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Clusterin levels are increased in Alzheimer's disease and influence the regional distribution of Aβ

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Short title: Clusterin influences the regional distribution of Aβ

Keywords

Alzheimer's disease, clusterin, apoJ, amyloid-β, amyloid-β clearance, plaque, cerebral amyloid angiopathy

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Abstract

Clusterin, also known as apoJ, is a lipoprotein abundantly expressed within the CNS. It regulates Aβ fibril formation and toxicity and facilitates amyloid-β (Aβ) transport across the blood-brain barrier. Genome-wide association studies have shown variations in the clusterin gene (*CLU*) to influence the risk of developing sporadic Alzheimer's disease (AD). To explore whether clusterin modulates the regional deposition of Aβ, we measured levels of soluble (NP40-extracted) and insoluble (guanidine-HCl-extracted) clusterin, Aβ40 and Aβ42 by sandwich ELISA in brain regions with a predilection for amyloid pathology – mid-frontal cortex (MF), cingulate cortex (CC), parahippocampal cortex (PH) – and regions with little or no pathology – thalamus (TH) and white matter (WM). Clusterin level was highest in regions with plaque pathology (MF, CC, PH and PC), approximately mirroring the regional distribution of Aβ. It was significantly higher in AD than controls, and correlated positively with Aβ42 and insoluble Aβ40. Soluble clusterin level rose significantly with severity of cerebral amyloid angiopathy (CAA), and in MF and PC regions was highest in *APOE* ε4 homozygotes. In the TH and WM (areas with little amyloid pathology) clusterin was unaltered in AD and did not correlate with Aβ level. There was a significant positive correlation between the concentration of clusterin and the regional levels of insoluble Aβ42; however, the molar ratio of clusterin:Aβ42 declined with insoluble Aβ42 level in a region-dependent manner, being lowest in regions with predilection for Aβ plaque pathology. Under physiological conditions clusterin reduces aggregation and promotes clearance of Aβ. Our findings indicate that in AD, clusterin increases, particularly in regions with most

abundant Aβ, but because the increase does not match the rising level of Aβ42, the molar ratio of clusterin:Aβ42 in those regions falls, probably contributing to Aβ deposition within the tissue.

Introduction

Alzheimer's disease (AD) is believed to be initiated by the accumulation and aggregation of amyloid-β (Aβ) peptides (the so-called amyloid cascade hypothesis (1)). The steady-state level of Aβ reflects the balance between its production and removal from the brain (2). Aβ peptides are produced by sequential cleavage of amyloid-β protein precursor (APP) and mostly end at amino acid 40 or 42. Aβ42 is the more amyloidogenic form – relatively insoluble in the interstitial fluid and prone to parenchymal deposition. Aβ40 is more soluble, less prone to parenchymal deposition but more likely to accumulate in the walls of cortical and leptomeningeal blood vessels (3, 4). Most mutations in familial AD are associated with increased amyloidogenic processing of APP and elevated Aβ42 or an increase in the Aβ42:Aβ40 ratio (5-8). In sporadic AD, which accounts for most cases, the accumulation of Aβ is thought largely to reflect alterations in the pathways responsible for the removal of Aβ (reviewed in (2)) or altered expression of chaperone proteins, such as apoE and clusterin (also known as apoJ) that regulate the structure, toxicity, and clearance of Aβ (reviewed in (9)).

Aβ peptides are produced throughout life (10, 11) but begin to accumulate and aggregate in the brain more than a decade before the onset of AD

(12, 13). Risk factors for AD, such as ageing and *APOE* genotype (14), accelerate the parenchymal deposition of Aβ. The deposition of Aβ within the brain follows a hierarchical sequence first appearing in the neocortex and spreading to limbic areas, deep cerebral grey matter and brain stem regions and finally the cerebellum (15). The determinants of regional variability in the susceptibility of different brain regions to Aβ deposition remain unclear. No link was found between the distribution of plaque pathology and the regional distribution of enzymes involved in the amyloidogenic processing of APP (APP, APP-CRFB, BACE-1, PS-1) (16-19). However, Shinohara and (19) found a strong inverse relationship between apolipoprotein E (apoE) level and Aβ deposition in brain tissue from cognitively normal elderly people and those with mild cognitive impairment (MCI). The authors suggested that apoE had a role in preventing Aβ accumulation and was reduced in brain regions that would later develop significant plaque pathology.

Clusterin, also known as apolipoprotein-J, is a 78-80 kDa heterodimeric glycoprotein that is abundantly expressed in the CNS (20). Genome-wide association studies have identified several single nucleotide polymorphisms (SNP) within the clusterin gene (*CLU*) that are risk factors for AD (21-24). Clusterin is up-regulated in the brain in AD (25, 26) and is present in plaques (27, 28). *In vitro* studies suggest that clusterin influences Aβ fibril formation and neurotoxicity (reviewed in (29, 30)) and can facilitate the transport of Aβ across the blood-brain barrier (31). We have undertaken a comprehensive analysis of the regional distribution of clusterin, soluble and insoluble Aβ40 and Aβ42 in post-mortem brain tissue across a number of brain regions that vary in their

predilection to amyloid pathology. Our findings indicate that clusterin level rises with the accumulation of insoluble Aβ42 but the molar ratio of clusterin:Aβ42 falls, which probably influences the regional distribution of Aβ deposition.

Materials and Methods

Case selection

Brain tissue was obtained from the South West Dementia Brain Bank (SWDBB), University of Bristol, UK with local research ethics committee approval. All brains had been retrieved within 72 h of death. The right cerebral hemisphere had been fixed in 10% formalin for three weeks before the tissue was processed and paraffin blocks were taken for pathological assessment. The left cerebral hemisphere had been sliced and frozen at -80°C until used for biochemical assessment. According to the NIA-AA guidelines AD neuropathological change was considered an adequate explanation for the dementia in all cases in the AD group (32). Controls were defined by an absence of clinical history of cognitive decline or other neurological disease and a lack of neuropathological abnormalities apart from sparse neuritic or diffuse plaques in some of the older cases, all of which were of Braak tangle stage III or lower. *APOE* genotyping and assessment of severity of cerebral amyloid angiopathy (CAA) had been performed as previously reported (33, 34). Demographic information, neuropathological findings and MRC identifiers for each case are shown in supplementary Tables 1 and 2.β

Brain tissue

Brain tissue (200 mg) was dissected from the midfrontal, cingulate, parahippocampal and medial parietal cortex, thalamus (pulvinar) and white matter underlying the parietal cortex. Brain tissue samples were prepared using a Precellys 24 homogenizer (Stretton Scientific, Derbyshire, UK) with 2.3 mm ceramic beads (Biospec, Stratech, Suffolk, UK) as previously described for Aβ measurements in human post-mortem tissue (10, 11, 35, 36). Soluble and insoluble extracts were prepared sequentially following initial homogenization in 1% NP-40 buffer containing 140 mM NaCl, 3 mM KCl, 25 mM TRIS, 5 mM ethylenediaminetetraacetic acid (EDTA) and 2 mM 1,10 phenanthroline). The homogenates were spun at 13,000 × g for 15 min at 4°C and the supernatant was removed and stored at −80°C. Insoluble extracts were prepared by homogenisation of pelleted insoluble material in 6 M GuHCl and were left for 4 h at room temperature (RT) before storage at −80°C.

Measurement of clusterin levels by sandwich ELISA

Clusterin level was measured by sandwich ELISA (duoset kit # DY5874, R&D systems, Oxford, UK) according to the manufacturer's guidelines. 96-well Maxisorp plates (R&D systems, Oxford, UK) were coated at room temperature (RT) overnight with mouse anti-human clusterin. We washed the plates in phosphate-buffered saline (PBS)/tween-20 (0.01%), added 1% PBS/BSA at room temperature for 1 h to block non-specific binding, then added recombinant human clusterin (62.5-4,000 pg/ml) and tissue homogenates (2.5 μl supernatant diluted in 3.125 ml PBS, and 1.8 μl insoluble extract diluted in 10 ml PBS) for 2 h

at room temperature. After a further wash, the plate was incubated for 2 h at room temperature with biotinylated mouse anti-human clusterin. The plate was again washed and incubated with streptavidin-horseradish peroxidase (HRP) (1:200 in 0.01% PBS:Tween-20) for 20 min at RT in the dark, washed, and incubated for 10 min with chromogenic substrate (TMBS, R&D systems, Oxford, UK). Absorbance was read at 450 nM in a FLUOstar Optima plate reader (BMG Labtech, Ayelsbury, UK) after the addition of 50 μl of 2 N sulphuric acid. Measurements were repeated in duplicate and across two plates to ensure that there was minimal plate-to-plate variation.

Measurement of Aβ40 and Aβ42 by sandwich ELISA

We measured Aβ40 and Aβ42 level in both soluble (NP1-40) and insoluble (guanidine-HCl-extracted) brain tissue fractions by sandwich ELISA as previously described (10, 11, 34-39). For the Aβ40 ELISA, mouse monoclonal anti-human Aβ (clone 6E10, raised against amino acids 1-16; Covance, Harrogate, UK), 2 μg/ml in PBS, was incubated overnight at RT, washed and then blocked with 300 μL protein-free PBS blocking buffer (Thermo Fisher Scientific, Loughborough, UK) for 2 h at RT. Samples of brain homogenate (diluted 1:49 for guanidine extracts and 1:3 for soluble extracts) or recombinant human Aβ1-40 (Sigma Aldrich, Dorset, UK) diluted in PBS containing 1% 1,10 phenanthroline (Sigma Aldrich, Dorset, UK) to prevent degradation of Aβ (40), were incubated for 2 h at RT on a rocking platform. After a further wash, the plates were incubated for 2 h at RT with mouse anti-human Aβ1-40 (1 μg/ml) (11A50-B10; Covance, Harrogate, UK) that had been biotinylated using Lightning-Link Biotinylation Kit (Innova Biosciences, Cambridge, UK) according to the

manufacturer's guidelines. Streptavidin-HRP (R&D Systems Europe, Abingdon, UK) diluted 1:200 was added to each well for 20 min at RT before they were washed and substrate solution (TMB; R&D Systems Europe, Abingdon, UK) was added for 30 min in the dark. The reaction was stopped with 2N sulphuric acid (R&D Systems Europe, Abingdon, UK) and the optical density of each well read at 450 nm in a FLUOstar plate reader (BMG Labtech, Aylesbury, UK).

For the Aβ42 ELISA, anti-human Aβ1-42 (12 F4, Covance) diluted 0.5 μg/ml in PBS was used as the capture antibody. Tissue samples (insoluble extracts diluted 1:9, soluble extracts diluted 1:3) were incubated at RT for 4 h. Biotinylated anti-human Aβ (Thermo Fisher Scientific) diluted to 0.1 μg/ml in PBS was used for detection and left overnight at 4°C. After washing, streptavidin-HRP was added for 1 h and chromogenic substrate for 20 min in the dark after a further wash. Aβ1-42 concentration in brain tissue was interpolated from a standard curve generated by serial dilution (16,000 to 1.024 nM) of recombinant human Aβ1–42 (Sigma Aldrich). Each sample was assayed in duplicate. The Aβ1-42 ELISA did not detect Aβ1-40, and the Aβ1-40 ELISA did not detect Aβ1-42.

Statistical analysis

Unpaired two-tailed t-test or ANOVA with Dunnett's post-hoc analysis was used for comparisons between groups, and Pearson's or Spearman's test was used to assess linear or rank order correlation, as appropriate, with the help of SPSS version 16 (SPSS, Chicago) and GraphPad Prism version 6 (GraphPad Software, La Jolla, CA). P-values < 0.05 were considered statistically significant.

Results

Regional distribution of soluble and insoluble Aβ40 and Aβ42 in AD

We examined the regional distribution of soluble (NP-40-soluble) and insoluble (after guanidine-HCl extraction) Aβ40 and Aβ42 in sequentially extracted brain homogenates from the following regions in AD and age-matched controls: midfrontal cortex (MF), cingulate cortex (CC), parahippocampal cortex (PH), medial parietal cortex (PC), thalamus (TH) and parietal white matter (WM) (Fig. 1). In CC, MF, PH and PC, the concentrations of insoluble Aβ40 and Aβ42 were significantly higher in AD than age-matched controls. The differences between AD and control brains in TH and WM did not reach significance.

In both control and AD groups, the concentration of insoluble Aβ40 and Aβ42 tended to decrease in the following order: CC > MF > PH > PC > TH and WM (Fig. 1A and 1B). In AD, the concentration of insoluble Aβ40 and Aβ42 was significantly higher in neocortex (MF and CC) than in other regions (Fig. 1A and Fig. 1B).

The regional distribution of soluble Aβ40 and Aβ42 differed substantially from that of insoluble Aβ40 and Aβ42. The concentration of the soluble forms of Aβ was lowest in MF and CC and tended to be higher in PC, PH, TH and WM (Fig. 1C and Fig. 1D). Soluble Aβ level did not differ to a statistically significant extent between AD and controls, with the exception of increased Aβ42 in AD within WM. In general the concentration of Aβ in grey matter regions was much lower in the soluble than the insoluble tissue fractions. In contrast, Aβ40 and Aβ42 concentration was higher in the soluble than the insoluble tissue fractions of

WM, and soluble Aβ level was several-fold higher in WM than cortex. Soluble Aβ42 level was also relatively high in PH.

The relative contribution of soluble and insoluble Aβ40 and Aβ42 to 'total' Aβ load in all regions studied is shown in supplementary Figure 1. 'Total' Aβ was highest in MF and CC and tended to decrease in PH > PC > TH. Total Aβ in MF and CC consisted almost entirely of insoluble Aβ40 and Aβ42. In contrast, in WM, most of the Aβ consisted of soluble Aβ40 with a small amount of insoluble Aβ40 and negligible Aβ42.

Regional distribution of clusterin in AD

We examined the regional distribution of clusterin in the same soluble and insoluble brain fractions in AD and age-matched controls that we had used to measure Aβ levels (Fig. 2). In AD, clusterin level within both the soluble and insoluble extracts was highest in CC, MF and PH and PC and lowest in the TH and WM (approximately mirroring the regional distribution of 'total' Aβ). The level of soluble clusterin was less variable between regions in the controls but was significantly higher in CC than PC or TH (Fig. 2A). Clusterin level in the insoluble extract was significantly higher in all grey matter regions than in the WM (Fig. 2A).

The level of soluble clusterin was significantly higher in AD than controls in most regions (CC, MF, PH and PC) (Fig. 2A) and was increased in the insoluble extract in MF and PH (Fig. 2B). Clusterin level within the soluble and insoluble extracts correlated significantly with soluble and insoluble Aβ42 and Aβ40 in MF

(with the exception of insoluble Aβ42) (Table 1). A similar trend was observed between clusterin and Aβ levels within the soluble extract in the PC but not the insoluble extract. A strong correlation was observed in the CC between clusterin and soluble and insoluble Aβ40. There was less correlation between clusterin and Aβ in the TH and WM (Table 1).

Regional association of clusterin and Aβ

To assess whether variations in clusterin concentration might influence the regional distribution of soluble and insoluble Aβ40 or Aβ42, we looked at the correlation between Aβ level and clusterin concentration across all regions. There was a significant correlation between the concentration of soluble clusterin and the level of insoluble Aβ42 within the AD cohort and a weaker, non-significant trend in the controls (Fig. 3 A-B). A trend approaching significance was observed between clusterin in the insoluble extract and insoluble Aβ42 in both controls and AD (Fig. 3C-D). We did not find significant correlations between clusterin concentration and the level of soluble Aβ42, soluble Aβ40 or insoluble Aβ40.

To investigate further whether clusterin might promote the accumulation of insoluble Aβ42, we calculated the molar ratio of insoluble clusterin to insoluble Aβ42 in the different regions. In both controls and AD, the molar ratio of insoluble clusterin:insoluble Aβ42 was lowest in regions with the highest concentration of insoluble Aβ42 (Fig. 4A-B) and *vice versa*.

Clusterin levels influenced by APOE genotype

Clusterin level was highest in *APOE* ε4 homozygotes in MF and PC (Figure 5) and rose significantly with severity of CAA. Post-hoc analysis revealed significantly higher clusterin level in ε4/4 than ε3/3 brains in PC ($P < 0.05$), and in ε4/4 than ε3/4 ($P < 0.01$) or ε3/3 ($P < 0.05$) in MF. Post-hoc analysis also showed clusterin level in MF and PC to be significantly higher in brains with severe than absent CAA ($P < 0.05$ for both regions). Insoluble clusterin level did not vary significantly in relation to *APOE* genotype or CAA severity.

Discussion

We have examined the relationship between clusterin/apoJ level and the regional distribution of Aβ within the brain. Although the concentration of clusterin was elevated in AD and was highest in cortical regions with the most abundant Aβ deposition, the molar ratio of clusterin:Aβ was lowest in those regions and was highest in parts of the brain with little or no amyloid pathology, such as in the thalamus and white matter. These findings in human brain tissue support experimental studies indicating that (i) clusterin level rises in association with increasing Aβ, (ii) within the physiological range of clusterin:Aβ, clusterin reduces aggregation and promotes clearance of Aβ, but (iii) when, despite a rise in clusterin, Aβ level increases to an extent that causes clusterin:Aβ to fall below the physiological range, Aβ-clusterin complexes tend to aggregate and deposit within the brain parenchyma. We have also shown that clusterin

concentration is influenced by *APOE* genotyope, being highest in brain tissue from ε4 homozygotes, and rises in relation to the severity of CAA.

Clusterin is highly expressed in the CNS, within which it is present at a similar concentration to that of apoE (20). Variations in the clusterin gene (*CLU*) are associated with sporadic AD (21), and previous studies showed that clusterin is increased in the CSF (41, 42) and brain tissue (25, 26) in AD. Clusterin is detectable immunohistochemically within plaques (27, 28) and increases in association with neuritic plaque density (43-45). In a transgenic APP/PS1 mouse model, clusterin level was elevated in plasma and brain tissue and found to co-localise with amyloid plaques (42). Present findings show that the concentration of clusterin in human brain tissue in AD is highest in regions with the greatest concentration of Aβ. Within those regions, clusterin concentration correlates closely with Aβ level (as was also shown in transgenic mouse models of AD expressing mutant APP (46)). These findings are in keeping with clinical evidence of a correlation between raised plasma clusterin level and accelerated clinical progression of disease (41, 42), and imaging studies showing that increased plasma clusterin levels were a strong predictor of brain amyloid load in AD patients (42). Within sub-cortical regions that have a much lower level of Aβ, clusterin concentration does not differ significantly between AD and controls.

In vitro studies indicate that clusterin binds to Aβ and influences both fibril formation and toxicity (47, 48). Yerbury and colleagues (49) reported that clusterin co-precipitates with Aβ as insoluble aggregates when Aβ is present in large molar excess. We have found the molar ratio of clusterin:Aβ42 to be lowest

in regions that have the greatest accumulation of Aβ (almost entirely in the insoluble tissue fraction) even though clusterin levels are highest in those regions. In contrast, within the white matter and thalamus, which had the highest ratio of clusterin:Aβ42, Aβ was almost all in a soluble form. It seems that when the clusterin:Aβ ratio falls low enough, clusterin actually promotes rather than simply fails to prevent the precipitation of Aβ, as evidenced by *in vitro* data (49). These data are consistent with experimental evidence in PDAPP mice (homozygous for the APP^{V717F} transgene) showing that clusterin stimulates amyloid aggregation when Aβ is present in excess. PDAPP mice homozygous for knock-out of the clusterin gene have significantly fewer fibrillar Aβ deposits and dystrophic neurites than PDAPP mice expressing clusterin (50). The rise in clusterin concentration that occurs with increasing Aβ seems likely to be a consequence of the latter. Thamsbietty et al. (42) reported that plasma clusterin level was elevated almost 10 years in advance of fibrillary Aβ deposition, suggesting that clusterin production is raised at an early stage in the disease process, although we know from other studies that Aβ starts to accumulate even earlier (51, 52).

ApoE is also highly expressed within the CNS and has been implicated in the pathogenesis of AD. The *APOE* gene is a strong risk factor for sporadic AD and individuals possessing the ε4 allele have more abundant plaque and cerebrovascular deposition of Aβ and a higher level of this peptide (33, 34, 53). *In vitro* studies demonstrated that apoE interacts with and influences Aβ fibrillogenesis and clearance (54-58). However, apoE and clusterin play somewhat divergent roles in the progression of AD. In contrast to clusterin, apoE

concentration shows a strong inverse correlation with regional Aβ load (19), and while fibril formation was reduced in clusterin-deficient PDAPP mice (50) it was significantly increased in PDAPP mice deficient in both clusterin and apoE (59). It is of interest that no regional association was found between Aβ level and molecules involved in APP processing (APP, APP-CTFβ, BACE-1 or PS-1) or enzymes involved in Aβ clearance (neprilysin and insulin-degrading enzyme). Together, these data suggest that the regional distribution of Aβ is influenced to a greater extent by apoE and clusterin expression than by pathways involved in the production or enzymatic degradation of Aβ.

Clusterin has also been shown to facilitate the clearance of Aβ at the blood-brain barrier (31) and is localised not only to plaques but also arterioles and capillaries within the brain (43-45). A recent immunohistochemical study showed that clusterin was associated with vascular Aβ, particularly Aβ40, in CAA (60). Co-localisation of clusterin with perivascular Aβ deposits and our finding of increased clusterin level in relation to CAA severity is supportive of a role in the perivascular drainage of Aβ, which is impaired in CAA (61, 62). Craggs and colleagues (60) also reported increased clusterin in the frontal white matter in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), suggesting that clusterin may also accumulate as a consequence of failed perivascular drainage of interstitial fluid.

In conclusion, we have shown that clusterin level is elevated in AD in regions with a predilection for plaque deposition. Yet despite that elevation, the molar ratio of clusterin:Aβ is lowest in those same regions, which is likely to

influence the regional distribution of Aβ by promoting its aggregation and precipitation.

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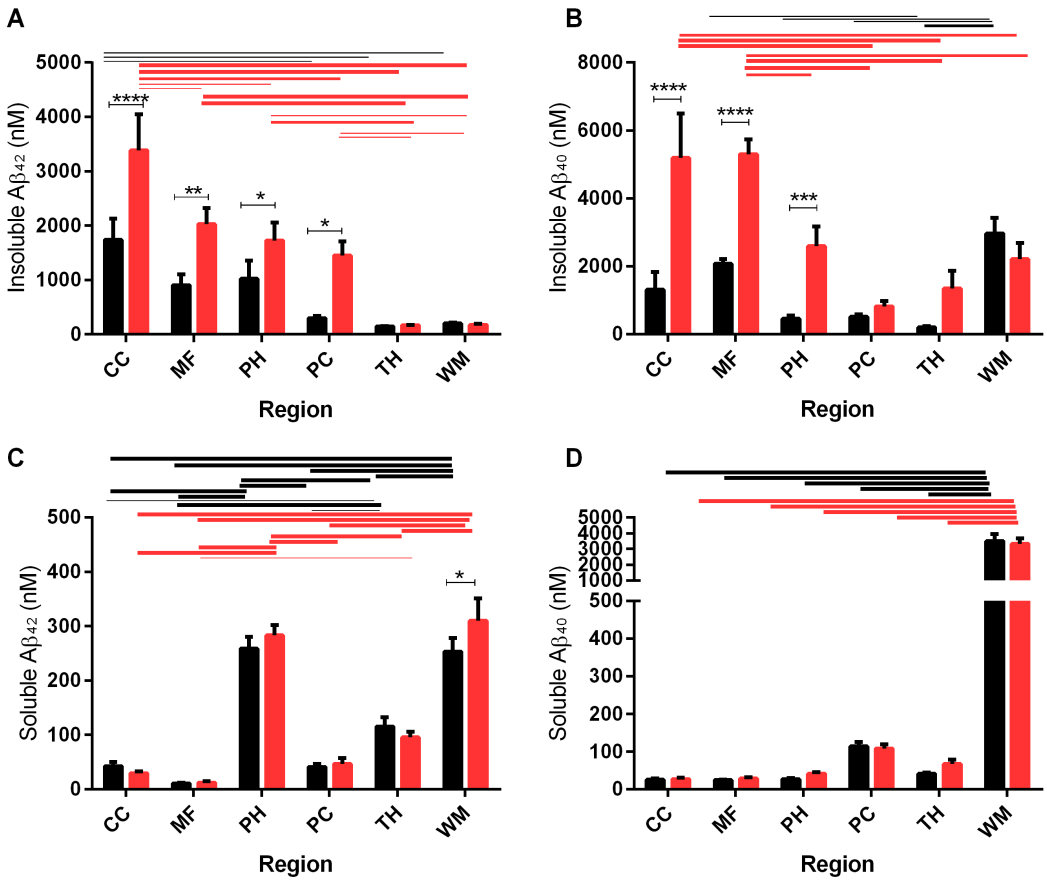


Figure 1. Bar charts showing regional levels of Aβ level in control (black bars) and AD brains (red bars), in mid-frontal cortex (MF), cingulate cortex (CC), parahippocampal cortex (PH), medial parietal cortex (PC), thalamus (TH) and parietal white matter (WM). Bars indicate the mean and SEM. *P < 0.05 **P < 0.01 ***P < 0.001 ****P < 0.0001. Lines indicate significant differences between regions, in the controls (black lines) and AD groups (red lines); the thickness of the line indicates the significance of the difference between the two regions, ranging from P < 0.01 to P < 0.0001.

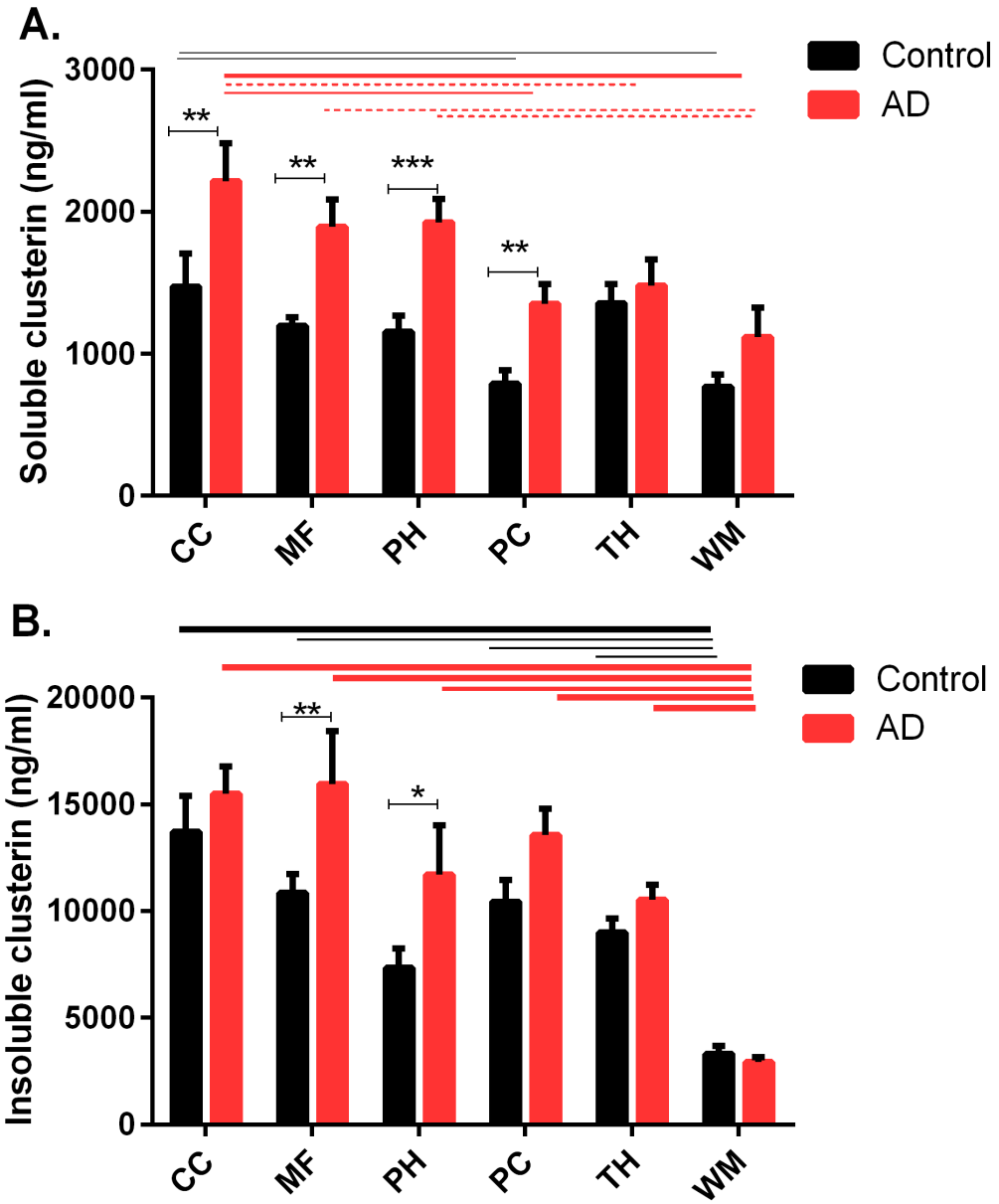


Figure 2. Bar chart showing regional levels of clusterin level in control (black bars) and AD brains (red bars), in the soluble and insoluble brain tissue fractions. Bars indicate the mean and SEM. *P < 0.05 **P < 0.01 ***P < 0.001. Lines indicate significant differences between regions, in the controls (black lines) and AD groups (red lines); the thickness of the solid lines indicates the significance of the difference between the two regions, ranging from P < 0.01 to P < 0.0001. The interrupted horizontal lines indicate differences significant at the P < 0.5 level.

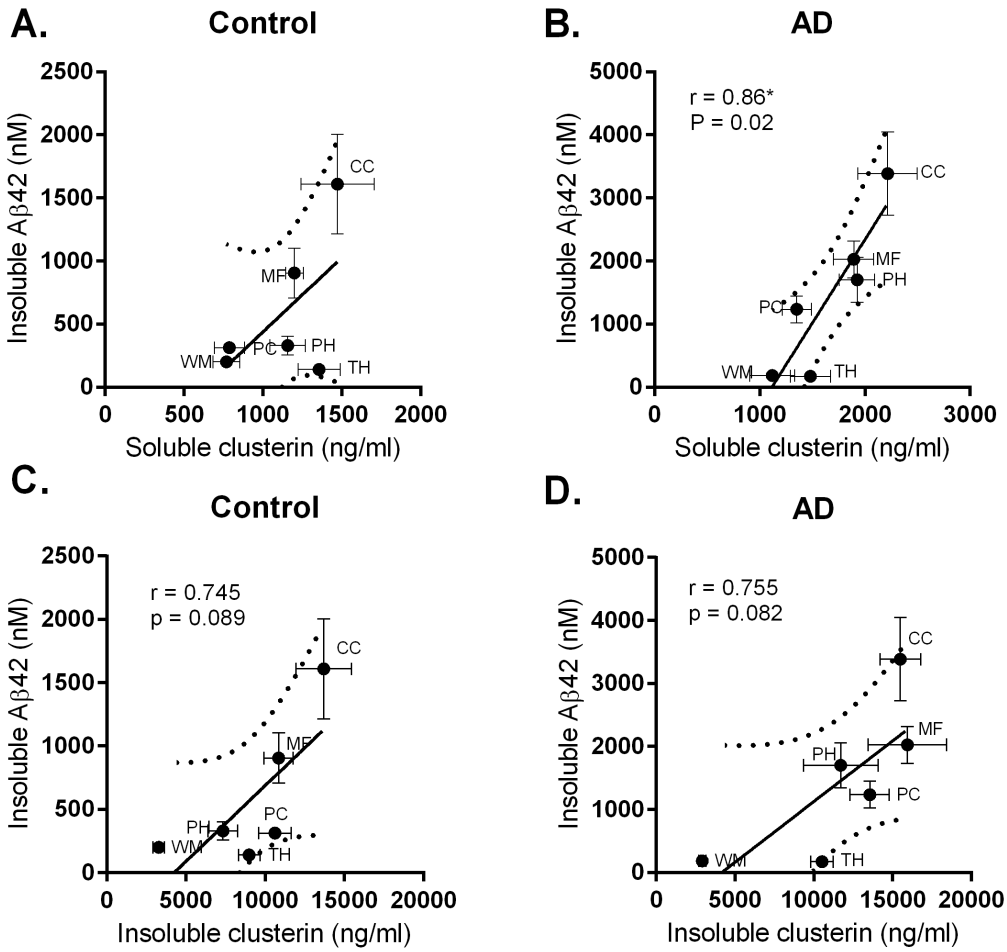


Figure 3. Regional association between clusterin and insoluble Aβ42 levels. The concentration of clusterin in soluble (A-B) and insoluble (C-D) brain tissue fractions was plotted against insoluble Aβ42 level in each region in controls and AD cases. The solid circles and thin bars indicate the mean values and SEM for clusterin (horizontal bars) and Aβ42 (vertical bars). The thick solid and dotted lines indicate the best-fit linear regression and 95% confidence intervals.

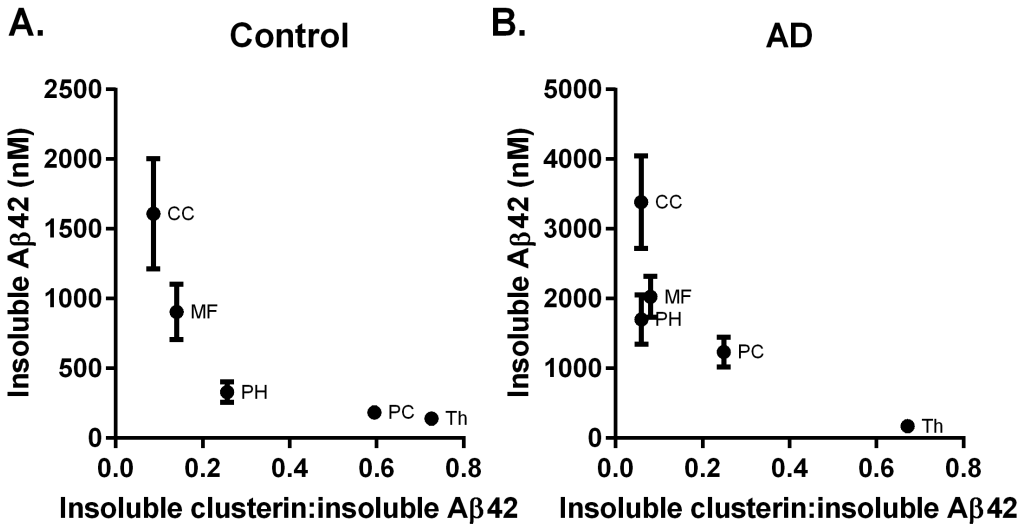


Figure 4. The ratio of clusterin:insoluble Aβ42 was lowest in regions with a predilection for Aβ42 deposition. The solid circles and bars indicate the mean values and SEM.

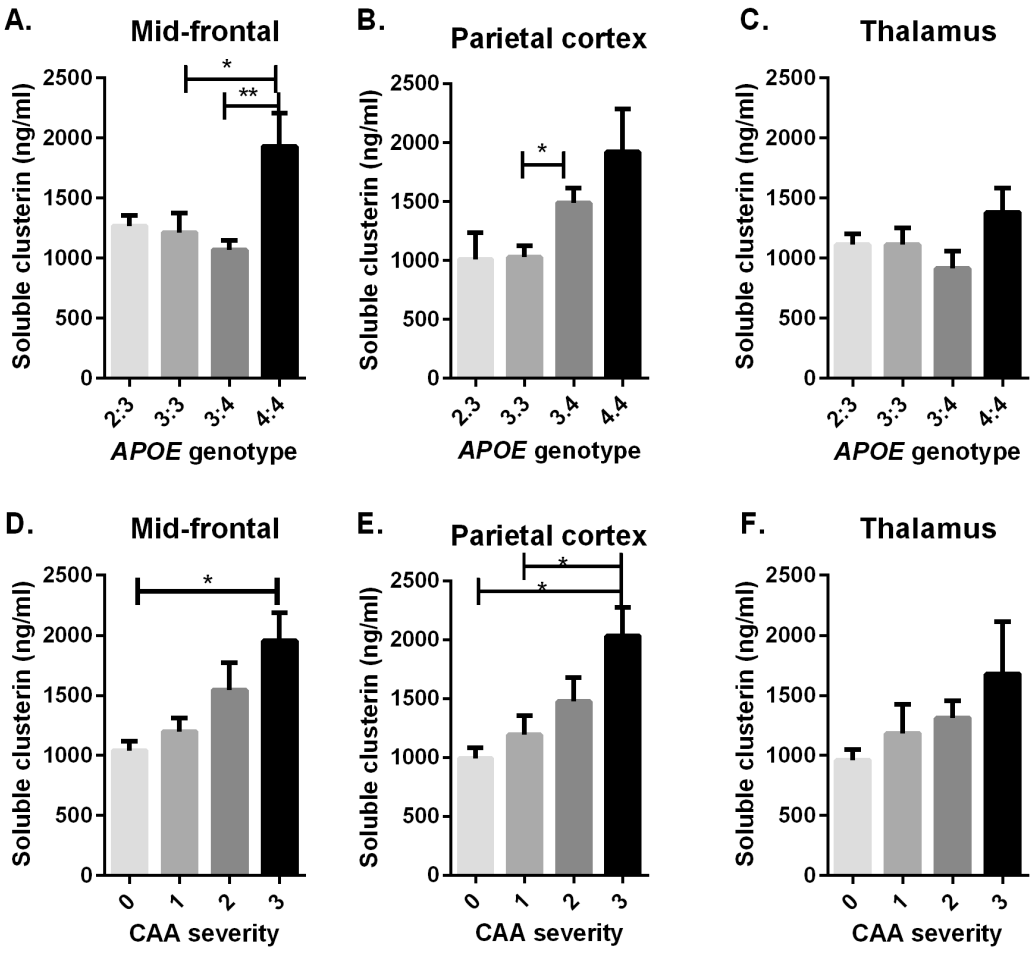
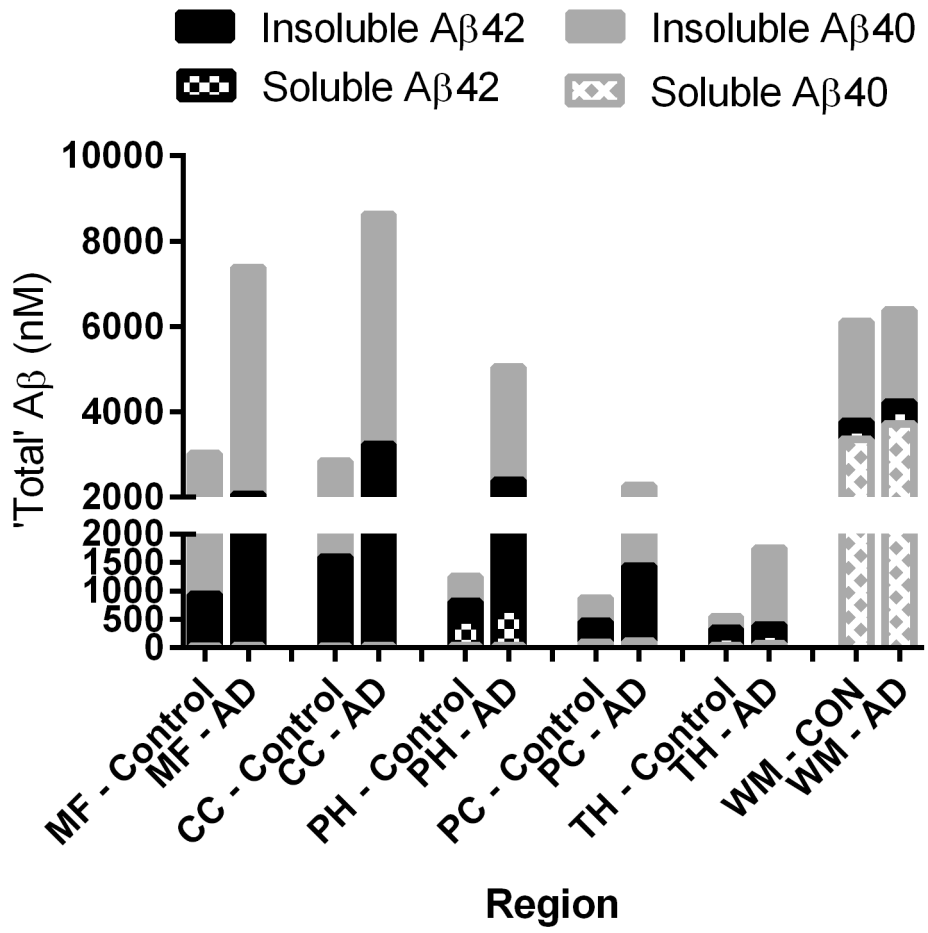


Figure 5. Bar charts showing clusterin level in relation to APOE genotype (A-C) and severity of CAA. Bars indicate the mean and SEM. *P < 0.05 **P < 0.01. Clusterin level was highest in *APOE* ε4 homozygotes in MF and PC and increased with severity of CAA.



Supplementary Figure 1. Stacked bar chart illustrating regional differences in the relative contributions of soluble and insoluble Aβ40 and Aβ42 to 'total' Aβ load in AD and control brains in MF, CC, PH, PC, TH and WM.