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

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Short title: Clusterin influences the regional distribution of A β

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 A β fibril formation and toxicity and facilitates amyloid- β (A β) 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 A β , we measured levels of soluble (NP40-extracted) and insoluble (guanidine-HCl-extracted) clusterin, A β 40 and A β 42 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 A β . It was significantly higher in AD than controls, and correlated positively with A β 42 and insoluble A β 40. Soluble clusterin level rose significantly with severity of cerebral amyloid angiopathy (CAA), and in MF and PC regions was highest in *APOE* ϵ 4 homozygotes. In the TH and WM (areas with little amyloid pathology) clusterin was unaltered in AD and did not correlate with A β level. There was a significant positive correlation between the concentration of clusterin and the regional levels of insoluble A β 42; however, the molar ratio of clusterin:A β 42 declined with insoluble A β 42 level in a region-dependent manner, being lowest in regions with predilection for A β plaque pathology. Under physiological conditions clusterin reduces aggregation and promotes clearance of A β . Our findings indicate that in AD, clusterin increases, particularly in regions with most

abundant A β , but because the increase does not match the rising level of A β 42, the molar ratio of clusterin:A β 42 in those regions falls, probably contributing to A β deposition within the tissue.

Introduction

Alzheimer's disease (AD) is believed to be initiated by the accumulation and aggregation of amyloid- β (A β) peptides (the so-called amyloid cascade hypothesis (1)). The steady-state level of A β reflects the balance between its production and removal from the brain (2). A β peptides are produced by sequential cleavage of amyloid- β protein precursor (APP) and mostly end at amino acid 40 or 42. A β 42 is the more amyloidogenic form – relatively insoluble in the interstitial fluid and prone to parenchymal deposition. A β 40 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 A β 42 or an increase in the A β 42:A β 40 ratio (5-8). In sporadic AD, which accounts for most cases, the accumulation of A β is thought largely to reflect alterations in the pathways responsible for the removal of A β (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 A β (reviewed in (9)).

A β 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 A β . The deposition of A β 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 A β 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 A β 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 A β 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 A β fibril formation and neurotoxicity (reviewed in (29, 30)) and can facilitate the transport of A β across the blood-brain barrier (31). We have undertaken a comprehensive analysis of the regional distribution of clusterin, soluble and insoluble A β 40 and A β 42 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 A β 42 but the molar ratio of clusterin:A β 42 falls, which probably influences the regional distribution of A β 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.

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 A β 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 \times 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 μ l supernatant diluted in 3.125 ml PBS, and 1.8 μ l 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 μ l 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 A β 40 and A β 42 by sandwich ELISA

We measured A β 40 and A β 42 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 A β 40 ELISA, mouse monoclonal anti-human A β (clone 6E10, raised against amino acids 1-16; Covance, Harrogate, UK), 2 μ g/ml in PBS, was incubated overnight at RT, washed and then blocked with 300 μ L 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 A β 1-40 (Sigma Aldrich, Dorset, UK) diluted in PBS containing 1% 1,10 phenanthroline (Sigma Aldrich, Dorset, UK) to prevent degradation of A β (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 A β 1-40 (1 μ g/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 A β 42 ELISA, anti-human A β 1-42 (12 F4, Covance) diluted 0.5 μ g/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 A β (Thermo Fisher Scientific) diluted to 0.1 μ g/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. A β 1-42 concentration in brain tissue was interpolated from a standard curve generated by serial dilution (16,000 to 1.024 nM) of recombinant human A β 1-42 (Sigma Aldrich). Each sample was assayed in duplicate. The A β 1-42 ELISA did not detect A β 1-40, and the A β 1-40 ELISA did not detect A β 1-42.

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 A β 40 and A β 42 in AD

We examined the regional distribution of soluble (NP-40-soluble) and insoluble (after guanidine-HCl extraction) A β 40 and A β 42 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 A β 40 and A β 42 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 A β 40 and A β 42 tended to decrease in the following order: CC > MF > PH > PC > TH and WM (Fig. 1A and 1B). In AD, the concentration of insoluble A β 40 and A β 42 was significantly higher in neocortex (MF and CC) than in other regions (Fig. 1A and Fig. 1B).

The regional distribution of soluble A β 40 and A β 42 differed substantially from that of insoluble A β 40 and A β 42. The concentration of the soluble forms of A β was lowest in MF and CC and tended to be higher in PC, PH, TH and WM (Fig. 1C and Fig. 1D). Soluble A β level did not differ to a statistically significant extent between AD and controls, with the exception of increased A β 42 in AD within WM. In general the concentration of A β in grey matter regions was much lower in the soluble than the insoluble tissue fractions. In contrast, A β 40 and A β 42 concentration was higher in the soluble than the insoluble tissue fractions of

WM, and soluble A β level was several-fold higher in WM than cortex. Soluble A β 42 level was also relatively high in PH.

The relative contribution of soluble and insoluble A β 40 and A β 42 to 'total' A β load in all regions studied is shown in supplementary Figure 1. 'Total' A β was highest in MF and CC and tended to decrease in PH > PC > TH. Total A β in MF and CC consisted almost entirely of insoluble A β 40 and A β 42. In contrast, in WM, most of the A β consisted of soluble A β 40 with a small amount of insoluble A β 40 and negligible A β 42.

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 A β 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' A β). 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 A β 42 and A β 40 in MF

(with the exception of insoluble A β 42) (Table 1). A similar trend was observed between clusterin and A β 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 A β 40. There was less correlation between clusterin and A β in the TH and WM (Table 1).

Regional association of clusterin and A β

To assess whether variations in clusterin concentration might influence the regional distribution of soluble and insoluble A β 40 or A β 42, we looked at the correlation between A β level and clusterin concentration across all regions. There was a significant correlation between the concentration of soluble clusterin and the level of insoluble A β 42 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 A β 42 in both controls and AD (Fig. 3C-D). We did not find significant correlations between clusterin concentration and the level of soluble A β 42, soluble A β 40 or insoluble A β 40.

To investigate further whether clusterin might promote the accumulation of insoluble A β 42, we calculated the molar ratio of insoluble clusterin to insoluble A β 42 in the different regions. In both controls and AD, the molar ratio of insoluble clusterin:insoluble A β 42 was lowest in regions with the highest concentration of insoluble A β 42 (Fig. 4A-B) and *vice versa*.

Clusterin levels influenced by APOE genotype

Clusterin level was highest in *APOE* $\epsilon 4$ homozygotes in MF and PC (Figure 5) and rose significantly with severity of CAA. Post-hoc analysis revealed significantly higher clusterin level in $\epsilon 4/4$ than $\epsilon 3/3$ brains in PC ($P < 0.05$), and in $\epsilon 4/4$ than $\epsilon 3/4$ ($P < 0.01$) or $\epsilon 3/3$ ($P < 0.05$) 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 $A\beta$ within the brain. Although the concentration of clusterin was elevated in AD and was highest in cortical regions with the most abundant $A\beta$ deposition, the molar ratio of clusterin: $A\beta$ 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 $A\beta$, (ii) within the physiological range of clusterin: $A\beta$, clusterin reduces aggregation and promotes clearance of $A\beta$, but (iii) when, despite a rise in clusterin, $A\beta$ level increases to an extent that causes clusterin: $A\beta$ to fall below the physiological range, $A\beta$ -clusterin complexes tend to aggregate and deposit within the brain parenchyma. We have also shown that clusterin

concentration is influenced by *APOE* genotype, being highest in brain tissue from $\epsilon 4$ 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 $A\beta$. Within those regions, clusterin concentration correlates closely with $A\beta$ 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 $A\beta$, clusterin concentration does not differ significantly between AD and controls.

In vitro studies indicate that clusterin binds to $A\beta$ and influences both fibril formation and toxicity (47, 48). Yerbury and colleagues (49) reported that clusterin co-precipitates with $A\beta$ as insoluble aggregates when $A\beta$ is present in large molar excess. We have found the molar ratio of clusterin: $A\beta_{42}$ to be lowest

in regions that have the greatest accumulation of A β (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:A β 42, A β was almost all in a soluble form. It seems that when the clusterin:A β ratio falls low enough, clusterin actually promotes rather than simply fails to prevent the precipitation of A β , 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 A β is present in excess. PDAPP mice homozygous for knock-out of the clusterin gene have significantly fewer fibrillar A β deposits and dystrophic neurites than PDAPP mice expressing clusterin (50). The rise in clusterin concentration that occurs with increasing A β seems likely to be a consequence of the latter. Thamsbierty et al. (42) reported that plasma clusterin level was elevated almost 10 years in advance of fibrillary A β deposition, suggesting that clusterin production is raised at an early stage in the disease process, although we know from other studies that A β 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 ϵ 4 allele have more abundant plaque and cerebrovascular deposition of A β and a higher level of this peptide (33, 34, 53). *In vitro* studies demonstrated that apoE interacts with and influences A β 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 A β 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 A β level and molecules involved in APP processing (APP, APP-CTF β , BACE-1 or PS-1) or enzymes involved in A β clearance (neprilysin and insulin-degrading enzyme). Together, these data suggest that the regional distribution of A β is influenced to a greater extent by apoE and clusterin expression than by pathways involved in the production or enzymatic degradation of A β .

Clusterin has also been shown to facilitate the clearance of A β 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 A β , particularly A β 40, in CAA (60). Co-localisation of clusterin with perivascular A β deposits and our finding of increased clusterin level in relation to CAA severity is supportive of a role in the perivascular drainage of A β , 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:A β is lowest in those same regions, which is likely to

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

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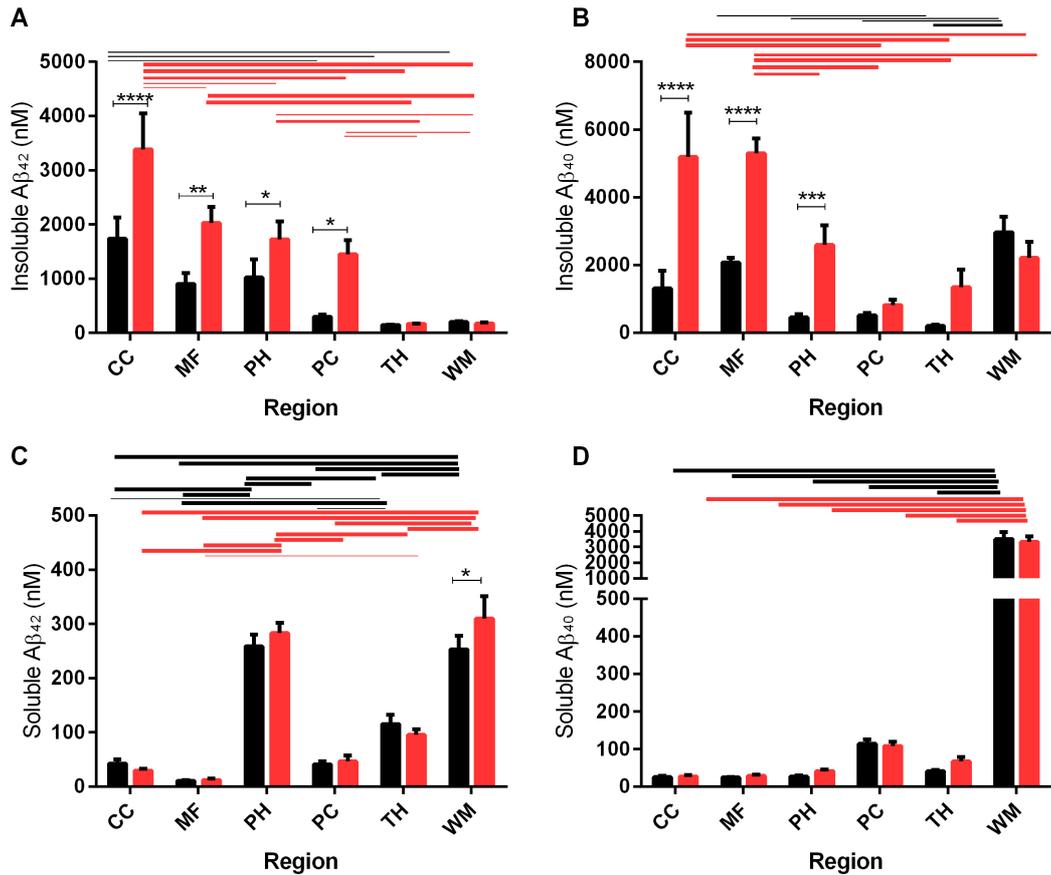


Figure 1. Bar charts showing regional levels of A β level in control (black bars) and AD brains (red bars), in mid-frontal cortex (MF), cingulate cortex (CC), parahippocampal cortex (PH), medial parietal cortex (PC), thalamus (TH) and parietal white matter (WM). Bars indicate the mean and SEM. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$ **** $P < 0.0001$. 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 $P < 0.0001$.

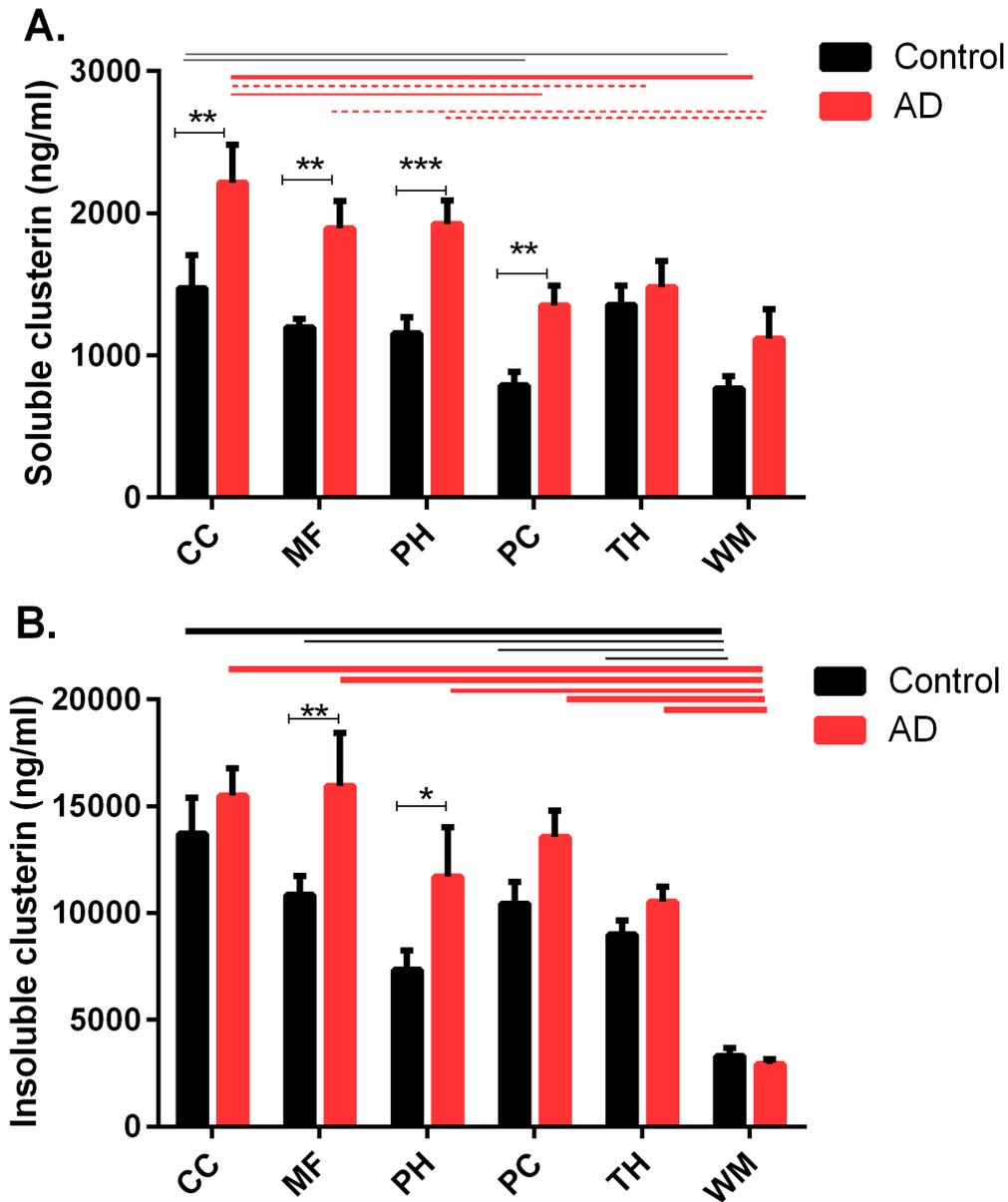


Figure 2. Bar chart showing regional levels of clusterin level in control (black bars) and AD brains (red bars), in the soluble and insoluble brain tissue fractions. Bars indicate the mean and SEM. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Lines indicate significant differences between regions, in 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.

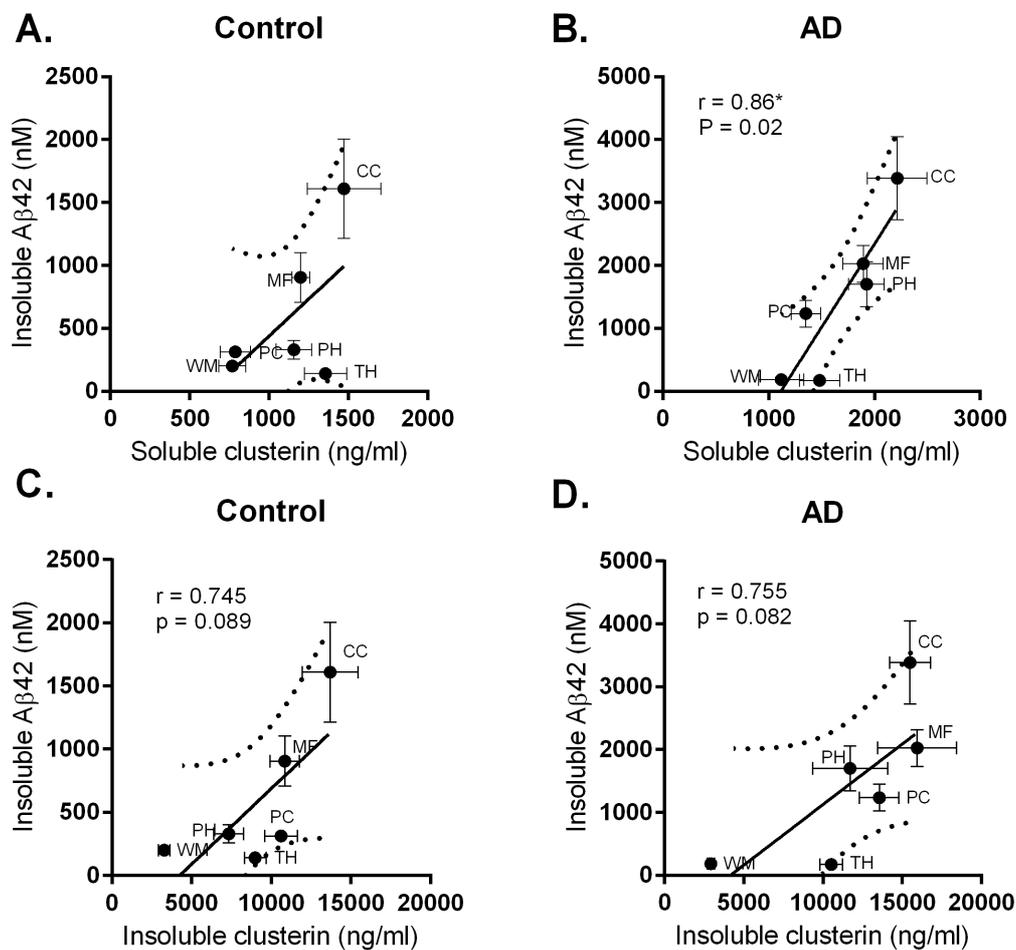


Figure 3. Regional association between clusterin and insoluble Aβ42 levels. The concentration of clusterin in soluble (A-B) and insoluble (C-D) brain tissue fractions was plotted against insoluble Aβ42 level in each region in controls and AD cases. The solid circles and thin bars indicate the mean values 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.

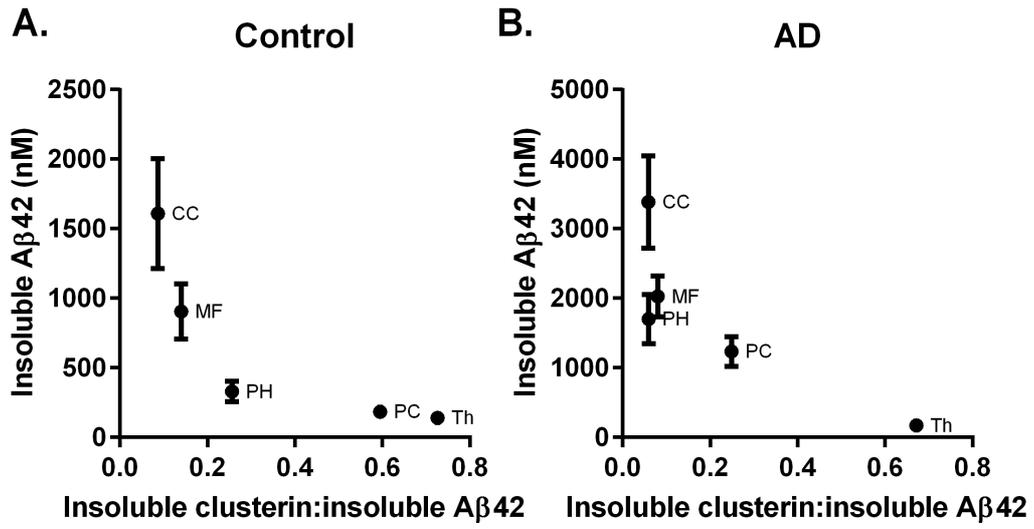


Figure 4. The ratio of clusterin:insoluble Aβ42 was lowest in regions with a predilection for Aβ42 deposition. The solid circles and bars indicate the mean values and SEM.

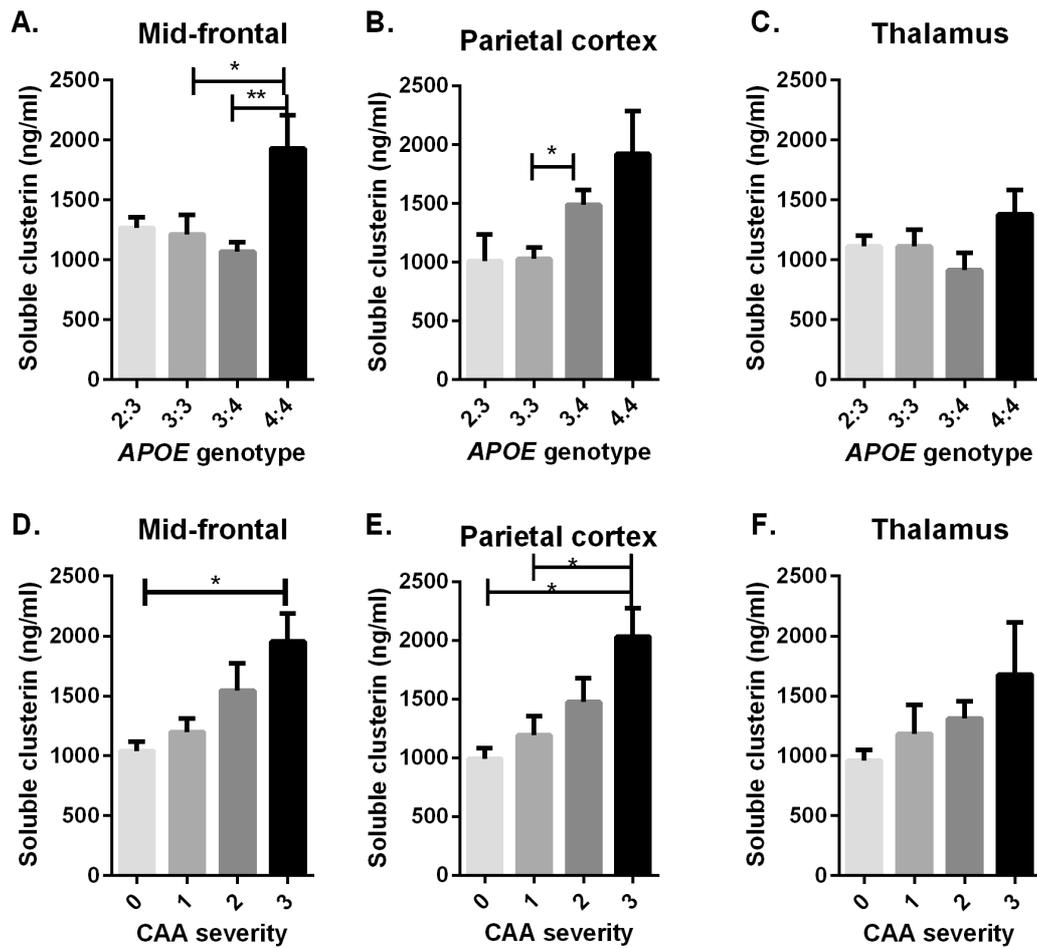
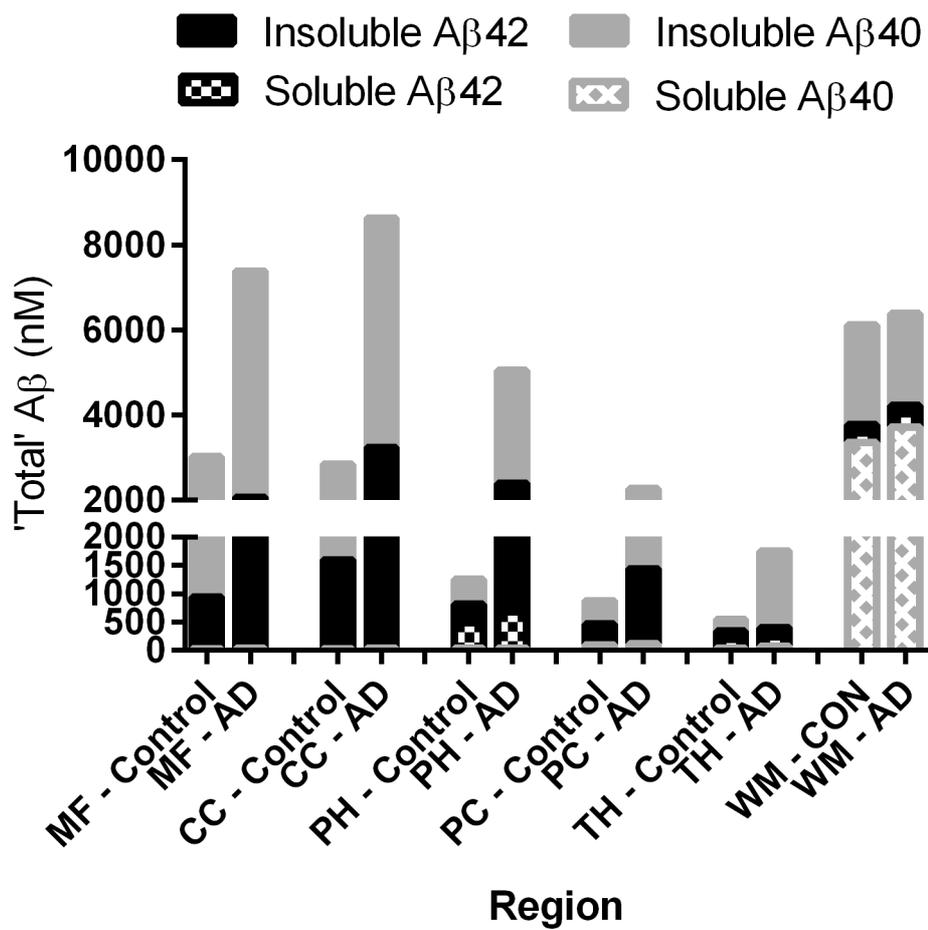


Figure 5. Bar charts showing clusterin level in relation to APOE genotype (A-C) and severity of CAA. Bars indicate the mean and SEM. * $P < 0.05$ ** $P < 0.01$.

Clusterin level was highest in *APOE* ϵ_4 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 A β 40 and A β 42 to 'total' A β load in AD and control brains in MF, CC, PH, PC, TH and WM.