Purification of EVs from human brain samples

The unfixed frozen frontal cortical grey matter tissue (0.5 g) from deceased AD or control cases were processed for EV extraction based on reported method with some modifications [1]. Briefly, frozen brain tissue was chopped on ice using a razor blade (# 12-640 Fischer Scientific) to generate 2-3mm^3 sections. The sections were transferred to 3mL of Hibernate E solution containing 20 units of Papain (# LK003178 Worthington-biochemical corporation) in Earle's Balanced Salt Solution (EBSS) (# 14155063 Gibco) and then incubated in a water bath at 37°C for 15 min by stirring once every 5 min. After incubation, the samples were immediately place on ice, and 6mL of ice-cold Hibernate E solution (# A1247601 Gibco). The dissociated brain tissue samples were gently homogenized (20 strokes) with a glass-Teflon homogenizer (# 89026-384 VWR). The homogenized tissue samples were filtered with 40μm mesh filter (# 22-363-547 Fisher scientific). After filtration, the tissue samples were centrifuged at 300 × *g* for 10 min at 4°C (# 5720R Eppendorf). The supernatant from 300 × *g* was transferred to new 15mL tube and then centrifuged at 2,000 × *g* for 10 min at 4°C (# 5720R Eppendorf). The supernatant from 2,000 × *g* was transferred to 30mL conical tube and then centrifuged at 10,000 × *g* for 10 min at 4°C (# Avanti J-E JA25-50 Beckman Coulter). The supernatant from this spin was filtered through a 0.22-μm mesh filter (# SLGP033RS EMD Millipore) into new a polyallomer ultracentrifuge tube with 13.2mL capacity (# 331372 Beckman Coulter) and then was centrifuged at 100,000 × *g* for 70 min at 4°C (# Optima-XE SW41 Beckman Coulter). After ultracentrifugation, the supernatant was completely discarded, and the pellet was resuspended in 2mL of 0.475M of sucrose solution (# S5-3 Fisher science). All sucrose solutions were diluted in double-filtered PBS (dfPBS) with 0.22-μm pore size (# SLGP033RS EMD Millipore). The

sucrose step gradient was created with six 2-mL steps starting from 2.0M to 1.5M, 1.0M, 0.825M, 0.65M, and 0.475M (containing the resuspended pellet) in a polyallomer ultracentrifuge tube. Each layer was colored with commercially available food coloring to facilitate capture of the EV-rich interphase present between certain steps. The gradient was centrifuged at 200,000 × *g* for 20 h at 4°C (35,000 rpm with # Optima-XE SW41 Beckman Coulter), after centrifugation, the gradient was collected in 2mL fractions, except for the first and last fractions, which were 1mL each. Fraction III corresponded to the 1mL removed from the tube, and particles in this area had a buoyant density of approximately 1.08 g/cm^3. The area between the second (0.65M) and third (0.825M) steps was collected and corresponded to fraction "V", with a buoyant density of 1.10 - 1.12 g/cm^3, while the interphase between the third and fourth steps corresponded to fraction "VI", with a buoyant density of 1.12 - 1.15 g/cm^3. The V and VI fractions were then diluted separately to a total volume of 12mL with dfPBS and centrifuged at 100,000 × *g* for 70 min at 4°C (# Optima-XE SW41 Beckman Coulter). The final pellet from each fraction was resuspended in 30μL of dfPBS. For all biochemical analyses, these fractions were combined such that an equal volume of each fraction (V and VI) was used. For proteomics, an equal amount of total protein from each fraction (V and VI) was used.

Protein concentrations

The bicinchoninic acid (BCA) assay was used to determine protein concentration for each sample. The Pierce BCA protein assay kit was used (# 23225 Pierce). Due to the limited amount of sample, EVs were diluted 1:10 before loading into the assay, and a 10:80 ratio of sample to reaction components was used. All assays were allowed to incubate at 60°C for 30 min before protein concentration was read in a Biotek SynergyMix at 562nm. For all protein assays, raw readings were adjusted by dilution of the sample for the assay, and then by the final dilution of

the sample and starting weight of the material. The average concentration was then taken for fractions V and VI.

Enzyme-Linked Immunosorbent Assay (ELISA)

EVs were diluted in TET buffer (50mM Tris HCl pH 7.5, 2mM EDTA, 1% Triton X-100) supplemented with Pierce HALT inhibitor (# 78425 ThermoFisher) for t-tau and ANXA5 or TENT buffer (50mM Tris HCl pH 7.5, 2mM EDTA, 150mM NaCl, 1% Triton X-100) supplemented with phosphatase inhibitors (either addition of 0.5mM PMSF, 10mM NaF (# S7920 SIGMA), 200mM glycerol-2-phosphate (# 50020 SIGMA), and 1mM Na_3VO_4 (# S6508 SIGMA) or Pierce HALT inhibitor for pT181 tau, pS196 tau, and pS396 tau. ELISAs were performed to assess levels of t-tau, p-tau, and ANXA5. Commercially available kits from ThermoFisher (t-tau: # KHB0042, pT181: # KHO0631, pS199: # KHO0631, pS396: # KHO0631, ANXA5: # BMS252) were used according to manufacturer's instructions. For Aβ1-40 and Aβ1-42, the levels were measured using the Single Molecule Counting (SMC®) Immunoassay Technology using commercially available kits from Millipore / Sigma (Aβ1-40: # 03-0145-00, Aβ1-42: # 03-0146-00). The limit of detection (LOD) was estimated at 9.97pg/mL and 0.368pg/mL and the limit of quantification (LOQ) was estimated at 25pg/mL and 3.13pg/mL and the coefficient of variation (CV) is less than 5% and 10% in Aβ1-40 and Aβ1-42.

Nanosight Tracking Analysis (NTA)

All samples were diluted in dfPBS at least 1:1000 or more to get particles within the target reading range for the Nanosight 300 machine (Malvern Panalytical Inc), which is 10-100 particles per frame. Using a syringe pump infusion system (Harvard Laboratories/Malvern), five 60-second videos were taken for each sample at 21°C. Analysis of particle counts was carried out in the Nanosight NTA 3.2 software (Malvern Panalytical Inc) with a detection threshold of 5.

Particle counts were normalized for dilution on the machine, dilution of the final pellet, and starting material for EVs extraction. The average count was then taken for fractions V and VI.

Transmission electron microscopy (TEM)

The EV isolated from AD or control brain tissue were analyzed by TEM. 5µl of the EV sample was adsorbed for 1 min to a carbon-coated mesh grid (# CF400-CU EMS www.emsdiasum.com) that had been made hydrophilic by a 20-sec exposure to a glow discharge (25mA). Excess liquid was removed with a filter paper (# 1 Whatman), the grid was then floated briefly on a drop of water (to wash away phosphate or salt), blotted on a filer paper, and then stained with 0.75% uranyl formate (# 22451 EMS) for 30 sec. After removing the excess uranyl formate with a filter paper, the grids were examined and random fields were photographed using a JEOL 1200EX TEM with an AMT 2k CCD camera.

Mass spectrometry

Sample preparation

The EV samples were mixed with 2,2,2-Trifluoroethanol (TFE) (# 75-89-8 Millipore) to a final concentration of 50%. The samples were sonicated for 2 min on an ice-water bath (VWR Scientific) and then incubated at 60°C for 2 h. After cool down, the 5mM dithiothreitol (# 3483-12-3 Sigma Aldrich) was added to the samples, and then reduced for 30 min at 60°C. Further, the samples were alkylated with 10mM iodoacetamide (# 163-2109 BioRad) for 30 min at room temperature in the dark. The samples were digested with mass spectrometry grade trypsin (# V5280 Promega) in 50mM ammonium bicarbonate (pH 7.5) for protein digestion (1:30 w/w trypsin-to-protein) for 16h at 37°C. The digested peptides were dried by vacuum centrifugation (# SPD1010 Speedvac system, Thermo Savant). The dried samples were resuspended in 2% acetonitrile/water/0.1% trifluoroacetic acid (TFA) and desalted using C-18 spin columns (#

89870 ThermoScientific); the cleaned peptides were eluted using 60% acetonitrile (ACN) /water/0.1% TFA, dried by vacuum centrifugation (# SPD1010 Speedvac system, Thermo Savant) and further resuspended in 1% ACN/water/0.1% formic acid (FA) and analyzed by nano-liquid chromatography and tandem mass-spectrometry (Nano-LC-MS/MS).

Liquid chromatography (LC)- electrospray ionization (ESI) tandem mass-spectroscopy (MS/MS) Analysis

Nano-LC-MS/MS analysis was conducted by a Q-ExactiveHF mass spectrometer (Thermo-Fisher Scientific) equipped with a nano ultra-performance liquid chromatography (UPLC) (Water Technology) Peptides were trapped on a trapping column (180 µm × 20 mm) and separated on a reversed phased C-18 analytical column (BEH C18, 150 µm × 100 mm) (Waters technology). We loaded 1µL onto the column and separation was achieved using a 75 min gradient of 2 to 98% ACN in 0.1% FA at a flow rate of ~500nL/min. Data-dependent acquisition tandem MS was acquired in the positive ionization mode for the top 20 most abundant precursor ions. The scan sequence began with an MS1 spectrum (Orbitrap; resolution 60,000; mass range 300-2000m/z; automatic gain control (AGC) target 1×10^6; maximum injection time 100 ms). MS2 analysis consisted of higher energy collisional dissociation (Orbitrap; resolution 15,000; AGC 1×10^5; normalized collision energy (NCE) 30; maximum injection time 100 ms, dynamic exclusion time of 8 s).

Sequence database

The raw LC-MS/MS data were converted into mZML format using ProteoWizard msConvert [2]. The data were searched using PeaksDB and PeaksPTM using Peaks Studio version 8.0 (Bioinformatics Solutions, Inc., Waterloo, ON, Canada) against the Uniprot/Swissprot database for Homo sapiens with a 0.1% false discovery rate (FDR) and at least two unique peptides. A 10-

ppm error tolerance for the precursor (MS1) and 0.02 Da mass error tolerance for fragment ions (MS2) were specified. A maximum of 3 missed cleavages per peptide was allowed for the database search, permitting non-tryptic cleavage at one end. Trypsin was specified as the enzyme and carbamidomethylation as a fixed modification. A PeaksPTM search was queued after the peaksDB search, using advanced settings of a larger set of variable modifications, including hydroxylation P, oxidation M, hydroxylation K, hydroxylation-Hex K, hydroxylation-Hex-Hex K, HexNAc ST, HexHexNAc ST, phosphorylation STY, ubiquitination K, deamidation N, methoxy K, and nitrotyrosine Y. The final protein list generated was a combination of peaskDB and peaksPTM searches. The label-free quantification was achieved using PEAKS Studio Quantification-label-free module with a setting of mass error tolerance of 10 ppm and a retention time shift tolerance of 2.0 min. For data filtering for label-free peptide quantification following parameters were used: significance 15, fold change 1, quality 0, average area 1E4, charges from 1-10. The protein quantification following settings was used: significance 0, fold change 1, at least 2 unique peptides, significance method PEAKS.

Machine learning

The protein biomarkers to distinguish patients with Alzheimer's from controls were selected using Least Absolute Shrinkage and Selection Operator (LASSO) on the proteomics data from the training set (n = 21), where each patient's true state is labeled. An ensemble machine learning classifier to evaluate the performance of the selected proteins was developed. The ensemble machine learning classifier consists of five individual machine learning algorithms to mitigate overfitting, including Linear Discriminant Analysis, Logisitic Regression, Naïve Bayes, Support Vector Machine, and K-Nearest-Neighbours [3,4]. The machine learning generated model's performance was evaluated on a separate, user-blinded test set (n = 17).

References

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