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RESEARCH ARTICLE

Lysosomal cathepsin D is upregulated in Alzheimer's disease neocortex and may be a marker for neurofibrillary degeneration

Yuek Ling Chai[1], Joyce R. Chong[1,2], Jiaju Weng[1], David Howlett[3], Andrea Halsey[3], Jasinda H. Lee[1], Johannes Attems[4], Dag Aarsland[5], Paul T. Francis[3], Christopher P. Chen[1,2] and Mitchell K.P. Lai[1,2,3]

[1] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Kent Ridge, Singapore.
[2] Memory Aging and Cognition Centre, National University Health System, Kent Ridge, Singapore.
[3] Wolfson Centre for Age-Related Diseases, King's College London, London, UK.
[4] Institute of Neuroscience, Campus for Ageing and Vitality, Newcastle University, Newcastle upon Tyne, UK.
[5] Department NVS, Center for Alzheimer Research, Division for Neurogeriatrics, Karolinska Institute, Huddinge, Sweden.

Keywords
Alzheimer's disease, cathepsin D, Lewy body dementia, lysosome, neurofibrillary degeneration.

Abbreviations
AD, Alzheimer's disease; catD, cathepsin D; CIND, cognitive impaired no dementia; CNS, central nervous system; DLB, dementia with Lewy bodies; NCI, non-cognitively impaired participants; NFT, neurofibrillary tangles; NINCDS-ADRDA, National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association; PDD, Parkinson's disease dementia; AP, amyloid plaques.

Corresponding author:
Mitchell K.P. Lai, PhD, Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Unit 09-01, Centre for Translational Medicine (MD6), 14 Medical Drive, Kent Ridge, Singapore 117599 (E-mail: *mitchell.lai@dementia-research.org*)

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Abstract

Alzheimer's disease (AD) is characterized by accumulation of β-amyloid plaques (AP) and neurofibrillary tangles (NFT) in the cortex, together with synaptic loss and amyloid angiopathy. Perturbations in the brain lysosomal system, including the cathepsin family of proteases, have been implicated in AD where they may be involved in proteolytic clearance of misfolded and abnormally aggregated peptides. However, the status of cathepsin D (catD) is unclear in Lewy body dementia, the second most common form of neurodegenerative dementia after AD, and characterized by Lewy bodies (LB) containing aggregated α-synuclein. Furthermore, earlier reports of catD changes in AD have not been entirely consistent. We measured CatD immunoreactivities in the temporal (Brodmann area BA21) and parietal (BA40) cortices of well characterized AD brains as well as two clinical subtypes of Lewy body dementia, namely Parkinson disease dementia (PDD) and dementia with Lewy bodies (DLB), known to show varying degrees of concomitant AD pathology. Increased catD immunoreactivities in AD were found for both neocortical regions measured, where they also correlated with neuropathological NFT scores and phosphorylated pSer396 tau burden, and appeared to co-localize at least partly to NFT-containing neurons. In contrast, catD was increased only in BA40 in DLB and not at all in PDD, did not correlate with LB scores, and did not appreciably co-localize with α-synuclein inclusions. Our study suggests that catD upregulation may be an adaptive response to AD-related processes leading to neurofibrillary degeneration, but may not be directly associated with formation of α-synuclein inclusions in Lewy body dementia.

INTRODUCTION

Alzheimer disease (AD) is the most common cause of dementia, and represents a substantial, global healthcare burden (3,33). Although the "amyloid cascade hypothesis" of AD, which attributes pathogenicity to processes favoring the accumulation and aggregation of β-amyloid (Aβ) peptides into extracellular senile plaques has provided much research impetus (27), doubts remain as to the exact nature of pathogenic Aβ species as well as their precise roles of Aβ

species in the disease process (37,49). Furthermore, therapeutic strategies based on reducing cortical Aβ deposition have, to date, been disappointing (15). This underscores the need to further understand the complex interactions between multiple processes, including amyloidogenesis, tau hyper-phosphorylation forming neurofibrillary tangles (25), neuroinflammation (16), alternative splicing dysregulation (40), cell cycle dysregulation (51) as well as lysosomal and autophagic dysfunction (44,52), within which the cathepsin family of proteases are important due to their critical roles in the degradation and turnover of abnormally aggregated or damaged proteins. In particular, cathepsins B and D have been implicated in various cancers as well as in cellular processes which may be relevant in neurodegeneration, including cell cycle regulation, autophagy and neuroinflammation (5,22,41,46,65). Cathepsin B, a cysteine protease, has been extensively studied in AD for its potential anti-amyloidogenic and neuroprotective effects (50,60). In contrast, previous studies on the aspartyl protease cathepsin D (CatD) in AD are less clear. Whilst a number of studies report activation or upregulation in vulnerable neuronal populations (10–12), there were also reports of decreased monocyte CatD expression in peripheral blood (62) contrasting with unchanged levels in the cortex (38), or increased mRNA in histologically normal neurons but decreased mRNA in degenerating neurons (10). Furthermore, the status of CatD is unclear in the neocortex of Lewy body dementias, whose major clinical subtypes, dementia with Lewy bodies (DLB) and Parkinson's disease dementia (PDD), comprise the second commonest cause of neurodegenerative dementias after AD (1,2). Whilst DLB and PDD are characterized by cortical Lewy bodies consisting of abnormally aggregated α-synuclein, both conditions also manifest variable burden of AD pathological hallmarks, including senile plaques and neurofibrillary tangles (24,32,34,57). Because CatD function has been implicated in the clearance of various proteins, including Aβ, tau and α-synuclein (26,36,58), we aimed to measure CatD immunoreactivity in the postmortem neocortex of AD, PDD and DLB and investigate its association with AD and Lewy body dementia neuropathological burden

METHODS

Patients, clinical and neuropathological assessments

Tissues for the postmortem study were obtained from subjects recruited into longitudinal studies with postmortem follow up from the University Hospital Stavanger, Newcastle Brain Tissue Resource, the London Neurodegenerative Diseases Brain Bank and the Thomas Willis Brain Collections at Oxford University, the UK sites being part of the Brains for Dementia Research network (http://brainsfordementiaresearch.org.uk). All dementia subjects for this study were selected on the basis of clinicopathological diagnosis. Diagnostic criteria included the Consortium to Establish a Registry for Alzheimer's disease (CERAD) criteria for AD (47), and the Dementia with Lewy bodies Consortium's "one-year rule" (45) together with the Movement Disorders Society criteria (18) to distinguish between DLB and PDD. Annual cognitive assessments with MMSE (21) were also available, and the average decline per year (MMSE decline) from the time of dementia diagnosis to death was used as an indicator of dementia severity. At death, informed consent was sought from next-of-kin before removal of brains, which were divided into hemispheres, with one formalin fixed for neuropathological assessments. Neuropathological diagnoses were based on Thal Aβ phases (61), neurofibrillary tangle Braak stages (7) and CERAD criteria for AD (47) which are all combined in the National Institute on Aging – Azheimer's Association guidelines (48) and the Newcastle/McKeith criteria for Lewy body disease (45). In addition, semi-quantitative scoring for amyloid plaques (AP, immunostaining with the 4G8 antibody), neurofibrillary tangles/neuropil threads (NFT, immunostaining with the AT8 antibody), and α-synuclein-containing inclusions, that is, Lewy bodies/Lewy neurites (LB, α-synuclein immunostaining) were performed as previously described (32) by neuropathologists blinded to clinical diagnosis on a four point scale: 0 = None, 1 = Sparse, 2 = Moderate and 3 = Abundant. The contralateral hemisphere was coronally sectioned before further dissection to obtain 1 cm^3 blocks from selected regions, followed by fresh freezing and storage at −80°C. Brains from controls were neurologically and cognitively normal, had only age-associated neuropathological changes and no history of psychiatric diseases.

Tissue processing

Frozen blocks of tissues from the middle temporal gyrus (Brodmann area, BA21) and parietal lobe (BA40) were thawed on ice and dissected free of meninges and white matter, then homogenised with an Ultra-Turrax homogeniser (IKA, Staufen im Breisgau, Germany, on highest setting, 10 s) in ice-cold buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) with cOmplete™ protease inhibitor cocktail and PhosSTOP™ phosphatase inhibitor tablets (Roche Life Science, Penzberg, Germany) at the concentration of 50 mg tissue wet weight/mL. Protein content of the homogenates was measured using Pierce Coomassie Plus Reagent (ThermoFisher Scientific, Waltham, MA, USA) before further processing for immunoblotting.

Immunoblotting

Brain homogenates (see above) were added to Laemmli samples buffer (1:1 vol./vol., Bio-Rad, Hercules, CA, USA) with heating (95°C for 5 min), followed by loading onto 10–12% SDS-polyacrylamide gels. Proteins were electrophoresed using a Mini-PROTEAN® system (Bio-Rad, Hercules, CA, USA) and transferred to nitrocellulose membranes. Membranes were blocked in phosphate-buffered

saline with 0.1% Tween® 20 (PBST) and 5% skimmed milk for 1 h, then incubated with primary antibodies in PBST containing 5% BSA at 4°C overnight. Primary antibodies used were as follows: anti-cathepsin D (C-20, goat polyclonal, 1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA); anti-LAMP-1 (ab24170, rabbit polyclonal, 1:1000 dilution, Abcam, Cambridge, UK) and anti-β-actin (AC-74, mouse monoclonal, 1:5000 dilution, Sigma Aldrich, St Louis, MO, USA). After primary antibody incubation, membranes were washed three times with PBST (10 min, 25°C), then incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution, Jackson ImmunoResearch, West Grove, PA, USA) for 1h at 25°C. Subsequently, membranes were washed three times with PBST (10 min, 25°C), and immunoreactivities were visualized with Luminata™ Crescendo Western HRP substrate (Merck Millipore, Billerica, MA, USA) and quantified with the Alliance 4.7 image analyser (UVItec, Cambridge, UK).

Cortical Aβ and tau measurements

Aβ, total tau, and serine-396 phosphorylated tau (pSer396 tau) measurements by enzyme-linked immunosorbent assay (ELISA) kit (Invitrogen, Carlsbad, CA, USA) were performed as previously reported (13,55). Briefly, tissues from BA21 and BA40 were homogenized in Tris HCl buffer (pH 8.0) with 5M guanidine, and resultant homogenates were assayed in duplicates for the amyloidogenic, neurotoxic 42 amino-acid species of Aβ (Aβ42) (67), total tau, and pS396 tau according to manufacturer's instructions, and expressed in pg/mg brain protein.

Double labelling immunohistochemistry

Brain tissue was acquired and assessed pathologically as described above (see also Howlett et al. 32). Formalin fixed, paraffin-embedded blocks of neocortex (BA9 and BA21) were cut into 7μm sections and mounted onto slides, then dewaxed and rehydrated using Histoclear® (ThermoFisher Scientific, Waltham, MA, USA) and alcohol dilutions. Antigen retrieval was carried out by microwaving for 10 min in citrate buffer (pH 6.0) for catD and phosphorylated tau, and by autoclaving for 10 min in EDTA buffer (pH 8.0) for α-synuclein; all of which were followed by immersion for 15 min in 98% formic acid. Tissue sections were then treated with 0.3% hydrogen peroxide in PBS (30 min) to inhibit endogenous peroxidases prior to addition of primary antibodies: 20G10 anti-Aβ42 monoclonal antibody, 1:10 000 dilution (31); AT8 phosphorylated (pSer202 and pThr205) tau monoclonal antibody, 1:200 dilution (ThermoFisher Scientific, Waltham, MA, USA); anti-α-synuclein monoclonal antibody, 1:100 dilution (Novacastra™ Leico Biosystems, Newcastle, UK); anti-cathepsin D (C-20, goat polyclonal, 1:1000 dilution, Santa Cruz Biotechnology, Dallas, TX, USA); with the sections incubated overnight at 4°C. Development of the sections was performed using biotinylated secondary antibodies, ABC reagents and a DAB kit, with NovaRED™ (all from Vector Laboratories, Peterborough, UK) used as a second chromogen for double labelling. In control experiments, the secondary biotinylated antibody was omitted. Sections were imaged on a Leica DMRB microscope equipped with DC420 digital camera. Ten random images were captured from each section and positively labelled cells identified by ImageJ analyses.

Table 1. Demographic and disease variables of a cohort of patients with postmortem confirmed AD and synucleinopathies

Variable	Control	PDD	DLB	AD	*P* Value
Maximum available *n*	18	22	21	16	NA
Age at Death, years	82.7 ± 1	82.4 ± 1	83.1 ± 2	87.2 ± 2	0.086
Female, *n* (%)	9 (50.0)	11 (50.0)	11 (52.4)	10 (62.5)	0.867
Postmortem Interval, h*	41.5 ± 6	37.3 ± 4	44.6 ± 6	37.1 ± 6	0.715
Brain pH	6.44 ± 0.07	6.46 ± 0.07	6.23 ± 0.08	6.31 ± 0.08	0.098
MMSE decline/year†	NA	2.29 ± 0.3	3.00 ± 0.5	3.77 ± 1.0	0.218
Duration of dementia, years‡	NA	3.4 ± 0.6	6.6 ± 0.8	9.9 ± 0.7	**<0.01***
Duration of PD symptoms, years§	NA	12.5 ± 1.2	2.9 ± 0.7	0.0 ± 0	**<0.01***
Braak stage‖					
0–II	13	15	4	0	NA
III–IV	0	6	8	3	NA
V–VI	0	1	9	12	NA

Data are expressed as mean ± SEM, with significant *p* values of *one-way analyses of variance (ANOVA) listed in **bold type**. Abbreviations: AD, Alzheimer's disease; BA, Brodmann area; DLB, dementia with Lewy bodies; LB, Lewy bodies; MMSE, Mini Mental State Examination; NA, not applicable/not measured; PDD, Parkinson's disease dementia.

*Information was not available for one PDD patient.

†Mean MMSE decline per year from study commencement to the last interview before death (available for 21 PDD, 15 DLB and 14 AD patients) and used as an indicator of dementia severity.

‡Years of dementia at death, available for 21 PDD, 15 DLB and 15 AD patients.

§Years of PD motor symptoms at death, available for 21 PDD, 14 DLB and 15 AD patients.

‖Braak staging (7) data not available for five controls and one AD patient.

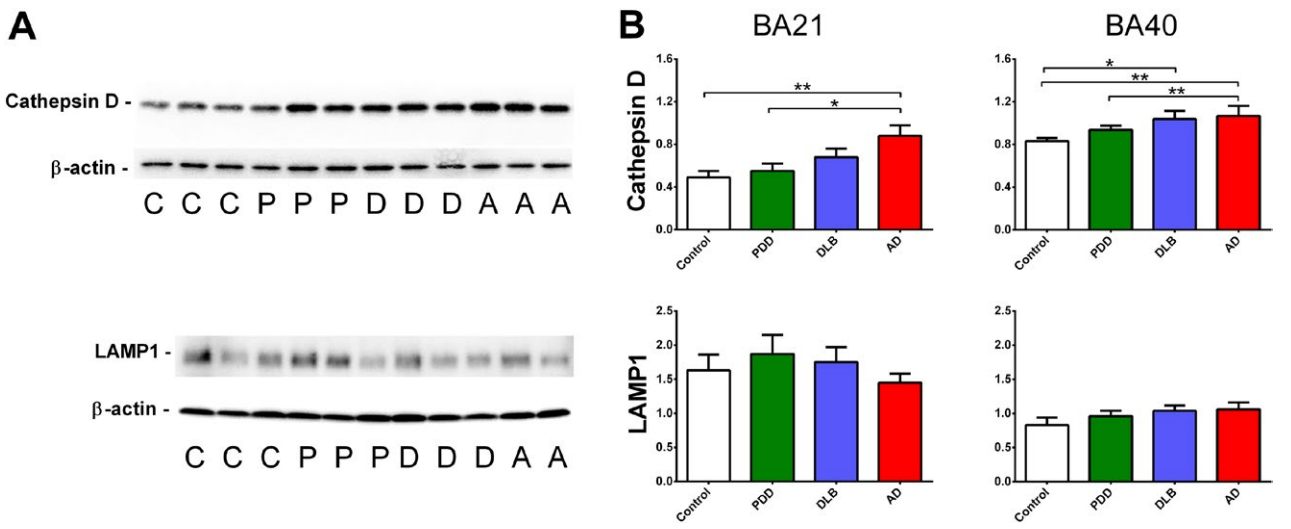

Figure 1. Cathepsin D (catD) immunoreactivities in the postmortem neocortex of Lewy body dementias and AD. **A** Representative immunoblots and **B** bar graphs of catD (top) and LAMP-1 (bottom) immunoreactivities (expressed in mean ± SEM arbitrary units) in Brodmann area 21 (BA21) and BA40 of 18 controls (C), 22 PDD (P), 21 DLB (D) and 16 AD (A) patients, normalised to loading control β-actin. $*p < 0.05$ and $**p < 0.01$; significant pairwise difference using Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests.

Statistical analyses

All analyses were performed using SPSS Statistics (version 23, IBM Inc., Armonk, NY, USA). All differences of demographic and disease variables in Table 1 were compared by one-way one way-analyses of variance (ANOVA) followed by *post-hoc* Tamhane's T2 tests, except for gender which was compared by Pearson χ^2 tests. On the other hand, differences in western blot immunoreactivities normalised to loading control β-actin, neuropathological scores as well as levels of Aβ42 and tau species for the postmortem studies were compared by Kruskal-Wallis ANOVA followed by *post-hoc* Dunn's tests for pair-wise comparisons. Inter-correlations between variables were performed using Spearman's rank correlation. For all tests, $p < 0.05$ were considered statistically significant.

RESULTS

Disease and demographic characteristics of study cohort

Table 1 shows the available controls as well as community-based PDD, DLB and AD patients in the cohort were closely matched in terms of age at death, sex distribution, postmortem interval, brain pH (as an indicator of tissue quality (29)) and MMSE decline per year (as a measure of dementia severity). For the mean duration of dementia, there were significant differences among the groups, with the longest in AD and shortest in PDD. In contrast, PDD patients had longer mean duration of parkinsonism symptoms than DLB, while no parkinsonism symptoms were apparent in the AD group. Braak staging for extent of AD pathological changes (7) showed all except one PDD with Braak stage 0–II (with the PDD having Braak VI), while all except three AD were stage V–VI (with three AD at stage IV). For DLB, around 57% were stages 0–IV, and 43% were stages V–VI. Taken together, these data suggest a relative lack of AD pathology and short dementia duration in PDD, high burden of AD pathology and long dementia duration in AD, with intermediate levels in DLB. These clinical and neuropathological features are in line with previous observations of AD and Lewy body dementias (17,24,32,64).

Cathepsin D immunoreactivities were increased in AD and DLB neocortex

We first performed catD immunoblotting in two defined areas of the neocortex (BA21 and BA40) known to play important roles in cognition, and are affected in AD and Lewy body dementias (32). Figure 1 shows that catD immunoreactivities were significantly increased in both areas for AD, and only in BA40 for DLB, whilst catD immunoreactivities were not altered in PDD. In AD, DLB and PDD, immunoreactivities of lysosomal associated membrane protein-1 (LAMP-1), a major component of lysosomes not directly involved in degradation of protein aggregates, and used commonly to define the lysosome compartment (20,39), were not significantly altered. Our data therefore suggest that in AD and DLB, increased catD was a specific alteration within the lysosomal compartment rather than a result of changes in lysosomal biogenesis.

Cathepsin D immunoreactivities were associated with neurofibrillary tangles

Because CatD may be involved in the clearance of Aβ, tau and α-synuclein (26,36,58), we compared catD

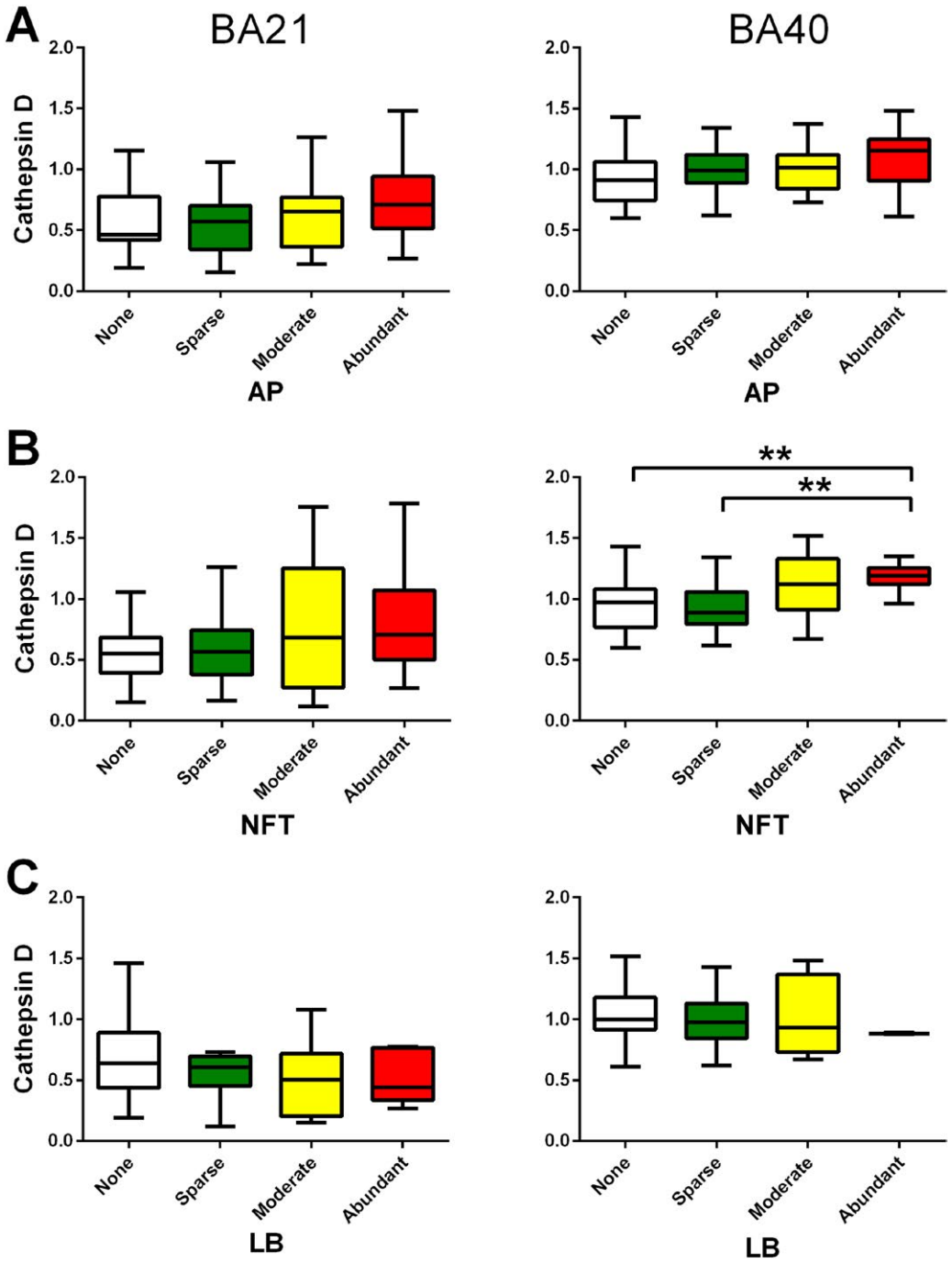

Figure 2. Cathepsin D (catD) immunoreactivities across a range of neuropathological burden. Semi-quantitative scoring for **A** amyloid plaques (AP), **B** neurofibrillary tangles/neuropil threads (NFT) and **C** α-synuclein inclusions e.g., Lewy bodies or Lewy neurites (LB) ranged from "None" (score = 0) to "Abundant" (score = 3) were performed according to Howlett et al. (32) (see Methods). Immunoreactivities of catD (expressed in mean ± SEM arbitrary units) in BA21 (left) and BA40 (right) are depicted in box and whiskers plots using Tukey's method. $^{**}p < 0.01$; significant pairwise difference using Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests.

immunoreactivities from immunoblotting across the range of semi-quantitative neuropathological scores in our cohort for amyloid plaques (AP), neurofibrillary tangles/neuropil threads (NFT) and Lewy bodies/Lewy neurites (LB) to investigate if the observed catD changes may be associated with neuropathological burden. Interestingly, only NFT scores in BA40 associated with catD, with immunoreactivities in the "Abundant" group significantly higher than those in the "Sparse" or "None" groups (Figure 2B). CatD immunoreactivities appeared to trend towards increases

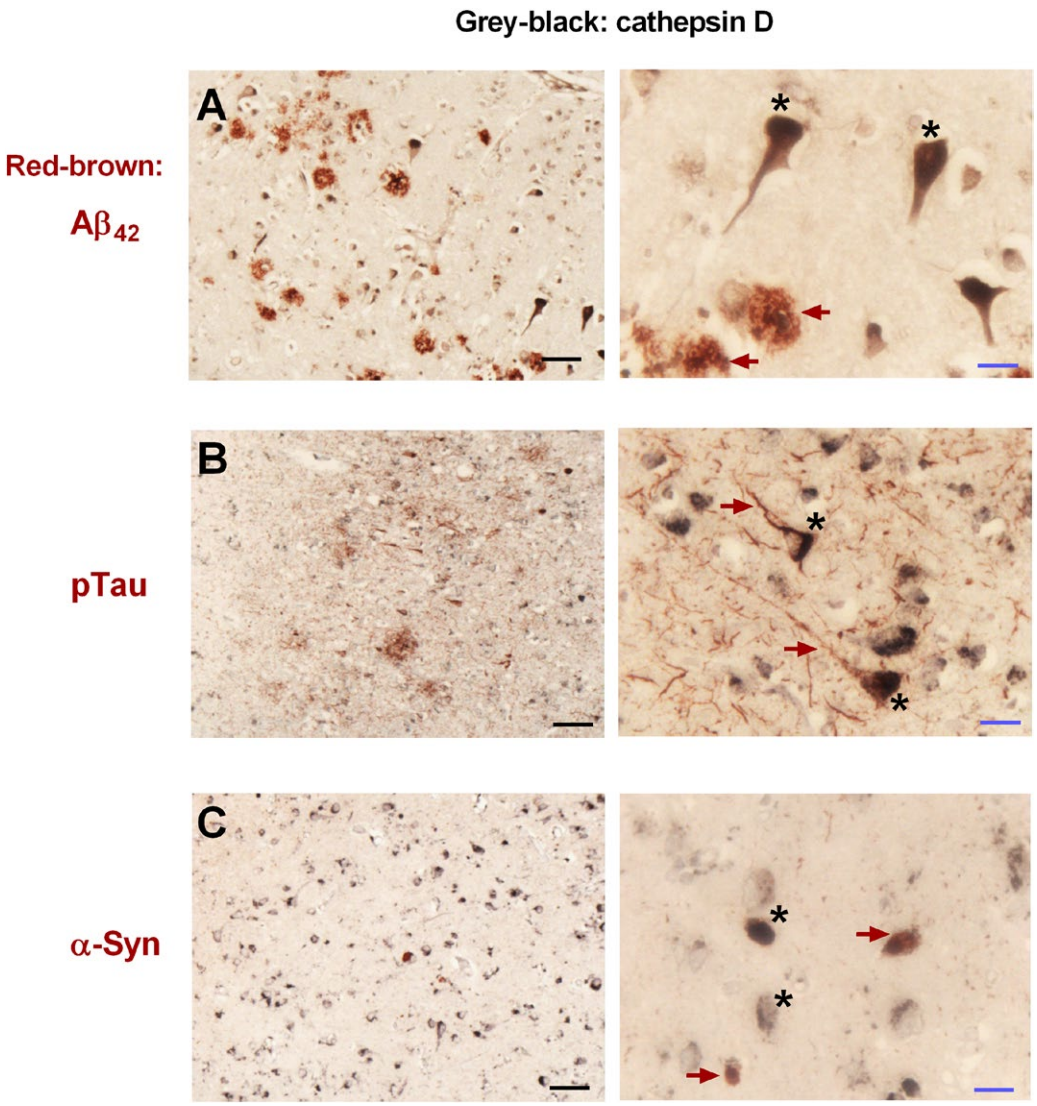

Figure 3. Immunohistochemical staining of neuropathological features and cathepsin D (catD) in postmortem human neocortex. Images of **A** Aβ42, **B** phosphorylated Tau reocognized by the AT8 antibody (pTau) and **C** α-synuclein (α-syn) co-stained with catD (see Methods) in the neocortex of an AD (**A**, **B**) and DLB (**C**) subject at 200x (left) and 630x (right) magnifications. Micrographs were representative images of three AD and three DLB subjects. Scale bars: 50 microns (black), 15 microns (blue). Red-brown arrows: **A** Aβ42, **B** pTau and **C** α-syn immunoreactivities. Black asterisks: catD immunoreactivity.

with NFT in BA21, and with AP in both regions, but did not reach statistical significance (Figure 2A, B). In contrast, catD did not appear to be associated with LB counts (Figure 2C). To further investigate spatial associations between catD and neuropathological hallmarks, double labelling immunohistochemistry was performed on neocortical sections stained for catD and AP, NFT or LB. Figure 3 shows representative micrographs suggesting that at least some of the catD immunoreactivities were co-localized with NFT-containing neurons (as indicated by AT8 phosphorylated tau antibody staining, Figure 3B). In contrast, α-synuclein immunoractivities (putatively labelling LB) typically showed minimal overlap with those of catD immunoreactivities (Figures C). Similarly, whilst occasional catD immunoreactivity was apparent in Aβ42-postitive plaques, the preponderance of catD staining was localized to pyramidal neurons (Figure 3A).

Increased cathepsin D immunoreactivities correlated with phosphorylated (pS396) tau

To corroborate the findings above showing associations between catD and specific AD hallmarks (Figures 2 and 3), we further measured the biochemical substrates of AD, namely guanidine-treated Aβ42 (previously shown to consist mainly of plaque-associated Aβ (13,55)) and tau phosphorylated at serine-396 (pSer396 tau, previously shown to be a specific marker of hyperphosphorylated tau, the major

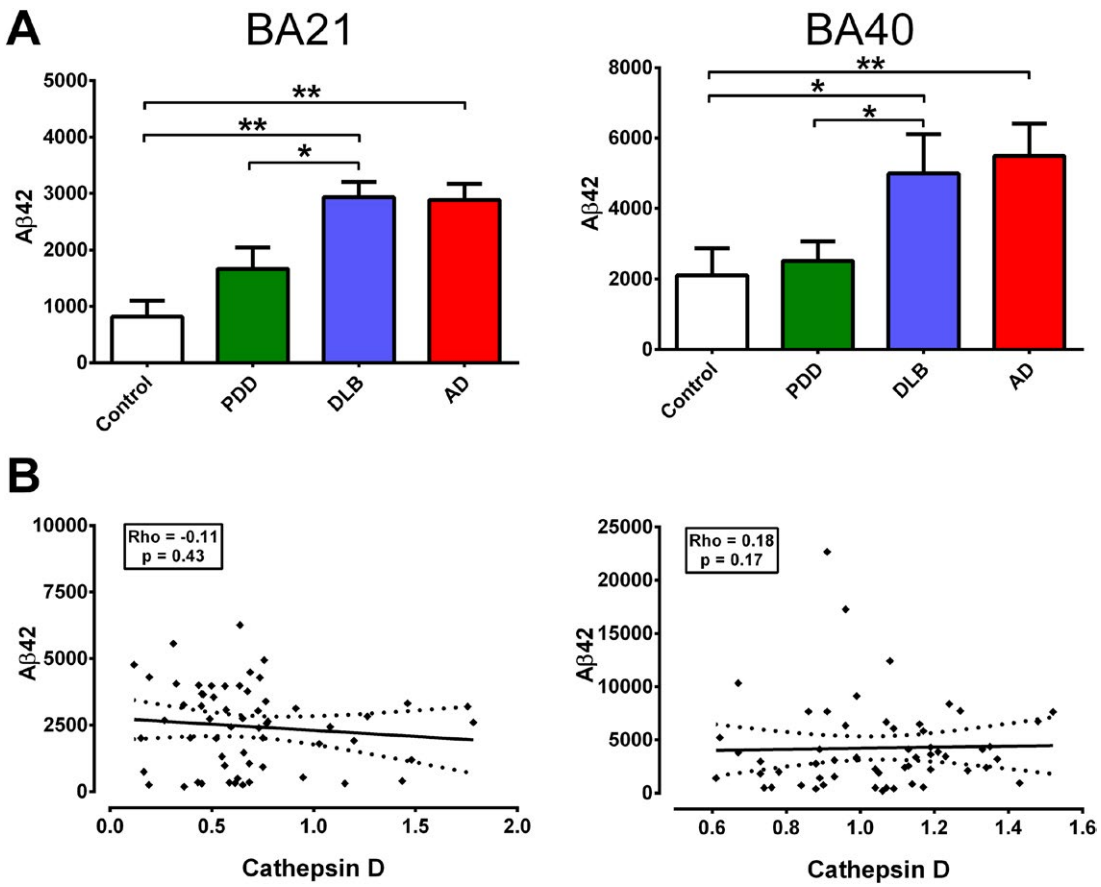

Figure 4. Correlations of cathepsin D (catD) immunoreactivities with Aβ42 concentrations in the postmortem neocortex of Lewy body dementias and AD. **A** Bar graphs of mean ± SEM Aβ42 concentrations (pg/mg brain protein) in BA21 (left) and BA40 (right). **B** Scatter plots of Aβ42 concentrations against catD immunoreactivities (arbitrary units) in BA21 (left) and BA40 (right) of the combined dementia cohort (22 PDD, 21 DLB, 16 AD). The solid lines indicate linear regressed best-fit curves while dashed lines indicate their respective 95% confidence intervals. No significant correlations were found. $*p < 0.05$ and $**p < 0.01$; significant pairwise difference using Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests.

constituent of NFT (4)). Similar to previous reports of higher amyloid burden in DLB compared to PDD (11,17,24), we found that Aβ42 concentrations in DLB were significantly higher than PDD, and reached levels comparable to AD in both BA21 and BA40 (Figure 4A). However, Aβ42 did not correlate with catD immunoreactivities within the combined dementia group (Figure 4B). In contrast, while pSer396 tau concentrations were higher in DLB compared with PDD, reaching significance in BA21, AD showed the highest pSer396 tau levels, being higher than both DLB and PDD in BA40 (Figure 5A). Changes in pSer396 tau were unlikely to be due to changes in tau expression, as total tau were unchanged among diagnostic groups (Figure 4B). Interestingly, pSer396 tau concentrations positively correlated with catD immunoreactivities in both brain regions (Figure 5C). On the other hand, catD immunoreactivities did not correlate with either pre-death MMSE scores or with MMSE decline per year (see Supplementary Figure S1). This suggests that catD is a marker of disease processes such as tau hyperphosphorylation rather than directly associated with neuronal function or synaptic plasticity events.

DISCUSSION

Cathepsin D (catD)'s role in neurodegeneration has been suggested by lysosomal storage disorders such as neuronal ceroid-lipofuscinosis associated with catD mutations which abolished enzymatic activity, leading inevitably to progressive neurodegeneration and brain atrophy (59). It is therefore not surprising that considerable research have been carried out to elucidate the role of lysosomal constituents, including cathepsins, in neurodegenerative conditions like AD. Previous studies on catD in AD have yielded inconsistent results (see Background). Using postmortem tissues from well-characterized cohort of AD patients together with Lewy body dementia subgroups with incidental high (DLB) and low (PDD) amyloid pathology (17,24,64), but which are otherwise matched in dementia severity (see Table 1), the current study reports robust increases in catD

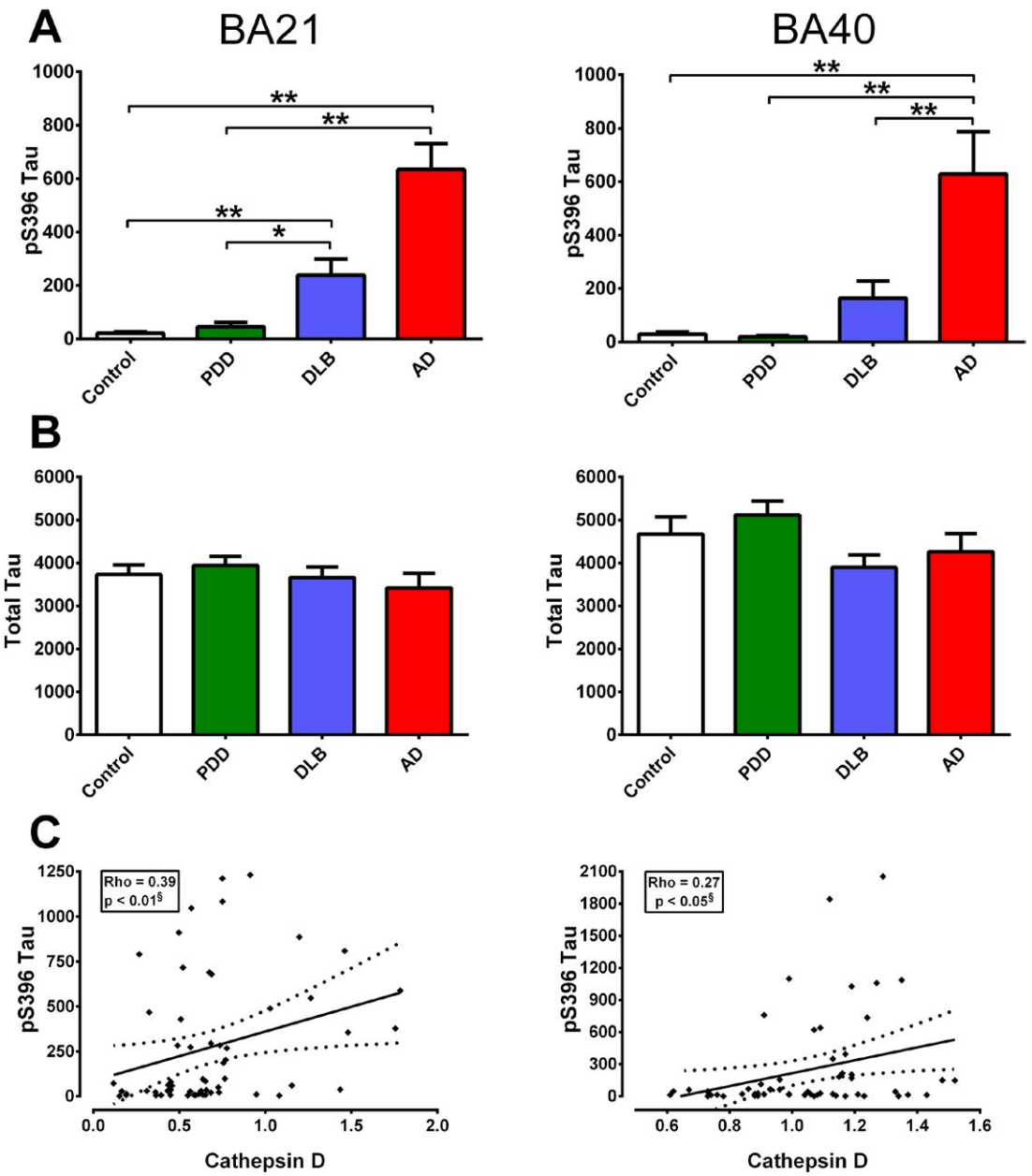

Figure 5. Correlations of cathepsin D (catD) immunoreactivities with pS396 Tau concentrations in the postmortem neocortex of Lewy body dementias and AD. Bar graphs of mean ± SEM **A** pS396 Tau and **B** total tau concentrations (pg/mg brain protein) in BA21 (left) and BA40 (right). **C** Scatter plots of pS396 Tau concentrations against catD immunoreactivities (arbitrary units) in BA21 (left) and BA40 (right) of the combined dementia cohort (22 PDD, 21 DLB, 16 AD). The solid lines indicate linear regressed best-fit curves while dashed lines indicate their respective 95% confidence intervals. $*p < 0.05$ and $**p < 0.01$; significant pairwise difference using Kruskal-Wallis ANOVA with Dunn's *post-hoc* tests. §Significant Spearman correlation.

immunoreactivities in AD which correlated with neurofibrillary tangle scores and phosphorylated tau burden, as well as co-localize at least in part within tangle-bearing neurons. This is also, as far as we know, the first study to measure catD immunoreactivities in relation to LB pathology in DLB and PDD. In our cohort, catD was unchanged in PDD and increased only in one of the two cortical regions measured in DLB. CatD was also not correlated with LB scores or demonstrated substantial co-localization with α-synuclein inclusions.

Taken together, the postmortem data therefore suggest that catD changes may be driven primarily by AD processes, as it is relatively unchanged in PDD characterized by low AD pathological burden, but demonstrated increases in DLB with higher AD pathological burden. In contrast, rodent, nematode or cell culture-based work have shown

protective effects of catD against α-synuclein aggregation and toxicity (56), suggesting either species differences in how catD interacts with α-synuclein, or biochemical differences in human LB-associated α-synuclein, including higher proportions of phosphorylated forms (63). However, follow up studies are needed to corroborate catD associations with cognitive function as well as investigate underlying mechanisms, the most straightforward of which is the enhanced clearance of abnormally aggregated proteins forming AP and NFT (26,36). In this context, various studies have previously reported catD association with vulnerable neurons, including those with Aβ deposits (10–12), although associations were more apparent with NFT than with AP in the current study (Figures 2 to 4). Nevertheless, the available data (including those reported here) suggest the upregulation of catD as a response to abnormal protein aggregation and subsequent formation of AP and NFT, two closely related processes (35,53).

Besides direct action on abnormally aggregated peptides, catD or its zymogen (pro-catD) may regulate other cellular responses implicated in AD pathogenesis and progression, including cytokine secretion and initiation of apoptosis (14,23,43). Conversely, pro-inflammatory cytokines like tumor necrosis factor and interferon-γ, known to be activated in AD by misfolded and aggregated peptides (30), may upregulate pro-catD and increase catD activities (19,66). Therefore, follow-up studies are needed to better characterize the intertwining relationships between protein misfolding, neuroinflammatory responses and catD upregulation in AD.

There are a few limitations in the current studies which requires discussion. First, the cellular basis of catD changes reported in this study are at present unclear, although the current immunohistochemical data suggest localization of catD immunoreactivities to neurons, in agreement with previous work (8,11). However, catD expression can also be found in astrocytes, microglia and pericytes (6,8,42,54), and further studies are needed to elucidate cell type- as well as brain region-specific catD changes in AD, together with their relative contributions to AD neuropathological and neurochemical features. Additionally, there remains a divergence between *in vitro* studies showing involvement of catD in α-synuclein processing (see above), and our postmortem data suggesting no significant associations between catD and α-synuclein lesions in Lewy body dementias. Findings of relatively unchanged catD in Lewy body dementias are also in need of corroboration from more detailed studies on potential interactions between catD and α-synuclein, for example, with different α-synuclein species (63). Furthermore, the regional specifity of associations with catD (both BA21 and 40 for AD versus only BA40 in DLB) may reflect differential vulnerabilities or regional involvement in the neurodegenerative dementias, but further work is needed to validate the regional differences and elucidate the underlying mechanisms. Lastly, the present study adds to growing literature on the involvement of cathepsins in neurological diseases, and gives impetus to further research in this area, both in the role of cathepsin D in other neurodegenerative conditions characterized by tauopathy (eg, progressive supranuclear palsy, corticobasal ganglionic degeneration and primary ageing related tauopathy), and in how other cathepsins shown to be involved in degenerative conditions (eg, cathepsin A-related arteriopathy with strokes and related leukoencephalopathy, CARASAL) (9) might also be affected in AD and Lewy body dementias.

CONCLUSION

Using postmortem brain cortical tissues and blood from separate cohorts of aged non-dementia and dementia patients, the current study reports increased catD immunoreactivities in the neocortex of AD patients which appear to be most strongly associated with neurofibrillary degeneration. In contrast, catD is relatively preserved in the neocortex of Lewy body dementias, and seemed to be associated with concomitant AD pathology rather than with α-synuclein inclusions. Further studies will help elucidate the mechanisms underlying catD upregulation, as well as its suitability as a prognostic biomarker or therapeutic target for AD.

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AUTHORS' CONTRIBUTIONS

YLC, PTF, CPC, and MKPL conceived the study and designed the project; YLC, JW, JRC, DH, AH, JHL performed the experiments; JA, DA, CPC, PTF provided postmortem and clinical data; YLC, JW, DH, MKPL analyzed the data; YLC, JW, MKPL wrote the first draft. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

For the postmortem study, ethics approval for the collection and study of brain tissues received Institutional Review Board approval in both the UK (08/H1010/4) and Singapore institutions (NUS 12-062E), and informed consent was obtained from participants' next-of-kin prior to removal of brain. For the clinical study, Institutional Review Board approval from the National Healthcare Group Domain-Specific Review Board (reference: 2010/00017; study protocol number: DEM4233) and written informed consent had been obtained from participants or their next-of-kin before study recruitment and blood collection procedures.

CONSENT FOR PUBLICATION

All authors gave consent for publication.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Scatter plots of cognitive status (Pre-death MMSE score and MMSE decline per year; see Methods) against cathepsin D immunoreactivities (arbitrary units) in BA21 (left) and BA40 (right) of the combined dementia cohort. The solid lines indicate linear regressed best-fit curves while dashed lines indicate their respective 95% confidence intervals. No significance was found using Spearman correlation.