

SUPPLEMENTARY SECTION

METHODS

Western Blotting

For Western blotting, 2.5 ml pooled CSF was used for EV isolation as described above. The pellet obtained after the final ultracentrifugation step was dissolved in 50 µL of 1X protein loading buffer containing SDS. The sample was then boiled at 70°C for 10 minutes prior to loading onto a 10% polyacrylamide gel for SDS-PAGE. Western blotting was performed according to standard protocols. Next, proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA) that were subsequently blocked for 30 min with blocking buffer (4% w/v nonfat dried milk in TBS-Tween 0.5% v/v (TBS-T)), and incubated with primary antibodies overnight at 4°C. The membranes were incubated with secondary antibodies (diluted 1:2000 in 4% milk in TBST) for 2 hours at room temperature. Protein bands were detected with the ECL Western Blotting detection kit (GE Healthcare, Amersham, UK). The primary antibodies were anti-Calnexin (Cat. Number C4731, Sigma-Aldrich, Darmstadt, Germany, 1:2000 dilution), anti-Flotillin-2 (Cat. Number 610384, BD Biosciences, San Jose, CA, USA, 1:500 dilution), and anti-Annexin A1 (EPR19342, Cat. Number ab214486, Abcam, Berlin, Germany, 1:500 dilution). Secondary antibodies against mouse and rabbit IgG were obtained from Dako at 1:5000 dilution.

Nanoparticle Tracking Analysis (NTA)

EVs in the ultracentrifugation pellet were resuspended in 100 µl PBS and vortexed at full speed for 2 minutes. 20 µl from this solution was resuspended in 480 µl of PBS for NTA measurement. EVs isolated using Vn96 peptide were processed according to³⁸ before NTA measurement. NTA was performed with a NanoSight LM10 instrument and a LM14 viewing unit equipped with a 532-

1 nm laser (NanoSight, Malvern Instruments Ltd, Malvern, UK). Samples were recorded in
2 quadruplicates for 30 seconds. Particle numbers and particle sizes were then analyzed with the
3 Nanoparticle Tracking Analysis (NTA) 2.3 software.

4 **Transmission Electron Microscopy (TEM)**

5 TEM was performed as described in our previous study.⁸ Briefly, TEM grids (copper, 150
6 hexagonal mesh, Science Services, Munich, Germany) were coated with a formvar film and
7 incubated for 10 min on top of 10 µl droplets containing the EV fraction. The grids were then
8 washed 5 times with PBS followed by incubation on droplets of water. The grids were then stained
9 for 5 min on droplets of uranylacetate-oxalate and incubated for 5 min on droplets of a 1:9 dilution
10 of 4% uranylacetate in 2% methylcellulose. Methylcellulose was removed from the grids with
11 filter paper. Samples were then imaged with a LEO912 transmission electron microscope (Carl
12 Zeiss Microscopy, Oberkochen, Germany) and an on-axis 2k CCD camera (TRS, Moorenweis,
13 Germany).

14 **Label-free protein quantification Cohort 1**

15 Sample preparation, data acquisition, and data analysis were essentially performed as previously
16 described in detail for synaptic protein fractions from mouse brain.³ EVs in the ultracentrifugation
17 pellet were dissolved in 200 µl lysis buffer (2% CHAPS, 7 M urea, 2 M thiourea, 10 mM DTT,
18 0.1 M Tris pH 8.5) and subjected to automated in-solution digestion by filter-aided sample
19 preparation (FASP). Briefly, samples were loaded on centrifugal filter units (30 kDa MWCO,
20 Merck Millipore), detergents were removed with wash buffer (8 M urea, 10 mM DTT, 0.1 M Tris
21 pH 8.5), proteins were alkylated with 50 mM iodoacetamide in 8 M urea, 0.1 M Tris pH 8.5, and
22 excess reagent was removed with wash buffer. After buffer exchange with 50 mM ammonium

1 bicarbonate (ABC) containing 10 % acetonitrile, proteins were digested overnight at 37°C with
2 400 ng trypsin in 40 µl of the same buffer. Tryptic peptides were recovered by centrifugation and
3 extracted with 40 µl of 50 mM ABC and 40 µl of 1% trifluoroacetic acid (TFA), respectively. For
4 quantification according to the TOP3 approach⁴, aliquots of combined flow-throughs were spiked
5 with 10 fmol/µl of yeast enolase-1 tryptic digest standard (Waters Corporation)⁵. Samples were
6 directly subjected to LC-MS analysis using nanoscale reversed-phase UPLC separation
7 (nanoAcquity system, Waters Corporation) coupled to quadrupole time-of-flight mass
8 spectrometry with ion mobility option (Synapt G2-S, Waters Corporation). Peptides were trapped
9 for 4 min at a flow rate of 8 µl/min 0.1% TFA on Symmetry C18 5 µm, 180 µm × 20 mm trap
10 column and then separated at 45°C on a HSS T3 C18 1.8 µm, 75 µm × 250 mm analytical column
11 over 120 min at a flow rate of 300 nl/min with a gradient comprising two linear steps of 3-35%
12 mobile phase B in 105 min and 35-60% mobile phase B in 15 min (A, water/0.1% formic acid; B,
13 acetonitrile/0.1% formic acid). Mass spectrometric analysis was performed in the ion mobility-
14 enhanced data-independent acquisition mode with drift time-specific collision energies (referred
15 to as UDMSE) as introduced by Distler and colleagues^{6,7} and adapted by us to synaptic protein
16 fractions.³ Continuum LC-MS data were processed using Waters ProteinLynx Global Server
17 (PLGS) and searched against a custom database compiled by adding the sequence information for
18 yeast enolase 1 and porcine trypsin to a UniProtKB/Swiss-Prot human proteome (see below) and
19 by appending the reversed sequence of each entry to enable the determination of false discovery
20 rate (FDR)⁸. Precursor and fragment ion mass tolerances were automatically determined by PLGS
21 and were typically below 5 ppm for precursor ions and below 10 ppm (root mean square) for
22 fragment ions. Carbamidomethylation of cysteine was specified as fixed and oxidation of
23 methionine as variable modification. One missed trypsin cleavage was allowed. Minimal ion

matching requirements were two fragments per peptide, five fragments per protein, and one peptide per protein. The FDR for protein identification was set to 1% threshold. For label-free protein quantification, the freely available software ISOQuant (www.isoquant.net) was used for post-identification analysis including retention time alignment, exact mass and retention time (EMRT) and ion mobility clustering, peak intensity normalization, isoform/homology filtering and calculation of absolute in-sample amounts for each detected protein.^{6,7,9} Only peptides with a minimum length of seven amino acids that were identified with scores above or equal to 5.5 in at least two runs were considered. FDR for both peptides and proteins was set to 1% threshold. Only proteins reported by at least two peptides (one of which unique) were quantified using the TOP3 method⁴ and expressed as parts per million (ppm) abundance values, i.e. the relative amount (w/w) of each protein in respect to the sum over all detected proteins.

Analysis of proteome data

A FASTA file of the UniProtKB/Swiss-Prot human proteome comprising canonical and isoform sequences (version 2020_04, 42347 entries) was used for that purpose. Similarly, the same statistical analysis was applied to the protein abundance values from both cohorts. First, the ppm (Cohort 1) or LFQ (Cohort 2) values were log2-transformed and normalized by subtraction of the median derived from all data points for the given protein. Transformed data were analyzed for significant changes in protein abundance by moderated t-statistics with an empirical Bayes approach and false discovery (FDR)-based correction for multiple comparisons¹⁰, using the Bioconductor packages "limma"¹¹ and "q-value"¹². Volcano plots were plotted using bioinfokit.

Supplementary TABLE

Table S3: Levels of CSF C1q, ALDOC, and CTSB

	Cohort 3 Controls	Cohort 3 AD	Cohort 3 Controls	Cohort 3 AD
N	16	24	43	100
CSF C1q (ng/mL)	148 [135.9-167.1]	221.9 [187.2-245.6] ^c	147.2[137.2-166.8]	253.9[224.2-284] ^c
EV ALDOC (pg/mL)	2771[2482-3157]	1170[1010-1556] ^b	2683 [2367-2939]	1248 [1029-1654] ^c
EV ALDOC /Annexin	5.260 [4.147-6.301]	2.389 [1.846-3.639] ^b	5.62 [4.66-9.04]	2.30 [0.80-3.52] ^c
CSF ALDOC (ng/mL)	12.61 [7.58-19]	12.96 [6.58-21]	13.34 [6.80-21.50]	12.59 [4-20.65]
EV CTSB (pg/mL)	1305 [1175-1563]	720 [600-818] ^c	1265 [1148-1419]	792 [668-986] ^b
CTSB/Annexin	2.47 [1.90-3.12]	1.47 [1.10-2.37] ^c	2.75 [2.15-4.00]	1.5 [0.61-2.45] ^c
CSF CTSB (ng/mL)	21.82 [14.60-30.54]	23.35 [12-25]	21.31 [9-32.1]	23.48 [14-27.6]

^sMMSE, Mini Mental State Examination

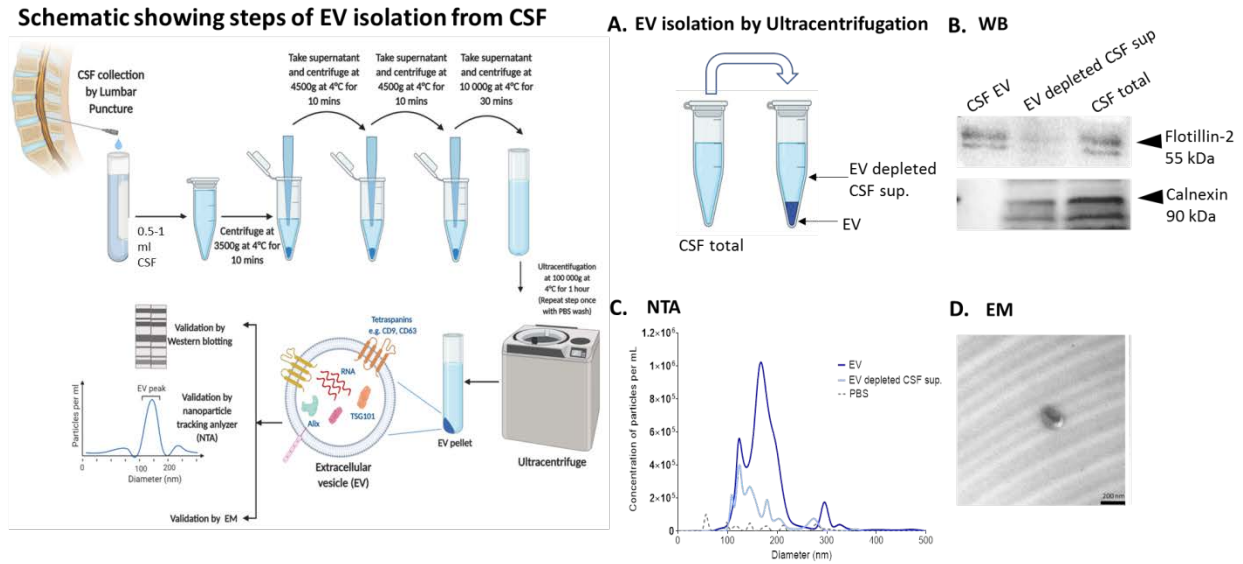
^a $p < 0.05$ AD versus controls

^b $p < 0.01$ AD versus controls

^c $p < 0.001$ AD versus controls

Supplementary Figures and Supplementary Figure Legends

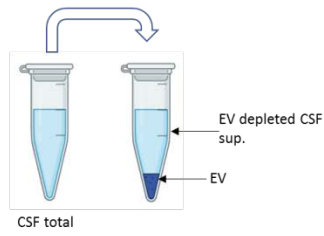
Figure S1



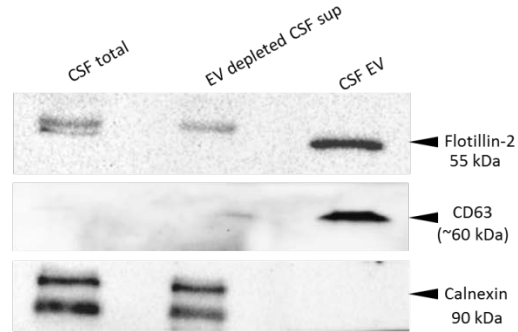
Supplementary Fig S1. Schematic showing EV isolation protocol using serial centrifugation and ultracentrifugation. Same protocol was used for all cohorts except Cohort 2. Characterization of EVs isolated from human CSF using Western blotting (**B**), nanoparticle tracking analysis (**C**) and transmission electron microscopy (TEM) (**D**). This figure was created using BioRender.com.

Figure S2

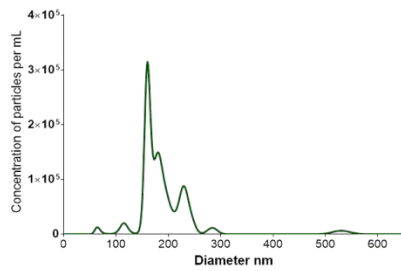
A. EV isolation by Vn96 peptide



B. WB

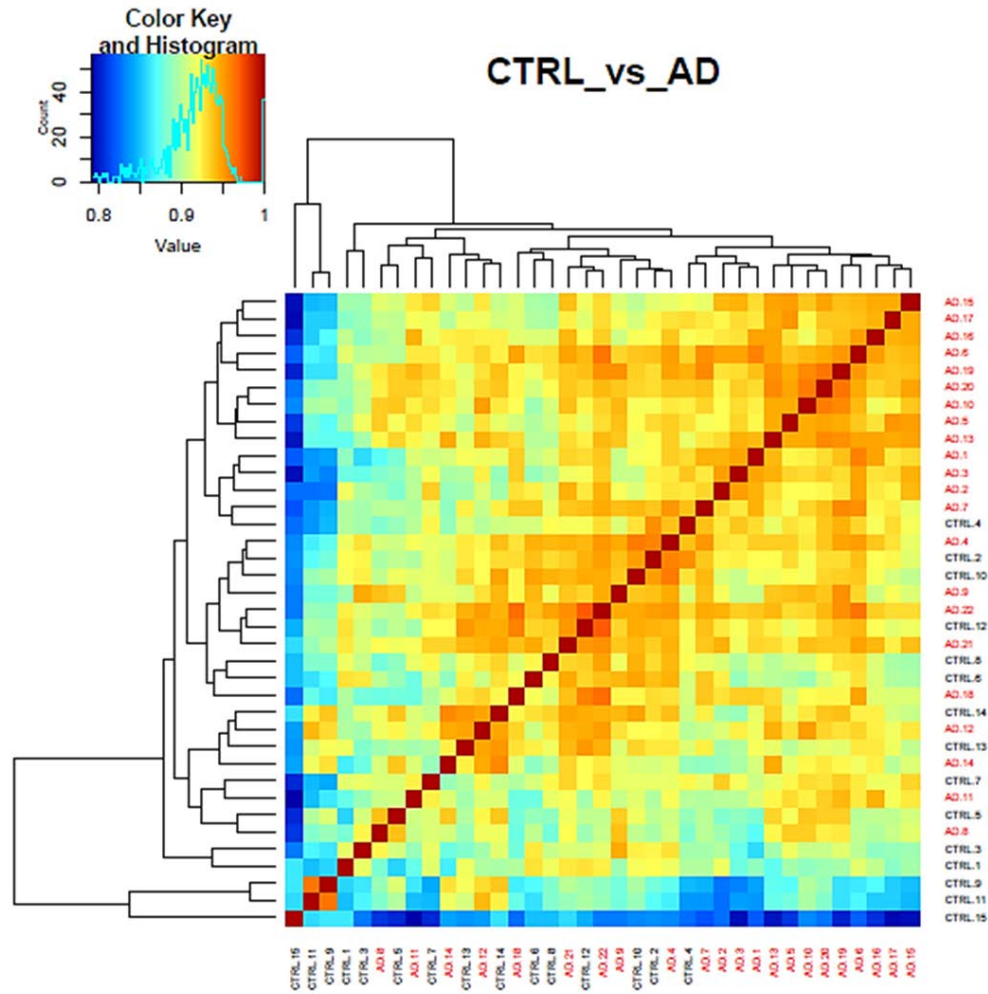


C. NTA



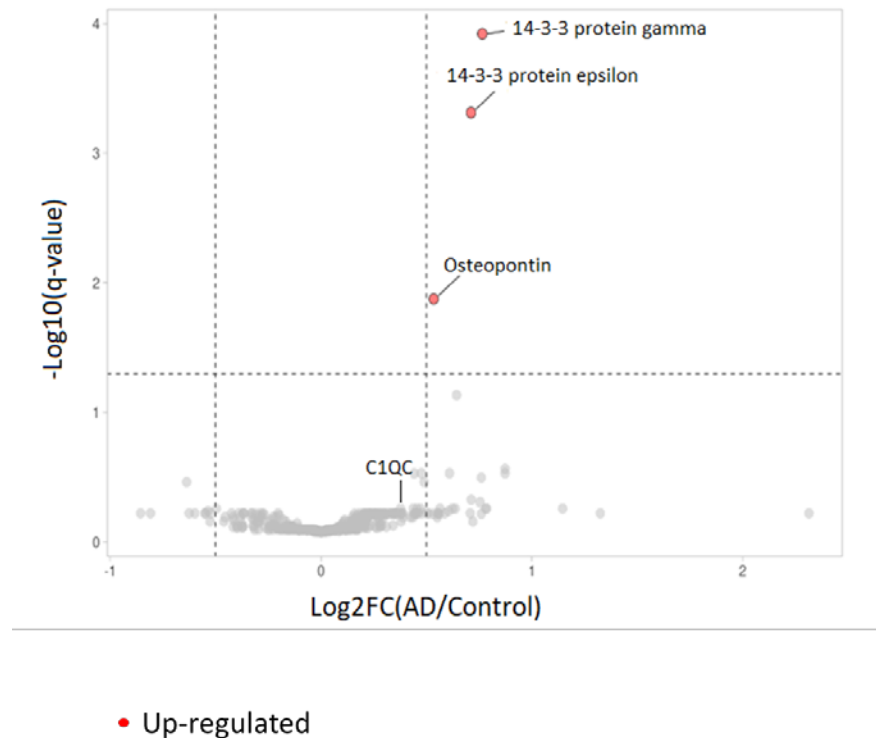
Supplementary Fig S2. Characterization of EVs isolated using Vn96 peptide (**A**) from human CSF (Cohort 2) using Western blotting (**B**) and nanoparticle tracking analysis (**C**).

Figure S3



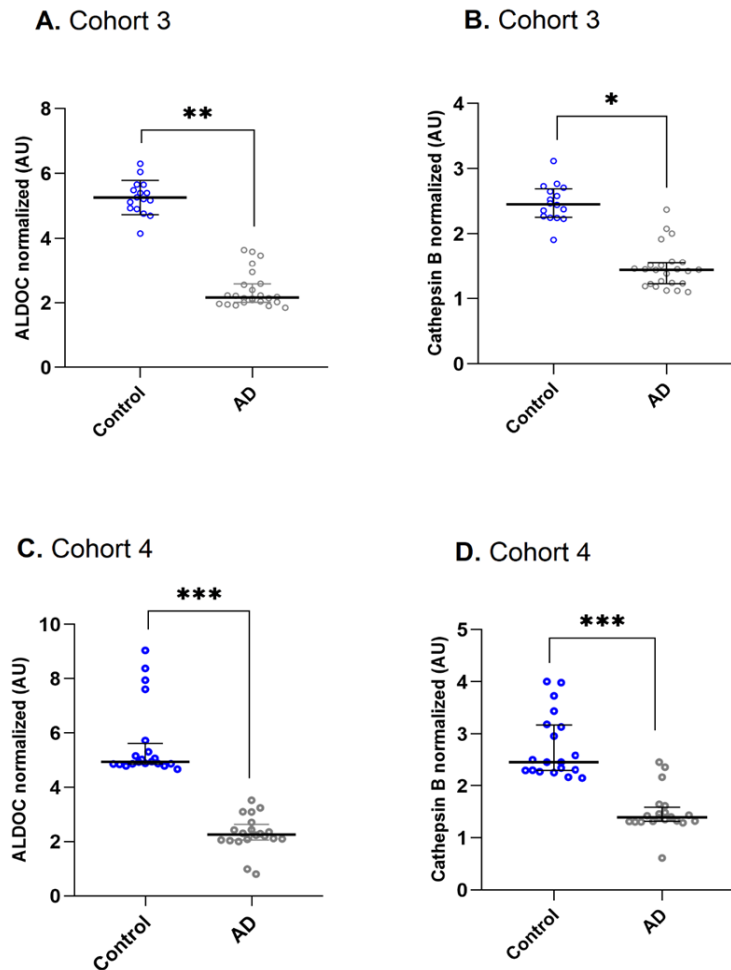
Supplementary Fig S3. Heatmap showing clustering of AD patients and controls based on Pearson correlation in Cohort 1. Correlation coefficients derived from log2-transformed ppm abundance values were clustered and visualized with the tool heatmap.2 contained in the R package gplots (CRAN.R-project.org/package=gplots). Only pairwise complete observations were considered to reduce the influence of missing values on clustering behavior.

Figure S4



Supplementary Fig S4. Volcano plot of differentially expressed proteins for the AD vs Controls in Cohort 2. Fold change (log2FC) cut off=0.5, Significance (-Log10)=1.3. C1q was also identified among up-regulated proteins but the change was not significant. Fold change (log2FC) cut off=0.5, Significance (-Log10)=1.3.

Figure S5

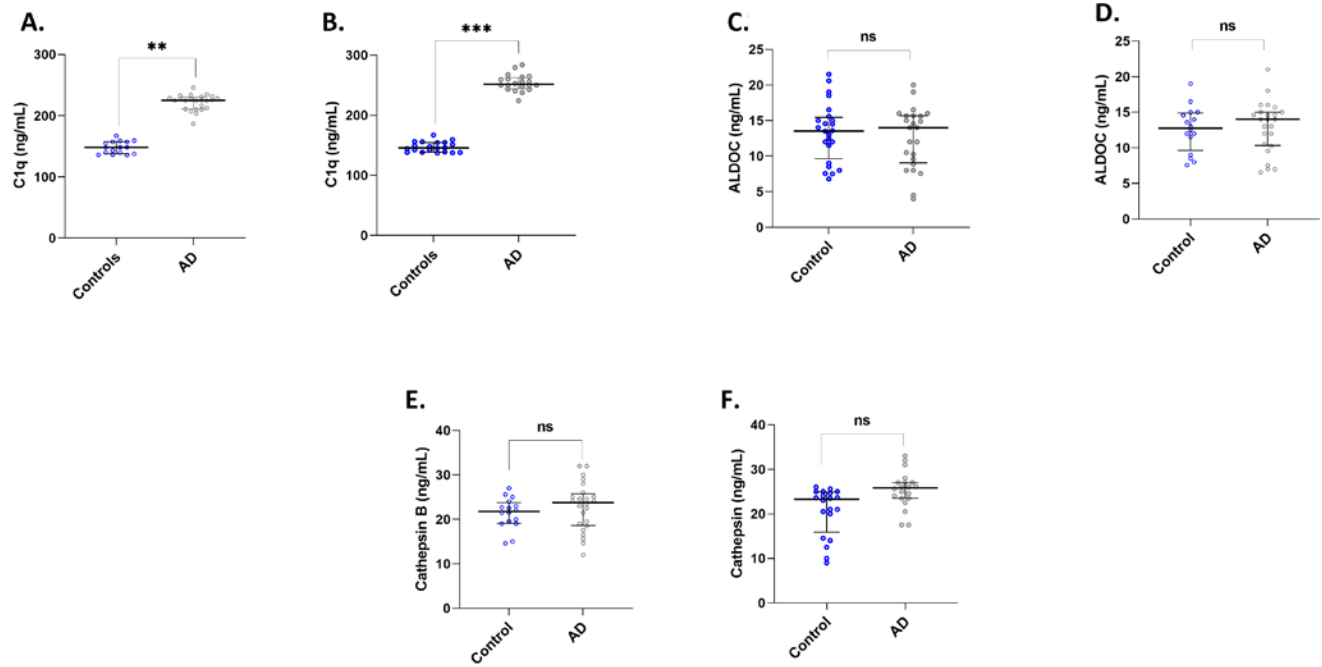


Supplementary Fig S5. (A) Levels of CSF EV associated ALDOC levels in Cohort 3. (B) CSF EV Cathepsin B levels in Cohort 3. (C) CSF EV ALDOC levels in Cohort 4. (D) CSF EV Cathepsin B levels in Cohort 4. CSF EV ALDOC and Cathepsin B levels were normalized to CSF EV Annexin levels to account for potential differences in EV preparation yields.

The long horizontal lines represent median and short horizontal lines represent inter-quartile range.

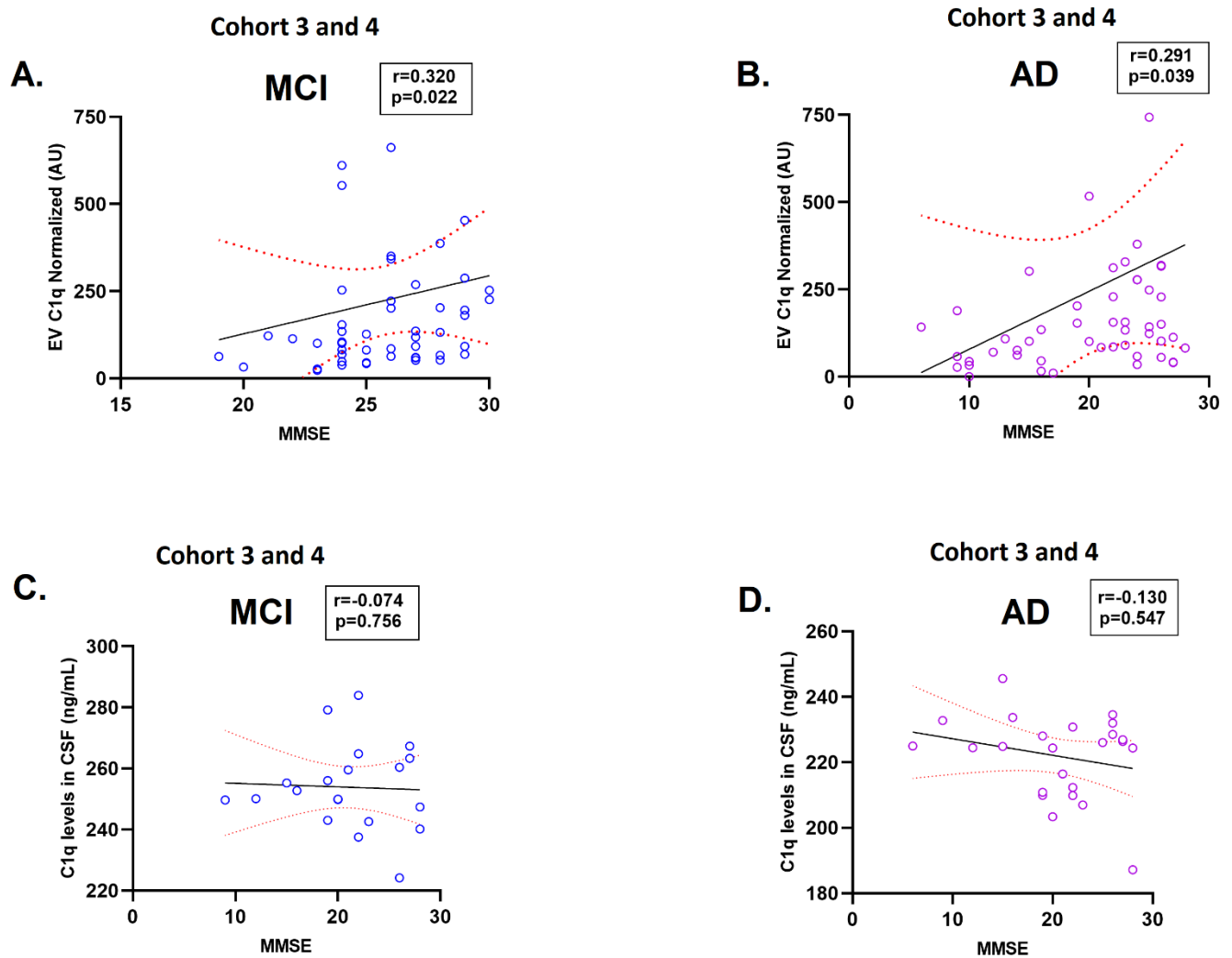
Mann-Whitney U test. ** $p < 0.01$, *** $p < 0.001$.

Figure S6



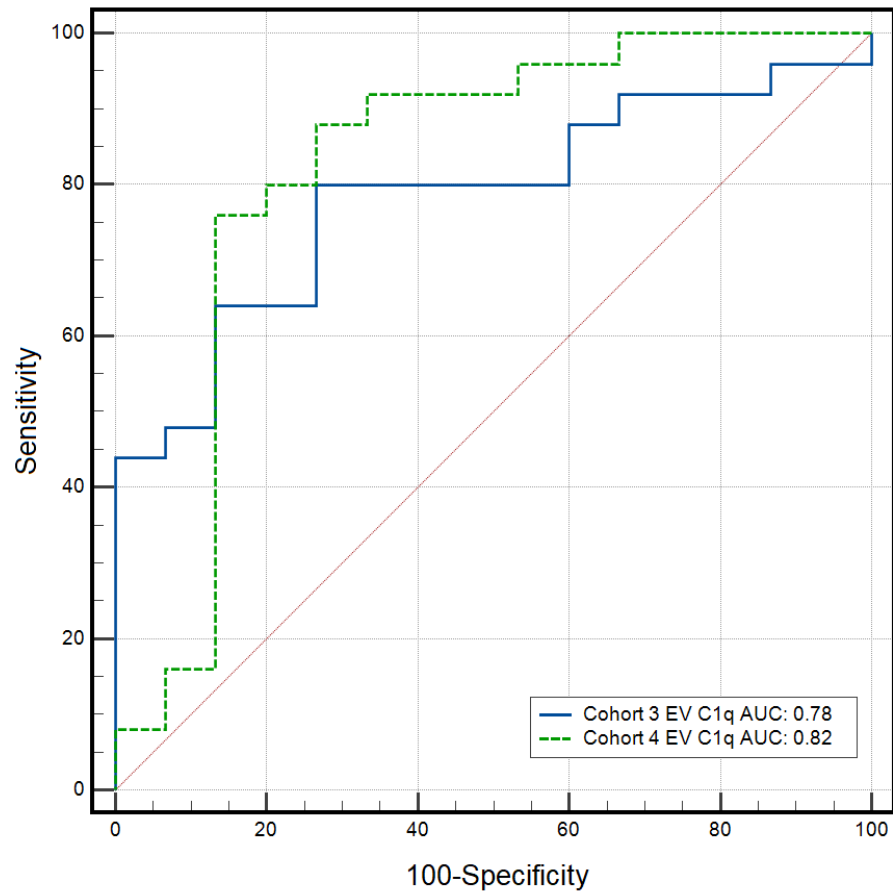
Supplementary Fig S6. Levels of CSF C1q in **(A)** Cohort 3 and **(B)** Cohort 4. **(C)** Levels of CSF ALDOC in Cohort 3 and **(D)** Cohort 4. **(E)** Levels of CSF Cathepsin B in Cohort 3 and **(F)** Cohort 4. The long horizontal lines represent median and short horizontal lines represent inter-quartile range. Mann-Whitney U test. ** $p < 0.01$, *** $p < 0.001$, ns: non-significant.

Figure S7



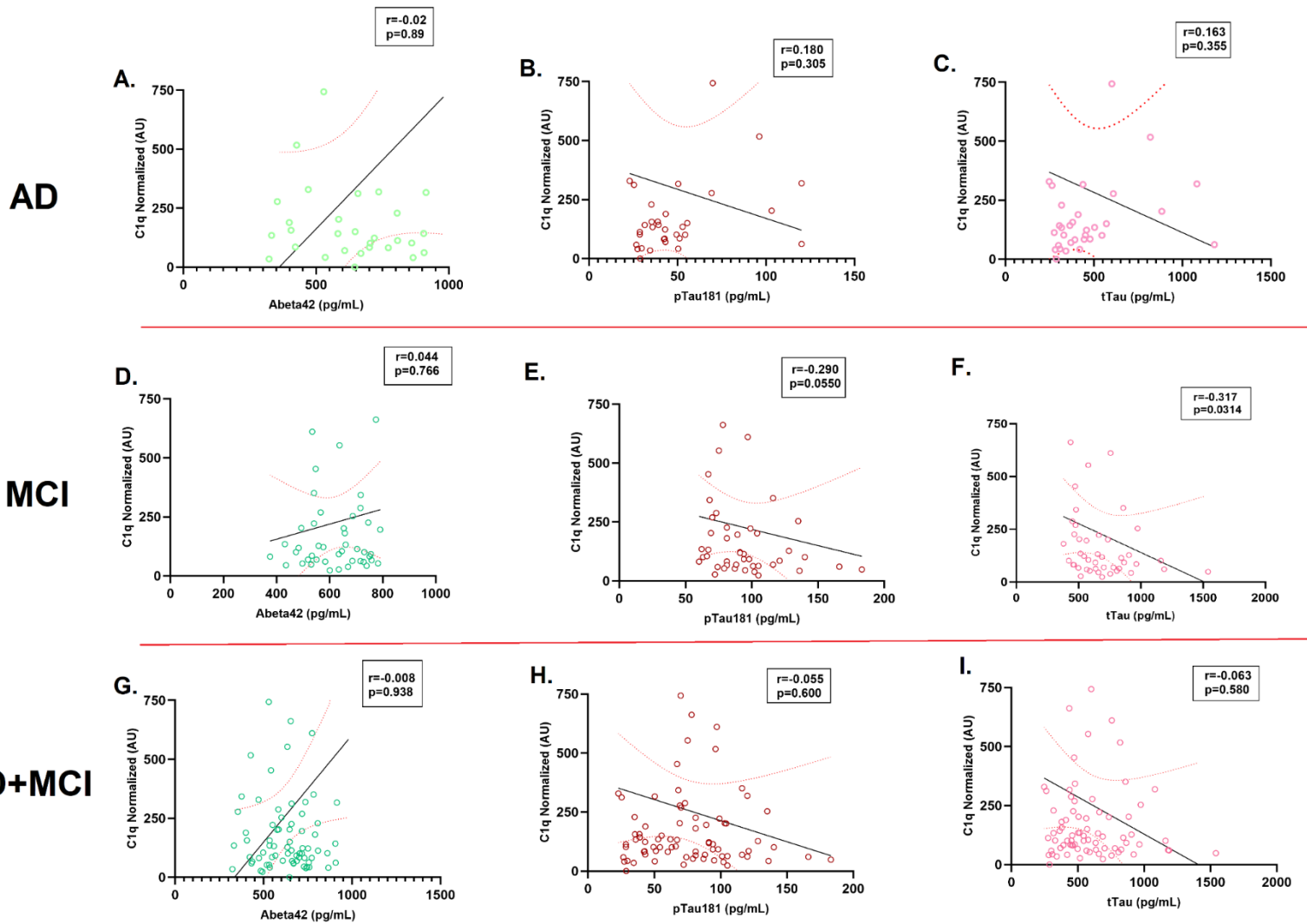
Supplementary Fig S7: Correlation analysis between CSF EV C1q levels and MMSE scores in Cohort 3 and Cohort 4 (pooled cohorts). **(A)** MCI group ($n=62$) **(B)** AD dementia group ($n=62$). Correlation analysis between CSF C1q and MMSE in **(C)** MCI group ($n=62$) and **(D)** AD dementia group ($n=62$) **(D)**. Spearman correlation coefficient (r) and p -values are shown.

Figure S8



Supplementary Fig S8: ROC curve analysis of CSF EV C1q in Cohort 3 (blue) and Cohort 4 (green, dashed). AUC Cohort 3= 0.7840 ($p<0.0001$); AUC Cohort 4=0.8280 ($p<0.0001$).

Figure S9

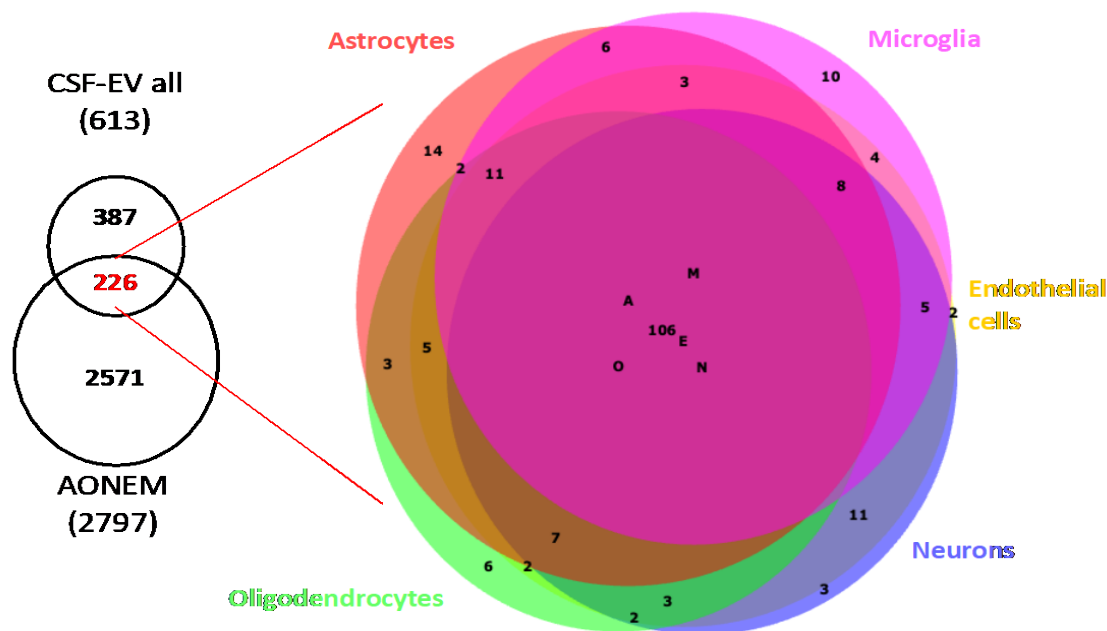


Supplementary Fig S9: Spearman rank correlation analysis and linear regression line fitting

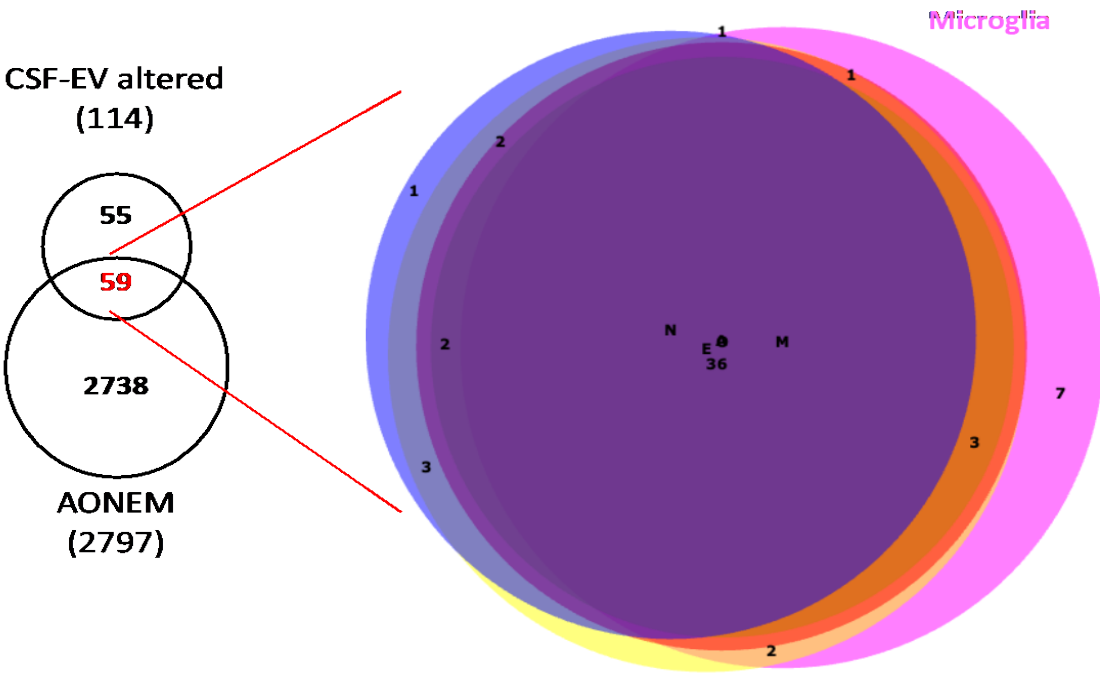
between CSF EV C1q levels and Aβ₄₂, pTau181 and tTau in MCI ($n=50$), AD ($n=50$) and combined AD+MCI ($n=100$) groups in Cohort 4. Spearman r and p -values are shown.

Figure S10

A.



B.



Supplementary Fig S10: Overlap between a cell type-specific mouse brain proteome and all quantified CSF EV proteins in Cohort 1 (**A**) or CSF EV proteins with altered abundance in AD (**B**). Data for the resource proteome covering astrocytes, oligodendrocytes, neurons, endothelial cells, and microglia (referred to as AONEM; 3541 proteins) were taken from Düking et al.¹³ and filtered further to increase cell type specificity (only proteins with eight out of eight possible data points were assigned to a certain cell type; 2797 proteins). Proteins from the intersections (red numbers) were analyzed for cell-type specificity by DeepVenn, a web application for the creation of area-proportional Venn diagrams using the deep learning framework Tensorflow.js (<https://www.deepvenn.com/>). The seven altered CSF EV proteins showing potential specificity for microglia in (**B**) included the C1q isoforms C1QA, C1QC, and C1QB, as well as PPGB, GCC2, RPB1, and PKP1.

Supplementary References

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