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Supporting Online Material for

A Recessive Mutation in the APP Gene with Dominant-Negative Effect on Amyloidogenesis

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Supporting Online Material

Materials and Methods

Neuropsychological evaluation. General intellectual function was assessed using Raven's Progressive Colored Matrices, and visuospatial perception by the Street's Completion Test. Auditory and visual memory were investigated using the immediate and delayed recall (Short Story Recall, Verbal Selective Reminding, Corsi Supraspan and Rey Figure Delayed Reproduction). Attention capabilities were assessed using the Attentive Matrices, Digit Span and the Trail Making. Language skills were measured by the Word Fluency, the Boston Naming Test and the Token Test. Executive functioning was evaluated by the Tower of London, the Weigl Sorting Test and Cognitive Estimation Test. Praxies were analyzed using the Imitating Gestures and Orofacial Expression Test and the Rey Figure Copying. Theory of Mind was evaluated by the Faux Pas Test. All tests were administered according to standardized procedures.

Genetic analysis. The exons 16 and 17 of the APP gene were amplified using the polymerase chain reaction (PCR) with previously described primers (*S1*). Sequencing of both sense and complementary strand of the PCR product was performed with ABI PRISM model 310 using the ABI PRISM BigDyeTM terminator cycle sequencing ready reaction kit (Perkin-Elmer). The mutation A673V was confirmed by restriction fragment length polymorphism analysis. The exon 16 of APP was amplified by PCR with the following primers: 5'-TACTTTAATTATGATGTAATAC-3' and 5'-GGCAAGACAAACAGTAGTGG-3'. The PCR product was digested with HpyCH4 V (New England Biolabs) and resolved using 10% polyacrilamide gels. The normal allele was characterised by two fragments of 91 and 78 bp, and the mutant allele by one fragment of 169 bp.
The Apoliprotein E (ApoE) genotype was determined using restriction isotyping, as described (*S2*). Briefly, genomic DNA was amplified by PCR using the following primers: 5'-TCCAAGGAGCTGCAGGCGGCGCA-3' and 5'-ACAGAATTCGCCCCGGCCTGGTACACTGCC A-3'. The PCR products were digested with CfoI enzyme (Roche) and the resulting fragments were resolved by electrophoresis on 15% polyacrilamide gels.
The proband was also subjected to the analysis of the entire coding sequence of PSEN1, PSEN2, and the genes encoding the microtubule-associated protein tau, progranulin, prion protein and huntingtin as described (*S3*-*S7*).
Total RNA was extracted from whole blood of the proband, a A673V heterozygous carrier and a control individual using the QIAamp RNA blood Mini Kit, according to the manufacturer's protocol. The RNA was reverse-transcribed with the Cloned AMV First-Strand cDNA Synthesis Kit using random primers. A 240-bp cDNA fragment comprising the mutated region was obtained by PCR with the following primers: 5'-CTGGGTTGACAAATATCAAGACGG-3' (forward) and 5'-CCACACCATGATGAATGGATGTG-3' (reverse). The amplified fragment was purified and subjected to direct sequencing in both directions.

Generation of DNA constructs and cell transfections. Human APP751 cDNA was HindIII subcloned into pcDNA3.1(-) plasmid vector (Invitrogen) and mutagenized using QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions with the following primers to introduce the A673V substitution: 5'-GATCTCTGAAGTGAAGATGGATGTAGAATTCCGACA TGAC-3' (forward) and 5'-GTCATGTCGGAATTCTACATCCATCTTCACTTCAGAGATC-3'(reverse).

COS7 and CHO cells were maintained in D-MEM (Gibco) and ALPHA-MEM (Cambrex), respectively and both supplemented with 10% foetal bovine serum (FBS). When a 80% confluency was reached, the cells were transiently transfected with either wild-type or A673V APP by electroporation. Six h post-transfection, media were changed with OPTIMEM (Gibco) that was conditioned for 48 h and then collected for ELISA assays in the presence of a protease inhibitor cocktail (Roche). The efficacy of transfections was determined by immunoblot analysis of transfected cell lysates using the antibodies 22C11 (Chemicon, 1:10,000 dilution) and A8717 (Sigma, 1:2,000 dilution) that recognize all three isoforms of APP (*S8, S9*). CHO or COS7 cells expressing the wild-type or mutated APP were lysed 48 h after transfection in a cold lysis buffer (100 mM NaCl, 10 mM EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM Tris-HCl, pH 7.4) containing protease inhibitors (Roche), briefly sonicated, and boiled for 10 min. Fibroblasts were grown until reaching 80% confluence, conditioned for 5 days in highly enriched OPTIMEM and then lysed following the same protocol. Equal amounts of protein were separated on a 12.5% Tris-Tricine gel and transferred to PVDF membranes (Millipore), which were blocked in Tris-buffered saline with 0.1% tween-20 (TBST) containing 5% non-fat dry milk, and incubated with the appropriate primary antibody. The signal intensity of APP was evaluated by densitometric analysis of blots, that were developed using enhanced chemoluminescence (Amersham) and visualized on autoradiography films.

sAPP, Aβ, total tau and phospho-tau determinations. Plasma levels of Aβ1-40 and Aβ1-42 were determined in the proband, his A673V homozygous sister, six A673V heterozygous carriers, three family members without the A673V mutation and six unrelated healthy subjects. Plasma samples were collected and stored at -80°C until use. The plasma levels of Aβ1-40 and Aβ1-42 were measured by ELISA (The Genetics Company) following manufacturer's instructions. CSF levels of Aβ1-42, total tau and T181-phosphorylated tau were measured in the proband, 10 sporadic AD patients and 10 control subjects by ELISA (Innogenetics) as described (*S10*). All determinations were performed in triplicate and were replicated six times.
sAPPα, sAPPβ, and Aβ peptides, including Aβ1-40, Aβ1-42 and N-terminal truncated Aβ species, were measured in conditioned media from APP-transfected COS7 and CHO cells and fibroblasts obtained by skin biopsies from the proband and four unaffected unrelated subjects using ELISA (Immuno-Biological Laboratories and The Genetics Company). The conditioned media were stored at -80°C in presence of a protease inhibitor cocktail (Roche) until analysis. The experiments were performed in triplicate and repeated three times in fibroblasts and seven times in transfected cells, following manufacturer's instructions.

Immunoblot analysis of APP C-terminal fragments. Lysates of fibroblasts and transfected cells were analyzed by immunoblot with the A8717 antibody, to determine the levels of APP carboxy-terminal fragments C99 and C83. The blots were developed using enhanced chemoluminescence (Amersham) and visualized on autoradiography films.
Quantification of immunoreactive bands was carried out by densitometry of the scanned autoradiograms under conditions of non-saturated signal, using the Quantity One-image software (Biorad Laboratories Inc.). The experiments were repeated six times for transfected cells and three times for fibroblasts, and the densitometric values in each experiment were the mean of three determinations.

Aβ peptide synthesis and purification. Synthetic peptides homologous to residues 1-40, 1-42 and 1-6 of Aβ either with or without the A-to-V substitution in position 2 ($A\beta1\text{-}40_{mut}$, $A\beta1\text{-}40_{wt}$, $A\beta1\text{-}42_{mut}$, $A\beta1\text{-}42_{wt}$, $A\beta1\text{-}6_{mut}$, $A\beta1\text{-}6_{wt}$) were prepared by solid-phase synthesis and purified as described (*S11*).

The purity and identity of peptides were determined by reverse-phase HPLC, amino acid sequencing and MALDI-TOF analysis. The purity of peptides was above 95%.

Laser light scattering. Aβ1-40_{mut}, Aβ1-40_{wt} and equimolar mixtures thereof were dissolved in 10 mM NaOH and then diluted in an equal volume of 100 mM Tris-HCl, pH 7.0, to the final concentration of 0.125 mM. The short-time (0-24 h) kinetics of aggregate formation was analyzed by laser light scattering, using an equipment designed at the Department of Medical Chemistry, Biochemistry and Biotechnology, University of Milan, that has been described in detail previously (*S12*). Both independent static (SLS) and dynamic (QELS) laser light scattering measurements were performed on the same samples at 37°C. The average scattered intensity (SLS) increases with the average molecular mass of macromolecules in solution. The scattered intensity correlation function (QELS) decays are proportional to the translational diffusion coefficients of macromolecules and then, via the Stokes-Einstein relation, their hydrodynamic diameter can be obtained (*S13*). To assess the stability of aggregates generated by Aβ1-40_{mut}, Aβ1-40_{wt} and peptide mixtures, the samples were diluted five folds after 48 h incubation using 50 mM Tris-HCl, pH 7.0, and light intensity scattered by peptide assemblies was recorded for 90 min.

Polarized-light microscopy and electron microscopy. Aβ1-40_{mut}, Aβ1-40_{wt} and equimolar mixtures thereof were dissolved in 10 mM NaOH and then diluted in an equal volume of 100 mM Tris-HCl, pH 7.0, to final concentrations of 0.250 and 0.125 mM. The samples were incubated at 37°C, and the tinctorial and ultrastructural properties of peptide assemblies were determined at various interval of times ranging from 1 h to 20 days (*S14*). At each time point (1, 4, 8 h, and 1, 2, 3, 4, 5, 7, 10, 15 and 20 days) 10 µl aliquots of peptide suspensions were air-dried on poli-L-lysine-coated slides (Bio-Optica) stained with the amyloid-binding dye Congo red and viewed under polarized light (Nikon Eclipse E-800 microscope). At the same time points, 5 µl aliquots were applied on Formvar-Carbon 200 mesh nickel grids for 5 min, negatively stained with uranyl acetate, and observed with electron microscope (EM109 Zeiss) operating at 80 KV at a standard magnification, calibrated with an appropriate grid. At day 20, samples were centrifuged at 13,000 x g for 30 min. The pellets were fixed in 2.5% glutaraldehyde in 50 mM phosphate buffer, pH 7.4, post-fixed in 1% acqueous solution of osmium tetroxide, dehydrated in graded acetone and embedded in epoxy resin (Spurr, Electron Microscopy Sciences). Ultrathin sections (500 Å) were collected on 200-mesh copper grids, positively stained with uranyl acetate and lead citrate, and observed with the electron microscope. The experiments were repeated five times. A similar study was carried out with the synthetic peptides Aβ1-42_{mut}, Aβ1-42_{wt} and equimolar mixtures thereof. Peptides solutions were prepared as described above at final concentration of 0.125 mM, and analysed at different time points ranging from 1 h to 10 days. To assess the anti-amyloidogenic effect of the hexapeptide Aβ1-6 carrying the A-to-V substitution in position 2, we prepared solutions of Aβ1-6_{mut} and Aβ1-40_{wt} in 50 mM Tris-HCl, pH 7.0, at final concentration of 0.125 mM, as well as mixture of Aβ1-6_{mut} and Aβ1-40_{wt} at 1:1 (0.125 mM concentration of each peptide) or 4:1 (0.5 mM Aβ1-6_{mut}, 0.125 mM Aβ1-40_{wt}) molar ratio. The solutions were incubated at 37°C and sample aliquots were analyzed by polarized light and electron microscopy after 4, 8 and 24 h, and 3, 5 and 7 days as described above.

Surface plasmon resonance (SPR). Binding of Aβ1-40_{mut}, Aβ1-40_{wt}, Aβ1-6_{mut} or Aβ1-6_{wt} to Aβ1-40_{wt} fibrils was assessed by SPR using the Proteon XPR36 equipment (Bio-Rad Laboratories, Inc.). Fibrils were obtained following incubation of 50 µM Aβ1-40_{wt} in 50 mM phosphate buffer, pH 7.4, for three days at 37°C under agitation. The fibrillary structure of Aβ1-40_{wt} assemblies was determined by atomic force microscopy. Fibrils were covalently immobilized on a sensor chip using amine-coupling chemistry (immobilization level ≈ 2000 resonance units) (*S15*). Reference cells were prepared in

parallel using the same immobilization procedure but without addition of peptide (*S16*). Freshly prepared solutions of Aβ1-40$_{wt}$ (1 μM concentration), Aβ1-40$_{mut}$ (1 μM), Aβ1-6$_{wt}$ (500 μM) and Aβ1-6$_{mut}$ (500 μM) in phosphate buffered saline, pH 7.4, were injected onto the immobilized fibrils for 2-10 min at a flow rate of 30 μl/min. These solutions did not contain aggregates as assessed by atomic force and electron microscopy. The sensorgrams -i.e. the time course of the SPR signal, expressed in resonance units (RU)- observed in the cell with covalently bound Aβ1-40$_{wt}$ peptide were corrected by subtracting the response detected in the reference cells (*S16*). The experiments were replicated three times.

Size Exclusion Chromatography. Aβ1-40$_{mut}$, Aβ1-40$_{wt}$ and equimolar mixtures thereof were dissolved in 10 mM NaOH and then diluted in an equal volume of 100 mM Tris-HCl, pH 7.0, to final concentration of 0.125 mM. To determine size and concentration of the peptide aggregates prior and following denaturation with urea, three set of samples were analyzed by size exclusion chromatography: (i) freshly-prepared peptide solutions; (ii) samples after 72 h incubation at 37°C; (iii) samples prepared as in (ii) and then incubated for 24 h after addition of urea at final concentration of 1M. Size exclusion chromatography was performed using an FPLC apparatus (Biologic FPLC system, Biorad) equipped with a precision column pre-packed with Superdex 75 with a separation range of 3-70 kDa (GE Healthcare). The mobile phase flow rate was set at 0.5 ml/min and the elution peaks were detected at UV absorbance 214 and 280 nm. The composition of the mobile phase was 1M urea in 50 mM Tris-HCl, pH 7.0. The column was calibrated using insulin chain B (3.5 kDa), D-JNK1 (3.8 kDa), ubiquitin (8.5 kDa), ribonuclease A (13.7 kDa), carbonic anydrase (29.0 kDa), ovalbumin (43.0 kDa) and BSA (67.0 kDa). The void volume was determined by Blue dextrane 2000 (200 kDa).

Neurotoxicity and MTT assay. The SH-SY5Y human neuroblastoma cell line was grown in D-MEM/F12 culture medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% foetal bovine serum (FBS). Cells were plated at 2×10^4 cells/well into 48-well plates, and differentiated for seven days by the addition of 10 μM retinoic acid in cell culture medium containing 0.5% DMSO. Freshly-prepared solutions of Aβ1-42$_{wt}$, Aβ1-42$_{mut}$ and equimolar mixtures thereof in 50 mM Tris-HCl, pH 7.4, were then added to the culture medium to the final concentration of 5 μM. After peptide treatment for 24 h, cell viability was determined by MTT assay (Sigma) following manufacturer's instructions. Results were expressed as percentage of the values obtained for control cells.

Statistical analysis. Student t-test was used to calculate the statistical difference of experimental versus control values in ELISA tests, immunoblot analysis of APP-transfected cells, and MTT assay. A difference was considered statistically significant if $P < 0.05$. The Prism program (GraphPad Software) was used to analyze the correlation and draw the standard curve in all the ELISA tests.

Supporting Text

Clinical and genetic study

The study was approved by the ethical committee of Carlo Besta Institute and written informed consent was obtained from the legal representative of the proband and participating relatives.

Case Report. The proband is a 44-year-old man with no family history of neurological disorders. At the age of 36 years, he developed progressive memory deficits, impairment of verbal initiative, difficulties in daily planning, mood depression and irritability with episodic aggressiveness. Three years later, neuropsychological assessment revealed severe impairment of attention, working memory, episodic memory, and executive functions (initiative, fluency, abstraction, set shifting, and planning). The Mini-Mental State Examination gave an adjusted score of 17/30, and the Wechsler Adult Intelligence Scale provided a total IQ of 47, with all sub-test scores under cut-off values. The clinical picture evolved towards a severe dementia, with loss of self-awareness, awareness of disease and interpersonal cognition, and disturbances of behavioural control including disinhibition, perseverations and echolalia. Myoclonic jerks, spastic tetraparesis and sphincteric incontinence were later additional features. The patient showed complete loss of autonomy in about eight years from clinical onset, and is presently unable to communicate, stand and walk.
Serial EEG recordings showed progressive slowing of background activity and occurrence of pseudoperiodic triphasic complexes. Serial Magnetic Resonance Imaging documented progressive cortico-subcortical atrophy, mainly involving the frontal, temporal, insular and parietal cortex, anterior cinguli and basal ganglia, with enlargement of the lateral ventricles (Fig. S3 A,B,C). The atrophy was more pronounced on the right hemisphere, and was associated with moderate hyperintensity in the subcortical white matter on T2-weighted and Fluid Attenuated Inversion Recovery (FLAIR) images (Fig. S3 D,E).

Genetic analysis. ApoE genotyping showed that the patient and the family members whose DNA was available (Fig. S2) were ε3/ε3 homozygous except for an A673V heterozygous carrier who had the ε2/ε4 genotype.
In addition to APP and ApoE genotyping, the patient was subjected to the analysis of PSEN1, PSEN2, and the genes encoding the microtubule-associated protein tau, progranulin, prion protein and huntingtin. No defects were found in any of these genes.

Neuropsychological evaluation of the patient's relatives. The A673V homozygous sister of the patient (Fig. S2, subject III-18) as well as five heterozygous (II-11, III-1, III-6, III-24, and III-26) and two non-mutated (III-14 and III-22) family members underwent neuropsychological assessment. At the interview, the proband's sister complained of mild attention and memory deficits, such as retaining newspaper articles or short novels. These difficulties became apparent in the last year, as confirmed by her cousins. The neuropsychological tests revealed that she had significant deficits in delayed recall of new verbal stimuli, picture naming and "theory of mind", i.e. the ability to attribute mental states, such as believes, intents and desires, to oneself and others. Moreover, her performances in word fluency and verbal episodic memory were at lower limits of normal values. Overall these features were consistent with the diagnosis of multiple domain MCI (*S17*). By contrast, the five A673V heterozygous and the two non-mutated relatives performed well on all tests (Table S1). Of particular significance was the excellent performance of the 88-year-old aunt of the proband (Fig. S2, II-11), despite she was non-educated. Moreover, the interview of several members of family A enabled us to rule out the presence

of progressive cognitive decline in the expected obligatory heterozygous carriers belonging to the I (I-1 or I-2) and II generation (II-1).

Supplementary Figures S1-S5

FIGURE S1

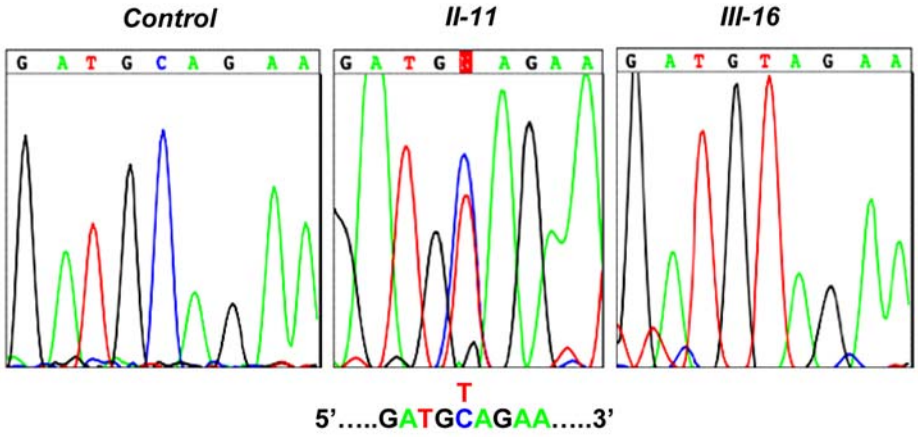

Fig. S1. Sequencing of the APP gene. Chromatograms of APP exon 16 showing the C-to-T transition resulting in A673V mutation in heterozygous (II-11) and homozygous (III-16) carriers, and the wild-type sequence in a control subject.

FIGURE S2

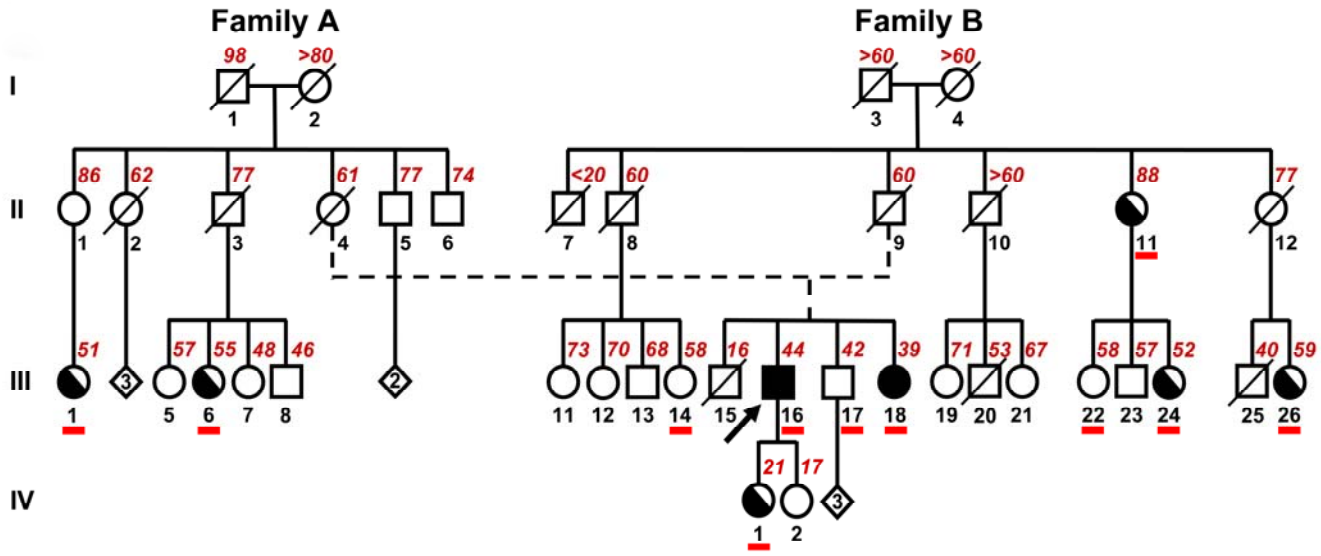

Fig. S2. Family trees of patient's (arrow) parental lineages showing the A673V homozygous (filled symbols) and heterozygous (half-filled symbols) carriers. Underscored numbers denote individuals whose DNA was analyzed. Red numbers indicate the age.

FIGURE S3

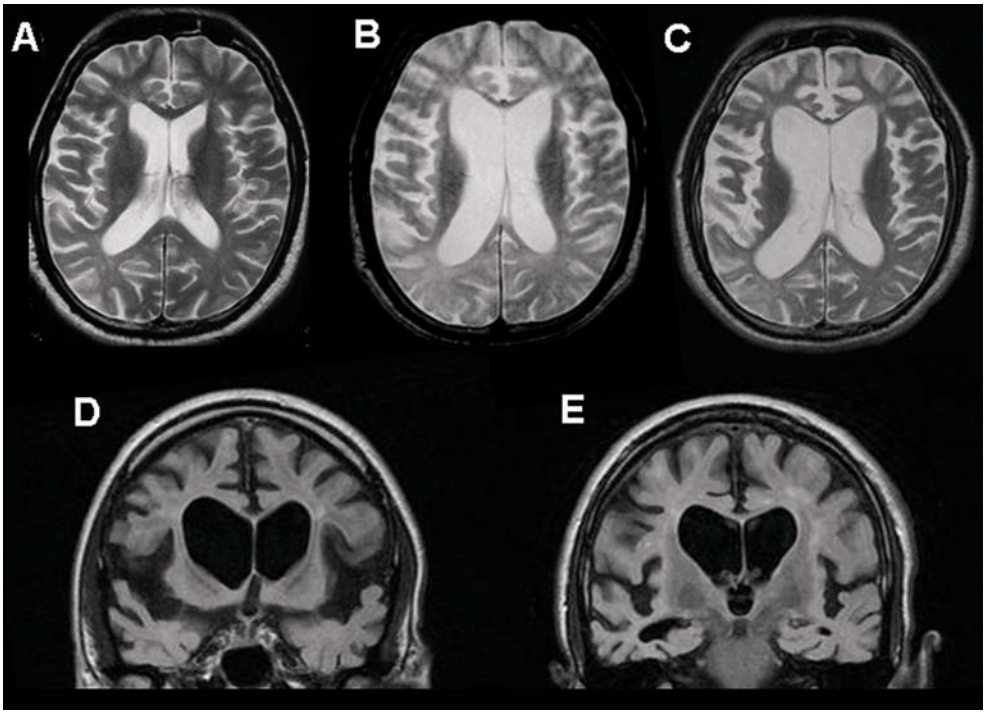

Fig. S3. Brain Magnetic Resonance Imaging of the proband. (**A**,**B**,**C**) Axial T2-weighted images in the early clinical stage of disease (**A**), after three years (**B**), and after eight years from onset (**C**).
(**D**,**E**) Coronal FLAIR images eight years after the onset of symptoms. The scans show the progression of the cortico-subcortical atrophy and subcortical white matter changes.

FIGURE S4

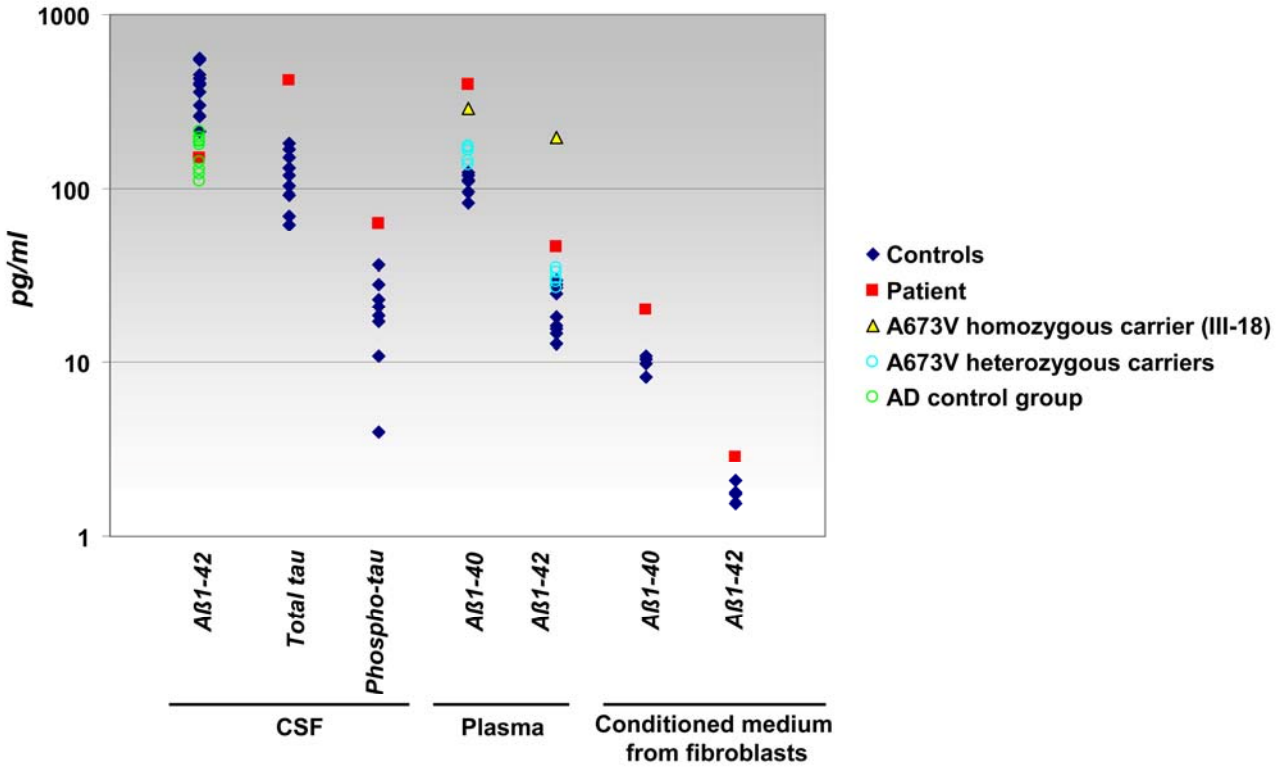

Fig. S4. Levels of Aβ, total tau and phospho-tau in CSF, and Aβ measurements in plasma and conditioned medium from cultured fibroblasts. The values of controls (N = 10 for CSF analysis, N = 9 for plasma analysis, and N = 4 for fibroblasts analysis), sporadic AD patients (N = 10) and A673V heterozygous carriers (N = 6) represent each the mean of three determinations. The values of the proband and his homozygous sister are the mean of 6 single determinations. The data are reported on a logarithmic scale.

FIGURE S5

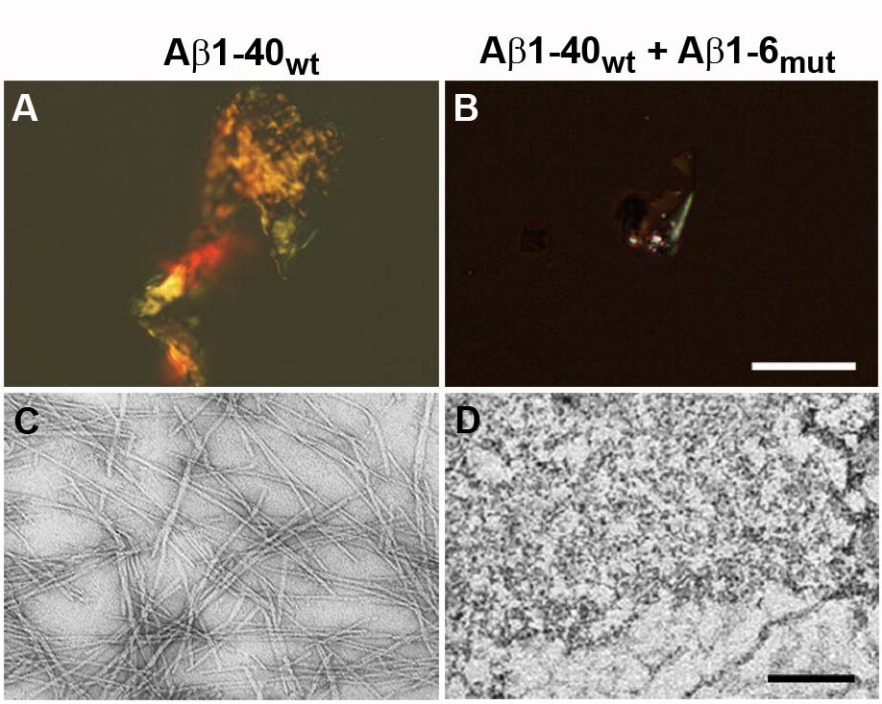

Fig. S5. Effects of Aβ1-6_{mut} on the aggregation properties of Aβ1-40_{wt}. (**A**,**B**) Polarized light microscopy after Congo red staining and (**C**,**D**) electron microscopy after negative staining of Aβ1-40_{wt} after 5-day incubation in the absence (**A**,**C**) or the presence (**B**,**D**) of Aβ1-6_{mut} at 1:4 molar ratio. In the absence of Aβ1-6_{mut}, Aβ1-40_{wt} generates large congophilic aggregates (**A**), that are formed by long, straight, unbranched fibrils (**C**). Amyloidogenesis is hindered by the presence of Aβ1-6_{mut} (**B**,**D**).
Scale bars indicate 250 μM (**A** and **B**), and 125 nm (**C** and **D**).

Supplementary Tables S1 and S2

Supplementary Table 1. Neuropsychological assessment of patient's relatives.

Test	**Subject III-18** *Age:39y* *Schooling: 8y*			**Subject III-1** *Age:51y* *Schooling: 13y*			**Subject III-6** *Age:55y* *Schooling: 8y*			**Subject III-14** *Age:58y* *Schooling: 5y*		
	Raw scores	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*
Digit Span (*S18*)	5	5	*3*	7	6.75	*4*	5	5	*3*	4	4.5	*2*
Attentive Matrices (*S18*)	59/60	52.75/60	*4*	60/60	60/60	*4*	50/60	48/60	*3*	49/60	50.75/60	*4*
Street Completion Test (*S18*)	6/14	4/14	*2*	10/14	7.75/14	*4*	6/14	5/14	*2*	8/14	8/14	*4*
Imitating Gestures (*S18*)	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*
Imitating Orofacial Expressions (*S18*)	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*
Trail Making Test Part A (*S19*)	33"	29"	*4*	29"	26"	*4*	84"	71"	*2*	52"	31"	*4*
Trail Making Test Part B (*S19*)	64"	47"	*4*	111"	95"	*4*	204"	156"	*2*	126"	50"	*4*
Tower Of London (*S20*)	26/36		*Cut-off 24*	30/36		*Cut-off 24*	24/36		*Cut-off 24*	26/36		*Cut-off 24*
Weigl Sorting Test (*S18*)	9/15	7.75/15	*2*	15/15	15/15	*4*	12/15	11.25/15	*4*	10/15	10.25/15	*4*
Rey Figure Copying (*S20*)	36/36	36/36	*4*	35/36	34.25/36	*4*	32/36	32.5/36	*4*	35/36	36/36	*4*
Raven's Progressive Colored Matrices (*S21*)	29/36	28.5/36	*3*	33/36	32/36	*4*	29/36	30/36	*3*	29/36	33.5/36	*4*
Word Fluency On Phonemic Cue (*S22*)	**19**	**20**	***1***	32	29	*3*	27	30	*3*	30	37	*4*
Word Fluency On Semantic Cue (*S22*)	33	32	*2*	42	40	*4*	29	31	*2*	40	46	*4*
Token Test (*S18*)	33/36	31.75/36	*3*	35/36	33/36	*4*	32/36	31.25/36	*2*	32/36	32.25/36	*3*
Corsi Bloks Span (*S18*)	5	5	*4*	5	4.75	*4*	4	4.25	*2*	5	5.5	*4*
Short Story (*S22*)	**10/28**	**8.5/28**	***1***	15.5/28	13/28	*3*	11/28	10.5/28	*2*	11.5/28	13.5/28	*4*
Rey Figure Delayed Reproduction (*S19*)	13.5/36	12.75/36	*2*	18/36	18.25	*4*	10/36	12.5/36	*2*	17.5/36	21.75/36	*4*
Faux Pas Test (*S23*)	**8/20**		***Cut-off 18***		-			-		20/20		*Cut-off 18*
Cognitive Estimation Test (*S24*)	14/42		*Cut-off 18*		-			-		10/42		*Cut-off 18*
Boston Naming Test (*S25*)	**43/60**		***Cut-off 56***		-			-		57/60		*Cut-off 56*
Verbal Selective Reminding (*S18*)	**17/160**	**0/160**	***0***		-			-		112/160	112/160	*4*
Corsi supraspan (*S18*)	22.14/29	15.39/29	*3*		-			-		15.5/29	15.5/29	*4*

Test	**Subject III-22** *Age:58y* *Schooling: 8y*			**Subject III-24** *Age:52y* *Schooling: 8y*			**Subject III-26** *Age:59y* *Schooling: 5y*			**Subject II-11** *Age:88y* *Schooling: 3y*		
	Raw scores	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*	*Raw scores*	*C.S.*	*E.S.*
Digit Span (*S18*)	5	5	*3*	6	6	*4*	5	5.5	*4*	4	4.75	*3*
Attentive Matrices (*S18*)	52/60	51.25/60	*4*	58/60	54.5/60	*4*	55/60	56.75/60	*4*	27/60	37.75/60	*2*
Street Completion Test (*S18*)	7/14	6.5/14	*3*	9/14	7.75/14	*4*	8/14	8/14	*4*	7/14	9.5/14	*4*
Imitating Gestures (*S18*)	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*
Imitating Orofacial Expressions (*S18*)	20/20	20/20	*4*	20/20	20/20	*4*	20/20	20/20	*4*	18/20	19/20	*3*
Trail Making Test Part A (*S19*)	37”	20”	*4*	20”	10”	*4*	40”	19”	*4*	102”	68”	*2*
Trail Making Test Part B (*S19*)	130”	72”	*4*	53”	16”	*4*	112”	36”	*4*		-	
Tower Of London (*S20*)	30/36		*Cut-off 24*	32/36		*Cut-off 24*	28/36		*Cut-off 24*		-	
Weigl Sorting Test (*S18*)	12/15	11.5/15	*4*	12/15	11/15	*4*	8/15	8.5/15	*2*	7/15	10/15	*3*
Rey Figure Copying (*S19*)	33.5/36	34.5/36	*4*	35/36	35.25/36	*4*	33/36	34.5/36	*4*	26/36	30.25/36	*2*
Raven’s Progressive Colored Matrices (*S21*)	28/36	31/36	*4*	34/36	35.5/36	*4*	28/36	32.5/36	*4*	25/36	33.5/36	*4*
Word Fluency On Phonemic Cue (*S22*)	28	31	*3*	43	46	*4*	28	35	*4*	10	23	*2*
Word Fluency On Semantic Cue (*S22*)	38	40	*4*	40	42	*4*	40	46	*4*	30	44	*4*
Token Test (*S18*)	33/36	32.5/36	*3*	36/36	36/36	*4*	35/36	35.25/36	*4*	39/36	31.75/36	*3*
Corsi Bloks Span (*S18*)	5	5.5	*4*	5	5.25	*4*	5	5.5	*4*	5	6	*4*
Short Story (*S21*)	14.5/28	14/28	*3*	15.5/28	15/28	*4*	13.5/28	15.5/28	*4*	9.5/28	17/28	*4*
Rey Figure Delayed Reproduction (*S19*)	15/36	18.5/36	*4*	14/36	15.5/36	*4*	16.5/36	20.75/36	*4*	10/36	21/36	*4*
Faux Pas Test (*S23*)	20/20		*Cut-off 18*	20/20		*Cut-off 18*	20/20		*Cut-off 18*		-	
Cognitive Estimation Test (*S24*)	15/42		*Cut-off 18*	8/42		*Cut-off 18*	16/42		*Cut-off 18*		-	
Boston Naming Test (*S25*)	59/60		*Cut-off 56*	60/60		*Cut-off 56*	57/60		*Cut-off 56*		-	
Verbal Selective Reminding (*S18*)	132/160	123/160	*4*	150/160	127/160	*4*	156/160	156/160	*4*		-	
Corsi supraspan (*S18*)	27.6/29	26.94/29	*4*	21.69/29	17.94/29	*3*	24.61/29	24.61/29	*4*		-	

Data are reported as raw scores, corrected scores (C.S.) and equivalent scores (E.S.: 0= Impaired performance, 1= Lower-limit performance, 2-3-4= Normal performance). For tests where equivalent scores are not available (Tower of London, Faux Pas Test, Cognitive Estimation Test and Boston Naming Test) the cut-off values are reported. Higher raw and corrected scores indicate better performances, except for the Trial Making Test and the Cognitive Estimation Task, which are inversely associated with the level of performance. Since subject II-11 is non educated, her row scores have been corrected for the lowest educational level (3 years). Impaired scores are highlighted in red while lower-limit scores are highlighted in blue.

Supplementary Table 2. Determination of A*β* peptides in CSF, plasma and media from cultured fibroblasts and APP-transfected cells.

	CSF *(pg/ml)*	**Plasma** *(pg/ml)*	**Conditioned medium from fibroblasts** *(pg/ml)*	**Conditioned medium from APP-transfected CHO cells** *(pg/ml)*	**Conditioned medium from APP-transfected COS7 cells** *(pg/ml)*
Control*					
Aβ1-40	**392 ± 115.5**	**109.5 ± 12.7**	**9.8 ± 1.0**	**49.8 ±11.8**	**572.3 ± 11.8**
Aβ1-42		**20.8 ± 6.7**	**1.7 ± 0.2**	**4.6 ± 1.4**	**54.2 ± 17.3**
Aβ1-42/Aβ1-40 ratio			**0.1**	**0.1**	**0.1**
Aβ11-40			*nd*	**13.3 ± 3.4**	**152.5 ± 1.6**
Aβ11-42			*nd*	**33.7 ± 2.5**	**130.9 ± 0.8**
AβN3pE-42			*nd*	**4.3 ± 1.1**	**4.2 ± 0.3**
Patient					
Aβ1-40	**151**	**426.5**	**20.2**‡	**85.3 ± 8.6** *(P=0.004)*	**863.4 ± 32.6** *(P=0.002)*
Aβ1-42		**45.9**	**2.9**‡	**9.9 ± 1.3** *(P=0.033)*	**78.1 ± 22.9** *(P=0.006)*
Aβ1-42/Aβ1-40 ratio			**0.1**	**0.1**	**0.1**
Aβ11-40			*nd*	**27.3 ± 2.4** *(P=0.002)*	**233.8 ± 16.6** *(P=0.011)*
Aβ11-42			*nd*	**45.7 ± 8.4** *(P=0.017)*	**198.5 ± 6.6** *(P=0.004)*
AβN3pE-42			*nd*	**8.4 ± 2.2** *(P=0.028)*	**9.8 ± 2.6** *(P=0.066)*
A673V homozygous patient's sister					
Aβ1-40	*Nd*	**287.8**	*nd*	*nd*	*nd*
Aβ1-42		**200.1**			
A673V heterozygous carriers†					
Aβ1-40	*Nd*	**155.9 ± 16.9**	*nd*	*nd*	*nd*
Aβ1-42		**30.8 ± 2.8**			

*****Controls are: 1) non demented subjects for CSF analysis (N = 10); 2) healthy subjects for plasma analysis (N = 9); 3) age-matched non-demented subjects for cultured fibroblasts (N = 4); 4) cells transfected with wild-type APP for A673V transfected CHO and COS7 (N = 7 for both CHO and COS7). Aβ values were normalized to the number of cells for Aβ determination in fibroblasts, and to the APP signal intensity for Aβ analysis in transfected CHO and COS7 cells. Statistical analysis was carried out according to Student's t test. The significance of differences between A673V and control values is reported in bracket.

[†]A673V heterozygous carriers: N = 6.

[‡]Mean of four determinations.

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