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Quantitative proteomics of tau and Aβ in detergent fractions from Alzheimer's disease brains

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Abbreviations: ACN, acetonitrile; AD, Alzheimer's disease; ALS, amyotrophic lateral sclerosis; APOE, apolipoprotein E; Aβ, amyloid-β; BCA, bicinchoninic acid; CAM; carbamidomethylation; cryo-EM, cryogenic electron microscopy; CSF, cerebrospinal fluid; DCN, disease-control; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionization; FA, formic acid; FDR, false discovery rate; FLEXItau, full-length expressed stable isotope-labeled tau; FLNA, filamin-A; FTD, frontotemporal degeneration; HCD, higher-energy collisional dissociation; HPLC, high performance liquid chromatography; HRMS, high-resolution mass spectrometry; hTau441, full-length human tau; IAA, iodoacetamide; iBAQ, intensity based absolute quantitation; IPTG, isopropyl-β-D-thiogalactoside; LCD, low complexity domain; LC-MS, liquid chromatography–mass spectrometry; LFQ, label-free quantification; MART, microtubule associated protein tau; MOPS, 3-morpholinopropane-1-sulfonic acid; MRM, multiple reaction monitoring; MS, mass spectrometry; MTBR, microtubule binding region; MT-3, metallothionein 3; MYH1, MYL1, myosin H1 and L1; NEFL/NfL, neurofilament light; NMR, magnetic nuclear resonance; P, tau pellet; PAGE, native polyacrylamide gel electrophoresis; PBS, phosphate buffer saline; PET, positron emission tomography; PHF, paired helical filaments; PRR, proline rich region; PTM, post-translational modification; RBP, RNA binding protein; SDS, sodium dodecyl sulfate; SIL, stable isotope labelled; SF, straight filament; SM, starting material; SN, supernatant; SNRP, small ribonuclear proteins; TBS, tris-buffered saline; TCEP, tris(2-carboxyethyl)phosphine; TDP43, transactive response (TAR) DNA binding protein 43; TEAB, tri-ethyl ammonium bicarbonate buffer; ThT, thioflavin; TFA, trifluoroacetic acid; TMEM106B, transmembrane protein 106B; UBB, ubiquitin; UHPLC, ultra-high performance liquid chromatography; WB, western blot.

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Abstract

The two hallmarks of Alzheimer's disease (AD) are amyloid-β (Aβ) plaques and neurofibrillary tangles marked by phosphorylated tau. Increasing evidence suggests that aggregating Aβ drives tau accumulation, a process that involves synaptic degeneration leading to cognitive impairment. Conversely, there is a realization that non-fibrillar (oligomeric) forms of Aβ mediate toxicity in AD. Fibrillar (filamentous) aggregates of proteins across the spectrum of the primary and secondary tauopathies were the focus of recent structural studies with a filament structure based nosologic classification, but less emphasis was given to non-filamentous co-aggregates of insoluble proteins in the fractions derived from post-mortem human brains. Here, we revisited sarkosyl-soluble and -insoluble extracts to characterize tau and Aβ species by quantitative targeted mass spectrometric proteomics, biochemical assays, and electron microscopy. AD brain sarkosyl-insoluble pellets were greatly enriched with $A\beta_{42}$ at almost equimolar levels to N-terminal truncated microtubule binding region (MTBR) isoforms of tau with multiple site-specific post-translational modifications (PTMs). MTBR R3 and R4 tau peptides were most abundant in the sarkosyl-insoluble materials with a 10-fold higher concentration than N-terminal tau peptides. This indicates that the major proportion of the enriched tau was the aggregation prone N-terminal and proline rich region (PRR) of truncated mixed 4R and 3R tau with more 4R than 3R isoforms. High concentration and occupancies of site-specific phosphorylation pT_{181} (~ 22 %) and pT_{217} (~ 16 %) (key biomarkers of AD) along with other PTMs in the PRR and MTBR indicated a regional susceptibility of PTMs in aggregated tau. Immunogold labelling revealed that tau may exist in globular non-filamentous form (N-terminal intact tau) co-localized with Aβ in the sarkosyl-insoluble pellets along with tau filaments (N-truncated MTBR tau). Our results suggest a model that Aβ and tau interact forming globular

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aggregates, from which filamentous tau and Aβ emerge. These characterizations contribute towards unravelling the sequence of events which lead to end-stage AD changes.

Keywords: Alzheimer's disease, neurofibrillary tangles, Aβ plaques, tau, proteomics, sarkosyl extracts, cryo-EM, immuno-EM, aducanumab

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Introduction

The two pathognomonic hallmarks of Alzheimer's disease (AD) are cortical deposits composed of Amyloid-β (Aβ) peptides(Masters *et al.* 1985) and neurofibrillary tangles and neuropil threads of microtubule associated protein tau (MAPT) protein(Goedert *et al.* 1991). While tangles, neurites and cognitive decline appear to occur in conjunction, Aβ accumulation occurs much earlier(Hampel *et al.* 2021). The advent of therapeutics which target Aβ has supported the concept that Aβ drives tau accumulation and phosphorylation(Cummings *et al.* 2021; Budd Haeberlein *et al.* 2022). One of the many enigmas of the natural history of AD is how the extra-cellular accumulation of Aβ drives the aggregation of the very soluble intracellular/cytosolic protein tau. The soluble forms of $A\beta_{42}$ in the periphery (as reflected in CSF and plasma(Michno *et al.* 2021)) decrease as the aggregated forms increase in the brain (as seen by Aβ-PET(Roberts *et al.* 2017; Li *et al.* 2021c)). Several years after this soluble/insoluble Aβ kinetic equilibrium begins to change, aggregated tau, particularly post-translationally modified (PTM) forms of tau, phosphorylated tau (p-tau), accumulate, as judged by increases in both tau-PET and the release of soluble p-tau species into the CSF and plasma pools(Zetterberg *et al.* 2013). Notably, the biofluid (CSF and plasma) values of p-tau increase years in advance of the tau-PET signal(Barthélemy *et al.* 2020).

From the postmortem AD brain, it is difficult to separate completely the various Aβ and tau species. It has become conventional to extract tau filaments from the L-lauroylsarcosine (sarkosyl)-insoluble fraction of human AD brain homogenates(Falcon *et al.* 2018b; Fitzpatrick *et al.* 2017; Diner *et al.* 2017), which does not differentially remove aggregates of Aβ. Since the first report of the isolation of sarkosyl-insoluble tau from AD patients' brains(Greenberg & Davies 1990), multiple studies have indicated that ionic-detergent sarkosyl is strong enough to solubilize the majority of natively folded proteins in

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brain without solubilizing protein aggregates of Aβ, tau, transactive response (TAR) DNA binding protein 43 (TDP43), α-synuclein, ferritin, neurofilament proteins and others(Diner *et al.* 2017). Recent investigations have also shown the substantial enrichment of multiple RNA binding proteins (RBP) in the sarkosyl-insoluble fractions(Guo *et al.* 2021). As sarkosyl is milder than sodium dodecyl sulfate (SDS) it allows for the preservation of oligomeric non-filamentous forms of aggregated proteins(Ren & Sahara 2013) and antigenic epitopes, that can be conformationally changed by SDS treatment(Kondo *et al.* 1988). Sarkosyl-insoluble filamentous tau (paired helical filaments (PHFs) and straight filaments (SFs)(Goedert *et al.* 1988)), Aβ, α-synuclein, TDP43, and transmembrane protein 106B (TMEM106B) across the spectrum of the primary and secondary tauopathies has been a focus of recent cryogenic electron microscopy (cryo-EM) with a filament structure based nosologic classification, but less emphasis has been given to non-filamentous co-aggregates of insoluble proteins in these fractions(Falcon *et al.* 2018a; Falcon *et al.* 2018b; Fitzpatrick *et al.* 2017; Schweighauser *et al.* 2022; Schweighauser *et al.* 2020; Shi *et al.* 2021; Yang *et al.* 2021; Chang *et al.* 2022; Jiang *et al.* 2022; Li *et al.* 2018; Sawaya *et al.* 2021).

In this study, we revisit sarkosyl-insoluble extracts to characterize all species of aggregated tau and Aβ by biochemical assays, negative stain immunolabeling, and cryo-EM. Using quantitative proteomics on 11 AD and 10 disease-control (non-AD) brains, we estimate the total amount of tau and Aβ, and find up to equimolar ratios of $A\beta_{42}$ to R1 tau peptides, and sub-equimolar ratios of $A\beta_{42}$ to R3-R4 tau peptides in AD. Utilizing high resolution mass spectrometry, we also characterized the PTMs of tau. Hyperphosphorylation of the proline rich region (PRR) and the C-terminus along with extensive ubiquitination and acetylation of the microtubule binding region (MTBR) are the key features of the N-terminal truncated insoluble tau. Cryo-EM confirmed the general C-shape fold of the extracted PHF filaments with potential sites of ubiquitination. Immunogold labeling electron microscopy suggests co-

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location of N-terminal intact tau and Aβ peptides in globules of non-filamentous materials. The substantial amounts of both tau and Aβ in the sarkosyl-insoluble pellets have implications for structural and neurotoxicity assessments of human brain derived materials. Since the onset of AD and other tauopathies occurs earlier than tau filaments are found in the brain, it is important to characterize non-filamentous aggregates and co-aggregates with interaction partners along with the filamentous pathway which may be the most relevant therapeutic targets. This should also help to find what determines disease-specific differences of tau filament structures in human tauopathies.

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Materials and Methods

Reagents

All LC-MS grade chemicals: acetonitrile (ACN) (cat. 85188), formic acid (FA) (cat. 28905), trifluoroacetic acid (TFA) (cat. 85183), acetic acid (cat. A113-50), isopropanol (cat. 383920025), urea (cat. 434720010), dithiothreitol (DTT) (cat. R0861), iodoacetamide (IAA) (cat. 35603), tri-ethyl ammonium bicarbonate (TEAB) buffer (cat. 90114), ethylenediaminetetraacetic acid (EDTA) tetrasodium salt dihydrate (cat. 147851000) were purchased from ThermoFischer Scientific. N-dodecanoyl-N-methylglycine sodium salt (N-lauroylsarcosine sodium salt) (cat. 61745), sucrose (cat. S0389), NaCl (cat. S7653), and Tris buffers (cat. T5030) were purchased from Merck-Sigma. MS grade metalloprotease LysN from *Grifola frondosa* (cat. 90300) and Bond-Breaker™ TCEP Solution, neutral pH, (cat. 77720) were purchased from ThermoFischer Scientific. Biomasher was purchased from Omni International. The MS vials, Advanced Bio Peptide Mapping C_{18} Column (2.1 × 150 mm, 2.7 μm) and ESI low concentration tune mix used for instrument calibration were obtained from Agilent Technologies (Santa Clara, USA). Anti-Aβ antibody (cat. MO872 – clone 6F/3D) and peroxidase labelled streptavidin/biotin were purchased from Dako. Oasis HLB μElution 96 well-plates were purchased from Waters. Amino acid analysed tryptic stable isotope labelled (SIL) peptides of tau, α-synuclein, β-synuclein, neurofilament light (NEFL), metallothionine-1 and metallothionine-3 were purchased from New England Peptides (MA, USA). Similarly, amino acid analyzed Lys-N peptides Aβ peptides DAEF(R+10)HDSGYEVHHQ, F(R+10)HDSGYEVHHQ, (K+8)GAIIGLMVGGVV, (K+8)GAIIGLMVGGVVIA, K(+8)LVFFAEDVGSN were purchased from New England Peptides. Stock solutions of SIS tryptic peptides and Aβ peptides were prepared in 2 % ACN, 0.05 % TFA to a final concentration of 5000 fmol/μL and 200 fmol/μL, respectively and stored at -80 °C. All the isomerized and citrullinated Aβ peptide standards were

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commercially synthesized and purchased from JPT Peptide Technologies (Germany). All the SIS isomeric and/or citrullinated Aβ peptides were resuspended in 30 % ACN, 0.1 % FA at 0.2 nmol/μL which were subsequently diluted to ~ 2 pmol/μL in 15 % ACN, 0.1 % FA and stored at -80 ˚C.

Human brain tissues

Postmortem brain tissue samples were obtained from the Victorian Brain Bank (Australia). The cohort consisted of age-matched AD brains (n=11) and disease-control Amyotrophic Lateral Sclerosis (ALS) brains (n = 10). Disease-control brains were histopathologically analyzed to determine the number of plaques and tangles which were below the cut-off values for AD. The AD brains met the standard criteria for AD neuropathological diagnosis (Table S1).

Histology and immunohistochemistry

Following fixation in 10% neutral buffered formalin, blocks of frontal cortex from each case were processed for paraffin embedding and sectioning at 6 μm. Sections were deparaffinised in xylene, endogenous peroxidase blocked with 5% hydrogen peroxide (5 min), treated with 98 – 100% formic acid (5 min), rinsed with water and placed in a Tris buffer bath (0.5 M pH 7.6) prior to immunohistochemical labelling. Amyloid Aβ plaque material was labelled with a 60 min incubation in a 1/500 dilution of Dako anti- Aβ antibody (cat. MO872–clone 6F/3D). Positive labelling was detected with the Dako Real EnVision™ horseradish peroxidase system (cat. K5007). Sections were then counterstained with Harris's haematoxylin, dehydrated and cover slipped. Double labelling of Aβ and tau aggregates with 6F/3D (1/500) and AT8 (tau $pSer_{202}$ + $pThr_{205}$) (Thermo Fisher cat. MN1020) diluted 1/2000 were detected with the Dako EnVision™ GL2 Doublestain system (cat. K5361). Aβ and tau

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aggregates were labelled pinkish red (coral) and brown, respectively. Following labelling, sections were cover-slipped using an aqueous mounting media (Gurr, aquahere). Low and high magnification images were obtained with a Leica ICC50 HD camera on a Leica DM 750 binocular microscope.

Biochemical extraction of insoluble materials

1 g of grey matter from postmortem frontal cortex tissues were homogenized in 10 volumes (v/w) of lysis buffer (10 mM Tris-HCl, pH 7.4, 800 mM NaCl, 1 mM EDTA, 2mM DTT, 10% sucrose) using a probe sonicator (Branson, Sonifier® Cell Disruptor) in a series of 30 seconds at 30% power until complete homogenization. The starting brain homogenate was centrifuged (Eppendorf microcentrifuge, 5415D) at 16,000 *g* for 20 mins at 4°C. The crude supernatant was treated with N-lauroylsarcosinate (1 % (w/v) final concentration) and shaken at room temperature for 1 hour. The supernatant was then centrifuged at 100,000 g for 1 hour at 4 °C. The sarkosyl-insoluble pellet was resuspended in 300 µL washing buffer (10 mM Tris-HCl, pH 7.4, 800 mM NaCl, 5 mM EDTA, 2mM DTT, 10% sucrose) and centrifuged at 16,000 *g* for 30 mins at 4°C. After centrifugation, the supernatant was further centrifuged at 100,000 g (Beckman Coulter, Optima MAX-XP) for 1 hour at 4°C. Finally, the purified sarkosyl-insoluble pellet was resuspended in 50 µL 20mM Tris-HCl, pH 7.4, 100 mM NaCl and stored at - 80 °C. Further details are given in schematic presentation of protocol in Supplementary Fig. S1.

Total protein concentration

Total protein concentration was determined using the bicinchoninic acid (BCA) assay method(Smith *et al.* 1985).

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Quantification of Aβ, tau and p(phospho)-tau by ELISA

Initially, 4 µL of brain fractions were digested with 10 µL of 70% FA for 2 hours at room temperature. To determine the concentration of $A\beta_{1\text{-}42}$, tau, and p-tau, 2 µL of the formic acid digested material from step above were neutralized with 38 µL of 1M Tris-HCl pH 9 then diluted with the sample buffer, before loading onto the plate. Each sample was tested in duplicate, and the assay was performed according to the manufacturer's instruction as described previously(Li *et al.* 2015). Commercial ELISA for $A\beta_{1\text{-}42}$, tau and p-tau were purchased from Fujirebio (INNOTEST, Ghent, Belgium, cat. FRI14358, FRI99021, FRI31928). The INNOTEST reagents included monoclonal capture/detection antibodies 21F12($A\beta_{33\text{-}42}$)/3D6($A\beta_{1\text{-}5}$) for $A\beta_{1\text{–}42}$, AT120($tau_{218\text{-}224}$)/HT7($tau_{159\text{-}163}$) and BT2($_{tau194\text{-}198}$) for tau, and HT7($tau_{159\text{-}163}$)/AT270(pT_{181}) for pT_{181} tau. Molecular masses were assumed to be 4.5 kDa and 45 kDa for Aβ and tau/p-tau, respectively. Based on internal CSF control results(Doecke *et al.* 2018), the mean intra-assay percentage coefficient of variance was expected to be 7.4% for $A\beta_{42}$, 4.0% for tau, and 1.6% for p-tau. These uncertainties were included in the estimation of the standard deviations for the average quantities of Aβ, tau and p-tau (Table 2).

LC-MS/MS for label free proteomics

Purified sarkosyl-insoluble pellet from one AD brain was first used to optimize the extraction protocol for quantitative proteomics. 10 µL of sarkosyl pellet was digested with 10 µL of 70% FA for 2 hours at room temperature. For quantitative analysis of the rest of the 10 AD and 10 disease-control brains 4 µL of sarkosyl pellet were digested with 10 µL of 70% FA for 2 hours at room temperature, out of which 4 µL was used for ELISA and the rest was processed for proteomics. The digested sample was lyophilized in a speed-vacuum. The sample was resolubilized in 20 µL of sample buffer (BioRad) with sonication for 30 seconds.

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10 µL of 1:5 Laemlli buffer: TCEP (tris(2-carboxyethyl)phosphine) mix was added, and the sample was equally divided and loaded on a 4–20% Criterion© gel. The gel was electrophoresed on at 160V for 45 minutes. After electrophoresis the gel was stained with Oriole stain for 90 minutes, in darkness. Gel was imaged using Typhoon Imager machine. Gel image was printed out and regions to cut were noted and numbered (Fig. 1B). The gel bands were cut using a sterilized scalpel and corresponding in-gel digestion using either trypsin or Lys-N protease was performed. The gel bands were de-stained using 50 % ACN, 0.1 % FA solution, reduced with 10 mM DTT at 56 °C for 30 minutes and alkylated in the dark using 20 mM IAA solution at 37 °C for 45 min. Gel bands were dehydrated in the speed-vacuum and rehydrated using a 12.5 ng/µL trypsin solution (25 mM TEAB pH 8.5, 1.1 mM $CaCl_2$) on ice for 10 min. The gel bands were digested overnight at 37 °C. The reaction was quenched using 10 % FA solution and the supernatant was desalted on Oasis µElution HLB plates (Waters, cat. 186005518).

Peptides from in-gel digested samples were analyzed using a QExactive Plus mass spectrometer (Thermo Fischer Scientific, Bremen) coupled to Dionex UltiMate 3000 RSLCnano liquid chromatography system (Thermo Fischer Scientific, Bremen). Peptides resuspended in peptide loading buffer (2 % ACN, 0.05 % FA) were loaded onto Acclaim™ PepMap™ 100 C18 trap column (100 µm X 2 cm C_{18} column, Thermo) using 5 µL/min loading pump and separated on an Acclaim™ PepMap™ RSLC column (75 µm X 25 cm) on a stepped gradient of 45 min starting from 97 % buffer A (0.1 % FA in HPLC water) and 3 % buffer B (0.1 % FA in ACN) to 25 % buffer B, 28 min; 40 % buffer B, 30 min at 300 nL/min. The column was washed with 85 % buffer B before equilibration at 3 % buffer B. The proteolytic peptides were sprayed into MS inlet at 1900 V in positive ion mode using commercial PicoTip emitters (New Objective). A full mass spectrum scan with a resolution of 70,000 @ *m/z* 400 was acquired in the mass range *m/z* 375-1400 (AGC target 3 X 10^6,

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maximum injection time 50 ms). Top 15 monoisotopic peptide species were selected for fragmentation using higher-energy collisional dissociation (HCD) in the data dependent mode. MS2 scan settings were the following: resolution 17,500, AGC target 5 X 10^4, maximum injection time 50 ms, isolation window 1.2 m/z, normalized collision energy 30 %, filter intensity (intensity threshold $4e^4$), charge states 2-7 selected, undetermined charge states excluded, exclude isotopes on, and dynamic exclusion 30 s.

Raw data was analyzed in MaxQuant using human proteome database downloaded in January 2019 from Uniprot (only reviewed entries and appended with in-house Fasta containing Aβ sequences) using Andromeda search engine(Cox *et al.* 2011). The following settings were applied: trypsin (specificity set as C-terminal to arginine and lysine) with up to two missed cleavages, the mass tolerance was set to ± 20 ppm for the precursor. Fixed modifications included Cys carbamidomethylation (CAM, mass shift +57.0215 Da) with variable modifications including Ser/Thr/Tyr phosphorylation (+79.9663 Da), Lys ubiquitination (+114.0429 Da), Lys N-terminal acetylation (+42.420106 Da), Gln and Asp deamidation (+0.98 Da) and Met oxidation (+15.9949 Da). False discovery rate (FDR) was set to 1% on peptide and protein levels with a minimum length of seven amino acids and was determined by searching a reverse database. For all other search parameters, default settings were used. Similar parameters were used for the Lys-N digested samples with the setting for the corresponding enzyme. Label-free quantification was done using the in-built label-free quantification (LFQ) algorithm(Cox *et al.* 2014) integrated into MaxQuant. iBAQ (intensity based absolute quantitation) was used for data analysis that were performed with the Perseus software and GraphPad Prism.

High-resolution LC-MS/MS measurements and data analysis

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Proteomics of the sarkosyl-insoluble samples were performed on a high-resolution mass spectrometer (HRMS) Orbitrap Eclipse (Thermo Fischer Scientific, Bremen) coupled to Dionex UltiMate 3000 RSLCnano liquid chromatography system (Thermo Fischer Scientific, Bremen). Peptides resuspended in peptide loading buffer (2 % ACN, 0.05 % FA) were loaded onto Acclaim™ PepMap™ 100 C18 trap column (100 µm X 2 cm C_{18} column, Thermo Fischer Scientific) using 5 µL/min loading pump and separated on an Acclaim™ PepMap™ RSLC column (75 µm X 25 cm) on a stepped gradient of 120 min starting from 97 % buffer A (0.1 % FA in HPLC water) and 3 % buffer B (0.1 % FA in ACN) to 23 % buffer B, 95 min; 40 % buffer B, 105 min at 300 nL/min. The column was washed with 80 % buffer B for next 10 min and equilibrated for another 10 min at 3 % buffer B. The proteolytic peptides were sprayed into MS inlet at 1900 V in positive ion mode using commercial PicoTip emitters (New Objective). A full mass spectrum scan with a resolution of 120,000 @ *m/z* 400 was acquired in the mass range *m/z* 375-1500 (AGC target 4 X 10^6, maximum injection time 20 ms). Peptides were selected for fragmentation using higher-energy collisional dissociation (HCD) using MIPS (monoisotopic precursor selection) mode. MS2 scan settings were the following: resolution 15000, AGC target 5 X 10^5, maximum injection time 22 ms, isolation window 1.6 m/z, normalized collision energy 30 %, filter intensity (intensity threshold $5e^4$), charge states 2-7 selected, undetermined charge states filter on, exclude isotopes on, and dynamic exclusion 30 s. The raw data from sarkosyl-insoluble pellets from AD and DCN were first analyzed in MaxQuant (2.0.3) using human proteome database downloaded in January 2019 from Uniprot (only reviewed entries and appended with in-house FASTA sequence database containing 2N4R tau and Aβ sequences) using Andromeda search engine as previously described(Cox *et al.* 2011) using trypsin as the enzyme, two missed cleavages, the default mass tolerances were used for MS1 and MS/MS level. Fixed modifications included carbamidomethylation on cysteine (C, +57.0215 Da) with variable modifications including acetylation (N-term/K, +42.420106 Da), and methionine oxidation (M, +15.9949 Da). False discovery rate (FDR) was set to 1% on peptide and protein levels. Following removal of common contaminants, the proteins iBAQ intensities were $\log_2$-transformed, missing values were interpolated and then *t*-test was performed between the AD *vs* DCN groups. Volcano plot was generated to visualize the differential protein expression in AD *vs* DCN group. The *t*-test – $\log_{10}$(p) values represent statistical significance in the y-axis, while the $\log_2$ fold change (difference) on the x-axis is the $\log_2$ ratio of normalized iBAQ intensities in a pairwise comparison between AD and DCN groups. The heatmap colors of the differentially expressed proteins in the insoluble pellets were based on the range of the minimum to maximum average of the $\log_2$ iBAQ intensities. The raw data from the sarkosyl insoluble pellet from AD were searched with the same human database in Proteome Discoverer (PD 2.4) using Sequest search engine using the following settings: enzyme specificity using trypsin with up to two missed cleavages, the mass tolerance was set to ± 30 ppm for the MS1 and ± 0.6 Da ppm tolerance for the MS/MS. Along with fixed carbamidomethylation modification on Cys (mass shift +57.021 Da), we included Lys acetylation (+42.420106 Da), Ser/Thr/Tyr phosphorylation (+79.9663 Da), Lys ubiquitination (+114.0429 Da), Gln/Asn/Arg deamidation (+0.984 Da), Arg dimethylation (+28.031 Da) and Met oxidation (+15.9949 Da) as variable modifications. N-terminal modifications of acetylation, methionine loss (-131.040 Da) and methionine loss followed by acetylation (-89.030 Da) were also included. The sarkosyl-insoluble AD data was filtered for tau PTMs.

Absolute quantitation of tau and Aβ

To estimate the absolute quantity of tau and Aβ we used complementary tryptic and Lys-N enzymatic digestion, respectively. For estimating total tau in the sarkosyl-insoluble and soluble components of the brain derived tissues, these fractions were spiked with known

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amount of full-length protein ^{15}N labelled tau_{1-441} (2N4R) (rPeptide, Georgia, USA) and after tryptic digestion further SIL tau peptides TPPAKT(+79.99)PPSSGEPPK($^{13}C6$, $^{15}N2$), TPPSSGEPPK($^{13}C6$, $^{15}N2$), LQTAPVPMPDLK($^{13}C6$, $^{15}N2$), TPSLPT(+79.99)PPTR($^{13}C6$, $^{15}N4$), TPSLPTPPTR($^{13}C6$, $^{15}N4$) were added. This strategy provided additional benefit of estimating the absolute amount of different tau peptides and compare them to full length N^{15} labelled tau protein standard. Similarly, to estimate total $A\beta_{42}$ concentrations, modified LysN-digestion workflow was used(Mukherjee *et al.* 2021a; Mukherjee *et al.* 2021b). It should be noted that recent analysis of tau isoforms and post-translational modification (PTM) stoichiometry in AD brains (Wesseling *et al.* 2020) was based on FLEXItau (full-length expressed stable isotope-labelled tau) approach (Mair *et al.* 2016) which estimates of a probable modification of peptide site (a relative abundance of PTM containing and unmodified peptide), however stable isotope labelled (SIL) peptides are necessary for absolute quantitation of PTM containing and unmodified peptides. Therefore, to quantify and reproducibly measure two distinct peptides in biological samples the SIL peptide targeted workflow was utilized in our current study. Briefly, predetermined amount of isotopically labelled $A\beta_{4-42}$ at R ($^{13}C6$,$^{15}N4$) and K ($^{13}C6$, $^{15}N2$) was spiked into the same samples as full-length tau standard and after Lys-N digestion further SIL Aβ peptides were added. These complementary digestions were performed using urea digestion protocol. Samples were first digested with formic acid (80 %) and lyophilized, followed by resuspension with 1 M Tris-HCl pH 8.8. Then, 8 M urea, 5 mM TCEP, 100 mM Tris-HCl pH 8.5 buffer and the full-length tau and $A\beta_{4-42}$ standards were added. The samples were sonicated for 5 minutes followed by incubation at 54 °C for 30 minutes. After reduction, the samples were cooled and alkylated with 20 mM final concentration iodoacetamide (IAA) in the dark for 30 minutes at 37 °C. The samples were diluted to 100 µL with 100 mM TEAB pH 8.5 buffer before being equally split into two that were further diluted with 100 mM TEAB pH 8.5, 1.1 mM $CaCl_2$

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buffer to reduce the urea concentration < 1 M. One half of the protein samples was digested with 50 ng/µL trypsin while the other half was digested with 25 ng/µL Lys-N enzyme overnight at 37 °C. The digested peptides were quenched with 10 % to 0.1 % TFA in the solution and these acidified peptides were desalted using Oasis µElution HLB plates (Waters).

Multiple reaction monitoring (MRM) of proteolytic peptides and data analysis

An Agilent 1200 Infinity series UHPLC system connected to 6495 QQQ (Agilent Technologies, USA) was used for the LC-ESI-QQQ-MRM assay. Mobile phase A consisted of 0.1 % FA in water and mobile phase B of 0.1 % FA in 100 % ACN. Trypsin digested samples were separated using an Agilent Advanced Bio Peptide Mapping C_{18} Column (2.1 × 150 mm, 3 µm) maintained at 55°C in column compartment and eluted at 0.4 mL/min flowrate with the following stepped gradient, 2.5 % B, 0 min; 25 % B, 15 min; 34 % B, 17 min; 81 % B, 17.2 min; 81 % B, 18.75min; 2.5 % B, 18.8 min. The source ESI parameters as well the collision energies were optimized for these peptides in the positive ion mode in Skyline software (20.2.1.315, MacCoss Lab, Department of Genome Sciences, University of Washington, Seattle, WA) and the method was imported into Agilent Mass Hunter Workstation (version 10.0.142) for data acquisition. The typical parameters were the following: gas temperature 200°C, gas flow 15 L/min, nebulizer 40 psi, sheath gas temperature 250°C, and sheath gas flow 11 L/min. The capillary voltage was 4500 V and the nozzle voltage was set at 1000 V. The optimized iFunnel parameters were 200 V and 110 V for high and low-pressure RF respectively. To ensure no bias in acquisition, samples were run in randomized order. For absolute Aβ quantitation, the Lys-N digested samples were loaded onto an Advanced Bio Peptide Mapping C_{18} Column (2.1 × 150 mm, 2.7 µm) maintained at 55°C in TCC and peptides were eluted at 0.4 mL/min flow-rate with the

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following gradient: 2.5 % B, 0 min; 6 % B, 5 min; 9 % B, 20 min; 22% B, 25 min; 29 % B, 35 min; 34 % B, 37 min; 81 % B, 38 min; 81 % B, 40 min; 2.5 % B, 41 min with a post-run equilibration for 2 min. The source ESI parameters were same as described above. 20 µL of either tryptic or Lys-N digested samples were injected on to the columns. The targeted QQQ data were imported into Skyline with formula annotations of the targeted peptides added to the method. Data for each peptide was extracted in the software using the transition lists and co-elution of RT of the heavy SIL spiked standard. The peak area for the tryptic tau/phospho-tau peptides in this study were compared to their heavy analogues (^{15}N metabolically labelled total tau as well as $R={}^{13}C_6, {}^{15}N_4, K={}^{13}C_6, {}^{15}N_2$ for phospho-tau SIS peptides), while $A\beta_{16\text{-}27}$, $A\beta_{28\text{-}40}$ and $A\beta_{28\text{-}42}$ peptides were compared to their heavy analogues ($R={}^{13}C_6, {}^{15}N_4$, $K={}^{13}C_6, {}^{15}N_2$) to derive the light-to-heavy ratios. The absolute quantification was determined by using the known concentrations of the spiked in SIS peptides. To determine statistically significant differences between potential biomarkers, the adjusted *P* values were calculated using unpaired non-parametric one-way analysis of variance (ANOVA), corrected for multiple comparison false discovery rate ($P < 0.05$) with Benjamini–Hochberg correction. Percentage phosphorylation on respective p-tau (T_{181} and T_{217}) was calculated as the fraction of phosphorylated peptide divided by the sum of unmodified and phosphorylated peptide pairs.

Immunoblotting

The AD starting material, and AD and disease control (ALS) final tau pellets were treated in 70 % formic acid (1:2) for two hours at room temperature. Samples were then lyophilized, resuspended in PBS + TrisHCl and sonicated for 5 min. Samples were mixed with 4X Laemmli sample buffer (BioRad, cat. 161-0747) containing 20% TCEP 0.5M and boiled for 10 minutes at 90 °C. 10 µg of samples were loaded per well and electrophoresed at 180 V for

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one hour on 4–20 % Criterion© TGX stain-free precast gels (BioRad; Aus), and then transferred onto 0.45 µm nitrocellulose membrane (Biorad) using iBlot semi-dry transfer station from Invitrogen. Membranes were boiled and then blocked in tris-buffered saline with 0.05% Tween20 (TBS-T; 6.05 g Tris, 8.76 g NaCl per liter) containing 5% low-fat milk powder (Diploma, Anchor, Australia, cat. 3001742) for 1 hour at room temperature. All antibodies were diluted in TBS-T and 5 % milk. Membranes were probed with primary antibodies (Aβ: G2-10 and WO2 1:1000 (Sigma-Aldrich, cat. MABN11, MABN10), tau: Dako A0024(K9JA) 1:10000 (LI-COR, Australia, cat. K500711-2), and TDP43 polyclonal 1: 10000 (Proteintech, cat. 10782-2-AP)) overnight at 4 °C. The membranes were then washed in TBS-T for 21 minutes (3 X 7 minutes) before and after incubation for 1 hour with corresponding secondary antibodies (1:750). Immunoreactivity was detected using LI-COR Odyssey FC system (LI-COR, Australia) and analyzed *via* signal intensities (ImageStudio 5.2, LI-COR, Australia).

hTau441 expression and purification

The full-length human tau (hTau441) cloned into pET-28a (Sigma-Aldrich, cat. 69864) in frame with a C-terminal His6 tag was expressed in large quantities in BL21 (DE3) competent *E. coli* cells (New England BioLabs, cat. C2527H) at a bench reactor scale (1L). High density of *E. coli* expression was achieved using defined minimal medium with glycerol as the sole carbon source and kanamycin for selection. The induction of protein expression was done by adding isopropyl-β-D-thiogalactoside (IPTG) (Sigma-Aldrich, cat. I5502) to a final concentration of 1 mM once the optical density OD600 reached 16. Post-induction temperature was reduced to 20°C as required to prevent biological oxygen demand exceeding maximum aeration capacity. When an OD600 of 46 was reached the culture was harvested by centrifugation at 8000 x g, 4 °C, for 30 min. Biomass cell pellets (total 80g) were frozen in

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liquid nitrogen and stored at -80 °C. Tau was then purified as described previously(Liu *et al.* 2016). Briefly, cell pellets were resuspended in IMAC buffer (300 mm KCl, 50 mm KH_2PO_4, 5 mm imidazole, pH 8.0) containing 0.1% complete protease inhibitor and 0.1 mg/ml lysozyme (Sigma-Aldrich, cat. I1408) and subjected to repeated freeze/thawing after which they were sonicated at 60% amplitude for 1 min (Qsonica Sonicator, Newtown, CT). The lysate was centrifuged at 16,000 × g for 20 min at 4 °C and filtered through a 0.22-µm syringe filter (Millipore, Billerica, MA). The supernatant was passed over a 1-ml Bio-Scale Mini IMAC cartridge (Bio-Rad) equilibrated in IMAC buffer and eluted in 300 mM KCl, 50 mM KH_2PO_4, 250 mM imidazole, pH 8.0. Eluted proteins underwent buffer exchange into 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 88 mM KH_2PO_4, pH 7.4, using a Bio-Scale Mini Bio-Gel P-6 desalting cartridge (Bio-Rad) and were then subjected to size exclusion chromatography using an S200 10/30 GL column (GE Healthcare) equilibrated in 1× PBS. Fractions corresponding to the proteins of interest were combined and concentrated to 6.3 mg/ml using an Amicon Ultra Filter with a 3,000-kDa molecular mass cutoff (Merck Millipore). Purified tau was analyzed by SDS-PAGE/Coomassie blue staining and stored at -20 ^{o}C.

hTau441 aggregation with heparin

Purified hTau441 was fibrillized in the presence of heparin, and successful fibrillization was confirmed using a Thioflavin (ThT) fluorescence assay and EM as previously described approaches(Barghorn *et al.* 2005; Guo & Lee 2011; Iba *et al.* 2013; Stancu *et al.* 2015). Briefly, 50 µL reaction mixture containing 40 µM hTau441, 30 mM MOPS buffer (Sigma-Aldrich, cat. M1254), 4 mM TCEP (Sigma-Aldrich, cat. C4706), pH 7.4 of incubation buffer and 40 µM heparin (MW ~15KDa) (MedChemExpress, cat. HY-17567C) (tau : heparin ~ 3 : 1) were prepared. The reaction mixture was split into two fractions and placed in 96-well

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plates (Wallac Isoplate, PerkinElmer) with 2 µL of ThT (16 µM) added to the fractions. ThT fluorescence intensity was measured every 5 min at 37 °C for ~ 5 days using the EnSpire Multimode Plate Reader (PerkinElmer) until a plateau was reached.

Synthetic Aβ_{42} aggregation

Synthetic A$\beta_{1\text{-}42}$ was prepared by dissolution in 10% % (w/v) NH_4OH at 0.5 mg/ml as described(Ryan *et al.* 2013). Briefly, the peptide was incubated for 10 min at room temperature followed by sonication for 5 min. The NH_4OH was removed by lyophilization to yield a salt free fluffy white peptide. A$\beta_{1\text{-}42}$ was fibrillized and monitored using a ThT fluorescence as described above. Briefly, 14 µg of lyophilized aliquot of synthetic A$\beta_{1\text{-}42}$ was resuspended at 60 µM in PBS, sonicated briefly and centrifuged at 10,000 *g* for 5 min at 4 °C. Then a reaction mixture was prepared containing 10 µM Aβ in incubation buffer and 16 µM ThT. After completion of the incubations/reaction the samples were harvested and the positive A$\beta_{1\text{-}42}$ samples were centrifuged at 100,000 *g* for 1 hour at 4°C. The pellet was harvested and washed. The pellet was stored at – 80°C for EM analysis.

Negative stain EM

About 4 µL of each final pellet with sarkosyl-insoluble material were placed on 400 mesh formvar/carbon film-coated copper grids (ProSciTech), that were glow discharged for 30 s at 15 mA with a PELCO easiGlow™ cleaning system (TED PELLA). The samples were incubated on the grid for about 1 min at room temperature. Excess solvent was soaked away with filter paper and the grids were stained with either 2% (w/v) uranyl acetate or Nano-W® (methylamine tungstate) (Nanoprobes, cat. 2018) stain in water following standard staining procedures. Uranyl acetate potentially gives images with low grain, high contrast, and clearly defined boundaries, whereas tungstate is expected to give superior delineation of the

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substructure(Wischik *et al.* 1985). The air-dried grids were examined in a FEI Tecnai F30 FEG/TEM or in a FEI Tecnai F20 FEG/TEM both equipped with upper CETA 4k x 4k CMOS cameras and operated at 300 kV and 200 kV, respectively. The dimensions of PHF, selected at random, were determined from measurements using images taken at a higher magnification.

Immunogold labelling EM

Immunogold labelling of sarkosyl-insoluble materials was performed essentially as described(Goedert *et al.* 1992; Falcon *et al.* 2018b) using commercial and non-commercial primary and secondary antibodies listed in the supplementary Table S2. 4 µL of final pellet with sarkosyl-insoluble material were deposited on glow-discharged grids as above for ~60 s, blocked for 10 min with blocking buffer (PBS + 0.1% gelatine), and incubated with primary antibody in blocking buffer for 1h at room temperature. The grids were then washed with blocking buffer, blotted, and placed on a solution of the appropriate secondary antibody conjugated to gold for 1 hr at room temperature. The grids were washed with blocking buffer, blotted, stained with two drops of Nano-W® (methylamine tungstate) for 60 s, and allowed to air dry. For double immunogold labelling, secondary antibodies against each of the primaries conjugated to gold particles of two different sizes were used. Grids were incubated as described above with the two primary antibodies and then with two secondary antibodies diluted together at their respective dilutions. Images were acquired as above. To identify nonspecific primary antibody binding, a substitution of the primary antibody with isotype control IgG antibody as well as using anti-Aβ antibodies with tau recombinant sample and anti-tau antibodies with recombinant Aβ samples were used. To confirm gold probe specificity, a negative control with the secondary antibody only by omitting the primary antibody was also conducted.

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Cryo-EM imaging

For cryo-EM imaging, 4 µL of sample was plunge-frozen on glow-discharged Lacey Carbon film on copper 200 mesh (ProSciTech) using FEI Vitrobot Mark IV (Fig. S13A). In total, 890 micrographs were recorded on a FEI Talos Arctica Cryo-TEM at 200 kV using a K2 direct electron detector, yielding a pixel size of 1.31 Å, exposure/frame of 1.30 e/Å^2 and total exposure of 52 e/Å^2. Aligning movies was done using cisTEM software(Grant *et al.* 2018), then further analysis was performed using RELION3.1 package(Scheres 2019). Filament selection was performed manually and in total 127653 segments were used. Two-dimensional and 3D classification (Figs. S13B&C) was conducted on selected 9926 segments. A box size of 214 px, which corresponds to 280 Å, and a radial mask with a diameter of 200 Å were used. A 3D reconstruction was low-pass filtered to 60 Å and was used as an initial model for further low-defocus 3D refinements. For further processing steps, only micrographs that contained a signal beyond a resolution of 10 Å were used. The final resolution of ~5.7 Å was assessed by Fourier shell correlation (Fig. S13D). Molecular graphics was performed with UCSF Chimera(Pettersen *et al.* 2004).

Statistical analysis and study design details

Data analyses that were performed with the Perseus software and GraphPad Prism. For MRM proteomics the adjusted *P* values were calculated using unpaired non-parametric one-way analysis of variance (ANOVA), corrected for multiple comparison false discovery rate ($P <$ 0.05) with Benjamini–Hochberg correction. For statistical comparison of two groups, a two-tailed Student's *t*-test was used. A value of $P < 0.05$ was considered significant. Data are represented as mean±standard deviation (SD). No blinding was performed. Data were not assessed for normality. No exclusion criteria were pre-determined and no test for outliers was

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conducted. A priori sample size calculation was not conducted. However, following the approach (Meurs 2016) for calculation of the effect size for postmortem brain studies, the total number of post-mortem brain samples of 21 (2 groups) in our study provided the effect size of 0.37 (with the minimal significance $\alpha=0.05$ and statistical power $1-\beta=0.80$), which was close to the average effect size of 0.46±0.3 found for 22 studies of postmortem brains (Meurs 2016).

Ethics approval and consent to participate

Fresh frozen brain samples were received from the Victorian Brain Bank (VBB) that obtained informed consent from the donors. The study was approved by the ethics committee of the University of Melbourne (Ethics 1750801.3).

Results

Immunohistochemical assessment of AD brain samples

The immunohistochemistry of postmortem human AD cortical tissue sections demonstrated the presence of both diffuse and dense core plaques labelled with $A\beta_{9-14}$ - 6F/3D (pink) and p-tau ($S_{202}T_{205}$) - AT8 (brown/black) (Fig. 1A). The dense core (pink) Aβ plaques/aggregates were surrounded by tau-reactive neuritic elements. Scattered dense (dark brown) neurofibrillary tangles were present in the neuropil. Disease-control brains (ALS cases) did not show any Aβ/Tau immunoreactivity (data not shown).

Qualitative assessment of AD brain derived detergent-insoluble pellet

Firstly, a qualitative assessment of AD brain derived pellet was performed. Following formic acid solubilization and neutralization, the pellet proteins were separated by denaturing SDS-PAGE. Complementary in-gel digestion was performed (Fig. 1B) for label-free proteomics (Fig. S2A), which indicated that tau (MAPT) was one of the most abundant proteins in these

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pellets (Fig. 1B, left panel). However, other proteins such as ferritin (light and heavy chain), α-synuclein, calmodulin kinase (CAMK2A), neurofilament light (NEFL/NfL), U1 small nuclear ribonucleoprotein 70 kDa (SNRNP70), Apolipoprotein E (ApoE) and ubiquitin (UBB) were also observed which are known to be co-enriched in sarkosyl pellets (Fig. 1B). Most importantly, we found N-terminal truncated Aβ peptides in these pellets around the 4 kDa band (Fig. 1B, right panel), whereas the more aggregation prone $Aβ_{42}$ peptide did not migrate to the bottom of the gel. $Aβ_{40}$ also showed a similar smearing trend with most of the intensity at the top of the gel, albeit less than $Aβ_{42}$. Western blotting analysis of the same pellet using a polyclonal tau antibody was associated with smeared immunostaining, that was also seen with p-tau (T_{181}) antibody, a well-known feature of heterogenous aggregated insoluble tau. Western blotting with the G2-10 antibody corroborated our proteomic observation, where monomeric Aβ migrated into the gel, whereas aggregated Aβ was trapped at the top of the gel (Fig. 1C).

Tau and Aβ peptide concentrations in detergent-soluble and -insoluble fractions in AD and disease-control brains

We designed a quantitative approach based on spiking with SIL tau and Aβ peptides to estimate the molar ratio of tau and Aβ peptides and to understand the tau isoform distribution in various biochemical fractions from brain tissues. This targeted MRM assay included the following tau peptides: PRR ($tau_{181-190}$, $tau_{195-209}$ and $tau_{212-221}$), MTBR R1 ($tau_{243-254}$), MTBR R2 ($tau_{282-290}$), (R2)-R3 ($tau_{299-317}$) and the C-terminus ($tau_{396-406}$). We tested this targeted quantitative MS approach on one AD brain sample during the sarkosyl enrichment process. The molar ratios of $Aβ_{42} : tau_{243-254} \sim 3.5$ and $Aβ_{40} : tau_{243-254} \sim 1$ (Fig. S2B) were consistent with enrichment of both tau and Aβ in the sarkosyl-insoluble pellets. The enrichment of these two proteins was associated with the depletion of other proteins such as metallothionein (MT-

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3), α-synuclein, β-synuclein and neurofilament light (NfL) from the starting material to the final detergent-insoluble pellet (Fig. S2B). Using the sarkosyl extraction protocol (Fig. S1), we obtained sarkosyl-soluble and -insoluble materials from the frontal cortices of 11 sporadic AD brains and 10 age-matched disease-control (DCN) ALS brains. For quantitative estimation, fully ^{15}N-labelled tau and ^{13}C/^{15}N-labelled (at Lys/Arg residues)-Aβ_{4-42} were spiked into the starting material (SM), 1st 100 k supernatant/sarkosyl soluble supernatant (SN) and final tau pellet (P) fractions before proteomic digestion for targeted multiple reaction monitoring (MRM) assay (Fig. 2A). A panel of proline rich region (PRR) (P1 tau$_{181-190}$, P2 tau $_{195-209}$ and tau$_{212-221}$), microtubule binding repeats (MTBR) (MTBR-N (R1) tau$_{243-254}$, 4R (R2) tau$_{282-290}$, 4R (R2-R3) tau$_{299-317}$, and C-terminal (R') (tau$_{396-406}$) tau peptides(Sato *et al.* 2018), Aβ_{28-42}, Aβ_{28-40}, and A$\beta_{\text{mid-domain}}$ peptides along with peptides from NfL, MT-3, MT-1, α-synuclein, and β-synuclein proteins were quantified using targeted MRM assay. The heatmap (Fig. 2A) clearly demonstrated the enrichment of both tau and Aβ peptides in the sarkosyl-insoluble pellets from all AD brains while the DCN samples did not show an enrichment.

The starting homogenate/material (SM) contained significantly more ($P<0.001$) tau$_{212-221}$, tau$_{195-209}$, and C-terminal tau$_{396-406}$ peptides in DCN (4223±1970, 1129±436, and 424±161 fmol/mg, respectively) compared to AD (1162±392, 314±129, and 165±94 fmol/mg, respectively) brains (Figs. 2B and S4A, Table 1). But there were no significant differences between the PRR tau$_{181-190}$ ($P=0.361$), MTBR R1 tau$_{243-254}$ ($P=0.317$), R2 tau $_{282-290}$ ($P=0.494$), and (R2)-R3 tau$_{299-317}$ ($P=0.336$) peptides in AD compared to DCN brains (Table 1). As expected, there was significantly more ($P<0.001$) of Aβ_{28-42} (7460±4479 fmol/mg) in AD compared to DCN (743±732 fmol/mg). Although, 1345±2889 fmol/mg of Aβ_{28-40} was observed in the AD SM, but that was not significantly different ($P=0.160$) from the DCN SM fractions (6±7 fmol/mg). Notable many-fold higher concentration of PRR tau$_{212-221}$ compared

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to the 4R specific $tau_{282-290}$ and $tau_{299-317}$ peptides in both the AD and DCN SM fractions was possibly due to multiple tau isoform present in human brain.

Like the SM fractions, the sarkosyl-soluble (SN) fractions from DCN brains had significantly more $tau_{195-209}$, $tau_{212-221}$ ($P \leq 0.001$), and C-terminal $tau_{396-406}$ ($P=0.63$) peptides (474±252, 1615±750, and 148±93 fmol/mg, respectively) compared to AD (172±58, 614±154, and 68±34 fmol/mg, respectively) brains. Additionally, $tau_{243-254}$ peptides (968±417 fmol/mg) were also significantly enriched ($P=0.002$) in DCN brains compared to AD (456±188 fmol/mg) (Fig. 2B and S4B, Table 1). Like in the SM fractions, there was significantly ($P<0.001$) more $A\beta_{42}$ in the AD SN (1631±915 fmol/mg) compared to the DCN SN (308±104 fmol/mg) fractions. Also, in the SN fractions there were no statistically significant differences in the levels of PRR $tau_{181-190}$ ($P=0.776$), 4R tau $_{282-290}$ ($P=0.416$), $tau_{299-317}$ ($P = 0.324$) as well as $A\beta_{28-40}$ ($P=0.164$) in AD compared to DCN samples (Table 1).

Ideally, if the endogenous tau from brain samples were only of full length (all six isoforms), the concentrations of nonmodified PRR $tau_{181-190}$, $tau_{212-221}$, and MTBR-N $tau_{243-254}$ peptides were expected to be similar, therefore the average value of these three peptides were used as an estimate of the total tau and referred to as t-tau from herein. Indeed, in the SM fractions the quantities of $tau_{181-190}$ (1388±721 fmol/mg), $tau_{212-221}$ (1162±392 fmol/mg, $P=0.372$) and $tau_{243-254}$ (1751±1244 fmol/mg, $P=0.431$) peptides in AD brains were not significantly different (Table 1, Figs. 2A and S4A). Therefore, the average value of 1433±743 fmol/mg was used as an estimate of t-tau in the SM fractions (Table 1).

The observed in Table 1 difference in the $tau_{195-209}$ peptides quantities compared to the other unmodified tau peptides in the SM and SN fractions could be explained by high degree of phosphorylation of the potential phosphorylation sites (S199, S202 and T205) as previous

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proteomics data(Horie *et al.* 2020) observed doubly and triply phosphorylated tau$_{195-209}$ peptides in the AD sarkosyl-insoluble pellets.

While tau$_{212-221}$ (4223±1970 fmol/mg) peptide level was significantly different from that for tau$_{181-190}$ (1651±543 fmol/mg, $P<0.001$) and tau$_{243-254}$ (2247±815 fmol/mg, $P = 0.009$) peptides in DCN brains, an average value of 2707±828 fmol/mg was used as an estimate of t-tau in DCN, which was still significantly ($P=0.002$) higher compared to AD brains. Similarly, in the DCN SN fractions had significantly more ($P<0.001$) t-tau (1097±333 fmol/mg) compared to the AD SN fractions (579±172 fmol/mg brain). In the AD SM and SN fractions, Aβ$_{28-42}$: t-tau ratio was ~ 5±4 and ~ 3±4, respectively (Table 1).

The total concentration of the MTBR R1 tau$_{243-254}$ peptides (412±324 fmol/mg) and R2 tau$_{282-290}$ (243±206 fmol/mg) (Figs. 2B and S4C, Table 1) in the AD P fractions were about 100-fold higher ($P<0.001$) than those (0.2±0.1 fmol/mg brain) and (0.1±0.1 fmol/mg brain) in the DCN P fractions, respectively. However, the concentration of PRR tau$_{212-221}$ (40±29 fmol/mg) in the AD P fractions was about 10-fold higher ($P<0.001$) that that (4±2 fmol/mg) in DCN brains. The difference in the tau$_{212-221}$ quantities indicating that soluble PRR peptides were decreased in the P fractions, as expected from high degree of phosphorylation of this epitope in AD (phospho tau pT$_{217}$, discussed later). This also showed that MTBR peptides were most abundant in the AD P fractions, while certain PRR peptides with higher levels of PTM had very low concentration of their nonmodified forms. Therefore, the concentrations of MTBR R1 tau$_{243-254}$ and R2 tau$_{282-290}$ peptides were averaged to approximate the t-tau (328±274 fmol/mg) in the AD P fractions (Table 1). However, considering that the R1 tau$_{243-254}$ peptide represents both the 4R and 3R tau isoforms, while the R2 tau$_{282-290}$ peptide represents the 4R isoform only, the 4R/3R ratio can be estimated as R2/(R1-R2) ~ 1.4, thus supporting that AD aggregated tau contained mixed 4R and 3R tau with more 4R than 3R isoforms. Noteworthy, the concentration of Aβ$_{28-42}$ (477±444

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fmol/mg) was approximately equimolar to the t-tau concentration (328±274 fmol/mg) with Aβ_{42} : tau ratio of 1.4±1.7, as well as to the concentration of MTBR R1 tau$_{243-254}$ peptide (412±343 fmol/mg) with Aβ_{28-42} : tau ratio of 1.2±1.5 in the AD P fractions (Fig. 2B, Table 1). Overall, about 23 % of the MTBR tau was recovered as insoluble tau fractions.

Further targeted quantitative estimations indicated almost two-fold NfL in AD (5464±1818 fmol/mg) *vs* DCN (2854±1539 fmol/mg) brains in the SM fractions ($P<0.001$) (Fig. 2A), whereas no change was observed for α-synuclein ($P=0.72$), β-synuclein ($P=0.77$), MT-1 ($P=0.08$) and MT-3 ($P=0.89$) concentrations between these two groups. Similar significant increase was observed in the SN fractions for NfL ($P=0.02$) in AD (725±513 fmol/mg) *vs* DCN (333±172 fmol/mg), and MT-1 ($P=0.01$) in AD (581±293 fmol/mg) *vs* DCN (170±82 fmol/mg) brains, while β-synuclein, MT-1 and MT-3 concentrations did not substantially change in the AD *vs* DCN SN fractions. The concentrations of Nfl, α-synuclein, β-synuclein, and MT-1/MT-3 proteins in the P fractions were very low in both AD and DCN, indicating that sarkosyl fractionation enriched mostly aggregated proteins.

As our MRM approach was not designed to measure N-terminal and R4 tau peptides, we used HRMS (high-resolution mass spectrometry using Orbitrap-MS) to further quantify changes observed for tau and Aβ peptides. The absolute concentrations of 22 tau peptides (encompassing htau441 from N- to C-terminus) in the AD pellets from the HRMS indicated that there were substantially less N-terminal peptides than MTBR fragments of tau (Fig. 2C and Table S3). The concentrations of four N-terminal tau peptides (tau$_{25-44}$, tau$_{45-67}$, tau$_{68-87}$ and tau$_{88-126}$) were in the range of ~ 2-10 fmol/mg in the sarkosyl-insoluble pellet (Fig. 2C). The concentrations of MTBR R1 tau$_{243-254}$, R2 tau$_{282-290}$, (R2)-R3 tau$_{299-317}$ and Aβ_{28-42} peptides were 394±298, 249±218, 446±395 and 444±378 fmol/mg, respectively (Figs. 2C and S4C and Table S3). These results further confirmed the equimolar ratio of MTBR specific tau to Aβ obtained from the MRM assay in AD sarkosyl-insoluble fraction (Fig. 2B)

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and the R2/(R1-R2) ratio of ~ 1.7 supported the MRM data that AD aggregated tau contained more 4R than 3R isoform.

Furthermore, the concentrations of the MTBR R3-R4 (AD tau filaments core(Fitzpatrick *et al.* 2017)) $tau_{322-340}$, $tau_{341-347}$, $tau_{344-349}$, and $tau_{354-369}$ peptides were 1240±1121, 1069±970, 1307±1427, and 1269±1027 fmol/mg in AD, respectively (Fig. 2C). This result is consistent with the recent estimate of ~1000 fmol/mg for the total insoluble tau in AD (Wesseling *et al.* 2020), which was based on the same tryptic peptides given in Table S3. The concentrations of the middle domain and the R3-R4 tau fragments were almost equimolar and 2.5-fold higher, respectively, than those of the $A\beta_{28-42}$ peptides, while the ratio of the N-terminal peptide fragments of both proteins were retained close to equimolar (Fig 2C and Table S3).

Along with targeted proteomics, we also performed western blotting of the sarkosyl-soluble and -insoluble fractions using anti-tau Dako A0024(K9JA) and anti-Aβ WO2 antibodies (Table S3). While all the sarkosyl-insoluble pellets from AD brain were immunoreactive for tau and Aβ, these immunoreactivities were absent in disease-control brains, as expected (Fig. S3A). However, disease control (ALS) pellets were immunoreactive to TDP43 (TARDBP gene and a pathological feature in ALS(Arseni *et al.* 2022)) antibodies (Fig. S3B).

PTMs of tau in detergent-soluble and -insoluble fractions in AD and disease-control brains

In addition to quantification of total tau in brain fractions, the concentrations of the clinically relevant(Barthelemy *et al.* 2020a; Horie *et al.* 2021; Barthelemy *et al.* 2020b) monophosphorylated-tau at T_{181} and T_{217} were measured (Fig. 3A, left panel). The absolute pT_{181} and pT_{217} concentrations were not significantly higher in AD *vs* DCN brains in the SM

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and SN fractions (P=0.422 and P=0.225 for pT_{181} in SM and SN, respectively, and P=0.216 and P=0.342 for pT_{217} in SM and SN, respectively) (Table 1, Fig. S4 D&E). For better understanding the AD disease specific phosphorylation changes, the percentage fractional phosphorylation occupancies of T_{181} and T_{217} tau were calculated. The percentage of phosphorylation at T_{181} (pT_{181}) of 24.1±6.5 % in the AD SM fraction was somewhat greater (P=0.028) than that of 17.9±5.3 % in the DCN SM, while phosphorylation at T_{217} (pT_{217}) of 15.7±8.3 % in the AD SM was significantly greater ($P < 0.001$) than that of 3.9±2.2 % in the DCN SM brains (Fig. 3A, center). In the sarkosyl-soluble SN fractions the pT_{181} occupancy of 21.7±9.2 % in AD was not significantly higher than that of 15.2±7.5 % (P=0.094) in DCN brains, while the pT_{217} occupancy of 11.3±3.4 % in AD was significantly greater (P<0.001) than that of 3.7±2.2 % in DCN brains (Fig. 3A, middle). The T_{217} phosphorylation increased by ~6-4 -fold in the AD SM and SN fractions, whereas the T_{181} phosphorylation increased by ~1.6-fold only in the same fractions, when compared with DCN samples. Similar pT_{217} (6-fold) *vs* pT_{181} (1.3-fold) hyperphosphorylation was shown to be a characteristic feature of AD cerebrospinal fluid (CSF) compared to non-AD CSF(Barthelemy *et al.* 2020a). While the total concentration of the PRR tau peptides was substantially lower compared to the MTBR tau peptides in the AD P pellets (Fig. 2, Table 1), the occupancies of pT_{181} and pT_{217} in the AD pellets were quite high: 22.4±3.1 % (pT_{181}) and 16.0±4.3 % (pT_{217}) (Fig. 3A, right) and significantly different (P<0.001). However, they did not significantly change in comparison with the corresponding SM fractions (P=0.443 and P = 0.916 for pT_{181} and pT_{217}, respectively). This suggested that the relative hyperphosphorylation of both T_{181} and T_{217} generally maintained in soluble and insoluble fractions, while higher occupancy of pT_{217} in the P (16.0±4.3 %) *vs* the SN (11.3±3.4 %) fractions (P=0.01) indicates increased deposition of pT_{217} phosphorylated tau species during tau aggregation.

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It has been shown (Wang *et al.* 2015) that tau protein in mouse brains could be rapidly dephosphorylated site-specifically (mainly at T_{205}, S_{214} and S_{396}) within 2-10 min post-mortem intervals. However, we did not observe any significant correlations between the percent phosphorylation occupancies of T_{181} or T_{217} and the post-mortem interval (PMI) or the age of the patients (Table S1) for the AD and DCN post-mortem brain samples.

Next, the heterogenous PTMs of tau in the AD P fractions were assessed, which showed sequence and region specific PTMs of the tau MTBR (Fig. 3B&C). While the PRR and the C-terminus of tau were highly phosphorylated, the tau MTBR had considerable ubiquitination and acetylation as previously documented(Arakhamia *et al.* 2020). In addition to T_{181} and T_{217}, the T_{212}, S_{214}, T_{231}, and S_{238} residues were highly phosphorylated in the PRR with S_{214}, T_{217}, T_{231}, and S_{238} occasionally doubly phosphorylated. Also, S_{262} and T_{263} in the MTBR R1 region showed relatively high phosphorylation occupancy (Fig. 3C), while K_{267} and K_{274} were found to have high ubiquitination occurrence with relatively lower acetylation. K_{311}, K_{317}, K_{321} and K_{343} were mostly ubiquitinated, whereas K_{369} and K_{370} were highly acetylated in the MTBR R3-R4. Ubiquitination at K_{254}, K_{257}, K_{281}, K_{298}, and K_{395}, and acetylation at K_{311}, K_{321}, K_{331}, K_{347}, and K_{385} were also observed at lower frequency (intensity). Finally, the high phosphorylation occupancy of S_{396}, S_{400}, T_{403}, and S_{404} (PHF1 antibody epitope) in the tau C-terminal region was recorded with doubly and triply phosphorylated peptides. We also identified a novel PTM, citrullinated (deiminated) R_{349}, that could also be methylated as shown before(Balmik & Chinnathambi 2021).

Based on the above quantification the average amounts of Aβ, t-tau, and p-tau (average of pT_{181} and pT_{217}) in ~520 g of the AD grey matter (based on the AD grey matter volume(Roberts *et al.* 2017) of 496.7±30.0) cm^3 and the density(Hasgall *et al.* 2022) of 1.045±0.008 g/cm^3) were estimated to be of 18±11, 34±18, and 8±5 mg in AD SM fractions, respectively, whereas in aggregated material (P) included 1±1, 8±6, and 2±1 mg, respectively

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(Table 1). Interestingly, the average amount of tau was significantly higher (*P*=0.004) in the SM fractions from DCN (63±19 mg) *vs* AD (34±18 mg) brains, then that difference reversed in the P fraction where the AD tau amount of 8±6 mg was much higher (*P*<0.001) with virtually no tau detected in the DCN pellets (~ 0.001) mg in DCN brains. This suggested that tau in disease-control (ALS) brains was mainly in sarkosyl-soluble form, as also evident from the SN fractions which showed significantly more tau (*P*<0.001) in DCN (26±8 mg) *vs* AD (14±4 mg) brains (Table 1).

Label free quantitative proteomics of insoluble fractions in AD and disease-control brains

To assess the differential enrichment of other proteins presented along with tau and Aβ in the sarkosyl-insoluble pellets, label-free quantitative proteomics was performed. Significant variations in abundance within a group were those beyond 50% change at *P*<0.05. The robustness of the enrichment of insoluble proteins in AD *vs* DCN was based on their false discovery rate (FDR) as follows: (i) FDR<0.001, (ii) FDR<0.01, and (iii) FDR<0.05. We could identify ~ 4000 unique proteins across all the sarkosyl-insoluble pellets, where only 636 proteins were significantly enriched (FDR<0.05). Out of 636 proteins that were significantly enriched (FDR<0.05) in the AD and DCN P fractions, we found only 170 proteins with FDR<0.001 (Fig. S6) that were only enriched in the AD insoluble P fractions. Similar, to the SDS-PAGE in-gel digested P fractions, tau (MAPT) and ferritin (light and heavy chain) were the most abundant proteins in these pellets (Fig. 4A), along with ubiquitin (S27a 40S ribosomal protein), tubulin, and calmodulin kinase (CAMK2A). Aβ (A4-amyloid β) was the third most abundant protein (Fig. 4A, right panel). NfL, NfH, and Apolipoprotein E (ApoE) were detected among the top 100 proteins. Heterogenous ribonuclear proteins (HNRNP) and small ribonuclear proteins (SNRP) were also highly represented in the AD

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sarkosyl-insoluble pellets. Tau (MAPT), ubiquitin, FLH, myosin (MYH1, MYL1), Aβ along with ApoE, complement C3 and C4B were substantially enriched in AD *vs* DCN with FDR<0.001 (Fig. 4B). TDP43 (TARDBP) and Filamin-A (FLNA) showed slightly significant (FDR≤0.05) enrichment in the AD insoluble pellet (Fig. 4B and Fig. S5). While α-synuclein (SNCA) was also significantly enriched (FDR<0.01), neurofilament light (NEFL) remained unchanged. RNA binding (RBPs) U1 snRNP proteins (snRNPs) such as U1-70K (snRNP70) were highly enriched (FDR<0.001) in the AD insoluble pellets (see further details in Figs. S5-S7). Similarly, other snRNP components of RBPs belonging to the Sm ring of the spliceosome and including snRNPD1, snRNPD2, snRNPD3, snRNPE and snRNPG exhibited considerable enrichment in the insoluble AD proteome. In addition, another group of RBPs - heterogenous ribonuclear protein (hnRNP) including hnRNPA3, hnRNPA2B1, hnRNPH3 also increased in the AD insoluble pellets (Fig. 4C).

Quantification of Aβ, tau, and p-tau from ELISA

The established INNOTEST immunoassay, validated only for CSF(Dakterzada *et al.* 2021), was utilized for complementary quantification of $A\beta_{33\text{-}42}$, tau (mainly $tau_{159\text{-}224}$ region) and, and p-tau (pT_{181}) antibodies in sarkosyl fractions (Table 2). It is notable that the DCN samples with insignificant levels of $A\beta_{42}$ in all fractions (Table 2) showed significant amounts of tau in the starting (SM) (2123±1163 fmol/mg) and soluble (SN) (2075±1243 fmol/mg) fractions, but very low levels in the insoluble (P) fractions (0.09±0.07 fmol/mg) consistent with MS results for DCN samples.

Since ELISA captured and detected mainly full length $A\beta_{1\text{-}42}$, then ~17-fold amount of Aβ peptides detected by MS *vs* ELISA ($A\beta_{28\text{-}42}$ 7460±4479 *vs* $A\beta_{1\text{-}42}$ 434±302 fmol/mg, respectively) in the AD SM fractions suggested high levels of the N-terminal truncated $A\beta_{42}$, $A\beta_{40}$, $A\beta_{\text{mid-domain}}$ peptides along with $A\beta_{1\text{-}42}$ in those fractions. The N-terminal truncated Aβ

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peptides contributed mostly to insoluble aggregates as indicated ~50-fold increase of Aβ_{28-42} in the MS data compared to Aβ_{1-42} in ELISA (447±408 *vs* 9±12 fmol/mg) in the AD P fractions (Table 1 & 2).

The ELISA detection of tau$_{159-224}$ fragments (Table 2) included all other fragments with this range and was consistent with the MS data for tau$_{181-190}$, tau$_{195-209}$, and tau$_{212-221}$ peptides (Fig. 2B, Table 1). The difference between the average amounts of the MS peptides in the range 181-221 (954±480 fmol/mg) and the ELISA tau$_{159-224}$ peptides (809±433 fmol/mg) was not significant (*P*=0.466) in AD SM fractions. Insignificant differences were maintained in both AD SN (averaged MS 483±254 *vs* ELISA 571±288 fmol/mg, *P*=0.456) and P fractions (averaged MS 59±80 *vs* ELISA 33±40 fmol/mg, *P*=0.347) (Table 1 & 2).

Like MS results, the ELISA showed no significant difference in phosphorylation pT$_{181}$ levels between AD and DCN brains in both SM (AD 22±11 vs DCN 18±5 fmol/mg, *P*=0.3) and SN (AD 16±5 vs DCN 15±5 fmol/mg, *P*=0.652) fractions, while the DCN P fractions showed significantly (*P*=0.005) lower (negligible) levels of pT$_{181}$ compared to AD (0.4±0.4 fmol/mg) brains. The ELISA average amount of pT$_{181}$ (22±11 fmol/mg) was significantly less (*P*<0.001) than that determined by MS (456±275 fmol/mg) in the AD SM fractions. Further, significantly lower the ELISA *vs* MS pT$_{181}$ levels were maintained in both AD SN (*P*<0.001) and P (*P*=0.002) fractions (Tables 1 & 2).

The ELISA resulted in Aβ_{42} : tau ratio of (0.5±0.5 in the AD SM fraction which was significantly less than that obtained from MS (5.2±4.2, *P*=0.002). Similar significant difference was maintained in the AD SN fractions (MS 2.8±1.8 *vs* ELISA 0.1±0.2, *P*<0.001). However, Aβ_{42} : tau ratio of 0.3±0.5 for the AD P fraction (Table 2) was not quite statistically significantly different from that from the MS estimate of 1.4±1.7 (*P*=0.053) (Tables 1&2). Based on the ELISA, the average amounts of Aβ_{1-42}, tau$_{159-224}$, and p-tau (pT$_{181}$) in the AD brain grey matter (~520 g) were estimated to be of 1.0±0.7, 19±10, and

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0.5±0.3 mg in AD SM fractions, respectively, whereas aggregated material in P fraction included 0.02±0.03, 0.8±0.9, and 0.1±0.1 mg, respectively (Table 2). These estimates were much lower in all fractions compared to the MS estimations (Table 1). However, disease-control (DCN/ALS) brain grey matter estimates demonstrated high amounts of tau: 50±26 and 49±28 mg in the SM and SN fractions, respectively, and negligeable amounts of insoluble tau and Aβ in the P fractions (Table 2). This was again consistent with MS results for DCN brains and indicated that potential aggregates in DCN/ALS brains would unlikely be formed by tau or/and Aβ species.

Negative staining electron microscopy (EM)

Negative staining EM of sarkosyl-insoluble (P) pellets revealed numerous filaments among considerable numbers of globular aggregates (Fig. S8). Although populations of tau-like filaments (PHF and SF) were observed in all AD samples examined, the purity and yield depended upon the case. Occasional scattered filamentous material was found after an extended search in the 1st and 2nd 16K g pellets (Fig. S8). Filaments were not detected in preparations from disease-control brain samples (Fig. S9) Most AD brain filaments displayed the characteristic morphology of PHF-like helically twisted tau fragments(Kidd 1963; Fitzpatrick *et al.* 2017) with an average diameter of 23.4±1.4 nm and a crossover repeat of ~70 nm (Fig. S8). Filaments similar to another structural polymorph, the SF(Crowther 1991; Fitzpatrick *et al.* 2017) with an average diameter of 10.7±2.5 nm ware also observed. Thus, tau filaments isolated from extracts of 11 AD brains displayed similar parameters as described before(Goedert *et al.* 1992). 10 -15 nm in diameter possibly longitudinally split filaments(Wischik *et al.* 1985) without a well pronounced helical turn were also occasionally observed.

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Immunolabeling EM of recombinant Aβ_{1-42} and hTau441 aggregates

To validate reactivity of antibodies used, recombinant Aβ_{1-42} fibrils and hTau441 (human MAPT 2N/4R tau$_{1-441}$) filaments were prepared from recombinant proteins and imaged by gold immunolabelling EM. All anti-Aβ N-terminal monoclonal antibodies WO2(Aβ_{2-8}), 6E10(Aβ_{3-8}) 1E8(Aβ_{1-2}) and aducanumab(Aβ_{3-7}) (Arndt *et al.* 2018) (Table S2) were reactive with synthetic Aβ_{1-42} fibrils decorating them equally well as shown in Fig.s 5A and S10A-C. Sarkosyl (~1%) treatment for 24 hours did not affect the Aβ fibril morphology and were well detected by WO2(Aβ_{2-8}) (Fig. S10A). Also, these N-terminal Aβ antibodies were reactive to Aβ in non-fibrillar globular material surrounding fibrils. These results suggest that the N-terminus was accessible in fibrillar and non-fibrillar recombinant Aβ_{1-42} aggregates and consistent with all available *in vitro* structures of Aβ_{1-42}(Creekmore *et al.* 2021). Similarly, all anti-tau N-terminal monoclonal antibodies TAU1 (tau$_{1-22}$), RNF5 (tau$_{35-44}$)(Bajracharya *et al.* 2021) and HJ8.5(Yanamandra *et al.* 2013) (tau$_{25-30}$) (Table S2) showed tight binding to recombinant hTau441 heparin-induced filaments (Figs.5B and S8D&E). That was again consistent with the structures of heparin-induced hTau441 filaments with accessible N-termini(Zhang *et al.* 2019). These antibodies were also reactive to non-filamentous aggregates.

Immunolabeling EM of sarkosyl-insoluble AD brain fractions

Contrary to results of immunolabeling EM for recombinant hTAu441 aggregates, imaging of sarkosyl-insoluble fractions from AD brains showed very limited reactivity of monoclonal N-terminal anti-tau TAU1 (tau$_{1-22}$), RNF5 (tau$_{35-44}$) and HJ8.5 (tau$_{25-30}$) antibodies (Table S2) to well-defined tau filaments (Figs. 5D and S11A-D for different samples). However, tau filaments remained reactive to Dako A0024(K9JA) polyclonal antibody (Table S2) which recognizes the 243-441 sequence of tau including MTBR (Figs. 5F and S11E for different

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samples). This was consistent with the quantitative MS results that N-terminal truncated MTBR R3-R4 tau was the main constituent of the AD tau filamentous aggregates. Reactivity of filaments was also observed with anti-ubiquitin antibody (Fig. S14F) and AT8 antibody [epitope around the phosphorylated pSer202-pThr205 region] (Fig. S11F) similar to other results(Arakhamia *et al.* 2020) and consistent with tau ubiquitination and phosphorylation PTMs indicated by MS (Fig. 3). However, all N-terminal monoclonal and polyclonal tau antibodies were reactive to globular non-filamentous aggregates in the brain samples suggesting that constituents of non-filamentous globular aggregates included the intact and accessible N-terminal tau species.

Immunolabelling with the N-terminal specific Aβ antibodies such as aducanumab (Figs. 5C and S12E&F), WO2 (Figs. S12A&B) and 6E10 (Figs. S12C&D) for different brain samples indicated that Aβ was present in the globular aggregates surrounding tau filaments. Aβ appeared to be localized in the same areas that were immunoreactive to monoclonal N-terminal anti-tau antibodies. To explore further co-localization of tau and Aβ, double immunogold labeling was performed utilizing primary monoclonal human anti-Aβ aducanumab and mouse anti-tau RNF5 antibodies incubated with secondary anti-human 6nm-gold and anti-mouse 10nm-gold conjugated antibodies, respectively. Representative images (Figs. 5E and S13A) showed reactivity of tau N-terminal and aducanumab antibodies to overlapping areas of non-filamentous globular aggregates of Aβ and tau. Imaging with primary polyclonal tau K9JA and monoclonal Aβ 6F/3D antibodies (Fig. S13B) showed reactivity of K9JA to tau filaments as expected, while the N-terminal Aβ 6F/3D antibody was reactive only to non-filamentous aggregates.

Cryo-EM imaging

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For higher-resolution examination of extracted PHF filaments, cryogenic electron microscopy (cryo-EM) was performed. Consistent with previous reports(Crowther 1991; Fitzpatrick *et al.* 2017; Falcon *et al.* 2018b) observed PHF's were composed of two protofilaments with C-shaped subunits. PHF thickness and crossover were refined to 244 Å and 689 Å, respectively. Successive rungs of β-strands along a protofilament were related by helical symmetry with the refined rise of 2.1 Å and the twist of – 0.68° (left-handed) with C_2 (2_1 two-fold screw axis) symmetry. The cryo-EM three-dimensional density reconstructed at resolutions of ~5.7 Å (Fig. S14) was generally consistent with higher resolution PHF tau model (PDB: 5osl)(Fitzpatrick *et al.* 2017) which was fitted to the density (Fig. S14E). Some additional low density was observed at the periphery of the filament core, particularly near the exposed K_{343} site (R4 tau region). This density could be either part of the folded back and disordered tau N-terminus(Fitzpatrick *et al.* 2017), or potential ubiquitination of K_{343} also observed as a large density attached to tau filaments in corticobasal degeneration (CBD)(Arakhamia *et al.* 2020). Since N-terminus antibodies did not show reactivity to tau filaments (Figs. 5D and S11A-D for different samples), a tentative ubiquitin molecule was accommodated into this density thus consistent with partial PTM of K_{343} in the MS analysis and reactivity of anti-ubiquitin antibody to tau filaments (Fig. S14F).

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Discussion

Emerging data suggests that sarkosyl-insoluble tau is N-terminal and C-terminal truncated, while the MTBR rich tau is the seeding active species extracted from neurofibrillary tangles of AD brains(Wesseling *et al.* 2020; Horie *et al.* 2021; Li *et al.* 2021a). The quantitative targeted mass spectrometric assessments of sarkosyl-insoluble pellets from postmortem AD brains revealed about equimolar amounts of Aβ and MTBR-N tau and 3R-4R tau specific peptides. Recent report(Wesseling *et al.* 2020) based on FLEXItau(Mair *et al.* 2016) indicated the presence of significantly more 0N tau peptides compared to either 1N or 2N tau peptides in the AD brain sarkosyl-insoluble fraction, while soluble brain-derived tau secreted into CSF and blood consists of N-terminal and mid-domain fragments (Snellman *et al.* 2022). Using high resolution MS, we found that the MTBR 4R tau peptides ($tau_{322-340}$, $tau_{341-347}$, $tau_{344-349}$, $tau_{354-369}$) were most abundant and 10-fold higher in concentration than the tau N-terminal tau peptides (tau_{24-44}, tau_{45-67}, tau_{68-87}, tau_{88-126}) in the sarkosyl-insoluble materials. This indicated that the major proportion of the enriched isoforms of tau were the aggregation prone N-terminal and PRR truncated 4R/3R mixed forms with the average of 1.6-fold incorporation ratio of 4R to 3R microtubule-binding repeats. This over-incorporation of 4R over 3R tau into the AD tau filament was consistent with recent solid-state NMR estimates of 1.5 from seeded 4R and 3R tau monomers with AD brain-derived tau(Dregni *et al.* 2022).

The MS data was supported by complementary ELISA quantification that the AD brain samples contained high levels of the N-terminal truncated Aβ and tau peptides which contributed mostly to insoluble aggregates. The N-terminal tau fragments were identified by immunogold EM in co-localized Aβ and tau non-filamentous globular aggregates. The average total amounts of Aβ, tau, and p-tau in the AD grey matter homogenates were estimated to be 18±11, 34±18, and 8±5 mg from MRM assay, respectively, where Aβ quantity was somewhat greater than other estimations of 6-10 mg(Gravina *et al.* 1995;

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Roberts *et al.* 2017) in AD brains. The corresponding ELISA estimations were much lower, possibly due to limited applicability of the classic INNOTEST CSF immunoassay kit for brain homogenates. However, the ELISA and MRM estimates of tau in detergent soluble fractions per total brain grey matter of 13-14 mg in AD and 26-49 mg in disease-control brains, respectively, were in line with estimates(Han *et al.* 2017) of frontal cortex soluble tau of ~ 27 and ~32 mg for AD and control brains, respectively.

The key PTMs of sarkosyl-insoluble N-terminal truncated tau included extensive and region-specific lysine ubiquitination (K_{311}, K_{317}, K_{321}) and acetylation (K_{369}, K_{379}) in the MTBR along with phosphorylation in the tau PRR (T_{181}, T_{217}, T_{231}), R1 (S_{262}, T_{263}) and the C-terminal (S_{396}, S_{400}, T_{403}, S_{404}) regions consistent with recent findings(Wesseling *et al.* 2020). Site-specific fractional phosphorylation occupancies pT_{181} (22.4±3.1 %) and pT_{217} (16.0±4.3 %) indicated a regional susceptibility of tau aggregates with higher hyperphosphorylation of T_{181} which is consistent with other MS estimates of p-tau occupancies in CSF: pT_{181} of 18.8±2.5 % and pT_{217} of 10.0±4.1 % in AD(Barthelemy *et al.* 2020b). Increased pT_{217} phosphorylation in the insoluble pellets (16.0±4.3 %) compared to soluble pools (11.3±3.4 %) is also consistent with pT_{217} being preferred biomarker of neurodegeneration associated with tau tangles(Barthelemy *et al.* 2020a) as well as with recent observation of hyperphosphorylation of multiple sites in the sarkosyl-insoluble fractions compared to the non-filamentous fractions (Li *et al.* 2021a). We observed that the fractional phosphorylation of T_{181} and T_{217} were not significantly different in the sarkosyl-insoluble pellets and close to the reported estimates(Han *et al.* 2017) obtained by using similar extraction protocol. This is in-line with the fact that phosphorylation epitopes in the PRR tau domain are susceptible to hyperphosphorylation in AD, while the level of mono-phosphorylation of the T_{181} and T_{217} tau sites can be similar in the insoluble fractions. The PTM analysis also suggested that multiple acetylation and ubiquitination of the lysine residues in the MTBR leads to charge neutralization, while phosphorylation in the PRR compensates for an acidic N-terminus of tau and may facilitate tau aggregation. Together, the R1 and C-terminal phosphorylation sites are at the flanks of tau filamentous cores(Shi *et al.* 2021) and may influence aggregation and structure of C-terminal fragments depending on whether tau is post translationally modified before or after aggregation. While our proteomics data identifying PTMs of the MTBR tau and the phosphorylation of the PRR and C-terminal tau regions in the sarkosyl-insoluble pellets were very similar to reported results, further detailed quantitative investigation of PTMs is required to understand their crosstalk during disease progression. The targeted mass spectrometry is limited in estimation of the quantity of unmodified peptides which are prone to modification such as the specific PRR tau domain. Using much larger numbers of brain samples to overcome our current study limitation of small sample set (11 AD and 10 DCN brains) and employing detailed analyses might provide the link between tau isoforms and PTM crosstalk in AD neurodegeneration.

Aβ was the third-most abundant protein in the label-free quantitative proteomics on the sarkosyl-insoluble pellets. Along with robust enrichment of tau, Aβ, ApoE, and ferritin light chain, there was enrichment of RBPs such as the snRNP70 (U1-70K), snRNPs, and hnRNPs. This is consistent with previous proteomic analyses of sarkosyl-insoluble fractions of AD(Diner *et al.* 2017), and especially with findings that showed insolubility of U1 snRNP components including snRNP70, accumulation of which occurs in the early stages of preclinical AD(Guo *et al.* 2021; Hales *et al.* 2014; Bai *et al.* 2014; Bai *et al.* 2013). α-synuclein, β-synuclein, and clusterin were marginally enriched in the AD pellets (~ 1-10 fmol/mg). Recent data showed that β-synuclein and pT_{181} performed equally well in diagnosing symptomatic AD in Down Syndrome and highlighted the value of serum β-synuclein as a potential early marker of AD (Oeckl *et al.* 2022). Filamin-A (FLNA) and TDP43 were also found to be somewhat enriched in the AD insoluble pellet (Figs. 4B and

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S5-S7). Recent study suggested that FLNA could accelerate the F-actin–mediated aberrant interaction with tau and thus could drives tau-induced neurodegeneration in AD(Tsujikawa *et al.* 2022).

On the other hand, disease-control (ALS) brains showed abundant non-filamentous aggregates containing TDP43 (Fig. S8), according to Western blotting analysis (Fig. S3B) and the MRM and ELISA assays with negligible levels of Aβ and tau in detergent-insoluble fractions (Table 1 & 2). As shown(Gruijs da Silva *et al.* 2022), ALS/FTD disease-linked hyperphosphorylation of serines at the C-terminal end of the TDP43 Low Complexity Domain (LCD) filament structure(Li *et al.* 2021b) suppresses TDP43 condensation and insolubility rendering it to non-filamentous form, which was observed in recent studies(Jiang *et al.* 2022; Schweighauser *et al.* 2022; Chang *et al.* 2022) of FTD with TDP43 inclusions. While neurofilament light (NfL) was not significantly enriched in the sarkosyl-insoluble pellet (Fig. 4B), our MRM data clearly indicated that there was considerably more NfL in both AD SM and SN pool compared to disease-control brains (Fig. 2A). Combining all these observations in a larger cohort would provide insights of the crosstalk between soluble and insoluble proteome during neurodegeneration.

Immunogold labelling confirmed tau aggregates along with filaments in the sarkosyl pellets as reported before(Maeda *et al.* 2006; Sahara *et al.* 2007; Berger *et al.* 2007), where non-filamentous aggregates were reactive to antibody specific to the 209-224 region of tau, N-terminal antibodies did not recognise tau filaments which were primarily comprised of MTBR (244-372 region)(Berger *et al.* 2007; Ward *et al.* 2014). It has been previously demonstrated that pathological tau-tau binding through the MTBR repeat domain initiates formation of the stable PHF core cross-β structure(Wischik *et al.* 2018). Our cryo-EM analyses confirmed the general C-shape fold of the extracted PHF filaments, with potential sites of ubiquitination. Recently, high resolution structures have been resolved by cryo-EM for *ex vivo* tau filaments from several neurodegenerative disease including AD(Falcon *et al.* 2018b; Fitzpatrick *et al.* 2017; Shi *et al.* 2021). While there are differences in protofilament structures between different primary and secondary tauopathies(Shi *et al.* 2021), they all have similar cores composed of the tau MTBR including R3, R4 and 10-12 residues C-terminal to R4 in AD(Creekmore *et al.* 2021). Acetylation and ubiquitination PTMs have also been observed by cryo-EM within the core region of filaments(Arakhamia *et al.* 2020). While ubiquitination at K_{343} was identified in tau filaments from CBD patients and K_{343} acetylation was also indicated in AD patients(Arakhamia *et al.* 2020).

Immunogold labelling also showed that aggregates of Aβ in our samples were not filamentous (fibril-like) and were co-localized with N-terminal intact tau peptides in globules. Aducanumab recognized the globular aggregates as well as recombinant fibrillar forms of $Aβ_{1-42}$ in agreement with findings(Arndt *et al.* 2018) that aducanumab selectively targets the aggregated species of Aβ, while also binding to the N-terminal $Aβ_{3–7}$ epitope. The possibility that the extraction method failed to capture Aβ fibrils is unlikely because we did not observe fibrils in other fractions throughout the extraction protocol. Also, sarkosyl treatment did not dissolve Aβ fibrils as tested with recombinant Aβ fibrils (Fig. S10A). It should be noted, that numerous forms and structures of Aβ fibrils have been obtained for *in vitro* derived peptide variants(Creekmore *et al.* 2021). Only recently $Aβ_{40}$ fibrils from the vasculature (meninges)(Kollmer *et al.* 2019) and two types of $Aβ_{42}$ fibrils have been described from AD brains(Yang *et al.* 2021) using cryo-EM analysis. Adding sarkosyl following the tissue homogenization step(Diner *et al.* 2017) was claimed to be a major difference in protocol(Yang *et al.* 2021) resulting in substantial enrichment of $Aβ_{42}$ fibrillar species(Yang *et al.* 2021). Of interest, the above mentioned cryo-EM studies(Jiang *et al.* 2022; Schweighauser *et al.* 2022; Chang *et al.* 2022) of FTD brain extracts revealed fibrils of TMEM106B instead of TDP43, which formed abundant non-fibrillar aggregates. All the

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TMEM106B fibrils were extracted using sarkosyl extraction protocols, but with variation in the stage of sarkosyl addition: (I) in homogenate before the first centrifugation step(Jiang *et al.* 2022; Schweighauser *et al.* 2022) or (II) later, after low-speed centrifugation(Chang *et al.* 2022). It was argued that the protocol modification from II to I was essential for detecting abundant TMEM106B filaments. None of these studies observed fibrils of TDP43, which have been identified before in ALS/FTD(Arseni *et al.* 2022)·(Nonaka *et al.* 2013) using the preferred sarkosyl extraction protocol I (sarkosyl in the first homogenate), while abundant non-filamentous aggregates of TDP43 were identified in FTD-TDP extracts(Jiang *et al.* 2022) as well as in disease-control ALS brains (Fig. S9) using our protocol type II. Additionally, our proteomics results generally agree with previous proteomics studies of detergent-insoluble protein aggregates from AD brains(Diner *et al.* 2014; Diner *et al.* 2017; Hales *et al.* 2016; Woltjer *et al.* 2005) including sarkosyl in the first homogenate (an abbreviated detergent-fractionation single step protocol) or similar to present here the more common sequential fractionation methodologies(Diner *et al.* 2017).

It is so far unclear whether Aβ and tau co-localize *in vivo* aggregates, and whether a cross-seeding mechanism is involved in AD. However, it has been demonstrated(Guo *et al.* 2006) that low nanomolar affinity between Aβ and tau form stable complexes that enhance tau phosphorylation, and the phosphorylation then promoted dissociation of the complex and self-aggregation of both into the insoluble forms. The probable sites of tau–Aβ interaction were N-terminal and mid regions of tau and the mid to C-terminal regions of Aβ. It was also claimed that phosphorylated tau and Aβ formed co-localised aggregates within the same neurons in AD brains(Guo *et al.* 2006). In what manner phosphorylation and other PTMs influence these interactions and aggregation type and structure also depend on many factors including processes such as protein localization and degradation(Alquezar *et al.* 2021). Negatively charged co-factors like heparin contribute to neutralising the tau N-terminus charge and

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accelerate tau aggregation(Zhang *et al.* 2019) as well as multiple co-factors, including Aβ itself, may have an influence on modulating fibrillar self-assemblies(Arakhamia *et al.* 2020). One model could be that Aβ and tau interact forming heterogeneous globular toxic aggregates(Meng *et al.* 2022; Sideris *et al.* 2021), possibly though droplet-gel condensation pathway generated by liquid-liquid phase separation(Vendruscolo & Fuxreiter 2022), modulated by PTMs including hyperphosphorylation levels(Gruijs da Silva *et al.* 2022) and other co-factors, from which filamentous tau and Aβ aggregates may emerge. While it remains to be shown how extracellular Aβ in the AD brain interacts with intracellular tau potentially engaging both neuronal connections and local contacts(Lee *et al.* 2022), it is becoming clear, through the results of clinical trials targeting Aβ(Cummings *et al.* 2021; Budd Haeberlein *et al.* 2022), that Aβ drives tau aggregation.

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Table 1. Aβ, tau and p-tau MS MRM quantification

Aβ, tau and p-tau quantification (fmol/mg brain tissue) and amounts per grey matter (mg) from the starting material (SM), the 1st 100K supernatant (SN) sarkosyl-soluble and the final pellet (P) sarkosyl-insoluble fractions for AD (n=11) and disease-control (DCN) (n=10) brain samples. Data are represented as mean±SD. All *P*-values were derived from *t*-tests.

	SM, fmol/mg brain			SN, fmol/mg brain			P, fmol/mg brain		
	AD	DCN	*P*-value	AD	DCN	*P*-value	AD	DCN	*P*-value
Aβ_{28-42}	7460±4479	743±707	<0.001	1631±915	308±104	<0.001	447±408	40±50	0.006
Aβ_{28-40}	1345±2889	6±7	0.160	316±678	5±4	0.164	41±63	0.4±0.5	0.056
tau$_{181-190}$(T$_{181}$, PRR, P1)	1388±721	1651±543	0.361	665±222	708±436	0.776	135±136	0.2±0.1	0.006
tau$_{195-209}$ (PRR, P2)	314±129	1129±436	<0.001	172±58	474±252	0.001	2±2	0.1±0.1	0.008
tau$_{212-221}$(T$_{217}$, PRR, P2)	1162±392	4223±1970	<0.001	614±154	1615±750	<0.001	40±29	4±2	<0.001
tau$_{243-254}$(MTBR-N, R1)	1751±1311	2247±815	0.317	456±188	968±417	0.002	412±343	0.2±0.1	0.001
tau$_{282-290}$ (4R, R2)	825±581	679±330	0.494	259±151	312±140	0.416	243±206	0.1 ±0.1	0.001
tau$_{299-317}$ (4R, (R2)-R3)	425±306	544±238	0.336	122±57	146±51	0.324	94±63	0.2±0.1	<0.001
tau$_{396-406}$ (C-term, R')	165±94	424±161	<0.001	68±34	148±93	0.015	10±11	0.1±0.1	0.011
t-tau	1433±743	2707±828	0.002	579±172	1097±333	<0.001	328±215	0.2±0.1	0.001
pT$_{181}$	456±275	370±193	0.422	158±74	118±72	0.225	39±36	0.1±0.1	0.029
pT$_{217}$	232±161	159±85	0.216	60±23	50±24	0.342	9±8	0.3±0.2	0.003
pT$_{181}$ (%)	24.1±6.5	17.9±5.3	0.028	21.7±9.2	15.2±7.5	0.094	22.4±3.1	-	-
pT$_{217}$ (%)	15.7±8.3	3.9±2.2 %	<0.001	11.3±3.4	3.7±2.2	<0.001	16.0±4.3	-	-
Aβ_{28-42} : t-tau	5.2±4.2	0.3±0.3	0.002	2.8±1.8	0.3±0.1	<0.001	1.4±1.7	200±269	0.024
Per total brain grey matter, mg									
Aβ_{28-42}	18±11	4±2	<0.001	1±1	2±2	0.158	1±1	0.1±0.1	<0.001
t-tau	34±18	63±19	0.002	14±4	26±8	<0.001	8±6	0.001±0.002	<0.001
p-tau	8±5	3±1	0.006	0.5±0.6	6±3	<0.001	2±1	0.004±0.003	<0.001

Table 2. Aβ, tau and p-tau ELISA quantification

Aβ, tau and p-tau quantification (fmol/mg brain tissue) and amounts per grey matter (mg) from the starting material (SM), the 1st 100K supernatant (SN) sarkosyl-soluble and the final pellet (P) sarkosyl-insoluble fractions for AD (n=11) and disease-control (DCN) (n=10) brain samples. Data are represented as mean±SD. All *P*-values were derived from *t*-tests.

*All fragments with this range were detected.

	SM, fmol/mg brain			SN, fmol/mg brain			P, fmol/mg brain		
	AD	DCN	*P*-value	AD	DCN	*P*-value	AD	DCN	*P*-value
$A\beta_{1-42}$	434±302	0.3±0.5	<0.001	73±95	0.4±1.1	0.026	9±12	0.01±0.01	0.029
$tau_{159-224*}$	809±433	2123±1163	0.002	571±228	2075±1243	0.001	33±40	0.09±0.07	0.018
pT_{181}	22±11	18±5	0.305	16±5	15±5	0.652	0.4±0.4	0.0±0.0	0.005
Aβ : tau	0.5±0.5	0.0001±0.0003	0.005	0.1±0.2	0.0002±0.0005	0.132	0.3±0.5	0.1±0.1	0.230
Per total brain grey matter, mg									
$A\beta_{1-42}$	1.0±0.7	0.001±0.001	<0.001	0.2±0.2	0.001±0.002	0.006	0.02±0.03	0.0±0.0	0.049
tau	19±10	50±26	0.002	13±5	49±28	<0.001	0.8±0.9	0.002±0.001	0.012
pT_{181}	0.5±0.3	0.4±0.1	0.329	0.4±0.1	0.4±0.1	1.000	0.1±0.1	0.0±0.0	0.006

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Figure legends

Fig. 1. **Immunohistochemistry and label free in-gel characterization of sarkosyl-insoluble pellet.** (A) Immunohistochemical colocalization of Aβ_{9-14} (6F/3D) (in pink with the black asterisks indicating the amyloid plaques) and pTau$_{S202T205}$(AT8) (in brown/black with black arrows pointing to the neuritic threads) is shown in two sections of human AD brain; the scale bars are 100 and 50 µm for left and right panels, respectively. The sarkosyl-insoluble fraction was separated on reducing SDS-PAGE following formic acid solubilization. Gel-bands were cut, and in-gel digested with proteases (trypsin/Lys-N) for label-free proteomics using nano-LC- tandem mass spectrometry. (B) Heatmaps depicting the top proteins (left) and Aβ peptides (right) identified in the bottom-up proteomic experiment from the gel bands (middle) that were cut after separating the detergent insoluble fraction on SDS-PAGE. Ubiquitin (UBB/RPS27A), 2N4R tau (MAPT) were the most abundant protein along with other known constituents (ferritin (FTH/FTL), NEFL, SNRPE, ApoE, α-synuclein (SNCA)), while most of the Aβ peptide intensity (left) indicated their migration to the bottom of the gel. However, Aβ_{28-42} peptide was predominantly detected on the top of the gel. (C) Western blot of tau, p-tau and Aβ using polyclonal anti-tau, p-tau (pT$_{181}$) specific and Aβ G2-10 antibodies, respectively, in the sarkosyl-insoluble pellet from the AD brain. Aβ immunoreactivity shows a highly polymerized fraction which does not enter the gel.

Fig. 2. **Quantitative estimation of tau and Aβ in sarkosyl-soluble and -insoluble fractions**. (A) Heatmap of the starting fractions of homogenate, sarkosyl-soluble and sarkosyl-insoluble of tau, Aβ, MT-1, MT-3, α-synuclein (SNCA), and β-synuclein (SCNB). (B) Scatter plots of mean ± SD concentration of tau (T$_{181-190}$, T$_{212-221}$, T$_{243-254}$) and Aβ (Aβ_{28-42}, Aβ_{28-40}) peptides in the starting homogenate, sarkosyl-soluble and sarkosyl-insoluble

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fractions in AD (red) and DCN (blue) brains. (C) Scatter plot of quantitative estimation ($\log_{10}$) of mean ± s.d. concentration 22 tau (from N-terminus, PRR, MTBR and C-terminus) and Aβ (Aβ_{1-15}, Aβ_{4-15}, A$\beta_{3pGlu-15}$, Aβ_{16-27}, Aβ_{28-42}, Aβ_{28-40}) peptides from AD sarkosyl-insoluble pellets using high-resolution MS. Scatter plot depicts that the concentration of R1 and R3 peptides of tau are almost equimolar to Aβ_{28-42} (~ 500 fmol/mg), while three R4 tau peptides are almost 2.5 times higher (~ 1000 fmol/mg) than Aβ_{28-42} in the sarkosyl-insoluble fraction. N-terminal peptides of tau are ~100-fold lower than the MTBR tau peptides (~ 10 fmol/mg) and comparable to the truncated N-terminal peptides of Aβ (~ 20 fmol/mg). ns>0.05; *$p < 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p < 0.0001$. The P values were calculated using unpaired one-way analysis of variance (ANOVA), corrected for multiple comparison false discovery rate ($P < 0.05$) with Benjamini–Hochberg correction (see also Statistical Analysis in Methods)

Fig. 3. **Quantitative estimation of PTM of tau.** (A) Scatter plots of percent fractional phosphorylation at T_{181} (pT_{181}) and T_{217} (pT_{217}) tau peptides in the starting material, sarkosyl-soluble, and sarkosyl-insoluble fractions in AD (red) and disease-control (DCN) brains (blue). (B) PTMs detected (phosphorylation, ubiquitination, acetylation, deimination and demethylation) depicted on the amnio acid sequence of 2N4R tau (MAPT) identified from the sarkosyl insoluble pellets from AD brains investigated in this study. (C) Heatmap of all the ($\log_{10}$abundance) PTMs associated with the most enriched tau peptides in the sarkosyl-insoluble pellet in AD brains using label-free quantitative proteomics, demonstrating hyperphosphorylation at the PRR and C-terminal region and high ubiquitination/acetylation in the MTBR domain. ns>0.05; *p< 0.05; **p≤ 0.01; ***p<0.001.

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Fig. 4. Quantitative proteomics of the sarkosyl-insoluble pellets. (A) Scatter plot of proteins found in sarkosyl-insoluble pellet ranked in decreasing order against their $\log_2$ (iBAQC) protein abundance found in the AD and DCN. The inset shows the top 100 proteins where ferritin, tau, Aβ were the among the most enriched proteins in the sarkosyl-insoluble pellets, while neurofilament medium (NEFL), neurofilament light (NEFL) and ApoE were also present in these pellets. RNA binding proteins (RBPs) such as SNRNP70 and HNRNP also ranked high in the insoluble proteome. (B) Volcano plots (differentially enriched proteins) of significantly enriched proteins in the sarkosyl-insoluble pellets in AD in comparison to DCN brains. The *t*-statistic ($-\log_{10}[P\text{-Value}]$) was calculated for all proteins in each pairwise group and displayed on the y-axis. Insoluble fraction proteins were considered significantly enriched with fold change of 2 and $P<0.05$. Ferritin heavy chain (FTH), tau (MAPT), Aβ, complement C3 and ApoE are significantly enriched in the sarkosyl-insoluble pellet of the AD brain (FDR < 0.001, depicted in red), while α-synuclein (SNCA), FTL, had FDR< 0.01 (blue). We noticed TDP-43 (TARDBP) and Filamin-A (FLNA) had significant enrichment in the AD sarkosyl-insoluble pellets (FDR < 0.05) while neurofilament light (NEFL) showed no enrichment. Several RBPs, including splicing factors such as SNRPNP70 (FDR < 0.001) and HNRNP (HNRNPA2B1, FDR < 0.01), showed increased insolubility in AD. (C) Heatmap of the z-score normalized protein abundance ($\log_2$-transformed iBAQ) for the proteins found significantly enriched in AD sarkosyl-insoluble pellets in comparison with the control brains that are labelled in (B).

Fig. 5. Filamentous *vs* globular aggregates. Negative immunogold staining EM for (upper row) recombinant (A) $A\beta_{1-42}$ fibrils with aducanumab antibody and (B) hTau441 heparin-induced filaments with RNF5 anti-tau antibody. Middle row: sarkosyl-insoluble fraction (pellet) from brain samples AD06 and AD11 with (C) aducanumab and (D) RNF5 antibodies.

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Bottom row: (E) double immunogold staining for AD11 sample with aducanumab (red arrows) and RNF5 (blue arrows) antibodies, respectively, and (F) AD09 sample with polyclonal rabbit K9JA anti-tau antibody. Secondary antibodies were anti-mouse 10nm-gold (tau RNF5), anti-rabbit 5nm-gold (tau K9JA), and anti-human 6nm-gold (aducanumab) labelled antibodies. Scale bar 50nm.

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Authors' contributions

S.M., C.L.M, and V.A.S. conceived the study. S.M. designed and performed the proteomics experiments, tandem mass spectrometry statistical analysis and interpretation of the MS data. C.M. brain bank access and diagnostics. S.M., C.D., K.P. M.L., and S.V. performed the brain homogenization and detergent extraction. C.D., K.P. and S.V. performed the Western Blot experiments. I.B. conducted immunohistochemical assessments. Q.-X.L. performed immunoassays, N.D, and R.N. produced recombinant tau protein. R.N. produced anti-tau TAU1 and RNF5 antibodies. V.A.S. performed all electron microscopy experiments and data processing, and final data interpretation. The manuscript was written with input from all the authors. All authors have read and approved the manuscript.

--Human subjects --

Involves human subjects:

If yes: Informed consent & ethics approval achieved:

=> if yes, please ensure that the info "Informed consent was achieved for all subjects, and the experiments were approved by the local ethics committee." is included in the Methods.

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Conflict of interest

The authors declare no competing financial interests.

Data availability statement

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE(Perez-Riverol *et al.* 2022) partner repository with the dataset identifier PXD032372 and PXD032389. Additional data are available as supplementary materials accompanying this article. The transition list and the MQ/proteomics outputs and other data supporting the findings of the study can be requested from the corresponding authors.

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