain constant region may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g. , effector cells) and the first component (Clq) of the classical complement system. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs arranged from amino-terminus to carboxy-

terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains

The terms "monoclonal antibody" or "mAb" as used herein refer to a preparation of isolated antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope. Accordingly, the term "human monoclonal antibody" refers to antibodies displaying a single binding specificity which have variable and constant regions derived from human germline immunoglobulin sequences. The human monoclonal antibodies may be produced by a hybridoma which includes a B cell obtained from a transgenic or transchromosomal non-human animal, such as a transgenic mouse, having a genome comprising a human heavy chain transgene and a light chain transgene, fused to an immortalized cell.

As used herein, "isotype" refers to the immunoglobulin class (for instance IgG1, IgG2, IgG3, IgG4, IgD, IgA, IgE, or IgM) that is encoded by heavy chain constant region genes.

The term "humanized antibody" as used herein, is intended to include isolated antibodies having variable and constant regions derived from human germline immunoglobulin sequences. The antibody or fragment thereof may be fully or partially humanized by methods known by the skilled artesian whereby, for example, the CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

As indicated herein-above, the term antibody herein may include fragments of an antibody that retain the ability to specifically bind to the antigen. It has been shown that the antigen-binding function of an antibody may be performed by fragments of a full-length antibody, e.g., Fab and $F(ab')_2$ fragments.

Antibody fragments can be obtained by conventional techniques, such as by fragmentation of full-length antibodies or by expression of nucleic acids encoding antibody fragments in recombinant cells (see, for instance Evans et al., J. Immunol. Meth. 184, 123-38 (1995)). The fragments can then be tested or

screened for their properties in the same manner as described herein for full-length antibodies. Examples of antibody fragments includes for examples the below:

$F(ab')_2$ fragments, which are bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region. These can be generated by, e.g., treating a full-length antibody with pepsin.

Fab' or Fab fragments, which are monovalent fragments consisting of the V_L, V_H, C_L and C_H1 domains. Fab fragments can be obtained, e.g., by treating an IgG antibody with papain. Fab' fragments can be obtained, e.g., by reducing the disulfide bridges of a $F(ab')_2$ fragment using a reducing agent such as dithiothreitol.

Fv fragments, which consist essentially of the V_L and V_H domains of a single arm of an antibody and single-chain antibodies thereof. Single-chain antibodies (also known as single chain Fv (scFv) antibodies) are constructs where the V_L and V_H domains of an Fv fragment are joined, using recombinant methods, by a synthetic linker that enables them to be expressed as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (see for instance Bird *et al.*, Science 242, 423-426 (1988) and Huston *et al.*, PNAS USA 85, 5879-5883 (1988)).

Domain antibodies (also called dAb fragments), which consists essentially of a V_H domain (see, e.g., Ward *et al.*, Nature 341, 544-546 (1989); Holt *et al*; Trends Biotechnol. 2003 Nov;21(11):484-90).

Other exemplary formats include camelids or nanobodies (see, e.g., Revets *et al*; Expert Opin Biol Ther. 2005 Jan;5(1):111-24

The term "epitope" means a portion of an antigen (*e.g.*, a protein determinant of an antigen) that is capable of specific binding by an antibody. The epitope may comprise amino acid residues which are directly involved in the binding, and other amino acid residues, which are not directly involved in the binding, such as amino acid residues which are effectively blocked or covered by the specific antigen binding peptide (in other words, the amino acid residue is within the footprint of the specific antigen binding peptide).

As used herein, the term "binding" in the context of the binding of an antibody to a predetermined antigen or epitope typically is a binding with an affinity corresponding to a K_D of about 10^{-7} M or less, such as about 10^{-8} M or less, such as about 10^{-9} M or less, about 10^{-10} M or less, or about 10^{-11} M or even less when determined by for instance surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using a soluble form of the antigen as the ligand and the antibody as the analyte. Typically, an antibody binds to the predetermined antigen with an affinity corresponding to a K_D that is at least ten-fold lower, such as at least 100-fold lower, for instance at least 1,000-fold lower, such as at least 10,000-fold lower, for instance at least 100,000-fold lower than its K_D for binding to a non-specific antigen (e.g., BSA, casein), which is not identical or closely related to the predetermined antigen. When the K_D of the antibody is very low (that is, the antibody has a high affinity), then the K_D with which it binds the antigen is typically at least 10,000-fold lower than its K_D for a non-specific antigen. An antibody is said to essentially not bind an antigen or epitope if such binding is either not detectable (using, for example, plasmon resonance (SPR) technology in a BIAcore 3000 instrument using a soluble form of the antigen as the ligand and the antibody as the analyte), or is 100 fold, 500 fold, 1000 fold or more than 1000 fold less than the binding detected by that antibody and an antigen or epitope having a different chemical structure or amino acid sequence.

The term "k_d" (sec^{-1}), as used herein, refers to the dissociation rate constant of a particular Ab-antigen interaction ([Ab] [antigen]/[Ab-antigen complex]). Said value is also referred to as the k_{off} value.

The term "k_a" ($M^{-1} \times sec^{-1}$), as used herein, refers to the association rate constant of a particular Ab-antigen interaction and is the reciprocal of the k_d.

The term "K_D" (M), as used herein, refers to the dissociation equilibrium constant of a particular Ab-antigen interaction and is obtained by dividing the k_d by the k_a.

The term "K_A" (M^{-1}), as used herein, refers to the association equilibrium constant of a particular Ab-antigen interaction and is obtained by dividing the k_a by the k_d.

Specific embodiments and aspects of the invention

The present invention relates to anti-Notch3 antibody therapy of CADASIL patients as exemplified by the isolated antibody 5E1 (Examples 1 and 2), which is a murine monoclonal antibody that binds to human Notch3 but neither to Notch1 nor Notch2. The isolated 5E1 antibody additionally has the ability to *i.a.* detect vascular NOTCH3ECD deposits in both human, mouse and rats, and thereby provide an antibody with exceptional good translation into human pathology from animal models. The 5E1 antibody was raised against human Notch3 and recognizes both human and rat NOTCH3 with close binding constants (KD=55 nM) (Figure 1, Example 3).

Peripherally administered anti-Notch3 antibodies acts directly on vascular Notch3ECD deposits and crosses the blood brain barrier prior to reaching the deposits, as shown for 5E1 (Figures 2 and 2 bis, Example 8). In an experiment in old TgPAC-Notch3R169C mice displaying abundant Notch3ECD deposits in both the brain vessels and peripheral arteries, ten to twelve months old TgPAC-Notch3R169C and control mice received a single injection of Alexa 488-conjugated anti-Notch3 antibody (5E1) or 5C9E7 control antibody (10mg/kg). Mice were subsequently sacrificed 3 days later and cryostat sections of the brain and kidney were examined by direct fluorescence. Brain vessels (including arteries and capillaries) of the anti-Notch3 antibody treated TgPAC-Notch3R169C mice were labelled whereas vessels of the 5C9E7-treated group were unstained (Figure 2 A, D and J). Notably, in the anti-Notch3 antibody treated TgPAC-Notch3R169C mice, peripheral arteries were far more strongly stained than the brain vessels (Figure 2, G compared to A and D). Co-immunolabelling of the sections with an anti-Notch3ECD antibody indicated that the 5E1 antibody had bound almost all Notch3ECD deposits in mutant mice in both the brain and kidney vessels (Figure 2 B,C, E,F, H,I). Thus the data indicated that anti-Notch3 antibodies can bind to vascular Notch3ECD deposits in vivo and that a small fraction can enter the brain to act directly on Notch3ECD deposits in the brain vasculature.

Old TgPAC-Notch3R169C mice might exhibit subtle blood brain barrier defects. This may favour the diffusion of antibodies into the brain. To investigate this the inventors of the present invention conducted experiments in younger TgPAC-Notch3R169C mice (Example 9). Two months old TgPAC-Notch3R169C mice received a single intraperitoneal injection of unconjugated an anti-Notch3 antibody (5E1) or control IgG1 (10 mg/kg) and were subsequently sacrificed 3 days later to study the target engagement. Immunofluorescence analysis of brain cryosections with an anti-mouse antibody revealed that the anti-Notch3 antibody robustly engaged Notch3ECD aggregates in the brain vessels (Figure 2bis, A). Moreover, coimmunolabeling with Alexa 488-conjugated anti-Notch3 antibody (5E1) was done to determine the total amount of Notch3ECD deposits and indicated that the percentage of target engagement was close to 100% (Figure 2bis–B,C). Hence, it can be concluded that anti-Notch 3 antibodies are suitable for a vaccinotherapy approach.

The observation that anti-Notch 3 antibodies binds to Notch3ECD deposits indicates a promising therapeutic application in CADASIL patients because it could also be efficacious at preventing or lowering vascular Notch3ECD or GOM deposits.

Brain vessels consist of a network of small arteries which travel on the brain surface (pial arteries), branch extensively into smaller arteries that penetrate into the brain parenchyma (penetrating arteries) and then terminate as an extensive capillary network (capillaries). In TgPAC-Notch3R169C mice, Notch3ECD aggregates are detectable in the pial arteries from birth, then aggregates extend gradually to the small penetrating arteries and the capillaries between 2 and 6 months of age. GOM deposits, which are scattered at 1 month, become readily detectable by 6 months of age in the pial arteries(Joutel A, Monet-Leprêtre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, Lemaire-Carrette B, Domenga V, Schedl A, Lacombe P, Hubner N. 2010. “Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease.”J Clin Invest 120(2):433-45). . Therefore, the inventors initiated a study, in which TgPAC-

Notch3R169C mice were treated from 2 to 6 months (Example 10), in order to explore the attenuation of preexisting NOTCH3 deposits in pial arteries and prevention of new NOTCH3ECD deposits in the intraparenchymal vessels as well as prevention of GOM deposits. On the basis of the results of the pharmacokinetics study showing that anti-Notch3 antibodies (as exemplified with 5E1) had a plasma half-life of 6-7 days (figure 6 and Example 7), mice were treated with weekly injections of anti-Notch3 antibodies (5E1) or control IgG1 at 10mg/kg. A group of TgPAC-Notch3R169C mice were sacrificed at 2 months of age (time zero) to determine the extent of existing NOTCH3ECD and GOM deposits prior to treatment. Brain sections were processed for quantitative immunohistochemical analyses of Notch3ECD aggregates, using an exogenous polyclonal antibody against the N-ter of NOTCH3, a domain which is not recognized by 5E1, or using a polyclonal antibody against the EGFR17-21 of rat NOTCH3 (Example 11). A comparison of the time zero (2 months) versus the control IgG1 (6 months) animals showed an age-dependent accumulation of Notch3ECD aggregates that increase in the pial and capillaries. Brain tissue was also processed for quantitative electron microscopy analyses of GOM deposits on ultrathin sections of the middle cerebral artery (Example 11). Importantly, quantification of GOM deposits revealed no significant difference between the anti-Notch3 antibodies and control antibodies treated mice (n=5 males per group) (Figure 3).

It was however observed that anti-Notch3 antibody treatment protects against in vivo cerebrovascular dysfunction, and thus provide a beneficial treatment option for CADASIL patients.

Using an open cranial window on the somatosensory cortex of anesthetized mice and laser Doppler flowmetry to measure cerebral blood flow (CBF), the CBF increase evoked by neocortical application of both the endothelium-dependent vasodilators Ach, bradykinin and the Ca2+ ionophore A23187, as well as responses to the smooth muscle relaxant adenosine were strongly reduced in TgPAC-Notch3R169C mice aged 6 months compared to both aged matched TgPAC-Notch3WT mice, which overexpress a similar amount of

wiltype Notch3, and non-transgenic littermates suggesting that smooth muscle reactivity was impaired in mutant mice (Example 10 and 12). It was also found that CBF increase produced by facial whiskers stimulation (functional hyperemia) was strongly attenuated in TgPAC-Notch3R169C mice at 6 months of age compared to control mice (Joutel A, Monet-Leprêtre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, Lemaire-Carrette B, Domenga V, Schedl A, Lacombe P, Hubner N. 2010. "Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease."J Clin Invest 120(2):433-45). Accordingly, this paradigm was a promising a mean to investigate whether or not anti-Notch3 antibody treatment could rescue the cerebrovascular dysfunction despite the lack of any detectable change in the load of Notch3ECD deposits.

First a parallel cohort of mutant and wildtype untreated mice at two months of age, ie time zero was assessed. It was found that at 2 months of age, functional hyperemia as well as the responses to both endothelium-dependent vasodilators and the smooth muscle vasorelaxant did not differ between TgPAC-Notch3R169C and the wildtype littermates (n=5 mice per group) Next TgPAC-Notch3R169C mice treated with weekly injections of anti-Notch3 antibodies (5E1) or control IgG1 at 10mg/kg from 2 months to 6 months of age was examined. Anti-Notch3 antibody treated TgPAC-Notch3R169C mice had significantly improved CBF responses evoked by topical neocortical application of endothelium-dependent vasodilators (Figure 4A) or smooth muscle relaxant adenosine (Figure 4B) that were not significantly different from untreated 6 mo WT mice but that were significantly different from IgG1-treated TgPAC-Notch3R169C mice. Moreover, functional hyperemia in anti-Notch3 antibody treated TgPAC-Notch3R169C mice was also markedly improved (n= 5-6 males per group) (Figure 4C). Therefore, these data shows that passive immunization with an anti-Notch3 antibody, such as 5E1, can restore CBF responses in TgPAC-Notch3R169C mice to wild-type levels.

Using an arteriograph system to measure active and passive diameter of pressurized posterior cerebral arteries, we previously reported that pressure-

induced constriction (myogenic tone) of brain arteries was markedly attenuated in TgPAC-Notch3R169C mice aged 6 months compared to both aged matched wildtype and TgPAC-Notch3WT mice. It was also found that the passive diameter of posterior cerebral arteries was markedly reduced in TgPAC-Notch3R169C mice aged 6 months compared to control mice whereas the wall thickness was unchanged indicative of an inward remodeling in these mice (Joutel A, Monet-Leprêtre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, Lemaire-Carrette B, Domenga V, Schedl A, Lacombe P, Hubner N. 2010. "Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease."J Clin Invest 120(2):433-45). Accordingly, this paradigm was a promising mean to investigate whether or not anti-Notch3 antibody treatment acts at the level of brain arteries to rescue the altered myogenic tone and the inward remodeling.

TgPAC-Notch3R169C mice treated with weekly injections of anti-Notch3 antibodies (5E1) or control IgG1 at 10mg/kg from 2 months to 6 months of age were examined. An additional group of untreated wild-type mice aged 6 months was tested in parallel. Anti-Notch3 antibody treated TgPAC-Notch3R169C mice had significantly improved passive diameters that were not significantly different from untreated 6 mo WT mice but that were significantly different from IgG1-treated TgPAC-Notch3R169C mice (Figure 4bis A). Moreover, anti-Notch3 antibody treated TgPAC-Notch3R169C mice had normalized myogenic responses that were comparable to untreated 6 mo WT mice (Figure 4bis B). Therefore, these data show that passive immunization with an anti-Notch3 antibody, such as 5E1, acts at the level of brain arteries to protect against inward remodeling and reduced myogenic tone.

Thus, without being bound to an underlying hypothesis the inventors of the present invention have shown that anti-Notch3 antibodies binding to the ectodomain domain of Notch3 (Notch3ECD) provide a disease modifying therapy that may help patients suffering from CADASIL.

As outlined in the background section, Notch 3 belongs to a larger receptor family that also includes Notch 1, Notch 2 and Notch 4.

By overexpressing Notch 1, Notch 2, Notch 3 and Notch 4 different biological responses has been observed. These effects include cell proliferation, apoptosis, and inflammatory and profibrotic responses, depending on the particular Notch receptor. For example Notch 1, Notch 2 and Notch 4 have been observed in glomerular and tubular cells in human and experimental kidney disease (Shanzes-Nino et al., J Pathol 2012:228:266-273). To avoid any side effects with a therapeutic antibody, such as 5E1, it desirable to have as a specific antibody to its target as possible.

To avoid these side effects, the invention therefore relates to an anti-Notch 3 antibody or a fragment thereof that essentially does not bind to Notch 1 or Notch 2, and more preferably that also essentially does not bind to other related Notch receptors such as Notch 4 (Example 2). Antibody 5E1 is illustrative of the invention in having these properties and is very specific for Notch 3. 5E1 binds neither to human Notch1 nor human Notch2 (Joutel, A et al. 2000. J Clin Invest 105 (5): 597–605).

In one embodiment the invention relates to a therapeutic Notch3 antibody or a fragment thereof having a 2 fold, 4 fold or 10 fold higher affinity to Notch 3 than Notch 1 or Notch 2. In another embodiment the Notch 3 antibody or fragment essentially does not bind Notch 1 or 2. In yet a further embodiment the Notch 3 antibody or fragment essentially does not bind Notch 1, 2 or 4.

Binding can be determined by for example surface plasmon resonance (SPR) technology in a BIAcore 3000 instrument using a soluble form of the antigen as the ligand and the antibody as the analyte.

In one embodiment the Notch 3 antibody or fragment is binding to an epitope comprising amino acids 40 – 1643 (SEQ ID NO 3) of human Notch3, such as an epitope comprised in amino acids 657-846 (SEQ ID NO 7) of human Notch3.

The invention relates to an antibody or fragment thereof for use in therapy. Thus, the invention relates to *i.a.* a method for treating a patient suffering from

CADASIL, or at risk thereof, wherein an antibody that binds to Notch3ECD is administered to such patient in an amount effective to treat such condition or risk, or wherein a fragment of Notch3 is administered to such patient in and amount and formulation sufficient to comprise a vaccine that elicits the production of antibodies that bind to Notch3ECD. Such a fragment may be or comprise a fragment of amino acids 40 – 1643 (SEQ ID NO 3), such as a fragment that is or comprises amino acids 657-846 (SEQ ID NO 7) of human Notch3.

In particular, the invention relates to an antibody or fragment thereof for use in therapy, wherein the antibody or fragment comprises:

a heavy chain variable region H-CDR1 comprising SEQ ID NO: 8

a heavy chain variable region H-CDR2 comprising SEQ ID NO: 9

a heavy chain variable region H-CDR3 comprising SEQ ID NO: 10

a light chain variable region L-CDR1 comprising SEQ ID NO: 12

a light chain variable region L-CDR2 comprising SEQ ID NO: 13 and

a light chain variable region L-CDR3 comprising SEQ ID NO: 14

In another embodiment the antibody or fragment thereof comprises a heavy chain variable region comprising SEQ ID NO: 11 and/or comprises a light chain variable domain comprising SEQ ID NO: 15

The antibody or fragment thereof may be fully or partially humanized by methods known by the skilled artesian whereby, for example, the CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The antibody or fragment thereof are useful in therapy and in particular in CADASIL patients.

It's also envisaged that amino acids 40 – 1643 (SEQ ID NO 3) of human Notch3, such as an amino acids 657-846 (SEQ ID NO 7) of human Notch3, may be used in an active vaccine strategy in therapy and to treat CADASIL. Fragments in the size of e.g. 4, amino acids, 5 amino acids, 7 amino acids 10 amino acids or larger or for example in a range of 5-10, 5-15, or 5-20 amino acids of said sequences 40 – 1643 or 657-846 of human Notch3 may be used to vaccinate

patients alone or in combination with an appropriate adjuvant such as e.g. such as aluminum hydroxide or phosphate.

Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from about 0.1 pg to 2,000 ug (even though higher amounts in the 1- 10 mg range are contemplated), such as in the range from about 0.5 ug to 1,000 ug, preferably in the range from 1 ug to 500 ug and especially in the range from about 10 ug to 100 ug. Suitable regimens for initial administration and booster shots are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable. These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated and the formulation of the antigen.

It also envisaged that the treatment may also comprise a step of diagnosing the progress of the disease therapy by measuring the amount of deposits using an anti-Notch 3 antibody labelled by an appropriate label. The choice of label depends on the means of detection. For example, a fluorescent label, such as a rare earth chelate (*e.g.*, a europium chelate), a fluorescein type label (*e.g.*, fluorescein, fluorescein isothiocyanate, 5-carboxyfluorescein, 6-carboxy fluorescein, dichlorotriazinylamine fluorescein), a rhodamine type label (*e.g.*, TAMRA or dansyl chloride), phycoerythrin; umbelliferone, Lissamine; cyanines; phycoerythrins, Texas Red, BODIPY-FL-SE, or an analog thereof, is suitable for optical detection. Chemoluminescent labels may be employed (*e.g.*, luminol, luciferase, luciferin, and aequorin). Paramagnetic labels and radioactive labels can also be employed, and are preferably detected using PET or SPECT. Such radioactive materials include, but are not limited to, bismuth (^{213}Bi), carbon (^{14}C), chromium (^{51}Cr), cobalt (^{57}Co), fluorine (^{18}F), gadolinium (^{153}Gd, ^{159}Gd), gallium (^{68}Ga, ^{67}Ga), germanium (^{68}Ge), holmium (^{166}Ho), indium (^{115}In, ^{113}In, ^{112}In, ^{111}In),

iodine (^{131}I, ^{125}I, ^{123}I, ^{121}I), lanthanium (^{140}La), lutetium (^{177}Lu), manganese (^{54}Mn), molybdenum (^{99}Mo), palladium (^{103}Pd), phosphorous (^{32}P), praseodymium (^{142}Pr), promethium (^{149}Pm), rhenium (^{186}Re, ^{188}Re), rhodium (^{105}Rh), ruthemium (^{97}Ru), samarium (^{153}Sm), scandium (^{47}Sc), selenium (^{75}Se), strontium (^{85}Sr), sulfur (^{35}S), technetium (^{99}Tc), thallium (^{201}Ti), tin (^{113}Sn, ^{117}Sn), tritium (^{3}H), xenon (^{133}Xe), ytterbium (^{169}Yb, ^{175}Yb), yttrium (^{90}Y), zinc (^{65}Zn); positron emitting metals using various positron emission tomographies, and nonradioactive paramagnetic metal ions.

Example 1

Generation of hybridoma clone 5E1

Human Notch3 cDNA coding for the extracellular epitope (amino acids 657-846) of the human Notch3 protein with a C-terminal stretch of 6 Histidine residues was inserted into the pACGP67B Baculovirus complementing transfer vector (Pharmingen) to be expressed as a gp67 signal peptide fusion protein under the control of the strong Baculovirus polyhedrin promoter. Sf9 cells grown as a monolayer were transfected with the transfer plasmid together with the modified baculovirus DNA (BaculoGold ^{TM} DNA , Pharmingen), which contains a lethal deletion, using the Insectin-Plus ^{TM} reagent (Invitrogen). Recombinant baculoviruses were identified and purified by virus plaque assay and the presence of Notch3 cDNA was confirmed by Southern blot. Recombinant baculoviruses carrying the *Notch3* cDNA were then amplified to obtain a high titer solution (10^8 -10^9 viral particles/ml). To produce the recombinant protein, 5. 10^7 Sf9 cells, grown in T175 culture flasks as a monolayer in Grace's medium with fetal calf serum (20ml) (Biowhittaker, Boehringer), were infected with 1.5 ml of high titer stock and incubated 3 to 4 days at 27°C. The supernatant, containing the recombinant protein, was then harvested, concentrated by ammonium sulfate (60%) precipitation. The pellet was resuspended in 300mM NaCl 50mM NaH_2PO_4 pH 8.0 and dialyzed overnight against the same buffer. The dialyzed supernatant was purified over Ni2+ agarose beads and the recombinant protein was eluted in dialysis buffer containing 250 mM Imidazole. Purity of the protein was checked by SDS-PAGE analysis and coomassie blue staining.

Hybridomas were obtained by fusing myeloma cells with the spleen cells from mice immunized with the recombinant protein. Hybridomas culture supernatant were screened by immunofluorescence and immunoblot, as described below in Example 2, to identify and select only those hybridomas producing antibodies that recognize Notch3 protein products but neither Notch1 nor Notch2 protein products. Positive hybridomas were further cloned generating several clones including one called "5E1".

Example 2

Screening assay for 5E1

293T cells were grown in Nunc® Lab-Tek® II chambered coverglass, 8 wells, and were transiently transfected by calcium phosphate precipitation with plasmids encoding for human full length Notch1, Notch2 or Notch3 cDNA or empty vector plasmids. Two days after transfection, cells were fixed in ethanol and processed for immunofluorescence using 5E1 anti-Notch3, anti-human Notch1, anti-human Notch2 primary monoclonal antibodies and appropriate FITC-conjugated antibodies. 5E1 antibody stained 293T cells transfected with the Notch3 construct but not the cells transfected with Notch1or Notch2 constructs, although the anti-Notch1 and anti-Notch2 antibodies labelled the later cells respectively.

293T cells were cultured in 6-well plates and were transiently transfected by calcium phosphate precipitation with plasmids encoding for human full length Notch1, Notch2 or Notch3 cDNA. Two days after transfection, cells were harvested and lysed in RIPA buffer. Samples were adjusted to 1× SDS-Laemmli buffer, run on a 6% SDS-PAGE gel and were transferred onto nitrocellulose membranes. Immunodetection was performed by sequential incubations with 5E1 antibody or specific antibodies to human Notch1 or Notch2 and horseradish peroxidase–conjugated goat anti-mouse antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA) followed by enhanced chemiluminescence detection (ECL; Pierce Chemical Co., Rockford, Illinois, USA). The 5E1 antibody detected the full-length Notch3 precursor (~280 kDa) as well as the Notch3ECD fragment (~210 kDa) in 293Tcells transfected with the Notch3 construct. Importantly, the 5E1 antibody in 293Tcells transfected with Notch1 or Notch2 constructs detected neither Notch1 nor Notch2 protein products respectively.

Example 3

Determination of 5E1 relative affinity to NOTCH3 using Octet binding system

Kinetic analysis was performed using the Octet RED technology from ForteBio according to the manufactures instruction. Biotinylated NOTCH3 657-848 was immobilized on streptavidin tips at a level of approximately 3 RU each. Subsequent association and dissociation of 5E1 antibody was analyzed at concentrations in the range from 333 nM to 3,3 nM. Data analysis was subsequently done using the ForteBio Data Analysis 7.0 software (Figure 1).

Example 4

Sequencing of 5E1

Total mRNA was extracted from the hybridoma cell pellets using RNeasy Mini Kit and following manufactures protocol (Quiagene Sciences, Valencia, CA)cDNA was created from the RNA by reverse-transcription with an oligo(dT) primer using Superscriptase III kit (Invitrogene, Carlsbad, CA). PCR reactions using variable domain primers to amplify both the VH and VL regions of the monoclonal antibody using several combinations of Ig variable domain primers (High Fidelity PCR systems, Roche)

The VH and VL products were cloned into the Invitrogen sequencing vector pCR2.1 and transformed into TOP10 cells and screened by PCR for positive transformants. Selected colonies were picked and analyzed by DNA sequencing on an ABI3130xl Genetic Analyzer.

VH Amino Acid Sequence:

EIQLQQSGTVLARPGASVKMSCKAS<u>GYTFTSYW</u>MHWVKQRPGQGLE WIGA<u>IYPGNGDT</u>SYNRKFNGKAKLTAVTSTSTAYMEFSSLTNEDSAVY FC<u>TRDYGSSYDYVMDY</u>WGQGTSVTVSS

VL Amino Acid Sequence:

DIQMTQSPSSLSASLGERVSLTCRASQDIGSSLNWLQQEPDGTIKRLIY ATSSLDSGVPKRFSGSRSGSDYSLTISRLESEDFVDYYCLQYISSPLTFG AGTKLELK

The variable domain is highlighted in **BOLD.**

The Complementarity Determining Regions (CDRs) are underlined as determined by the IMGT numbering system (Lefranc, M.-P. et al., Nucleic Acids Research, 27, 209-212 (1999))

Example 5

Mice for use in methods

TgPAC-Notch3^{R169C} (line 88) were maintained at the heterozygous state on a FVB/N background as previously described) (Joutel A, Monet-Leprêtre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, Lemaire-Carrette B, Domenga V, Schedl A, Lacombe P, Hubner N. 2010. "Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease."J Clin Invest 120(2):433-45).. These mice and their wild-type control littermates were bred at the animal facility on the Villemin site of Paris Diderot University (Paris, France). Mice were housed under a normal light/dark cycle (12 h) with standard rodent chow and tap water supplied *ad libitum*. All the experiments described here were conducted in full accordance with the guidelines of our local institutional animal care and use committee (n°9 Lariboisière-Villemin) with every effort to minimize the number of animals used.

Example 6

Injected antibodies used in methods

5E1 monoclonal antibody (isotype IgG1) was raised against a recombinant protein derived from EGFR17-21 (amino acid residues 657-846) of human NOTCH3; it recognizes human, mouse and rat Notch3 (Joutel, A et al . 2000. "The Ectodomain

of the Notch3 Receptor Accumulates within the Cerebrovasculature of CADASIL Patients." J Clin Invest 105 (5): 597–605). The 5C9E7 mouse monoclonal antibody (isotype IgG1) against human pyro-Glu Abeta and the mouse monoclonal antibody (isotype IgG1) against the hen egg white lysozyme were used as control antibodies. All antibodies were dialyzed against PBS.

Example 7

Determination of antibody plasma kinetics

TgPAC-Notch3R169C mice (n=10 males and 10 females; aged 6 weeks, weight range: 16 -26 g) received a single 10mg/kg intra peritoneal injection of 5E1. Mice were randomly assigned to 5 distinct groups (n=2 males and 2 females per group). In group 1, plasma sample was collected at 15 minutes and 24h after the injection; in group 2, plasma was collected 30 minutes and 48h after the injection; in group 3, plasma was collected 1 and 96h after the injection; in group 4, plasma was collected 4h and 1 week after the injection and in group5, plasma was collected 8h and 2 weeks after the injection. Five uninjected TgPAC-Notch3^{R169C} mice (n=5 females; aged 6 weeks, weight range 18-22g) were used as controls. All mice were sacrificed and tissues were harvested immediately after the second blood sampling. Antibody levels in plasma were determined. $T_{1/2}$ was determined to be 6-7 days and Cmax (4 hours) was about 500ug/ml . Quantitation of free plasma mAb level was performed using 5E1 as standard and Notch domain coated plates.

Example 8

Determination of in vivo antibody recognition of Notch3ECD deposits

The 5E1 and control 5C9E7 antibodies were concentrated and conjugated to the Alexa-488 molecule. At 10-12 months of age, TgPAC-Notch3R169C mice exhibit extensive Notch3ECD deposits in both the brain and peripheral vessels. Ten to twelve months-old TgPAC-Notch3R169C or wildtype mice (n= 4 per treatment) were injected intraperitoneally with the 5E1 or control IgG1 antibodies (Alexa conjugated or unconjugated) at 10 mg/ kg of body weight. After 3 days, mice were deeply anesthetized with sodium pentobarbital (80 mg/kg), flush-perfused

transcardially with phosphate buffer phosphate (PB). Brain and peripheral tissues were harvested, frozen in liquid nitrogen and stored at -80°C. Cryostat sections (12 µm) were then analyzed by direct fluorescence or were coimmunolabeled with Alexa 488-conjugated antibodies against murine immunoglobulin (1:500, Life Technology) together with antibodies against the Nter (Ala40-Glu468) of musNotch3 (1:2000 dilution; R&D AF 1308) and detected with secondary anti-goat Alexa 594 (1:500, Life technologies) or antibodies against a recombinant rat NOTCH3 protein (aa 649-859, sequence NP_064472) (1:16,000 dilution) and detected with secondary Alexa 594 anti-rabbit (1:500, Life Technology). Sections were washed, counterstained with DAPI (1:10,000; Sigma-Aldrich) in PBS for 5 minutes at room temperature, mounted in a drop of Dako fluorescence mounting medium and subjected to epifluorescence imaging (Nikon eclipse 80i). Results are shown in Figure 2 and 2bis.

Example 9

Histology in vivo target engagement

The acute target engagement of 5E1 or control egg white IgG1 was evaluated in 2 months old TgPAC-Notch3R169C mice. Mice (n=4 per treatment) were injected intraperitoneally with 10 mg antibody/kg of body weight; 72 hrs. later, mice were deeply anesthetized with sodium pentobarbital (80 mg/kg), flush-perfused transcardially with phosphate buffer phosphate (PB), the brain and kidney were harvested, frozen in liquid nitrogen and stored at -80°C. Acetone fixed cryostat serial sections (12 µm) were stained with an Alexa 594 conjugated anti-mouse antibody to visualize the murine antibody that had engaged the Notch3ECD deposits and coimmunolabeled either with the Alexa 488 conjugated 5E1 or with the antibody against the N-ter of musNotch3 (1:2000 dilution; R&D AF 1308) detected with secondary anti-goat Alexa 488 conjugated IgG , or the antibody against a recombinant rat NOTCH3 protein (aa 649-859, sequence NP_064472) (1:16,000 dilution) and detected with secondary Alexa 488 anti-rabbit (1:500, Life Technology), to determine the total amount of Notch3ECD deposits. Results are shown in Figure 2 and 2bis.

Example 10

Passive immunizations

Experimental mice were divided in two groups. For the first group, two months old TgPAC-Notch3R169C male mice were randomized and dosed weekly intraperitoneally with 10mg/kg of 5E1 or control egg white IgG1 for four months. At the conclusion of dosing, mice (n= 12-14 per treatment) were analysed for in vivo cerebrovascular reactivity or measurement of active and passive diameters of pressurized pial arteries, the brain was subsequently harvested to quantify vascular Notch3ECD and GOM deposits, and the plasma was collected to determine antibody levels. The second group included untreated TgPAC-Notch3R169C and wild type male mice (n=30) analysed at study initiation with a mean age of 2 months to determine the initial load of Notch3ECD and GOM deposits as well as the initial cerebrovascular function, and at study completion with a mean age of 6 months, to follow the extent of cerebrovascular dysfunction. Results shown in Figures 3 and 4.

Example 11

Quantitative analysis of Notch3ECD and GOM deposits

Mice were overdosed with isoflurane, decapitated, and the brain was harvested. For immunodetection of Notch3ECD deposits, half brain was frozen in liquid nitrogen and stored at -80°C. Acetone fixed cryosections (12µm) were stained with (1) goat polyclonal anti-Notch3ECD (1:2000 dilution; R&D AF 1308) or rabbit polyclonal antibody against a recombinant rat NOTCH3 protein (aa 649-859, sequence NP_064472) (1:16,000 dilution) (2) rabbit polyclonal anti-collagen IV (αColIV) (1:250 dilution; Novotec 20411) or rat monoclonal anti-perlecan (1:1000 dilution; clone A7L6, Millipore) and (3) FITC conjugated alpha smooth muscle actin (SMA) (1:2500 dilution, Sigma-Aldrich) followed by appropriate secondary antibodies (anti-goat Alexa 594, anti-rabbit Alexa 594, anti-rabbit Alexa 350 or anti-rat Alexa 350) (1:500, Life technologies). Stained sections were imaged using a Nikon 80i eclipse microscope at 40x magnification setting

and captured using a digital camera (Nikon) and NIS Elements BR v 3.0 sofware (Nikon), with identical settings across compared groups.

For quantitative analysis of Notch3ECD deposits, we used the ImageJ software: the mean parenchyma background intensity value of all images was set to a fixed value, vessel borders were manually delineated and vessel area was then analyzed by the "Analyze Particles" function. Arteries were identified as SMA positively stained vessels, capillaries were defined as collagen IV or perlecan positively stained and SMA negatively stained vessels with a diameter < 10 μm. The mean of 3 non consecutive sections was used to represent a Notch3ECD load for each mouse. Results are expressed as the number of deposits over the vessel area or length. All analysis were performed in a blinded manner.

For detection of GOM deposits, half brain was fixed in CARSON solution. The middle cerebral artery and surrounding brain tissue were dissected under the microscope and embedded in Epon E812 resin as previously described (Joutel A, Monet-Leprêtre M, Gosele C, Baron-Menguy C, Hammes A, Schmidt S, Lemaire-Carrette B, Domenga V, Schedl A, Lacombe P, Hubner N. 2010. "Cerebrovascular dysfunction and microcirculation rarefaction precede white matter lesions in a mouse genetic model of cerebral ischemic small vessel disease."J Clin Invest 120(2):433-45). Semi-thin sections were cut with an ultramicrotome (Leica EM EC7), stained with 1% toluidine blue and screened by light microscopy to select the region containing the middle cerebral artery. Ultrathin sections of regions of interest were cut, mounted on copper grids, contrast stained with uranyl acetate and lead citrate and examined by transmission electron microscopy (Philips CM100). Electron micrograph images were captured using a digital camera at magnification 7,400. For each mouse, an arterial ring (mean diameter 60-80 μm) was rebuild using PhotoShop. Number of GOM deposits was blind counted on the abluminal perimeter of smooth muscle cells. Results are expressed as a number of GOM deposits/ 100 μm and shown on figure 3.

Example 12

In vivo analysis of cerebrovascular reactivity

Mice were anesthetized with isoflurane (maintenance, 2%), mice were then intubated, and artificially ventilated with an O2/ N2 mixture adjusted to provide an arterial PO_2 of 120 to 130 mm Hg and $PC0_2$ of 33 to 36 mmHg. The femoral artery was cannulated for recording of arterial pressure, and blood sampling for blood gazes determination. Rectal temperature was maintained at 37°C, using a thermostatically controlled rectal probe connected to a heating pad. After surgery, isoflurane was gradually discontinued and anesthesia was maintained with urethane (750 mg/kg, i.p.) and α-chloralose (50 mg/kg, i.p.). The level of anesthesia was monitored by testing corneal reflexes and responses to tail pinch. The somatosensory cortex was exposed by drilling a small hole through the parietal bone (2x2 mm) the dura was removed, and the site was superfused with a modified Ringer's solution (37°C; pH 7.3–7.4). Cerebral blood flow (CBF) was monitored in this cranial open window by using a laser Doppler flowmeter probe (Moor Instruments, MBF3-Dual, Axminster, Devon, UK), positioned stereotaxically above the brain surface and connected to a computerized data acquisition system (Powerlab, Chart). The laser Doppler flowmeter probe detects microvascular blood flow in a 1-mm3 tissue volume. The outputs of the flowmeter and blood pressure transducer were connected to a computerized data acquisition system (Powerlab, Chart). Experiments were started 30 ± 5 minutes after the end of surgery and isoflurane discontinuation, when arterial pressure and blood gases were in a steady state. The whiskers, contralateral to the cranial window, were gently stroked for 1 minute with a cotton-tipped applicator at a frequency of 3 to 4Hz and CBF responses to whisker stimulation were recorded. CBF responses to acetylcholine (10 μM; Sigma), bradykinin (50 μM; Sigma), the calcium ionophore A23187 (3 μM; Sigma) and NO-independent vasodilator adenosine (400μM; Sigma) were also tested. CBF was expressed as percent increase relative to the resting level. Results shown in Figure 4.

Example 13

Ex vivo analysis of myogenic tone and arterial remodeling

After overdosing with CO2, mice were decapitated and their brains were harvested. Arterial segments of the posterior cerebral artery were dissected, cannulated on two glass micropipettes in an organ chamber containing physiological salt solution (PSS) maintained at 37°C (pH 7.4), and pressurized using an arteriograph system (Living Systems Instrumentation, Inc., VT, USA). Once prepared, arteries were allowed to stabilize for at least 30 minutes at a pressure of 50 mmHg before initiating basal tone analyses. Myogenic tone was determined by increasing intraluminal pressure in steps of 10 to 100 mmHg using a pressure-servo control pump. Vessel internal diameter was continuously recorded using a CCD camera and edge-detection software (Biopac MP150 and AcqKnowledge software, Biopac Systems Inc, CA, USA). Diameters measured in PSS were considered active diameters. At the end of each experiment, maximal dilation was obtained in nominally Ca2+-free PSS containing EGTA (2-5 mmol/L, Sigma) and sodium nitroprusside (10 μmol/L, Sigma). Pressure steps were repeated to determine the passive diameter of the arteries. Artery diameters are given in micrometers. Myogenic tone was expressed as the percentage of passive diameter ([passive diameter – active diameter]/passive diameter × 100). Results shown in Figure 4bis.

Claims

1. An anti-Notch3 antibody or a fragment thereof having a 2 fold, 4 fold or 10 fold higher affinity to Notch 3 than to Notch 1 or Notch 2 for use in therapy.
2. The antibody or fragment thereof for the use according to claim 1, wherein said antibody or fragment thereof is capable of binding to a Notch3ECD deposit and /or Wild Type Notch3ECD
3. The antibody or fragment thereof according for the use to claim 1 or 2, wherein said antibody or fragment essentially does not bind Notch 1 or Notch 2.
4. The antibody or fragment thereof for the use according to any one of the preceding claims wherein said antibody or fragment binds to an epitope comprising amino acids 40 – 1643 of human Notch3 (SEQ ID NO: 3).
5. The antibody or fragment thereof for the use according to claim 5 wherein said antibody or fragment binds to an epitope comprised in amino acids 657-846 of human Notch3 (SEQ ID NO 7).
6. The antibody or fragment thereof for the use according to claims 1-5, wherein said antibody or fragment thereof is isolated.
7. An antibody or fragment for the use according to claims 1-6 wherein said antibody or fragment thereof comprises at least one of:
   a heavy chain variable region H-CDR1 comprising SEQ ID NO: 8
   a heavy chain variable region H-CDR2 comprising SEQ ID NO: 9
   a heavy chain variable region H-CDR3 comprising SEQ ID NO: 10
   a light chain variable region L-CDR1 comprising SEQ ID NO:12
   a light chain variable region L-CDR2 comprising SEQ ID NO: 13 or
   a light chain variable region L-CDR3 comprising SEQ ID NO:14
8. The antibody or fragment thereof according to claim 9 comprising all of
   a heavy chain variable region H-CDR1 comprising SEQ ID NO: 8
   a heavy chain variable region H-CDR2 comprising SEQ ID NO: 9
   a heavy chain variable region H-CDR3 comprising SEQ ID NO: 10

a light chain variable region L-CDR1 comprising SEQ ID NO:12
a light chain variable region L-CDR2 comprising SEQ ID NO: 13 and
a light chain variable region L-CDR3 comprising SEQ ID NO:14

9. The antibody or fragment thereof for the use according to claims 7 or 8, wherein said antibody or fragment thereof comprises a heavy chain variable region comprising SEQ ID NO: 11.

10. The antibody or fragment thereof for the use according to claims 7 or 8, wherein said antibody or fragment thereof comprises a light chain variable domain comprising SEQ ID NO: 15.

11. The antibody or fragment thereof for the use according to claims 7 or 8, wherein said antibody or fragment thereof comprises both the heavy chain variable domain and light chain variable domain according to claims 12 and 13.

12. The antibody or fragment thereof for the use according to any one of the preceding claims wherein said antibody or fragment thereof is humanized.

13. A vaccine comprising:

(A) amino acids 40 – 1643 of human Notch3 (SEQ ID NO: 3) or a fragment thereof;

(B) amino acids 657-846 of human Notch3 (SEQ ID NO 7) or a fragment thereof;or

(C) (A) and (B);

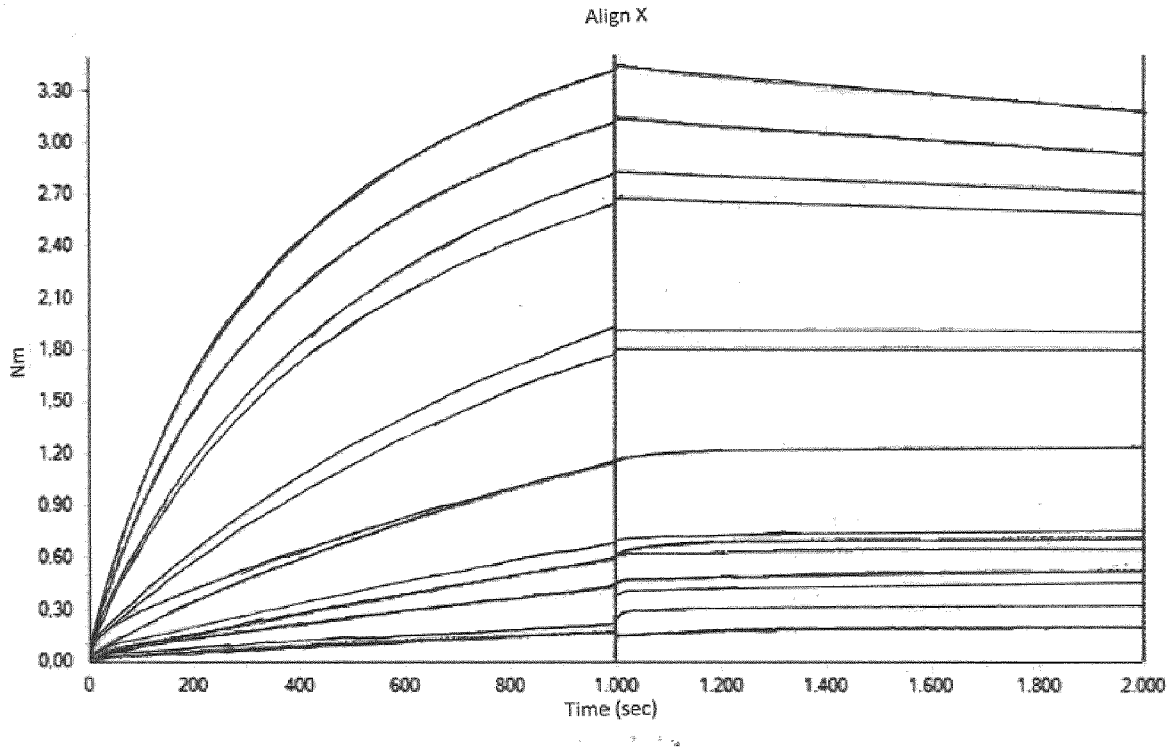
for use in therapy wherein administration of said vaccine to a patient elicits antibody capable of binding a Notch3ECD deposit.

14. A vaccine for the use according to claim 6 said vaccine further comprising an adjuvant.

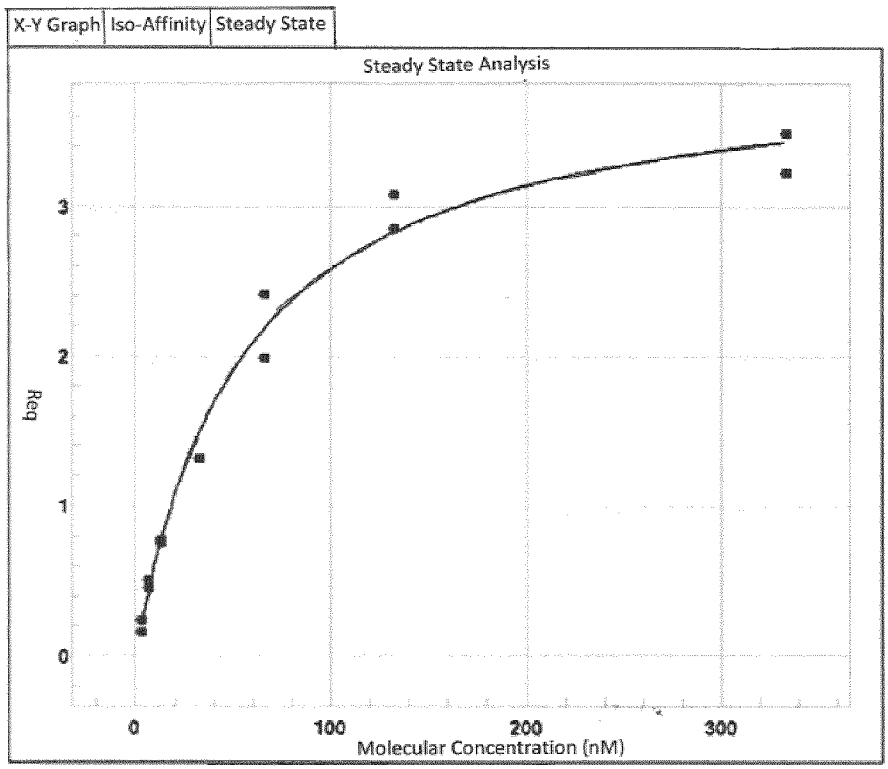
15. The antibody or vaccine according to claims 1-14 for use in treating Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL).

16. The antibody or vaccine for the use according to claim 16, wherein the treatment is chronic.

17. The antibody or vaccine for the use according to claim 17, wherein the chronic treatment is for at least 2 weeks, such as at least for 1 month, 6, months, 1 year or more.
18. Use of an antibody of any one of the claims 1-15 for the manufacture of a medicament for treatment of CADASIL.
19. A method for treating CADASIL by administering, to an individual in need thereof, an effective amount of an antibody of vaccine of any one of claims 1-15.
20. The method according to claim 20, wherein the treatment is chronic
21. The method according to claim 21, wherein the chronic treatment is for at least 2 weeks, such as at least for 1 month, 6, months, 1 year or more
22. A kit comprising the antibody or vaccine according to claims 1-15 for use in therapy.
23. A kit for the use according to claim 23 further comprising a labelled Notch 3 antibody or fragment thereof.



A



Fitting Results

Model Equation:

Response:
Rmax*Conc.
/(KD + Conc.)

Y Weighting: None

Results:

Chi^2/DoF = 0.0224

R^2 = 0.9867

RMax = 3.9954 ± 0.148

KD = 5.5E-08 ± 6.1E-09M

B

Figure 1

Figure 2

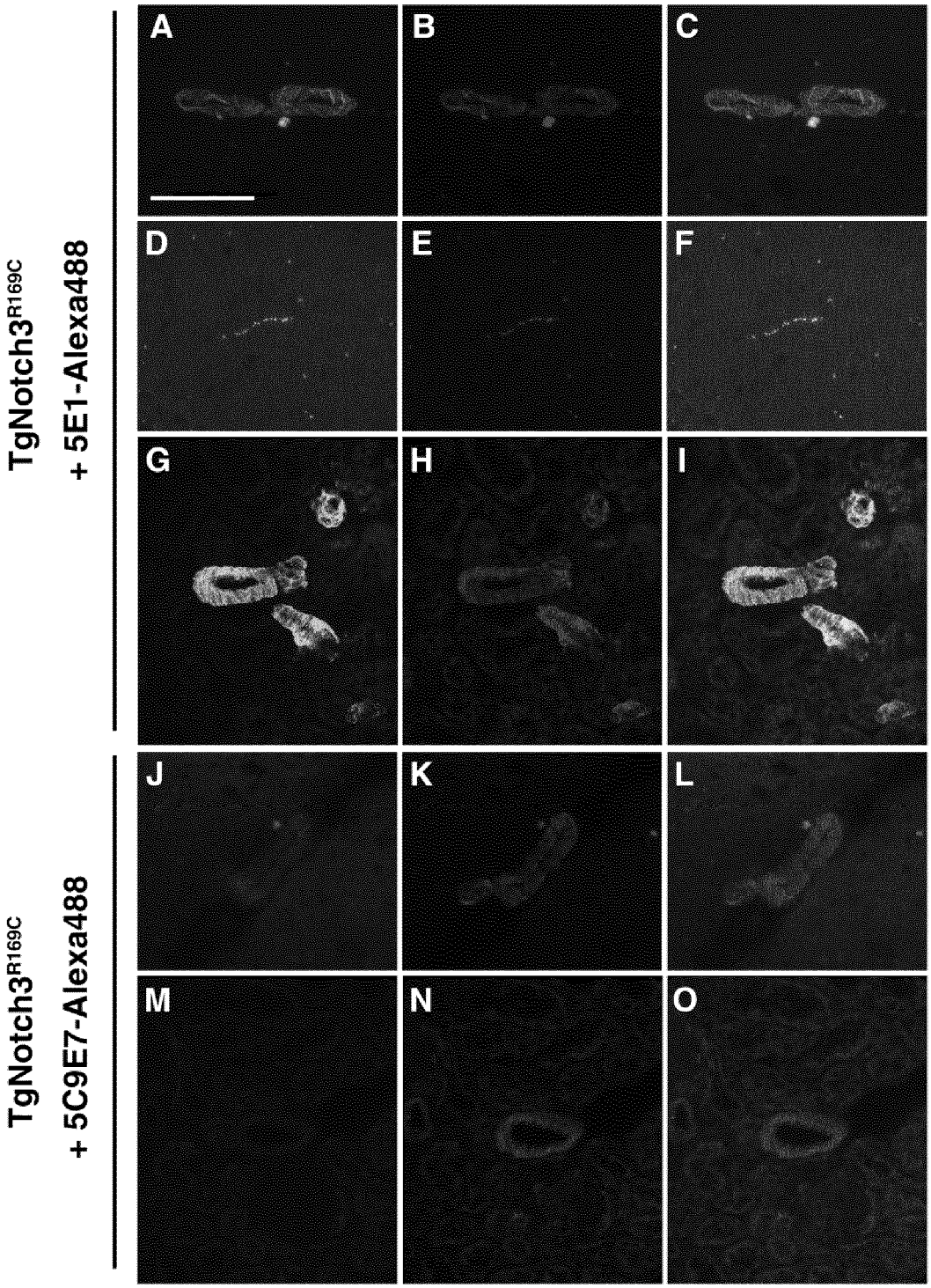


Figure 2

Figure 2bis

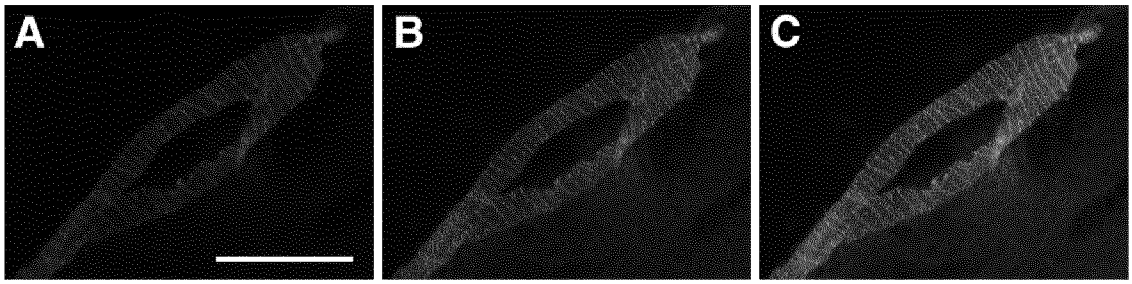
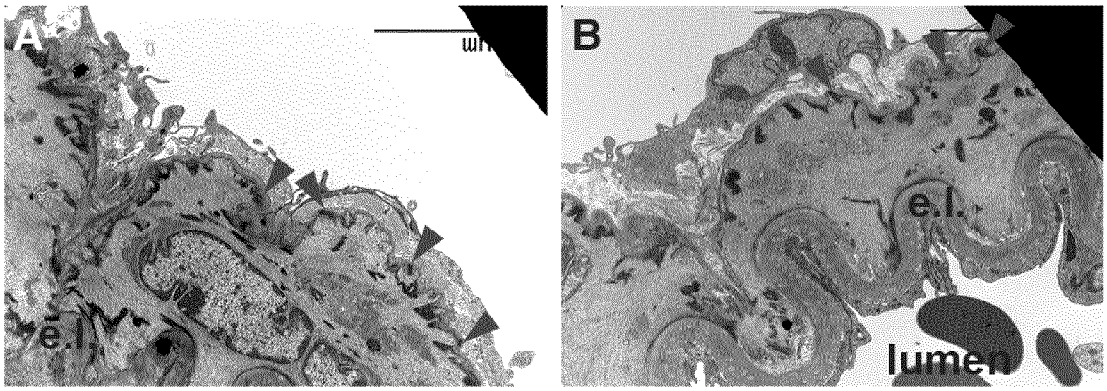


Figure 2bis

Figure 3



IgG1 5E1

C

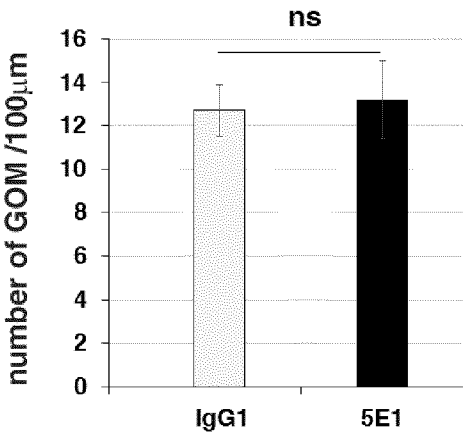


Figure 3

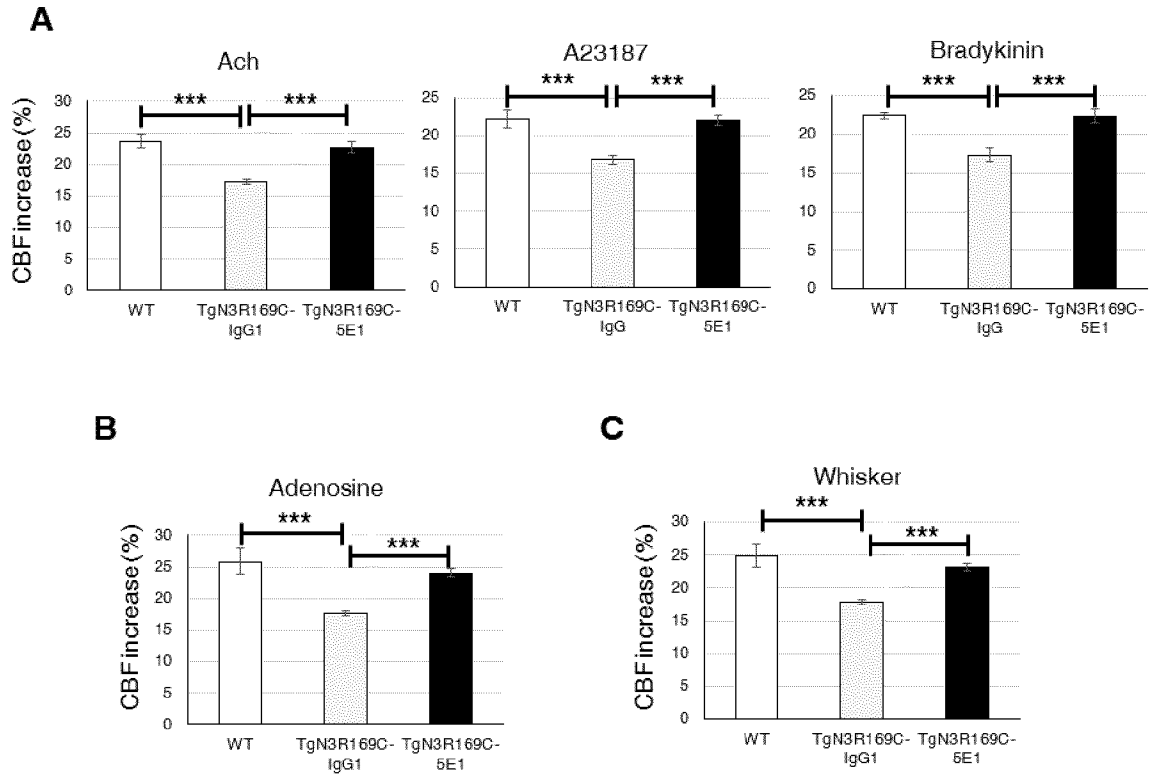
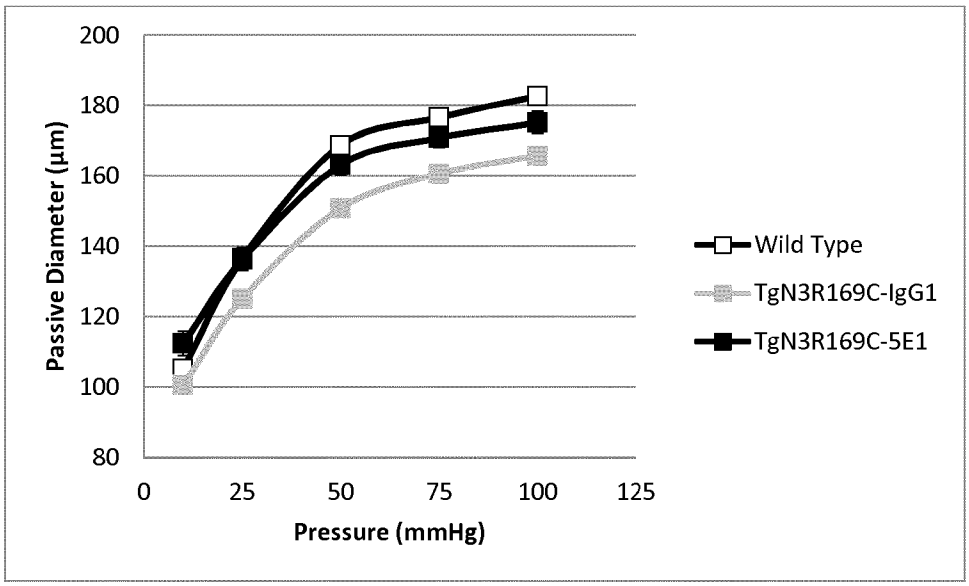

A
Ach
A23187
Bradykinin
CBF increase (%)
***
WT
TgN3R169C-IgG1
TgN3R169C-IgG
TgN3R169C-5E1
B
Adenosine
C
Whisker

Figure 4

A



B

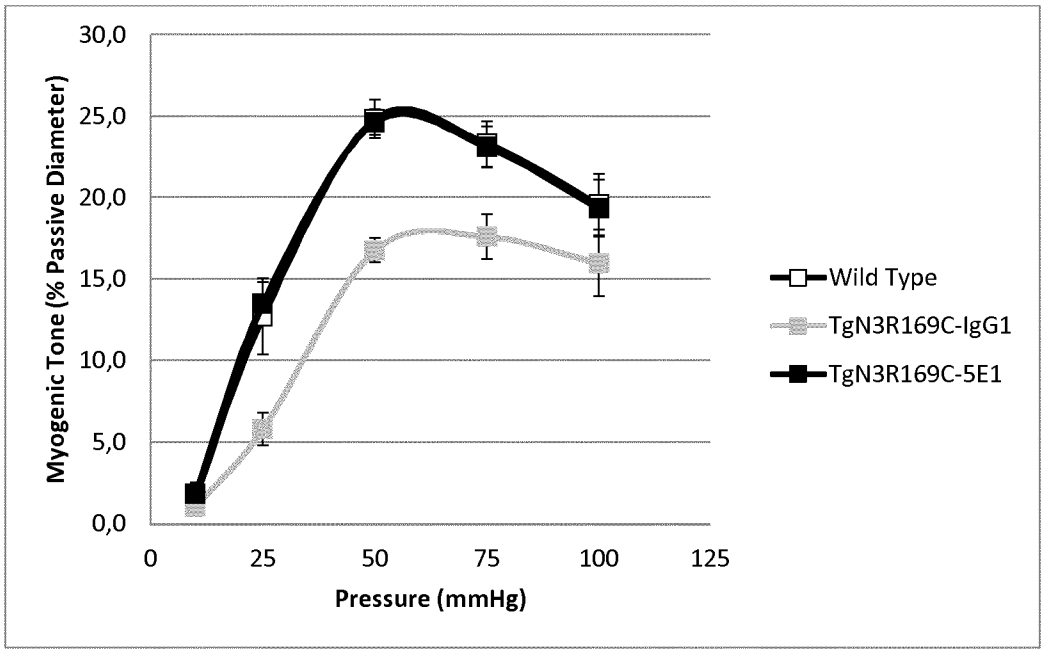
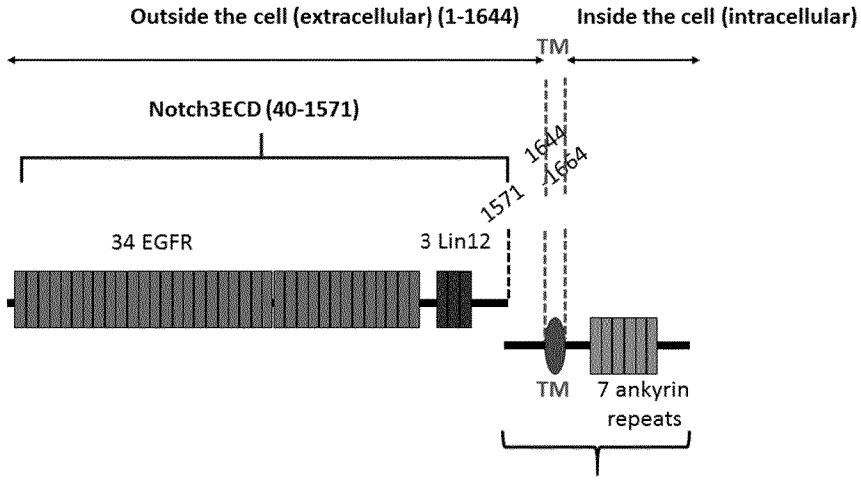


Figure 4bis

Human Notch3 (**UniProt number Q9UM47**)



(What does accumulate in CADASIL is the Notch3ECD)

TM (transmembrane domain)

Figure 5

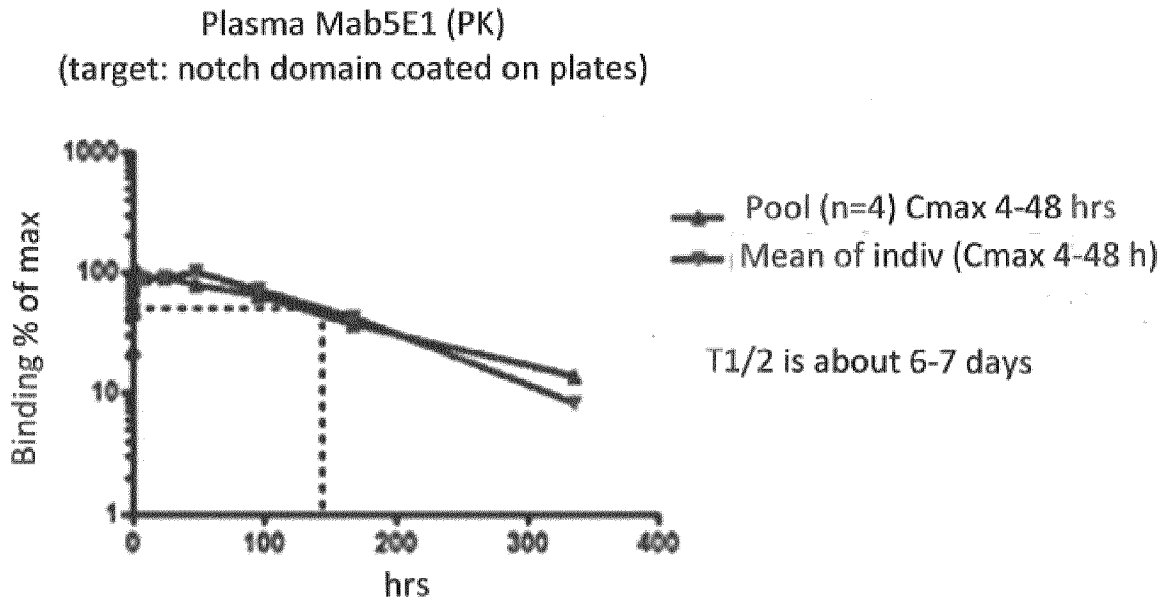

Plasma Mab5E1 (PK)
(target: notch domain coated on plates)
Binding % of max
1000
100
10
1
0
100
200
300
400
hrs
Pool (n=4) Cmax 4-48 hrs
Mean of indiv (Cmax 4-48 h)
T1/2 is about 6-7 days

Figure 6

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/071311

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 C07K16/28 A61K39/00
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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, EMBASE, PAJ, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2008/051797 A2 (GENENTECH INC [US]; LI KANG [US]; ZHOU BIN-BING STEPHEN [US]; WU WENJU) 2 May 2008 (2008-05-02) abstract, p. 3 [0009] - p. 4 [0014], p. 11 [0046], 23. [0084] - p. 47 [00157], examples 1-12 and claims ----- -/--	1-23

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 December 2015	17/12/2015

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

2

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOUTEL A ET AL: "THE ECTODOMAIN OF THE NOTCH3 RECEPTOR ACCUMULATES WITHIN THE CEREBROVASCULATURE OF CADASIL PATIENTS", JOURNAL OF CLINICAL INVESTIGATION, AMERICAN SOCIETY FOR CLINICAL INVESTIGATION, US, vol. 105, no. 5, 1 March 2000 (2000-03-01) , pages 597-605, XP001024826, ISSN: 0021-9738, DOI: 10.1172/JCI8047	13,14,23
A	abstract, p. 598 left-hand column last paragraph, paragraph bridging p. 601 to p. 602.	1-12, 15-22

X	LI KANG ET AL: "Modulation of Notch signaling by antibodies specific for the extracellular negative regulatory region of NOTCH3", JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, US, vol. 283, no. 12, 21 March 2008 (2008-03-21), pages 8046-8054, XP002481515, ISSN: 0021-9258, DOI: 10.1074/JBC.M800170200 abstract, p. 8047 right-hand column first full paragraph, p. 8053 right-hand column first full paragraph - p. 8054 left-hand column line 11	1-23

X	WO 2008/076960 A2 (GENENTECH INC [US]; LI KANG [US]; ZHOU BIN-BING STEPHEN [US]; LI YUCHE) 26 June 2008 (2008-06-26) abstract, p. 4-5 [0011] & [0012], p. 5 [0013] - p. 5-6 [0018], p. 13 [0057], examples 1-12 and claims	1-23

X	MOHAMMAD A. Y. ALQUDAH ET AL: "NOTCH3 Is a Prognostic Factor That Promotes Glioma Cell Proliferation, Migration and Invasion via Activation of CCND1 and EGFR", PLOS ONE, vol. 8, no. 10, 15 October 2013 (2013-10-15), page e77299, XP055231792, DOI: 10.1371/journal.pone.0077299 abstract, p. 8 left-hand column last paragraph	1-23

	-/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Marie Magdeleine Ruchoux ET AL: "Transgenic Mice Expressing Mutant Notch3 Develop Vascular Alterations Characteristic of Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy", , 1 January 2003 (2003-01-01), XP055231797, Retrieved from the Internet: URL:http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1851116/pdf/3469.pdf [retrieved on 2015-11-26] the whole document -----	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2015/071311

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