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Association of clusterin with the BRI2-derived amyloid molecules ABri and ADan

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Abstract

Familial British and Danish dementias (FBD and FDD) share striking neuropathological similarities with Alzheimer's disease (AD), including intraneuronal neurofibrillary tangles as well as parenchymal and vascular amyloid deposits. Multiple amyloid associated proteins with still controversial role in amyloidogenesis colocalize with the structurally different amyloid peptides ABri in FBD, ADan in FDD, and Aβ in AD. Genetic variants and plasma levels of one of these associated proteins, clusterin, have been identified as risk factors for AD. Clusterin is known to bind soluble Aβ in biological fluids, facilitate its brain clearance, and prevent its aggregation. The current work identifies clusterin as the major ABri- and ADan-binding protein and provides insight into the biochemical mechanisms leading to the association of clusterin with ABri and ADan deposits. Mirroring findings in AD, the studies corroborate clusterin co-localization

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with cerebral parenchymal and vascular amyloid deposits in both disorders. Ligand affinity chromatography with downstream Western blot and amino acid sequence analyses unequivocally identified clusterin as the major ABri- and ADan-binding plasma protein. ELISA highlighted a specific saturable binding of clusterin to ABri and ADan with low nanomolar Kd values within the same range as those previously demonstrated for the clusterin-Aβ interaction. Consistent with its chaperone activity, thioflavin T binding assays clearly showed a modulatory effect of clusterin on ABri and ADan aggregation/fibrillization properties. Our findings, together with the known multifunctional activity of clusterin and its modulatory activity on the complex cellular pathways leading to oxidative stress, mitochondrial dysfunction, and the induction of cell death mechanisms – all known pathogenic features of these protein folding disorders – suggests the likelihood of a more complex role and a translational potential for the apolipoprotein in the amelioration/prevention of these pathogenic mechanisms.

Keywords

amyloid; Alzheimer's disease; apo J; apolipoprotein J; chromosome 13 dementias; familial British dementia; familial Danish dementia

INTRODUCTION

Clusterin, also known as apolipoprotein J (ApoJ), is a ubiquitous protein present in nearly all body fluids (1, 2) and the most highly expressed apolipoprotein in the brain after ApoE (3). A single gene located on chromosome 8 produces a single mRNA that codes for a 449 amino acid chain containing the leader sequence of 22 amino acids and the α- and β- subunits of 205 and 222 residues, respectively. The final clusterin structure is generated by post-translational cleavage at peptide bond Arg^{205}-Ser^{206} and stabilized by the presence of five interchain disulfide bonds (1, 2, 4, 5). As a consequence of the presence of glycosyl moieties at six Asn-glycosylation sites, the experimental and theoretical molecular masses of the protein widely differ. The multifunctional protein clusterin has been ascribed anti-inflammatory and antiapoptotic properties (6, 7) and it is a known regulator of the complement system activation (8). But perhaps, its most recognized characteristics is its ability to act as a potent extracellular ATP-independent chaperone molecule capable of binding partially misfolded proteins and form stable complexes with different client proteins (9–11). Indeed, clusterin is among the group of proteins collectively known as "amyloid-associated proteins" which, in spite of not being structural parts of the fibrils, colocalize with vascular and parenchymal amyloid lesions in systemic and cerebral amyloidoses including Alzheimer's disease (AD), the most common form of dementia, worldwide (12). Consistent with its molecular chaperone properties, clusterin has been reported to bind to the Alzheimer's amyloid-β (Aβ) protein preventing its fibrillization and toxicity *in vitro* (9, 13, 14). In addition, the presence of clusterin-Aβ complexes in plasma and CSF supports the role of the apolipoprotein as a major Aβ-transport molecule (15). In this context, it is important to highlight that a major route of Aβ transport at the cerebral vascular endothelium and choroid plexus epithelium involves the cellular uptake of clusterin-Aβ complexes by gp330/megalin, also known as LRP-2 receptor (16) with more recent *in vitro*

studies demonstrating enhanced Aβ40 trafficking through endothelial cell monolayers when the peptide is previously complexed to clusterin (17).

The relevance of clusterin for AD pathogenesis was boosted by two large independent Genome Wide Association Studies (GWAS) that identified the protein as a novel late onset Alzheimer's disease (LOAD)-risk gene (18, 19) with numerous single nucleotide polymorphisms (SNPs) characterized as susceptibility loci in these and subsequent studies (20, 21). Although little is known about the SNPs contribution to AD pathogenesis, some of them have been shown to have potential roles in regulating gene expression, influencing clusterin mRNA levels and the plasma concentration of the protein (12).

Interestingly, most of the significant SNPs appear to exist in a linkage disequilibrium block and may together influence disease risk at levels not achieved when acting alone. At the present time, although the exact contribution of these variants to AD remains unclear, clusterin -together with bridging integrator 1 (BIN1), phosphatidylinositol binding clathrin assembly protein (PICALM), ATP-binding cassette sub-family A member 7 (ABCA7), and Complement receptor 1 (CR1) among others- are considered as low but frequent genetic risk factors for LOAD in difference to APOE and TREM2, which are associated with a higher risk but show a lower frequency expression in the population (22).

Based on the significant role of clusterin in AD and the ability of the protein to interact with the Alzheimer's Aβ protein, it is of interest to investigate any potential participation of clusterin in the pathophysiology of two hereditary conditions, familial British and Danish dementias (FBD and FDD, respectively), originated by different genetic alterations in the chromosome13 *BRI2* gene (Figure 1). These disorders are also associated with neurodegeneration, including neurofibrillary tangle formation, and amyloid deposition in the central nervous system in the form of parenchymal plaques and pre-amyloid lesions, as well as abundant cerebrovascular deposits (23–25). Although the deposited amyloid proteins, ABri in FBD and ADan in FDD, are structurally unrelated to any previous known amyloid molecule, including Aβ, both disorders share striking clinical and neuropathological similarities with AD (25–27). The amyloidogenic peptides ABri and ADan, generated by furin processing of the respective elongated precursor molecules (Figure 1), show high tendency to aggregate as it is the case with Aβ and other amyloid molecules (28–33). Indeed, our previous work demonstrated that both ABri and ADan rapidly adopted β-sheet-rich conformations in aqueous buffers containing physiologic salt concentrations in contrast to the 23 amino acid-long peptide (Bri1–23 or p23) circulating in non-carrier individuals which exhibited a primarily random coil configuration. The ABri and ADan structural properties translated in a high proclivity to form oligomeric assemblies as demonstrated by Western blot analysis, thioflavin- and ANS-binding, as well as electron microscopy studies (28, 29). The accelerated and enhanced formation of oligomeric/pre-fibrillar assemblies correlated, in turn, with the initiation of neuronal cell death mechanisms involving oxidative stress, perturbation of mitochondrial membrane potential, release of mitochondrial cytochrome c, and downstream activation of caspase-mediated apoptotic pathways (28–31).

The current work expands on the similarities of BRI2-associated dementias and AD, highlighting the presence of clusterin in both parenchymal and vascular lesions in FBD and FDD brain tissues. The studies demonstrate the ability of the apolipoprotein to bind the ABri and ADan amyloid molecules in a high affinity interaction with Kd values in a comparable nM range as that of clusterin-Aβ. Through the demonstration of the capability of clusterin to prevent the aggregation of ABri and ADan, the current work highlights a modulating activity of the protein in the process of amyloid formation adding a potential new therapeutic target for these neurodegenerative diseases.

MATERIAL AND METHODS

Materials

Human plasma apolipoprotein J and mouse monoclonal anti-clusterin antibody (clone G7) were purchased from Quidel, Inc. (San Diego, CA). Rabbit polyclonal H330 and mouse monoclonal A9 anti-clusterin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies immunoreactive with ABri (Ab 338) and ADan (Ab 5282) molecules were raised in New Zealand White rabbits by immunization with synthetic peptides comprising positions 22–34 of the respective amyloid molecules, as previously described (23, 24). Plasma samples were obtained from normal healthy subjects (age 25–40 years) after 12h fast with adequate understanding and written consent of subjects. Alkaline phosphatase- and horseradish peroxidase (HRP)-labeled goat F(ab')2 anti-mouse immunoglobulins were purchased from Thermo Fisher/Invitrogen (Carlsbad, CA) and GE Healthcare Life Sciences (Boston, MA), respectively.

Synthetic Peptides

FBD- and FDD-associated amyloid peptides were synthesized using N-tert-butyloxycarbonyl chemistry at the ERI Amyloid Laboratory (Oxford, CT, USA). The synthetic homologues, as illustrated in Figure 1, included the full-length amyloid subunits ABri (34 amino acids; EASNCFAIRHFENKFAVETLIC SRTVKKNIIEEN) and ADan (34 amino acids; EASNCFAIRHFENKFAVETLICFNLFLNSQEKHY), the BRI2 C-terminal fragment 244–266 (23 amino acids, p23; EASNCFAIRHFENKFAVETLICS), containing the first twenty-two common residues of both ABri and ADan molecules, as well as two peptides encompassing the pertinent C-terminal region of the respective amyloid subunits, ABri24–34 (11 amino acids, ABri Ct; RTVKKNIIEEN), and ADan23–34 (12 amino acids, ADan Ct; FNLFLNSQ EKHY). Aβ1-40, DEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVVGGVV, used as control, was also synthesized at the same facility. All synthetic homologues were purified by high performance liquid chromatography on a Vydac C4 column (Western Analytical, Murrieta, CA) and their molecular masses corroborated by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry, as previously reported (28, 29, 34). Prior to use, all peptides were incubated for 4 days at a concentration of 1 mg/ml with hexafluoroisopropanol (HFIP; Sigma Chemical Co., St. Louis, MO), a pretreatment that breaks down pre-existing secondary structures and disrupts hydrophobic forces (35) providing consistency among different batches and leading to monodisperse amyloid subunit preparations, a critical initial step for controlled aggregation studies (28, 29).

Immunohistochemistry

Single immunohistochemical analysis.—Formalin-fixed brain tissue samples from FBD, FDD, and AD cases, diagnosed according to established neuropathological criteria (36–40), were subjected to immunohistochemical analysis using standard procedures (23, 41). Briefly, sections were deparaffinized in xylene, endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol, and antigen retrieval achieved by 10 min incubation with 98% formic acid followed by 10 min pressure cooker treatment in 0.1 M citrate buffer pH 6.0, as described (41). After blocking nonspecific binding with 10% nonfat milk in PBS, tissue sections were subsequently incubated with rabbit polyclonal anti-clusterin antibodies (H330; 1/600 dilution) and a standard ABC protocol (Dako, Denmark). For the immunohistochemical assessment of the FBD, FDD, and AD amyloid proteins, rabbit polyclonal antibodies Ab338 and Ab5282 (both at a 1/1000 dilution) recognizing the C-terminal residues of ABri and ADan, respectively, as well as a mouse monoclonal anti-Aβ antibody recognizing amino acids 17–26 of the molecule (Dako, Denmark; 1/200) were employed using previously reported protocols (41). In all cases, diaminobenzidine/H_2O_2 was used as the chromogen and was followed by hematoxylin counterstaining.

Double immunohistochemical analysis.—The immunohistochemical protocol was carried out as detailed above up to the incubation step with the primary antibodies. At this point, FBD sections were incubated with anti-ABri antibody (Ab338; 1:1000) and anti-clusterin (A9; 1:600) whereas FDD sections were incubated with anti-ADan (Ab5282; 1:1000) and the A9 anti-clusterin antibody at the same concentration (1:600). Following incubation with the respective primary antibodies, sections were incubated with Alexa Fluor 488-conjugated anti-rabbit immunoglobulins (Molecular Probes; 1:1000) for 1 hour at room temperature. To visualize the clusterin staining, sections were also incubated with biotinylated anti-mouse immunoglobulins (Vector 1:200), followed by ABC (Vector; 30 minutes), and further incubation with TSA rhodamine (30 minutes, room temperature). Images were acquired on a Leica fluorescent microscope.

Thioflavin S staining.—Sequential sections from those used for the double immunohistochemical analysis were dewaxed and rehydrated as above. Tissue sections were incubated in 0.1% aqueous thioflavin-S for 7 minutes, differentiated in 70% alcohol, mounted in an aqueous mounting medium and images acquired as above. Sequential sections were used for this protocol as thioflavin staining is substantially reduced with the use of formic acid, a necessary pre-treatment for both ABri and ADan immunohistochemistry.

Identification of clusterin as a major ABri- and ADan-binding protein by ligand affinity chromatography

The full-length amyloid molecules ABri and ADan were coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (GE Healthcare) at a concentration of 2 mg/ml beads, using previously reported protocols from our laboratory (42). The affinity matrices were incubated with pooled normal human plasma (5ml plasma per ml beads; 3 h at room temperature) by end-over-end rotation followed by extensive washes with phosphate buffered saline (PBS) to remove unbound material, and subsequent elution of bound proteins with 0.1 M Acetic

acid. As a control for non-specific binding, human plasma was incubated under identical conditions with plain Sepharose prepared by blocking the active sites of the CNBr-activated matrix for 2h with 0.1M Tris-HCl buffer, pH 8.0, in the absence of peptides. In all cases, eluted bound proteins were separated on 10% Tris-tricine SDS-PAGE in the absence of any reducing agent and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore Corp., Bedford, MA) using 10 mM CAPs (3-cycloexylamino-1-propanesulfonic acid, Sigma) buffer, pH 11 containing 10% methanol, as we previously described (28, 29, 42). After transfer, membranes were stained with 0.1 % Coomassie Blue R250 in 50% methanol, the bands of interest, were excised from the PVDF membrane and subjected to automatic N-terminal Edman degradation on a 494 Procise Protein Sequencer (Applied Biosystems; Foster City, CA), as previously described by our laboratory (43, 44).

Assessment of clusterin interaction with ABri and ADan by solid-phase enzyme-linked immunosorbent assays

The binding interaction of clusterin to BRI2-related peptides was studied by ELISA following previously described methodology from our laboratory (9). Briefly, polystyrene microtiter plates were coated overnight at 4°C with ABri, ADan, and Aβ1-40 peptides (400 ng/100 μl/well) in 0.1 M $NaHCO_3$, pH 9.6. Typically, under these conditions ~ 2.5% of the peptide offered remained bound to the wells, as determined by a modification of Quantigold assay (Diversified Biotech., Boston, MA) adapted for microtiter plates (9, 45). After blocking with 1% bovine serum albumin (BSA), amyloid-coated wells were subsequently incubated with increasing concentrations of clusterin [0–20 nM in Tris-buffered saline (TBS); 100 μl/well; 3h at room temperature], followed by monoclonal anti-clusterin antibody [1/1000 in TBS containing 0.1% Tween (TBST)] and alkaline phosphatase-conjugated F(ab')2 anti-mouse immunoglobulins (1/5000 in TBST). The reaction was developed for 30 min with p-nitrophenyl phosphate in diethanolamine buffer (BioRad, Richmond, VA), stopped with 0.4 M NaOH, and quantitated at 405 nm in a Spectracount microplate reader (Packard, Meriden, CT). Non-specific binding was determined using BSA-coated wells. Binding data were analyzed by non-linear regression with GraphPad Prism (GraphPad Software Inc., San Diego, CA).

Mapping the clusterin binding activity in ABri and ADan amyloid molecules

The clusterin binding regions within ABri and ADan subunits were localized through the use of synthetic peptides representing different regions of the respective amyloid molecules on affinity chromatography and ELISA-based approaches. The peptides tested in the experiments consisted of the common N-terminal region of both the amyloid subunits (p23), as well as the respective C-terminal fragments ABri24–34 and ADan23–34 (Figure 1).

For the ligand affinity chromatography experiments, pooled normal human plasma was separately incubated, under identical conditions as described above, with affinity matrices containing the full-length ABri and ADan peptides, as well as their respective N- and C-terminal regions coupled to Sepharose 4B. Immobilized Aβ40 prepared with the same protocol was used as a positive control. In all cases, following washing out of unbound material and elution of bound proteins with 0.1 M acetic acid, the respective bound fractions

were separated by electrophoresis and electrotransferred to PVDF membranes as above. Membranes were blocked with 5% nonfat milk in TBST, and subsequently immunoreacted with monoclonal anti-clusterin antibodies (Clone G7; 1:1000 in TBST) followed by HRP-labeled labeled F(ab')2 anti-mouse IgG (1/5000 in TBST). Fluorograms were developed by enhanced chemiluminiscence (ECL) with ECL Western blotting detection reagent (GE Healthcare), and exposed to Hyperfilm ECL (GE Healthcare Life Sciences).

The localization of the binding site for clusterin in ABri and ADan amyloid molecules was corroborated by ELISA using microtiter wells coated overnight at 4°C with the N-terminal ABri and ADan common peptide p23 and the C-terminal fragments of both molecules, ABri24–34 and ADan23–34, at a concentration of 400 ng/0.1 ml/per well. The wells were subsequently incubated with increasing concentrations of clusterin (0–20 nM in TBS) and the bound apolipoprotein detected as described above by incubation with monoclonal anti-clusterin antibodies followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulins. Binding data were analyzed by non-linear regression using GraphPad Prism (GraphPad Software).

Effect of clusterin on ABri and ADan aggregation evaluated by Thioflavin T fluorescence assays

The aggregation properties of ABri and ADan were assessed by thioflavin T binding following our previously described procedures (28, 29, 46). HFIP-treated peptides were thoroughly dissolved to 10 mM in dimethyl sulfoxide (DMSO, Sigma) then diluted to 1 mM in deionized water followed by further dilution in 1x phosphate-buffered saline (PBS) to reach a 50 μM concentration. Reconstituted ABri and ADan homologs were incubated at 37 °C for up to 24 h in the presence or absence of different clusterin concentrations representing clusterin to ABri/ADan molar ratios of 1:100, 1:50, and 1:10, estimated based on the molecular mass of the respective monomeric forms of the amyloid molecules. Binding of ABri/ADan to thioflavin T (ThT) and its modulation by clusterin was subsequently monitored by fluorescence evaluation, as previously described (28, 29, 46). Briefly, 6 μl aliquots from each peptide aggregation time-point were added to 184 μl of 50 mM Tris–HCl buffer, pH 8.5, and 10 μl of freshly prepared 0.1 mM ThT (Sigma). Fluorescence was recorded for 300 s in an LS-50B spectrometer (Perkin Elmer, Waltham, MA) with a slit width of 10 nm and excitation and emission wavelengths of 435 and 490 nm, respectively.

RESULTS

A number of amyloid-associated proteins or molecular chaperons have been found co-deposited with the parenchymal and vascular amyloid lesions in AD as well as in other forms of cerebral and systemic amyloid diseases (34, 41, 47–50). Figure 2 illustrates clusterin immunoreactivity in FBD and FDD postmortem brain tissues and, as a comparison, depicts clusterin presence in AD parenchymal plaques and cerebrovascular deposits. The anti-clusterin antibody strongly labelled blood vessels as well as parenchymal amyloid plaques and diffuse preamyloid deposits in FBD tissue, exhibiting a pattern closely overlapping that of the previously described for ABri deposition (38). In agreement with

our previous reports, clusterin immunostaining in FDD brain tissue also stained pathological structures previously described for ADan, highlighting vascular deposits as well as diffuse plaques, which are the predominant lesions in this form of cerebral amyloidosis (41, 51). Clusterin appeared to label the FDD vascular lesions with the same intensity as ADan, whereas the parenchymal diffuse deposits were less intensely stained. Double immunohistochemical analyses were performed to assess co-localization of clusterin with the respective amyloid molecules in the brain deposits while thioflavin S staining in sequential sections was used to allow the visualization of the amyloid and preamyloid lesions alongside clusterin deposition. As illustrated in Figure 3, thioflavin positivity in FDD was mainly confined to the CAA, whereas ADan was present in both CAA and parenchymal preamyloid deposits. Clusterin immunoreactivity in FDD, mirrored the thioflavin staining with the majority of the clusterin being found in the vascular amyloid deposits. In FBD large amyloid plaques and CAA were observed with the thioflavin staining. Amyloid plaques and the preamyloid deposits were highlighted by the ABri antibody whereas clusterin staining mirrored that of thioflavin, with the majority being found in the large amyloid plaques and CAA. To provide a semiquantitative assessment of clusterin association with FBD amyloid deposits 50 ABri and Thioflavin positive plaques in the hippocampus were identified in randomly chosen fields and colocalization with clusterin was evaluated. Indeed, all ABri plaques identified as thioflavin positive and therefore in amyloid conformation, were also positive for clusterin. Mirroring these findings, analysis of 50 ADan positive blood vessels selected from randomly chosen fields and exhibiting thioflavin S fluorescence signal indicated that all ADan deposited in the form of CAA was also positive for clusterin. Only the occasional ADan parenchymal deposits were weakly positive for clusterin, consistent with the low intensity signal in preamyloid lesions observed by light microscopy.

Identification of the circulating plasma proteins capable of interacting with ABri and ADan amyloid molecules was performed using solid-phase affinity matrices in which the respective peptides were covalently immobilized onto CNBr-activated Sepharose 4B, one of the most widely used pre-activated media for immobilization of ligands that contain primary amines. Normal human plasma was incubated with the ABri- and ADan-affinity matrices, unbound components removed by extensive washes, bound proteins eluted with a low pH solution, separated by electrophoresis, and electro-transferred to PVDF membranes, as described in Methods. Coomassie Blue staining indicated that the major component in each of the respective bound fractions, representing >95% of the total bound proteins, corresponded to a ~ 80 kDa band, as illustrated in Figure 4A. Two minor components exhibiting molecular masses of ~ 50 and 28 kDa and representing <5% of the retained material were not further studied. The main ~80 kDa bands were excised from the PVDF membrane and subjected to N-terminal Edman degradation analysis. The first twelve residues retrieved for the ABri-bound protein consisted of two equimolar sequences: SLMPFSPYEPLN and DQTVSDNELQEM. Identical N-terminal sequences were obtained for the first ten N-terminal residues of main protein bound to the ADan-matrix: SLMPFSPYEP and DQSTVSDNELQ. Homology search at the National Center for Biotechnology Information (NCBI) revealed a 100% match with the N-terminal amino acids of the α-, and β- chains of human clusterin, respectively (Figure 4B). Highlighting the relevance of clusterin binding to ABri- and ADan- peptides, it is notable the enrichment of

the apolipoprotein in the fraction bound to the immobilized peptides, particularly taking into consideration that clusterin is a minor component of the total plasma proteins, with reported mean plasma concentrations varying from 52.8 to 116 μg/ml depending on the methodology and type of study (52–55), and yet, it represented >95% of the total bound proteins.

Further validation of the specific interaction of clusterin with ABri and ADan peptides was achieved by solid-phase ELISA experiments. Incubation of increasing concentrations of purified human clusterin with the immobilized full-length ABri and ADan peptides resulted in a dose-response relationship that reached saturation in both cases, as illustrated in Figure 4C. The binding paralleled that of Aβ40 used as control, which exhibited practically overlapping curves with the BRI2-related amyloids. Non-linear regression analysis of the binding values indicated that the data fitted a rectangular hyperbola and estimated the corresponding dissociation constants as 1.15 ± 0.23 nM for ABri and 0.88 ± 0.08 nM for ADan, values indicative of a high affinity interaction and in a comparable range to the 2.0 ± 0.29 nM yielded by clusterin binding to Aβ. Validating the accuracy and reproducibility of the assay, the Kd value for Aβ40-clusterin interaction determined in the current work was identical to the one previously reported by our lab two decades ago (9) and within a comparable order of magnitude to the 1 nM value later demonstrated via surface plasmon resonance-based technology for a transient oligomeric population of Aβ1–42 oligomers (13). The difference between these two Kd values could certainly be attributed not only to the completely different methodologies employed in the two studies but to the nature of the peptides themselves, Aβ40 vs. Aβ42, as well as to variations in their oligomerization state consistent with our previous findings describing a differential clusterin-binding ability between monomeric and aggregated forms of Aβ40 (9). To what degree peptide oligomerization affects ABri and ADan clusterin-binding remains to be elucidated. In our assays, prior to their coupling to the solid-phase matrices, peptides were preincubated with HFIP, a treatment known to enhance their solubilization and disrupt pre-existing β-sheet-rich structures with potential to seed/enhance the aggregation process (28, 29). While coupling to Sepharose-matrices or ELISA wells have been shown to mimic the formation of protein aggregates (56, 57) the efficiency of the coupling procedures and final protein density on the solid matrices will certainly have a modifying effect. Overall, the data highlight the striking similarity among the binding affinity of clusterin for the ABri, ADan, and Aβ amyloid molecules, irrespective of the nature of the primary amino acid sequences, suggesting a binding mechanism related to a common folding structure rather than to the identity of a specific polypeptide substrate.

The use of affinity matrices containing covalently bound ABri- and ADan-truncated peptides allowed the identification of the region involved in clusterin binding within the respective amyloid molecules. As indicated in Figure 4D, Western blot analysis revealed the presence of the 80 kDa clusterin band in the fraction retained by the immobilized full-length ABri and ADan peptides further corroborating the sequence findings illustrated in Figure 4 (panels A and B). The clusterin immunoreactive band was also present in the fraction bound to the affinity matrix coupled to the peptide comprising the common N-terminal amino acid residues of both BRI2-related amyloids (p23). No clusterin binding was observed to the ABri24–34 Sepharose column and only a negligible immunoreactive band was retrieved from immobilized ADan23–34. Consistent with the known capacity of clusterin to interact

with Aβ (9), a strong immunoreactive clusterin band was observed in the fraction bound to Aβ40-matrices, used as a positive control.

The truncated p23 peptide containing the shared first 22 N-terminal residues of BRI2 related amyloid subunits showed a binding curve comparable to the full-length peptides (Figure 4E). On the contrary, and in agreement with the affinity chromatography data illustrated above, clusterin displayed no specific binding to the C-terminal regions of the two amyloid molecules, ABri24–34 and ADan 23–34. Non-linear regression analysis of the binding values indicated that clusterin binding to immobilized p23 fitted a rectangular hyperbola and estimated the dissociation constant as 1.94 ± 0.23 nM, value within the range of those obtained for intact ABri and ADan. The ability of clusterin to bind p23 which, as we previously reported lacks oligomerization/aggregation properties (28, 29), argues for the capability of clusterin to bind monomeric forms of BRI-2 related molecules, as previously shown for Aβ (9). Taken together, both the ligand affinity chromatography and solid phase binding experiments demonstrate a high affinity binding interaction between clusterin and BRI2-associated amyloids and map the binding site to the common N-terminal residues of the amyloid molecules. Notably, this N-terminal region of ABri and ADan was also identified as the area responsible for complement activation and the binding site of the C1q complement component (34) providing additional explanation for many of the commonalities exhibited by both amyloid proteins as well as the neuropathological similarities between FBD and FDD.

In an effort to determine whether clusterin binding exerted a modulatory effect on the aggregation of BRI-2 related peptides, their fibrillization propensity, in the presence and absence of the apolipoprotein, was evaluated using thioflavin T, a dye that displays enhanced fluorescence upon binding fibrillar and protofibrillar amyloid conformations (46, 58). In accordance to their previously described high β-sheet content under physiologic salt concentrations, both ABri and ADan showed thioflavin T fluorescence values that increased rapidly overtime. These values reached a plateau after only 1.5–2h and remained constant for the 24h duration of the experiment (Figure 5A) with ABri displaying higher thioflavin T fluorescence than ADan under the conditions tested, in agreement with our previous reports (28, 29). In contrast, Bri1–23 (p23) which exhibited an unstructured conformation in circular dichroism analysis, displayed negligible binding to thioflavin T, a trend that remained unaltered during the 24h of the experiment and even when followed up to 6 days (28). The presence of clusterin exerted a profound effect on ABri and ADan aggregation. As illustrated in Figure 5, panels B and C, co-incubation of ABri and ADan with increasing concentrations of clusterin that raised the clusterin to peptide-substrate molar ratios from 1:100 to 1:10 decreased thioflavin T fluorescence in a dose response manner. For both ABri and ADan peptides, the presence of clusterin translated in lower thioflavin T levels at all time points tested compared to values obtained in absence of the apolipoprotein.

DISCUSSION

FBD and FDD are two neurodegenerative conditions with clinical and neuropathological features remarkably similar to AD (25–27, 59) including cognitive impairment, parenchymal pre-amyloid and amyloid deposits, cerebrovascular amyloid lesions, and neurofibrillary

tangles (38). In spite of the similarities among the three neurodegenerative conditions, the ABri and ADan amyloid peptides generated by furin-like proteolytic processing, are structurally unrelated to Aβ. Both amyloid molecules are C-terminal proteolytic fragments of a larger-than-normal precursor molecule BRI2 originated in two different genetic defects, a Stop-to-Arg mutation in FBD and a ten-nucleotide duplication-insertion immediately before the stop codon in FDD (Figure 1) (25). Regardless of the nucleotide changes, the final outcome is common to both diseases: the genesis of an extended precursor featuring a C-terminal piece that does not exist under normal conditions. The *de novo* created amyloid peptides, with no sequence identity to any known amyloid protein, share the first 22 N-terminal amino acids but show no homology in the 12 C-terminal residues, characteristics that allowed the generation of disease-specific antibodies used in the topographical characterization of the deposited proteins (23, 24).

Similarly to data reported in AD, FBD and FDD brain tissues exhibit an abundance of activated microglia surrounding amyloid plaques and dystrophic neurites, characteristics that, in conjunction with the presence of inflammation-related cytokines and proteins of the complement system, highlight the relevance of pro-inflammatory processes for these neurodegenerative conditions (25, 34, 60). Certainly, complement proteins are integral components of amyloid plaques and cerebrovascular deposits in AD and activation products from both the classical and the alternative pathways together with regulatory elements of the activation cascade have been found co-deposited in FBD and FDD amyloid and pre-amyloid lesions (34, 61). The presence of clusterin in these lesions may reflect an attempt to counterbalance the detrimental activation of the system resulting from the known capability of clusterin to act as a soluble complement activation inhibitor at the level of the terminal complement complex assembly (62). Along this line, it should be mentioned that the limited studies available have demonstrated increased expression of the complement regulators C1INH and CD59 in AD brains albeit their moderate increment appears to be insufficient to compensate for the marked upregulation and activation of the complement proteins seen in the disease (63).

The presence of clusterin in the FBD and FDD lesions is not unique as multiple amyloid associated proteins have been shown colocalized with the lesions in both disorders, closely resembling findings in AD (41, 64). Among the proteins co-depositing with the amyloid subunits in these cerebral disorders as well as in other amyloidoses, serum amyloid P-component, apolipoprotein E, and clusterin, together with a number of proteases, and proteoglycans, are the most relevant (41, 50, 65). Pointing out to a broad role of clusterin in amyloid disorders in general, clusterin was identified associated with TTR and immunoglobulin-light chain amyloid deposits in cardiac and renal forms of systemic amyloidoses and light-chain deposition diseases, as described by our work and that of others (66, 67). While the deposition of some of these associated proteins may constitute a secondary event, in the case of clusterin, the current finding of its specific binding interaction with the ABri and ADan amyloid molecules suggest a more central role in the pathophysiology of these diseases. Along this line, it is known that several amyloid associated proteins are capable of influencing the conversion of soluble amyloid proteins to insoluble forms with high β-sheet content (49, 68). As such, clusterin in particular, was shown to bind Aβ with comparable affinity to the one demonstrated herein for

ABri and ADan, and as a result, influence Aβ fibril formation (69). Highlighting the translational potential of the versatile apolipoprotein, intraventricular administration of a short clusterin-derived peptide corresponding to a D-amino acid version of one of the predicted amphipathic helices of the protein (70) decreased both Aβ plaques and the severity of cerebral amyloid angiopathy while ameliorating cognitive defects in a widely used transgenic mouse model (71). The capacity of clusterin to inhibit the formation of amyloid aggregates in *in vitro* and *in vivo* models was additionally demonstrated for a broad range of unrelated proteins among them apolipoprotein C-II, α-synuclein, TAR DNA-binding protein 43 (TDP-43), prion protein, and transthyretin, all associated with pathological protein folding disorders (14, 72–75). The demonstration of the high affinity interaction of clusterin with the BRI2-related amyloid molecules as well as the reduced aggregation/fibrillization properties of ABri and ADan in the presence of the apolipoprotein reported herein, add to the increasing research highlighting a key role of clusterin in the pathophysiology of amyloid diseases. Notably, the association of clusterin with the FBD and FDD amyloid deposits contrasts with its capacity to reduce the fibrillization of the BRI2-related peptides *in vitro*, similarly as in the case of AD in which this dichotomy has been attributed to the dependance of clusterin chaperone activity on the molar ratio of the apolipoprotein with respect to Aβ (14). Sub-stoichiometric clusterin levels –as those employed in the current experiments– were shown to exert an inhibitory effect on amyloid formation while under conditions in which the amyloid protein was present at a very large molar excess, the apolipoprotein co-incorporated with the substrate protein into the insoluble aggregates (14). In our experiments, the thioflavin T values never reached complete inhibition at sub-stoichiometric clusterin-to-ABri/ADan molar ratios, providing further support to the idea that clusterin has the capability to bind to ABri or ADan monomers and/or to small oligomeric assemblies, as also suggested by the ability of clusterin to bind p23, a fragment that does not oligomerize, as indicated above. Whether the binding of clusterin to the common N-terminal of BRI2 peptides structurally interferes with the aggregation properties of the C-terminal ends of ABri and ADan, fragments that are necessary for the oligomerization and fibrillization of the intact peptides, remains to be investigated. Furthermore, the possibility of clusterin binding to N-terminal regions of the aggregates that are not masked by self-steric hindrance cannot be discarded and it may provide a mechanism for the recruitment of clusterin into fibrillar structures.

Mounting evidence continues to link clusterin to multiple neurodegenerative disorders. Increased levels of the apolipoprotein have been observed not only in the AD brain but also in other neurological conditions, such as amyotrophic lateral sclerosis, transmissible spongiform encephalopathies, Huntington’s disease, traumatic brain injury, and multiple sclerosis, (12, 76–80). Despite the initial research linking clusterin to these diseases, insight into the protein contribution to the pathogenesis of the disorders has been slow to unravel with multiple observations supporting the notion of clusterin as a neuroprotective molecule through its action as a chaperone molecule favoring solubilization and clearance of toxic protein aggregates. Recent work supports a more complex role for clusterin in the pathogenesis of neurodegenerative diseases extending beyond its chaperone activity. The ability of the protein to act as a novel ligand of TREM2 (triggering receptor expressed on myeloid cells 2) –a property shared with apoE, apoA2, and apoA1, influenced by the

degree of lipidation of the apolipoproteins, and decreased by the presence of TREM2 disease-associated mutations– has clear connotations for AD pathophysiology (81, 82). Through activation of the downstream PI3K/Akt axis, TREM2 has also been shown to mediate the negative regulation of proinflammatory responses, oxidative stress, and neuronal apoptosis (81, 83–85) adding to the underlying mechanisms by which loss-of-function TREM2 mutations are associated with increased AD risk while expanding on the potential role of clusterin, known to exert also a modulatory effect on PI3K/Akt activation (86, 87). Binding of clusterin-Aβ complexes to TREM2, a cell membrane protein highly expressed in brain microglial cells (88), was shown to facilitate microglial internalization and clearance of Aβ supporting also a key role of this path in the maintenance of Aβ homeostasis (89, 90).

The current work offers support for the close association of clusterin with FBD and FDD providing a mechanistic explanation for the protein colocalization to the amyloid lesions likely resulting from the capacity of the apolipoprotein to bind the BRI2-related molecules with high affinity. It should also be taken into consideration that clusterin is a multifaceted protein found linked to non-proteopathic neurological diseases, including schizophrenia, Rett syndrome, and hypoxic-ischemic conditions indicative of a more complex role for secreted clusterin than its sole chaperone activity. In this sense, clusterin has also been shown co-localized with ocular non-amyloid fibrillar aggregates in exfoliation syndrome, an age-related disorder that constitutes the most common identifiable cause of glaucoma. Proteomic and immunohistochemical analyses of exfoliation deposits revealed a complex glycoprotein/proteoglycan composition enriched in extracellular matrix, basement membrane, and elastic fiber proteins (91). The additional identification of clusterin together with vitronectin, both fluid phase inhibitors of the complement cascade, in conjunction with activation products of the classical pathway, suggests that the association of the apolipoprotein with exfoliation deposits may likely rely – at least in part – on the activity of clusterin as a modulator of complement activation (91, 92).

The contribution of clusterin to the pathophysiology of BRI2-related dementias may additionally reflect the widely demonstrated modulatory effects of the protein on the complex cellular pathways leading to oxidative stress, mitochondrial dysfunction, and the induction of cell death mechanisms. Along this line, our previous work has amply demonstrated that ABri-, ADan-, and Aβ-mediated toxicity proceeds through the initiation of apoptotic mechanisms, with severe mitochondrial compromise, release of cytochrome c to the cytoplasm, changes in mitochondrial membrane potential, induction of oxidative stress mechanisms, and downstream activation of terminal caspases (28, 29, 46, 93). Based on the reported activities of clusterin, the apolipoprotein has the potential to exert a protective effect at different points of these pathogenic cascades. Clusterin has been shown to hinder apoptosis and oxidative stress, pathways elicited by ABri and ADan, as demonstrated by ours and others previous work (28, 29, 31, 33). Along this line, clusterin was shown to inhibit BAX localization onto the outer mitochondrial membrane thereby preventing the release of cytochrome c and downstream induction of apoptotic mechanisms (94) while promoting cell survival and balancing oxidative stress through activation of PI3K/AKT/GSK-3 mediated signaling pathways (86, 87). Notably, a main cellular path activated via the PI3K/GSK-3 axis is the Nrf2-mediated anti-oxidative response (95). Indeed, pharmacologic activation of Nrf2-mediated mechanisms has been demonstrated to

exert a protective effect from amyloid-induced alterations ameliorating oxidative damage and restoring mitochondrial function as well as metabolic/bioenergetic alterations (93), findings that suggest a translational potential for clusterin in the amelioration/prevention of these pathogenic mechanisms. Overall, the complexity of clusterin biogenesis together with the lack of clarity over the high number of physiological functions attributed to this multifunctional molecule have contributed to the challenge of understanding the role of the apolipoprotein in the pathophysiology of neurodegenerative disorders. Further investigations are warranted to fully elucidate the contribution of clusterin to the pathophysiology of cerebral amyloidoses and its potential as a modulating agent in the development of alternative therapeutic strategies.

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Data Availability Statement:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

List of Abbreviations:

Aβ	amyloid-β
AD	Alzheimer's disease
apo J	apolipoprotein J
BSA	bovine serum albumin
CSF	cerebrospinal fluid
ELISA	enzyme-linked immunosorbent assay
FBD	familial British dementia
FDD	familial Danish dementia
HRP	horseradish peroxidase
Kd	dissociation constant
LRP-2	low density lipoprotein-related protein 2
PBS	phosphate buffered saline
SNPs	single nucleotide polymorphisms

TBS	Tris-buffered saline

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- FBD and FDD mutations originate the highly amyloidogenic ABri and ADan molecules
- Clusterin co-localizes with ABri and ADan amyloid lesions, mirroring AD deposits
- ABri- and ADan-clusterin binding interactions are saturable and of high affinity
- ABri and ADan aggregation/fibrillization is inhibited in the presence of clusterin
- Data argue for a translational value of clusterin in protein folding disorders

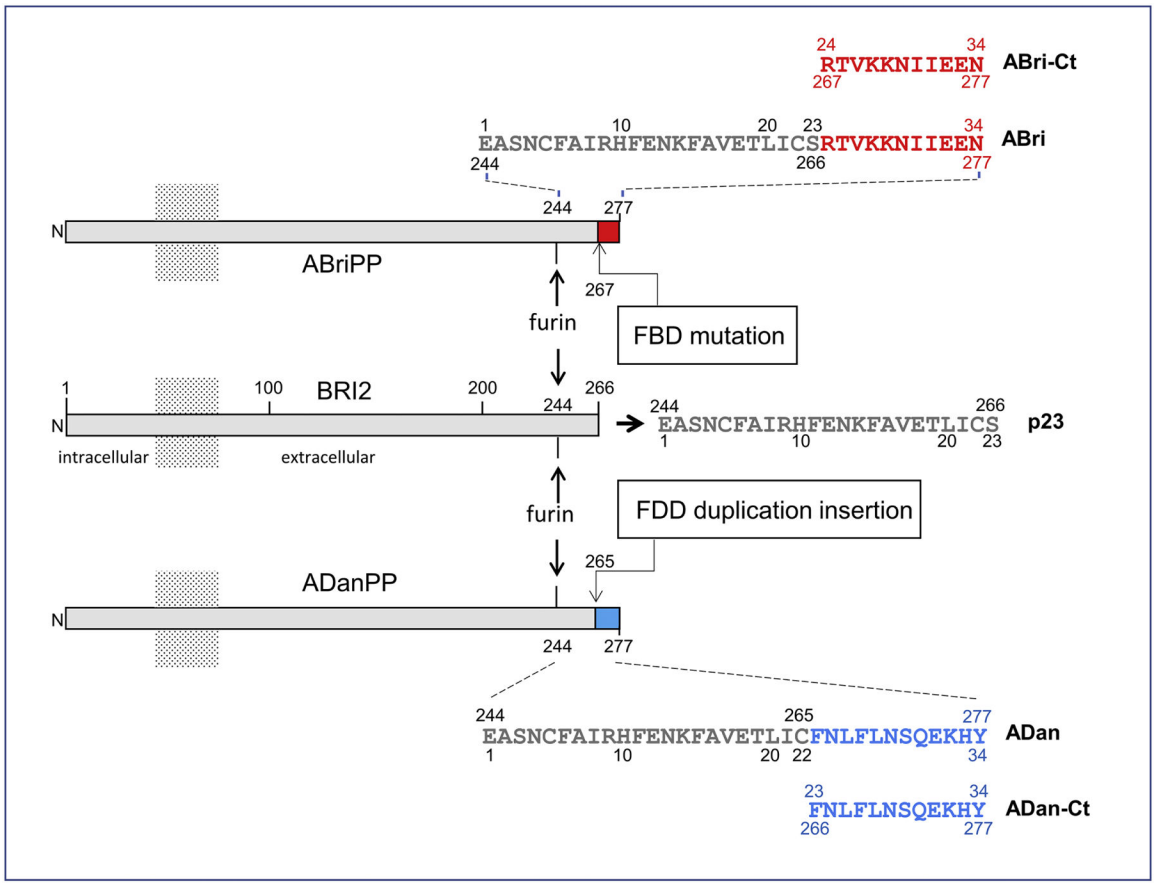

Figure 1: Schematic representation of the genetic alterations in the *BRI2* gene linked to familial British and Danish dementias.

A single nucleotide mutation at the Stop codon of the *BRI2* gene generates a longer than normal protein (ABriPP) that is the precursor of the ABri amyloid. Similarly, a 10-nucleotide duplication insertion one codon before the stop codon in the *BRI2* gene also generates a longer than normal protein (ADanPP), the precursor of the ADan amyloid. ABri and ADan are generated by furin-like proteolytic processing at position 244 of their respective elongated precursors whereas the same processing in the wild-type BRI2 releases the non-amyloidogenic shorter fragment labeled p23. ABri and ADan are identical in their first 22 residues and completely different in the 12 C-terminal amino acids (C-terminal ABri: red font; C-terminal ADan: blue font).

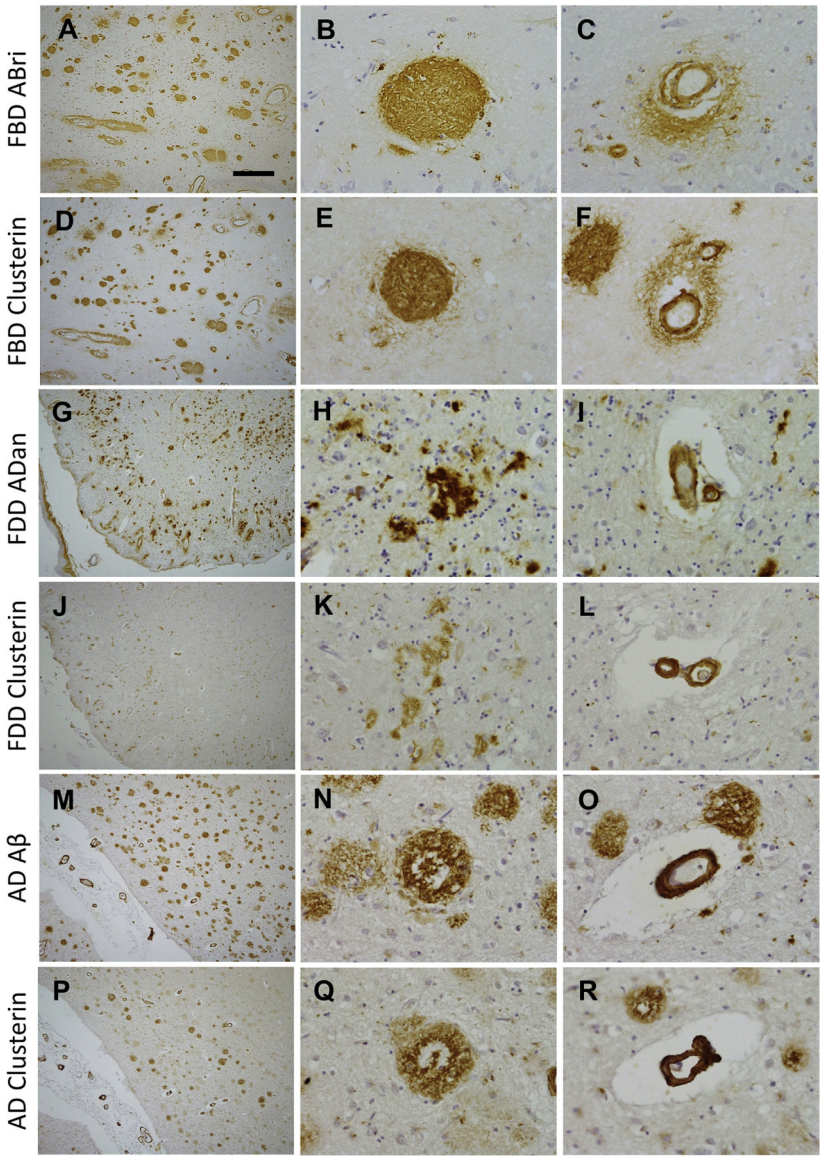

Figure 2: Immunohistochemical identification of clusterin and corresponding amyloid peptides in familial British dementia (FBD), familial Danish dementia (FDD), and Alzheimer's disease (AD) brain tissue.

In FBD (65 years old, female), ABri deposition (**A**) is found in parenchymal plaques (**B**) and cerebral amyloid angiopathy (CAA; **C**), which is mirrored by clusterin immunohistochemistry (**D–F**). In FDD (60 years old, female), parenchymal ADan deposits (**G**) are observed in the form of diffuse plaques (**H**) and CAA (**I**), which are also positively stained for clusterin (**J–L**). A similar immunostaining pattern in plaques and CAA is seen in AD (69 years old, female) for Aβ (**M–O**) and clusterin (**P–R**). Bar represents 500μm in **A**, **D**, **G**, **J**, **M**, and **P**; 50μm in all other images.

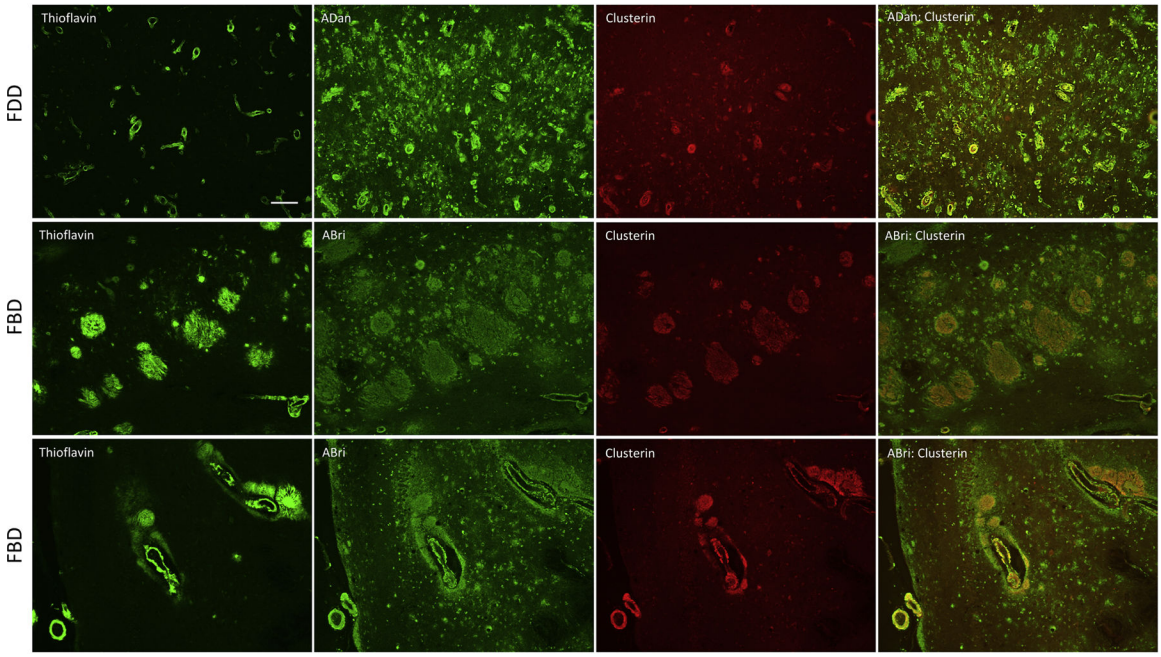

Figure 3: Thioflavin S and double immunofluorescence assessment of clusterin and the ABri/ADan amyloid molecules in FBD and FDD.

Sequential sections were stained with Thioflavin S and a combination of either ADan/Clusterin or ABri/Clusterin antibodies, to allow the visualization of the amyloid and preamyloid lesions alongside clusterin deposition. In FDD thioflavin positivity was mainly confined to the CAA, whereas ADan was present in both CAA and parenchymal preamyloid deposits. Clusterin immunoreactivity in FDD, mirrored the thioflavin staining with the majority of the clusterin being found colocalizing with the vascular amyloid deposits. In FBD large amyloid plaques and CAA were observed with the thioflavin staining, whereas amyloid plaques and the preamyloid deposits were highlighted by the ABri antibody. Clusterin also mirrored the thioflavin staining in FBD, with the majority of the immunoreactivity being found in the large amyloid plaques and CAA. Bar represents 50μm in all panels.

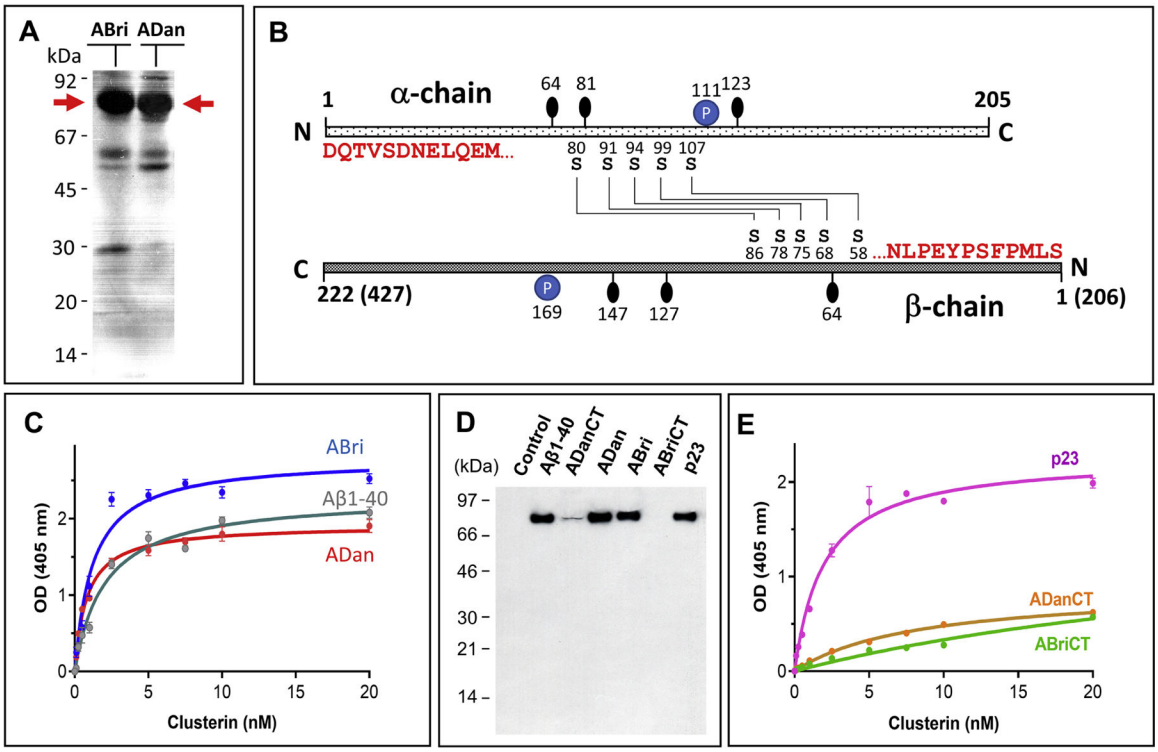

Figure 4: Identification of clusterin as a major ABri- and ADan-binding protein.

A) Pooled normal human plasma was incubated with ABri- and ADan affinity matrices followed by low-pH elution, electrophoretic separation of bound proteins, electro-transference to PVDF membranes, and Coomassie Blue staining. The eluted fractions were enriched in a component of ~80 kDa that represented more than 95% of the bound material to both affinity matrices. **B)** Amino acid sequence analysis of the major ~80 kDa bands retrieved, in both cases, two equimolar sequences (red font) corresponding to the intact N-terminal of the α- and β-chains of clusterin. The location of the five disulfide bonds (S-S), six N-glycosylation (🍍) and two phosphorylation (●) sites within the clusterin heterodimer are illustrated; amino acids are indicated in one-letter code. **C)** Clusterin-ABri and Clusterin-ADan binding isotherms were assessed by ELISA. Microtiter wells were coated with ABri, ADan, and Aβ1-40 synthetic homologues and incubated with increasing concentrations of clusterin (0–20 nM). Bound clusterin was subsequently detected by incubation with monoclonal anti-clusterin antibodies followed by alkaline phosphatase-labeled goat F(ab')2 anti-mouse Immunoglobulins, further detection with p-nitrophenyl phosphate, and quantitation of Absorbance at 405 nm. Data represent the mean ± SD of three independent experiments performed in duplicate. **D)** The binding site for clusterin in ABri and ADan amyloid molecules was localized by ligand affinity chromatography / Western Blot analysis. Pooled normal human plasma was incubated with separate affinity matrices containing the full-length ABri and ADan peptides, as well as synthetic homologues corresponding to their respective N- and C-terminal regions. Bound proteins, eluted with 0.1 M acetic acid, were separated by 10% SDS-PAGE, and electrotransferred to PVDF. Membranes were probed for clusterin with mouse monoclonal anti-clusterin antibodies followed by HRP-conjugated anti-mouse immunoglobulins and

subsequent assessment by enhanced chemiluminescence. Plain Sepharose was used as a control for non-specific binding. Clusterin bound to Aβ1-40 containing affinity matrices containing is also shown for comparison. **E)** Binding isotherms to ABri/ADan fragments were evaluated by ELISA. Microtiter wells were coated with the N-terminal ABri/ADan peptide (p23) and the C-terminal fragments of both molecules, ABri24–34 and ADan23–34, at a concentration of 400 ng/0.1 ml/well. Wells were subsequently incubated with increasing clusterin concentrations (0–20 nM) and bound clusterin detected as in panel **C**. Data represent the mean ± SD of three independent experiments performed in duplicate. Binding data in **C** and **E** were analyzed by non-linear regression using GraphPad Prism (GraphPad Software).

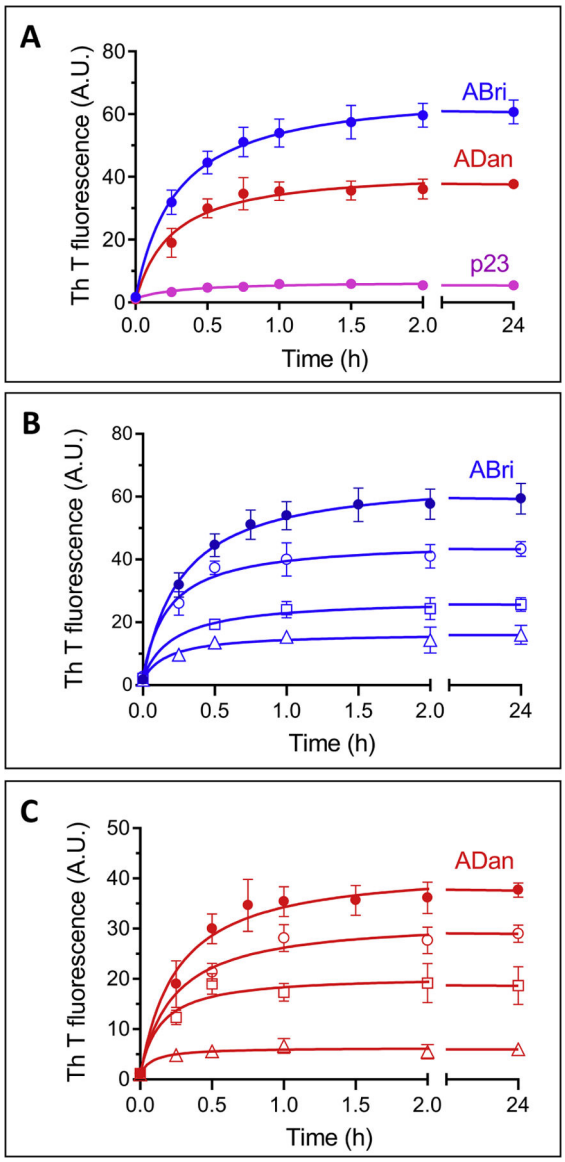

Figure 5: Aggregation/fibrillization of BRI2-related peptides and its modulation by clusterin. **(A)** Oligomerization/fibrillization of ABri, ADan, and Bri1–23 (p23) was assessed by fluorescence evaluation of thioflavin T binding to 50 μM peptide in 1× PBS. Fluorescence evaluation is expressed in arbitrary units (A.U.). **(B)** The effect of clusterin on the aggregation/fibrillization of ABri was evaluated assessing thioflavin T fluorescence signal after co-incubation of the amyloid peptide with the apolipoprotein at different clusterin to substrate molar ratios. ABri: full circles; CLU to ABri molar ratio of 1 to100: open circles; CLU to ABri molar ratio of 1 to 50: open squares; CLU to ABri molar ratio of 1 to10: open triangles. **(C)** Thioflavin T fluorescence signal of ADan in the absence and presence of clusterin at different apolipoprotein to substrate molar ratios. ADan: full circles; CLU to ADan molar ratio of 1 to100: open circles; CLU to ADan molar ratio of 1 to 50: open

squares; CLU to ADan molar ratio of 1 to10: open triangles. In all cases graphs illustrate mean ± SEM of three independent experiments after subtraction of blank levels.