

Stratification of Brain-Derived Extracellular Vesicles of Alzheimer's Disease Patients Indicates a Unique Proteomic Content and a Higher Seeding Capacity of Small Extracellular Vesicles

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Abstract

Alzheimer's disease (AD) is the most prominent form of dementia worldwide. It is characterized by tau lesions that spread throughout the brain in a spatio-temporal manner. This has led to the prion-like propagation hypothesis implicating a transfer of pathological tau seeds from cell-to-cell. Human extracellular vesicles isolated from the brain-derived fluid (BD-EVs) of AD patients contain seeds that contribute to this tau pathology spreading. Knowing the rich diversity of EVs, isolation of functional EVs sub-population is required to unravel their implication in the pathophysiology of AD. Here, enriched-small EVs (eSEVs) and enriched-large EVs (eLEVs) were isolated from frozen tissue after collagenase enzymatic brain dissociation to guaranty the best EVs' integrity. Both AD-derived eSEVs and eLEVs show the presence of GWAS-associated proteins and indicate a specific AD pathophysiological signature. Notably, AD eSEVs contain more proteins relative to the integrin-mediated synaptic signaling, while AD eLEVs proteins were more related to respiratory electron transport and brain-immunity. Injection of these vesicles in transgenic mouse brain revealed that AD-derived eSEVs are more prone than eLEVs to participate to the prion-like propagation and hence represent an interesting therapeutic target.

Introduction

Extracellular vesicles (EVs) are spherical nanoparticles comprised of a bilipid layer. EVs mainly contain proteins, nucleic acids and lipids. However, under this generic name, there is a huge diversity of EVs depending on their biogenesis, cell of origin, content and their surfaceome. This makes EVs secretion a complex spectrum of sub-populations, often classified according to their biogenesis as ectosomes, exosomes and apoptotic bodies.¹⁻⁴ Ectosomes, also known as microparticles, are generated by the outward budding of the plasma membrane, while exosomes are released by the exocytosis of multivesicular bodies filled with intraluminal vesicles.¹ Classically, exosomes are described as smaller (30–150 nm) than ectosomes (100–500 nm); however, this is a spectrum of secreted EVs that is cell-dependent.³ Currently, there is a growing interest in EVs research in neurodegenerative diseases due to their multiple roles in intercellular communications in both physiological and pathological contexts.⁵

Alzheimer's disease (AD), the most common neurodegenerative disease, has neuro-anatomical features which imply the presence of two hallmark lesions accompanied by an inflammatory reaction during pathology progression.^{6,7} These brain lesions are extracellular amyloid plaques formed by A β peptides and intraneuronal neurofibrillary tangles formed by aggregated tau protein. Neurofibrillary tangle spatial occurrence correlates with the cognitive decline observed in AD patients.^{8,9} A growing body of evidence explains the spreading of tau pathology by a prion-like propagation of pathological tau seeds from an affected cell to a healthy cell.^{10,11} This implicates transfer of tau seeds to the extracellular space before uptake by healthy cells can occur. Tau is a cytosolic translated protein and is also detected in the extracellular space such as the cerebrospinal fluid of healthy subjects and AD patients.^{12,13} These arguments suggest the existence of unconventional protein secretion (UPS) mechanisms of tau¹⁴ that can include HSPG mediated tau translocation, autophagy-mediated secretion or tau encapsulation into

extracellular vesicles.^{15–19} Recent evidences, including ours, indicate the presence of pathological tau seeds from brain-derived EVs (BD-EVs) of AD transgenic mouse models^{20–22} and AD brain patients.^{21,23} Interestingly, Ruan and collaborators demonstrated that tau within AD BD-EVs is even more seed competent than free form tau.²³

Our current interest is now to go further and to understand the involvement of EVs subtypes in the complex AD pathophysiology through their protein profiles and their contribution to tau propagation. At this day, more papers can be found on exosomes, which is partially due to a lack of robust EVs nomenclature and to the technical hurdles to distinguish large and small EVs based on their biogenesis.^{16,24} Consequently, a lack of comparative studies on EVs sub-populations stratified by their biogenesis exist. Technical challenges associated with segregating EVs into distinct categories of microvesicles and exosomes leads indeed to increased risk of cross-contamination. This directed our choice for a stratification based on size.^{3,4} Consequently, here, EVs are presently delineated into two main groups: enriched-small EVs (eSEVs $\leq$ 150 nm diameter) and enriched-large EVs (eLEVs $>$ 150 nm diameter). ESEVs and eLEVs from human brain-derived fluid (BDF) were separated by combining size-exclusion chromatography (SEC) and ultracentrifugation (UC). Here, pure and intact EVs are collected from frozen human brain samples after enzymatic dissociation. We aim to define the best enzyme for brain dissociation optimal for downstream proteomic analysis and functional studies. For this both papain (broad substrate specificity: L-Arginine and L-lysine)²⁵ and collagenase type 3 (specific substrate specificity: Triple helix structure of collagen)²⁶ were compared. Our results revealed an increased protein yield and preservation of transmembrane proteins for both EVs sub-populations by using the collagenase enzyme. Further, the proteomic signature of collagenase purified AD eLEVs and eSEVs have revealed a unique AD pathology signature where GWAS-associated proteins were also detected by proteomics. Interestingly, AD-derived eSEVs and eLEVs indicate unique and differentially expressed proteins, suggesting different implications of EVs sub-populations as mediators of dysregulated pathways of AD. This is illustrated here by the assessment of their tau seeding capacity, which demonstrates that AD-derived eSEVs, contrary to AD-derived eLEVs, exhibit an enhanced ability to promote tau spreading in the brain.

Results

Isolation of high quality small and large human BD-EVs

The high heterogeneity of EVs based on their biogenesis and the absence of biogenesis specific EVs biomarkers, prompt the development of a size-based segregation protocol for BD-EVs. This was achieved through a combination of SEC followed by UC, as illustrated in Figure 1a and 2a. This protocol was applied to prefrontal BDF of Alzheimer's disease (AD) patients and non-demented controls (demographic specifications presented in Table 1) and showed no significant loss in EVs quantity (Supplementary Fig. 1).

Two enzymatic dissociation protocols, namely papain and collagenase type 3, were compared to assess their impact on BD-EVs size, yield, purity, and integrity. Both enzymatic protocols successfully enriched the two size-based EVs populations: enriched-small EVs (eSEVs, 10–150 nm) and enriched-large EVs (eLEVs, >150 nm), which was confirmed by NTA (Figs. 1b, 2b). Papain-derived eSEVs and eLEVs contained 73% and 77% of vesicles in their respective size ranges (Fig. 1c), and collagenase-derived fractions contained 82% and 69% of respective size range enrichment (Fig. 2c). TEM confirmed intact EVs morphology with preservation of the EVs cup-shape (Figs. 1d, 2d).

The proteomic analyses validated by MISEV guidelines confirmed the presence of EVs-enriched proteins (categories 1 and 2) with minimal contaminants (category 3) (Figs. 1e, 2e; Supplementary Table 1).^{3,4} Gene ontology cellular component (GOCC) analysis indicated a highest enrichment in exosomes GOCC for both eSEVs and eLEVs (Figs. 1f, 2f). Unique and common protein profiles between eSEVs and eLEVs revealed a higher number of unique proteins in eLEVs compared to eSEVs for both papain (178 vs 30) and collagenase (457 vs 17). More than 50% of common proteins between eSEVs and eLEVs were found differentially expressed (Figs. 1g, 2g). The principal component analysis (PCA) confirmed distinct EVs sub-populations separation using our combinatorial SEC-UC protocol (Supplementary Fig. 2).

Comparison of the brain dissociation enzymes revealed that papain dissociation yielded a significantly higher number of EVs, yet collagenase-derived EVs showed superior downstream proteomic detection by mass spectrometry (Fig. 3a- b). A fold enrichment of the number of proteins detected within the MISEV guideline categories was done of collagenase EVs over papain EVs (Fig. 3c).⁴ This indicated an enrichment in collagenase eSEVs and eLEVs for EVs specific proteins (MISEV categories 1b and 2a) with low presence of contaminants (MISEV category 3). Next, a comparison of transmembrane (TM), luminal (non-TM) and total proteins unveiled more unique proteins in collagenase-derived EVs compared to papain-derived EVs (Fig. 3d- f). The collagenase-derived eSEVs and eLEVs demonstrated a 10.2- and 24.3-fold enrichment in TM proteins over papain-derived fractions, respectively (Fig. 3e). These TM proteins are crucial for better isolation and categorization of EVs sub-populations. Additionally, a higher abundance of non-TM proteins was also observed using collagenase enzyme (Fig. 3f).

Overall, the combined SEC-UC protocol effectively enriched size-specific EVs sub-populations for both papain and collagenase dissociation. However, collagenase dissociation demonstrated enhanced EVs purity (enrichment of EV-specific proteins) and integrity (higher TM and non-TM protein detection). Consequently, collagenase was selected as the optimal dissociation enzyme for investigating the role of BD-EV subpopulations in AD pathophysiology.

GWAS-associated proteins found inside eSEVs and eLEVs of AD patients reveal pathways dysregulated in AD patients

AD is a complex progressive disease overruled by sporadic cases compared to inherited cases. A growing field aims to define risk genes, which interlink AD patients compared to controls. This is done in Genome Wide Association Studies (GWAS) where peculiar single nucleotide polymorphisms (SNPs) are

compared between controls and AD patients.²⁷ These GWAS studies have already revealed numerous SNPs from genes with dysregulated pathways in AD. Our goal was to assess if proteins found in eSEVs and eLEVs of AD patients are coming from genes identified by GWAS. This was done by crossing our collagenase protein database with the GWAS genes list.²⁷ This analysis did show the presence of GWAS-associated proteins in both eSEVs and eLEVs from AD and controls (Fig. 4a). Some GWAS-associated proteins such as BIN1, EPDR1, FERMT2 and SNX1 were only detected in eLEVs. TSPAN14 and CTSH were found only within AD EVs (Fig. 4a, Supplementary Table 2). Interestingly, we showed a significant increase of LFQ values for the presence of clusterin (CLU) in AD eLEVs and eSEVs compared to controls (Fig. 4b). Western blots loaded with the same number of EVs (5×10^9 EVs) allowed to compare GWAS-associated proteins in eSEVs and eLEVs between AD and controls (Fig. 4e and Supplementary Fig. 3). An increased presence was clearly visualized for both the CLU precursor and cleaved protein in AD patients. Further, a strong tendency to increase of FERMT2 protein LFQ intensities in AD eLEVs compared to control eLEVs was observed (p-value 0.054) (Fig. 4c). The western blot confirmed the presence of FERMT2 in human eLEVs and eSEVs, but did not show a clear increase in AD eLEVs (Fig. 4e).

Taken together, our data unveils the presence of GWAS-associated proteins in eSEVs and eLEVs. As GWAS-associated proteins are implicated in numerous dysregulated pathways in AD, we seek to identify the biological pathways reflected within AD-derived EVs sub-populations.

Biological pathways profiling of eSEVs and eLEVs reveals a specific AD signature

We performed a gene ontology biological pathway (GOBP) analysis on our collagenase protein data. GOBP comparison between controls and AD patients demonstrated the presence of an AD signature in both AD-derived eSEVs and eLEVs (Supplementary Fig. 4). This showed that EVs content is clearly modified during the pathological course of AD. Knowing this, a proteomic comparison of AD eSEVs and AD eLEVs was done to assess whether both EVs sub-populations have specific protein profiles allowing to further precise their own role in AD pathophysiology (Fig. 5). We found 12 proteins unique to AD eSEVs (of which 1 TM proteins: AGRN) and 332 proteins (of which 49 TM proteins) unique to AD eLEVs (Fig. 5a). Within the 723 common proteins, 139 proteins were overexpressed in AD eSEVs and 336 proteins were overexpressed in AD eLEVs. The GOBP analysis indicate that unique AD eSEVs proteins are linked to the integrin signaling (Fig. 5b, supplementary table 3) and the AD eSEVs overexpressed proteins are more related to translation (Fig. 5d, supplementary table 4). On the other hand, unique AD eLEVs proteins are more connected with the respiratory electron transport (Fig. 5c, supplementary table 3), and their overexpressed proteins show an implication in the neuro-immune system (e.g. platelet activation, S1P pathways) (Fig. 5e, supplementary table 4).²⁸

We conclude that AD eSEVs and AD eLEVs have unique proteins and differentially expressed proteins involved in different biological pathways.

Assessment of tau seeding capacity of AD-derived EVs reveals a higher involvement of eSEVs in the seeding process

The distinct protein signatures found in AD eSEVs and AD eLEVs could indicate specialized functional roles. Therefore, we selected tau seeding as a readout of these specialized functions and compared the ability of AD eSEVs and AD eLEVs to induce tau nucleation *in vitro* and *in vivo*. First, AD eSEVs and AD eLEVs were lipofected onto the HEK-tau FRET biosensor cells (Fig. 6a). The flow cytometry analysis enabled quantification of the percentage of FRET positive cells after 72h incubation with the AD eSEVs or eLEVs. The results indicate a significantly higher *in vitro* seeding capacity of AD eSEVs compared to AD eLEVs (Fig. 6b). This HEK-tau FRET cellular model requires the use of lipofectamine for EVs internalization within the HEK biosensor cells. This circumvents EVs sub-population cellular internalization affinity. The EVs-mediated prion-like propagation hypothesis implicates both a successful cellular internalization of the EVs and a high seed-competent content. Hence, we used the THY-Tau30 transgenic mice model to assess the *in vivo* seeding capacity including both EVs cellular uptake affinity and seeding capacity.²⁹ For this, bilateral stereotaxic injections of 1.6×10^9 eSEVs or eLEVs from control or AD patients were done in the hippocampus of one-month old THY-Tau30 mice (Fig. 6c). At the time of injection, these mice have low endogenous tau pathology, allowing the evaluation of the ability of EVs to induce tau nucleation *in vivo*. The MC1 immunostaining was used to visualize misfolded tau lesions within neurons of the CA1 (Fig. 6d).³⁰ Blinded MC1+ cell quantification indicated that injection of CTRL eSEVs and eLEVs leads to a low level of basal MC1+ staining inherent to the THY-Tau30 model. Contrary, injection of AD-derived eSEVs shows a higher *in vivo* seeding capacity that is not observed for AD eLEVs (Fig. 6e).

Taken together, we demonstrated here that AD eSEVs are more prone to mediate tau nucleation in a prion-like manner.

Discussion

In this study, we explored the biological pathways and functional implications of AD EVs sub-populations to assess their role in AD pathophysiology. For this, we effectively stratified eSEVs and eLEVs from human BDF using a size-based separation protocol combining SEC and UC. The SEC efficiently removes soluble proteins of the interstitial fluid, including free tau and other pathological proteins, from EVs to enable specific study of BD-EVs in AD. The subsequent differential UC separated eLEVs and eSEVs based on sedimentation coefficient, which is size dependant.⁴

Here, collagenase type 3 was identified as the optimal dissociation enzyme for preserving EVs integrity and enriching EVs-specific proteins while minimizing multivesicular body contamination. Although papain has been widely used,^{31,32} recent findings, including ours and other retrospective research,³³ highlight superiority of collagenase to enhance EVs purity. We compared collagenase and papain dissociation on the same frozen brain samples to circumvent differences in sample origin, EVs isolation method or inter-laboratory bias. Both enzymes showed low cellular contamination based on the MISEV2023,⁴ with collagenase showing an enrichment in EVs associated proteins (TM and non-TM proteins) compared to papain. The well-defined substrate specificity of collagenase against collagen of

the extracellular matrix, may explain this protein enrichment and especially the preservation of TM proteins. These TM proteins, enriched with collagenase, are essential for EVs classification, immunoprecipitation and functional studies, particularly in the context of AD where EVs surfaceomes may influence cell vulnerability. We demonstrated that the dissociation enzyme significantly affects EVs quality and recommend collagenase type 3 for standardized characterization of human BDF EVs. This finding emphasizes the importance of harmonizing protocols to improve reproducibility across studies of BD-EVs.

Previous studies have been published which assessed protein profiles of whole BD-EVs population,^{34–38} while here we used this SEC and UC set-up after collagenase dissociation to investigate the EVs sub-population proteomic profiles. We started by assessing the presence of GWAS-associated proteins within brain eSEVs and eLEVs. Results show that CTSB is uniquely found within AD eLEVs and TSPAN14 only within AD EVs subtypes (Fig. 4a). Their relation with tau propagation remains to be elucidated along with the role of their transport within EVs sub-populations. Interestingly, the most known AD GWAS-associated protein, APOE was detected in all conditions and found with a tendency to significance to increase in AD eLEVs compared to control eLEVs ($p = 0.063$). Further, Clusterin (CLU/APOJ gene), an extracellular chaperone and also an AD risk gene²⁷ known to be increased in AD patient brains,^{39–41} was found significantly increased in both eLEVs and eSEVs of AD patients.

As GWAS-associated proteins are related to dysregulated pathways in AD, we seek to perform a proteomic profiling without prejudice of our well-characterized EVs sub-populations to reveal an AD eSEVs and eLEVs sub-population specific proteomic signature. AD eSEVs indicated biological pathways related to the integrin signaling synaptic pathway. The integrin signaling pathways comprises of numerous integrin mediated downstream molecular activations upon binding of a ligand which can be involved in brain immunity but also the focal adhesion (FA) complex located at the synapse.⁴² This FA complex is triggered by amyloid β and leads to multiple outcomes such as astrogliosis, microglia activation and increased tau phosphorylation in AD.⁴² AD eLEVs unveiled pathways related to the respiratory electron transport and to brain-immunity (platelet activation and S1P pathways). Both platelet activation and integrin signaling pathways are involved in the brain immunity. Therefore we defined the cellular origin of our EVs based on cell type enriched proteins of EVs derived from hiPSC.⁴³ We observed an increase in glial origin of both eSEVs and eLEVs in AD conditions (Supplementary Fig. 5) and a significant loss in neuronal eLEVs of AD (Supplementary Fig. 5a). This is in accordance with the advanced Braak stage VI of AD brain samples used in our study. In the future, it is also of interest to map brain area dependent EVs (sub-population) content as Huang and collaborators started for EVs of healthy individuals and to link this at different pathological stages of AD.³⁸

Biological pathway assessment indicated distinct pathways within the protein content of AD eSEVs and eLEVs. As this could reflect distinct roles of AD EV subtypes during AD pathophysiology, this triggered further investigation. As a readout, the seeding capacity of AD-derived eSEVs and eLEVs sub-populations was compared. Both *in vitro* and *in vivo* seeding capacity was higher for AD-derived eSEVs (Fig. 6). It is

important to mention that not only AD eSEVs are more seed competent, but also eSEVs are approximately 10 times more secreted than eLEVs in human BDF based on NTA quantification (Fig. 2b). Hence, injection of the same number of AD eSEVs and eLEVs *in vivo* revealed a higher seeding capacity of eSEVs, combined with their 10-fold higher presence in the BDF, suggests that among EVs, the ones playing a role in the propagation of tau pathology are the small ones. This makes AD eSEVs an interesting therapeutic target. In contrary, we speculate that AD eLEVs, that also contain tau seeds, could have implications in clearance of immunity triggering proteins or on the opposite stimulate brain-immunity. Clearance can include proteins directly linked to immunity such as the platelet activation proteins found enriched in AD eLEVs (Fig. 5) or indirect proteins such as FERMT2, a GWAS protein and mediator of the focal adhesion complex⁴⁴ known to stimulate astrogliosis and activation of microglia. AD eLEVs showed enriched proteins related to the immune system (Supplementary Fig. 5e), which could increase their internalization by glial cells and enhance clearance. In contrary, the presence of immune related proteins and platelet activation proteins in eLEVs could trigger the brain-immunity in a way that eLEVs could function as a chemokine. Overall, the findings on the tau propagation highlight distinct roles for AD eSEVs and eLEVs.

EVs-mediated seeding can be influenced by numerous factors. The first one, are the forms of tau present within each EVs sub-population. In AD eSEVs and eLEVs, we detected both 1N3R and 1N4R tau isoforms significantly enriched compared to controls (Supplementary Fig. 6). In the future, it is of interest to map the tau proteoforms found within AD EVs sub-population using tau targeted proteomics. A second element affecting EVs-mediated tau propagation may be their trans-membrane proteins, which can affect different stages such as EVs docking, EVs internalisation, intracellular fate or cell type affinity. We could hypothesize that the agrin (AGRN), the TM protein only detected in eSEVs, could explain the higher seeding capacity of AD eSEVs because (1) AGRN is a syndecan TM protein of which research demonstrated the implication of syndecans to stimulate EVs internalisation^{45,46} and (2) syndecans are known to be related to AD pathophysiology.^{47–50} Lastly, co-factors interfering with tau can be transported together and shield within EVs. This could explain the higher seeding capacity of EVs than free form secreted tau.²³ Based on previous research, co-factors could include RNA,⁵¹ lipids like cholesterol⁵² or proteins such as CLU³⁹ promoting tau seeding. Further studies on the relationship between proteomic signature and differential roles of EVs subtypes will allow to fully comprehend their AD pathophysiological implication.

Overall, our results show that collagenase-derived brain EVs sub-populations from AD patients, separated based on size, indicate specific protein profiles. Importantly, we found that AD eSEVs have a higher tau seeding capacity, highlighting their significant contribution to tau propagation and providing new insights into different EVs sub-population roles in Alzheimer's disease.

Material and methods

Human samples

Non-demented human control (control) and AD prefrontal, Brodmann Area 8/9 (BA8/9), fresh-frozen brain extracts were obtained from the Lille Neurobank (fulfilling French legal requirements concerning biological resources and declared to the competent authority under the number DC2008-642) with donor consent, data protection and Ethics Committee approval. Samples were managed by the CRB/CIC1403 Biobank, BB-0033-00030. The demographic data is listed in Table 1.

Our main goal is to investigate the place of EVs subtypes in AD pathophysiology. Therefore, the first part of the results (Figs. 1-3) is dedicated to the validation of the eSEVs and eLEVs separation protocol and the selection of the most suited brain dissociation enzyme. We aim to define an experimental procedure to recover well-preserved EVs applicable to both control- and AD-BDF. Therefore, AD and control samples have been isolated and analyzed separately. For ethical reason and because human samples are extremely valuable, we generated a post-analytical dataset by pooling the separate data from AD and controls. It should be noted that for transmission electronic microscopy (TEM) images, this post-analytical procedure was not possible and hence EVs from a BDF pool of AD and controls were analyzed directly by TEM. The second part of results (Figs. 4-5) is obtained from the separate proteomic analysis of eSEVs and eLEVs of AD and eSEVs and eLEVs of controls from collagenase-derived BDF. This allows us to compare AD EVs sub-populations to highlight differences that might participate in tau pathology progression. The functional study on the HEK-tau biosensor cell model of AD EVs subtypes was done for eSEVs and eLEVs of four individual AD patients (Fig 6b), while for the *in vivo* experiment eSEVs or eLEVs of a pool of four control or four AD patients was injected to avoid both animal and patient related variabilities (Fig. 6d-e). A recapitulative overview of the used human samples in the different experiments is provided below in Table 2.

Brain-derived fluid isolation

Purification of BD-EVs is a challenge since the preparation of BDF is done from frozen human prefrontal brain extracts. To isolate the BDF from this solid tissue, enzymatic dissociation is used. Here, two different protocols were applied. The first protocol uses papain for enzymatic digestion of the brain tissue as previously described²⁰ and adapted in our previous study.²¹ Briefly, brain tissue (80 mg for TEM; 200 mg for Nanoparticle Tracking Analysis (NTA) and proteomic analysis) was incubated on ice in Hibernate-A and then gently homogenized in a Potter before adding 2 mL of 20 units/mL papain (LS003119, Worthington). After a 20 min incubation at 37 °C with agitation, 15 mL of cold Hibernate-A (50 mM NaF, 200 nM Na₃VO₄, 10 nM protease inhibitor (E64 from Sigma) and protease inhibitor cocktail (Roche)) were added and mixed by pipetting to stop the enzymatic activity while on ice.

The second protocol used collagenase type 3 (LS0004182, Pan Biotech) for enzymatic tissue dissociation as previously described by Vella and collaborators.³⁴ Briefly, brain tissue (80 mg for TEM; 200 mg for NTA and proteomic analysis) was sliced on ice to generate smaller sections (~2 mm) before adding 75 units/mL of collagenase type 3 in Hibernate-E (800 µL per 100 mg of tissue, 10315538, Gibco). After 20 min incubation at 37 °C with agitation, PhosSTOP (4906837001, Roche) and Complete Protease Inhibitor including EDTA (4693124001, Roche) were added to a final concentration 1X on ice.

For both protocols, successive centrifugations of 300 x g, 2,000 x g and 10,000 x g were applied at 4°C to remove cells, membranes and debris, respectively. The final supernatant is entitled BDF and was consistently prepared freshly prior to each EVs isolation.

Brain-derived EVs (BD-EVs) isolation

First, size-exclusion chromatography (SEC) is used to isolate EVs from the BDF and to separate EVs from proteins contaminants.^{53,54} SEC allows quick isolation with little non-vesicular contaminants. Commercial SEC columns (IC0-70, IZON) packed with Sepharose resin CL-2B (CL2B300, Sigma-Aldrich) were used. After column equilibration with degassed phosphate-buffered saline (PBS, 12559069, Gibco), 500 µL of BDF were applied on the SEC column followed by elution in degassed PBS. After the void volume (3 mL), the first 2 mL (F1-4) were recovered as EVs fraction in protein low binding tubes (0030108132, Eppendorf protein LoBind). We previously characterized this fraction enriched in BD-EVs²¹ in accordance to MISEV 2018 guidelines.³

Separation of large from small BD-EVs

SEC is not able to fully separate EVs subtypes depending on their size. This results in a mix of both large and small EVs subtypes in the SEC-derived EVs fraction (2 mL). Hence, a centrifugation step at 10,000 x g for 30 min at 4°C was added to pellet eLEVs (Centrifuge 5424-R, 2519550, Eppendorf). The supernatant was transferred to an ultracentrifuge tube (344062, Ultra-Clear) and the pellet was suspended in 500 µL of ice-cold PBS. This 10,000 x g centrifugation step was repeated two more times to reduce contamination of the eLEVs pellet by eSEVs from the supernatant. The final pellet representing the eLEVs fraction was suspended in 50 µL PBS. At the end, the supernatant (3mL) from all three centrifugations was ultracentrifuged at 20,000 x g during 2 h at 4°C (Optima XE-90, rotor SW60Ti, Ultra-Clear) to pellet residual eLEVs and recovered only eSEVs in the final supernatant. This supernatant enriched in eSEVs was concentrated using ultrafiltration device 3 kDa Amicon (Amicon® Ultra-2 3 kDa, Millipore) at 4,000 x g (Multifuge X3R, Thermo Scientific) to a final volume of 100 µL.

Nanoparticle tracking analysis (NTA)

The concentration and size distribution of particles were measured by NTA (NanoSight NS300, Malvern Panalytical) immediately after isolation. eSEVs and eLEVs from two controls and one AD patient were passed separately on the NTA and were pooled post-analysis (Table 2). Samples were diluted in PBS and continuously infused into the NTA device by an automatic syringe pump at a flow rate of 20 µL/min. The focus was adjusted and the temperature was set to 25 °C. Three videos of 60 seconds were acquired at camera level 15 and processed at detection level 4 using the NTA software [v 3.2.16]. Samples were freshly used for TEM or stored at -20°C until proteomic analysis.

Transmission electron microscopy

TEM morphological visualization of eLEVs and eSEVs fractions were obtained from the BDF of a pool of two controls and one AD patient (Table 2). For this pool, 80 mg brain tissue resulted in approximately 600 μ L BDF of which 500 μ L were used for one SEC and downstream eSEVs and eLEVs separation. 5 μ L of eLEVs or 5 μ L eSEVs sample were deposited on a carbon grid (400 mesh) and incubated for 20 min at RT. Grids were rinsed twice in PBS and were fixed in PBS-glutaraldehyde (1%) for 5 min at RT and then rinsed 7 times in distilled water. The light-sensitive grids were incubated for 5 min in 1% uranyl acetate and for 10 min on ice in a mixture containing 4% uranyl acetate/2% methylcellulose (25 cP, 9004-67-5, Sigma) in the dark. Dry grids were observed under a transmission electron microscope (Zeiss EM900) with the 20,000 x objective.

Proteomic sample preparation

eLEVs and eSEVs were isolated from either a pool of four controls or a pool of four AD patients (Table 2). These were prepared with both brain-dissociation protocols, namely papain or collagenase dissociation and were further analyzed by label free quantification mass spectrometry at the *OrganOmics platform of PRISM Inserm U1192* (Lille, France). Using both dissociation enzymes, around 400 mg of brain tissue were used, resulting in 3 mL of BDF, which was suited for six SEC and allowed isolation of 5×10^{10} eSEVs or eLEVs for proteomic analysis. More precisely, the same number of eLEVs and eSEVs (5×10^{10}) was lysed in RIPA buffer for 15 min at 95°C and subsequently centrifuged at 16,000 x g for 10 min. For each sample, the collected supernatant was reduced using reduction buffer (Dithiothreitol, DTT 0.1M) for 40 min at 56°C, and diluted in the denaturing buffer (0.1M Tris/HCl, 8M urea, pH 8.5). The preparation of the samples by Filter Aided Sample Preparation (FASP)^{55,56} was carried out using 30 kDa Amicon® device (Millipore) to eliminate the denaturant buffer by centrifugation at 14,000 x g for 15 min. This rinsing step was repeated a second time. Next, the alkylation of the proteins was done by addition of IAA buffer (Iodoacetamide 0.05M) in the Amicon® and set for 20 min in the dark. This was followed by a centrifugation at 14,000 x g for 10 min. Samples were washed three times by addition of denaturing buffer and followed three times by addition of AB buffer (Ammonium bicarbonate 0.05M). At each washing step, centrifugation was carried out at 14,000 x g for 10 min. Samples were then incubated by adding 40 μ L of trypsin buffer (40 μ g/ μ L, in AB buffer) at 37°C overnight. The digested proteins were collected by centrifugation at 14,000 x g for 10 min and rinsed on Amicon® device with 0.5M NaCl. The digestion was stopped using 5% trifluoroacetic acid (TFA). The samples were desalted using Evotips-C18 (Evosep, Denmark) in accordance with the manufacturer's instructions provided by Evosep, immediately before data acquisition via mass spectrometry (MS).

LC-MS/MS analysis

The Evotips, housing the peptides, were introduced into the Evosep-One⁵⁷ liquid chromatography system (Evosep, Denmark). The system automatically engaged the tip and conducted elution directly within the liquid chromatography setup. Peptide separation occurred utilizing the C18 endurance column (15 cm x 150 μ m ID, 1.9 μ m) employing the extended method 15 SPD (Sample Per Day). Mobile phase A consisted of 0.1% formic acid (FA) in water, while phase B comprised 0.1% FA in acetonitrile. The chromatographic

system was linked to a Q-Exactive Orbitrap mass spectrometer (Thermo Scientific) through a nanospray source. The Q-Exactive operated in a data-dependent mode, targeting the top 10 most intense ions for MS analysis. MS analysis spanned a mass to range (m/z) of 300 to 1600, with a resolution of 70,000 full width at half maximum (FWHM), an automated gate control (AGC) of 3×10^6 ions and a maximum injection time of 120 milliseconds (ms). For MS/MS analysis, the m/z mass range extended from 200 to 2,000, with an AGC of 5×10^4 ions, a maximum injection time of 60 ms, and a resolution set at 17,500 FWHM. Higher Energy Collision Dissociation (HCD) was set to 30%, with precursor ions bearing charge states $> +1$ and $< +8$ selected for fragmentation, and a dynamic exclusion time of 20 s.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE ⁵⁸ partner repository.

Proteomic data analysis

Proteins were identified through comparison of all MS/MS data with the Homo sapiens proteome database (Uniprot, release August 2022, 75004 entries) using MaxQuant software version 1.6.0.5.^{59,60} An initial mass tolerance of 6 ppm was applied for MS mode, while a tolerance of 20 ppm was set for fragmentation data in MS/MS mode. Digestion parameters utilized trypsin with up to 2 allowed missed cleavages. Variable modifications included oxidation of methionine and N-terminal protein acetylation, while carbamidomethylation of cysteine was selected as a fixed modification. Label-free quantification (LFQ) was conducted with default parameters of the MaxLFQ algorithm.⁶¹ Protein and peptide identification adhered to a false discovery rate (FDR) of 1%, with a requirement of at least 2 peptides per protein, including 1 unique peptide. Statistical analysis was performed using Perseus software (version 1.6.10.43). Briefly, LFQ intensities for each sample were imported into Perseus after which the data matrix underwent filtering to remove potential contaminants, reverse entries and those identified by site only. Data transformation involved $\log_2(x)$ conversion. Prior to statistical analysis, groups were defined with 3 replicates per group. Two-sample tests using Student's T-test with a significance threshold of $p = 0.01$ were conducted for comparisons between 2 groups. Results were normalized by Z-score and only statistically significant proteins were subjected to hierarchical clustering.

The EVs quality was assessed using our proteomic data. For this our data was crossed with the 5 categories of the Minimal Information for Study of Extracellular Vesicles guidelines 2023 (MISEV2023)⁴ which were complemented with the protein lists of the MISEV guidelines 2018 (MISEV2018).³ Categories 1 and 2 are defined as EVs enriched proteins while category 3 was defined as EVs contaminants. The complete list of detected and non-detected proteins for each category is shown in Supplementary Table 1. To analyze the benefit of collagenase brain dissociation, a fold enrichment was calculated. For this, the number of present proteins in the collagenase data (eSEVs and eLEVs) over the number of proteins present in the papain database (eSEVs and eLEVs) was done for MISEV2023 categories 1 to 3.

The assessment of transmembrane proteins was done by crossing our identified proteins (Uniprot entry) with the UniProtKB reviewed (Swiss-Prot) human transmembrane protein database (5,232 proteins, KW-

0812). The enrichment analysis for the gene ontology “biological processes” (GOBP) and “cellular components” (GOCC) categories were obtained with the Funrich software (version 3.1.4). For GOCC the detected exosomes and lysosomes categories had around 64% of protein overlap due to common pathways between intraluminal vesicles and secretory vesicles. Further, only 21% were lysosome-specific proteins.

Western Blot

For western blotting, eSEVs and eLEVs were obtained from a pool of four controls or a pool of four AD patients (Table 2) following the protocol as described above. For western blot 125 mg brain tissue, resulting in 1 mL BDF allowed two SEC and further size separation. The eSEVs were additionally concentrated by an ultracentrifuge step at 100,000 x g for 2 hours at 4°C. The pellet of eSEVs was suspended in PBS (25 µL PBS for each SEC). Both concentrations of eSEVs and eLEVs were quantified by NTA. 5×10^9 EVs were diluted in RIPA 1X (150 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1% NP-40, EDTA free protease inhibitor, 50 mM Tris base at pH 8) and sonicated in a water bath (Bioruptor sonication system, Diagenode) for 5 min on high Intensity setting. The EVs were then diluted in lithium dodecyl sulphate (LDS 2X: NuPage LDS sample buffer 2X, NuPage reducing agent 2X) and heated for 10 min at 100°C. After this, eSEVs and eLEVs were loaded on 4–12% Bis-Tris NuPAGE Novex gels (Invitrogen) and transferred to a nitrocellulose membrane of 0.45 µm employing the Novex system from Life Technologies (XCell II blot module). Membranes were blocked in Tris-buffered saline with 5% skim milk for 1 h, at RT and incubated with the appropriate primary antibody overnight at 4°C in TNT 5% milk. Antibodies are listed in Table 3. Membranes were then rinsed and further incubated with horseradish peroxidase-labelled secondary antibodies and bands were visualized by chemiluminescence (ECL, Amersham Biosciences).

Cell culture

The stable Tau RD-P301S FRET Biosensor cells (ATCC CRL-3275) and HEK 293T cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, 13345364) with pyruvate and without HEPES complemented with 10% fetal bovine serum (FBS, A5256701, Gibco), glutaMax 1X (35050061, Gibco) and 1% penicillin-streptomycin. Cells were maintained in a humidified incubator with 5% CO₂. Twice a week the cells were split.

FRET biosensor cell assay

Upon addition of a tau seed, the tau-RD with a P301S pro-aggregative mutation coupled with a Yellow-fluorescent protein (YFP) or Cyan-fluorescent protein (CFP) come in close proximity and allow a Fluorescence Resonance Energy Transfer (FRET) measurement of these HEK-tau FRET cells. To assess AD eSEVs and eLEVs *in vitro* seeding capacity, the FRET biosensor cell assay was performed as previously described in Leroux and collaborators.²¹ Briefly, HEK-tau FRET and HEK 293T cells were plated (150,000 cells per well) 24 h before lipofection of 50 µL of eLEVs or eSEVs of individual AD patients (Table 2). 72 h after lipofection, cells were analysed on the Aria SORP (BD Biosciences;

acquisition software FACSDiva v7.0, BD Biosciences) flow cytometer. The FRET data were quantified using the Kaluza analysis software v2 and results were expressed as the percentage of FRET-positive cells. Three independent experiments were done in duplicate of eSEVs and eLEVs of four AD patients. At least 10,000 cells per replicate were analyzed.

Animals

The study was performed in accordance with French and European Community rules. The experimental research was performed with the approval of an ethics committee (agreement APAFIS #43474-2023050714441306 v6) and follows European guidelines for the use of animals. The animals (males and females) were housed in a temperature-controlled room (20°C–22°C) and maintained on a 12-h day/12-h night cycle with food and water provided ad libitum in a specific, pathogen-free animal facility (with five mice per cage). Animals were randomly allocated to the different experimental groups. The tau transgenic mice line THY-Tau30 expressing human 1N4R tau protein with two pathogenic mutations (P301S and G272V) under the control of the neuron-specific Thy-1.2 promoter was used.⁶²

Stereotaxic injections

To assess the *in vivo* seeding capacity of eSEVs and eLEVs, stereotaxic injections were performed in THY-Tau30 mice as described in Leroux and collaborators.²¹ Briefly, around 350 mg brain tissue from a pool of four control or four AD patients (Table 2) were dissociated using collagenase enzyme. The obtained 2.5 mL BDF allowed 5 SEC and downstream eSEVs and eLEVs separation through differential centrifugations. The eSEVs and eLEVs were maximally concentrated using 3K amicon. For each condition, 2.5 μ L (1.6×10^9 vesicles) were bilaterally injected into the hippocampi of 1-month-old anesthetized THY-Tau30 mice (n=4 for control eSEVs and n=5 mice for control eLEVs, AD eSEVs and AD eLEVs) at a flow rate of 0.25 mL/min followed by a 5 min syringe hold. Injections coordinates were anterior-posterior, - 2.5 mm; mediolateral, - 1 mm; dorsal-ventral, - 1.8 mm to bregma.²⁹ In contrary to the FRET assay, no lipofectamine was used for the stereotactic injected material.

Tissue processing and immunohistochemistry

Brain tissue processing and immunohistochemistry (IHC) of stereotaxic injected THY-Tau30 mice was done as explained in Leroux and collaborators.²¹ To resume, four weeks post-injection a trans-cardiac perfusion was done with 0.9% saline solution followed by 4% PFA perfusion. Extracted brains were post-fixed before isopentane freezing. Using the cryostat microtome, free-floating coronal sections (40- μ m thickness) were obtained. For IHC, the brain sections were washed, treated with 0.3% H₂O₂, rinsed and Mouse on Mouse blocking reagent was added. After three rinses, overnight incubation with the primary antibody MC1-biotin (recognising the pathological tau conformation³⁰) was done, followed by rinses and amplification using anti-mouse biotinylated IgG and application of the avidin-biotin-HRP complex. Visualisation of tau lesions was done using diaminobenzidine tetrahydrochloride (DAB). Next, brain sections were mounted, air-dried and dehydrated by passage through a graded series of alcohol and

toluene baths. Lastly, cover slips were mounted with VectaMount and images were acquired using a Zeiss Axio Scan.Z1 and scale bar were added using the ZEN Blue software (version ZEN 2.3 lite).

Tau lesion quantification

Mounted brain sections were visualized on the Mercator Leica DM5500 where a threshold of MC1-positive lesions was established manually to present a minimum background and remained constant throughout the analysis. For blinded quantification of MC1 immunoreactivity, the CA1 region of the hippocampus from bregma -1.06 to bregma -3.52 (based on the Mouse Atlas, George Paxinos and Keith B.J. Franklin, Second Edition, Academic Press) was chosen as the quantification zone. The number of MC1-positive (MC1+) somas were manually counted per brain section by two independent individuals. Results are presented as the mean number of neurofibrillary tangles per brain section where the left and right hemispheres of the mice were counted separately.

Statistical analyzes

Statistics and graphs were generated using GraphPad Prism 9 software (version 9.1.0). Data were represented as mean $\pm$ standard error of mean (SEM). Shapiro-Wilk normality test was used to assess normality for each group. For comparison of two independent groups with a normal distribution a t-test was done and for groups with non-parametric distributions, a Mann-Whitney U test was used. Comparisons of three or more independent groups, with a normal distribution were done using ordinary one-way Analysis Of Variance (ANOVA), while Kruskal-Wallis test was used for non-parametric samples. Statistical testing was done at the two-tailed p-value of 0.05.

Declarations

Availability of data and materials- The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE ⁵⁸ partner repository.

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Author contribution- E.L., M.O. and R.P. carried out the experiments. M.O., T.B and B.N. carried out the proteomic analyses. R.C. performed the stereotaxic injections. M.O. wrote the manuscript with support from M.C. and L.B. S.A. and C.L. performed the nano-HPLC MS/MS runs at the OrganOmics platform. C.A.M. and B.A. helped in the selection of the AD and CTRL cases and carried out brain sampling (Lille NeuroBank). EM was performed by A.L., D.M., M.O. and E.L. M.C. and L.B. conceptualized the study. All authors discussed the results and reflected on the manuscript.

Competing interests- The authors declare that they have no competing interests

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Tables

Table 1. Demographic, biological and clinical characteristics of the human brain sample donors. List of the brain samples used for BDF isolation of AD patients (AD, n=4) and non-demented controls (CTRL, n=5). PMD post-mortem-delay, NFT neurofibrillary tangles. N/A not available.

Patient ID	Sex	Age of death (y)	PMD (h)	Diagnosis	Tau lesions	Braak stage	Thal stage	Cause of death	ApoE status
A	F	66	16.5	AD	NFT	VI	4		E3/E3
B	M	78	10	AD	NFT	VI	4		N/A
C	F	60	24	AD	NFT	VI	5		E4/E4
D	M	64	20	AD	NFT	VI	4		E3/E3
E	F	81	N/A	CTRL	none	I	0	Pericarditis	
F	M	22	24	CTRL	none	0	0	Myocarditis	
G	M	59	13	CTRL	none	0	0	Septic shock	
H	M	41	11	CTRL	none	0	0	Suffocation	
I	M	78	19	CTRL	none	0	0	Invasive aspergillosis	

Table 2: Overview table of the used human samples for each experiment. For figures 1-3, non-demented controls (CTRL) and AD patients BD-EVs were analyzed separately and pooled post-analysis to define the best brain dissociation and separation protocol applicable on both AD and CTRL BD-EVs. An exception was made for TEM where AD and CTRL eLEVs or eSEVs were pooled directly before TEM. Analysis done in figures 4-5 were applied on the separate data of CTRL and AD eLEVs and eSEVs. In vitro assessment of seeding capacity was done on separate AD patients, while stereotaxic injections were done using a pool of four patients. The number of patients used for the pool are indicated between brackets.

Experiments	Sample	Patients
Fig. 1: eSEVs and eLEVs comparative proteomics- Papain procedure:		
NTA	CTRL (2) and AD (1) pooled post-NTA for analysis	A, E, F
TEM	One pool of CTRL (2) and AD (1)	A, E, F
Comparative proteomic	CTRL (4) and AD (4) pooled post-proteomics for analysis	F, G, H, I / A, B, C, D
Fig. 2: eSEVs and eLEVs comparative proteomics- collagenase procedure:		
NTA	CTRL (2) and AD (1) pooled post-NTA for analysis	A, F, G
TEM	One pool of CTRL (2) and AD (1)	A, F, G
Comparative proteomic	CTRL (4) and AD (4) pooled post proteomics for analysis	F, G, H, I / A, B, C, D
Fig. 3: Effect of dissociation enzyme on EVs yield, purity and integrity- Papain or collagenase procedure:		
NTA	CTRL (4) and AD (3) pooled post-NTA for analysis	F, G, H, I /A,C,D
Detected proteins	CTRL (4) and AD (4) pooled post-proteomics for analysis	F, G, H, I / A, B, C, D
Comparative proteomic	CTRL (4) and AD (4) pooled post-proteomics for analysis	F, G, H, I / A, B, C, D
Fig. 4: WB of GWAS proteins in eSEVs and eLEVs- collagenase procedure		
Comparative proteomic	One pool of CTRL (4) and one pool of AD (4)	F, G, H, I / A, B, C, D
Western blot	One pool of CTRL (4) and one pool of AD (4)	F, G, H, I / A, B, C, D
Fig. 5: AD eSEVs and eLEVs comparative proteomics- collagenase procedure:		
	One pool of CTRL (4) and one pool of AD (4)	F, G, H, I / A, B, C, D

Fig. 6: Tau seeding capacity of eSEVs and eLEVs-collagenase procedure

HEK-tau biosensor cells	Individual AD (4)	A, B, C, D
Stereotaxic injection THY-Tau30 mice	One pool of CTRL (4) and one pool of AD (4)	F, G, H, I / A, B, C, D

Table 3: List of primary and secondary antibodies used for western blotting of eLEVs and eSEVs.

Antigen 1° antibody	Manufacturer, reference	Dilution	2° antibody	Supplier	Dilution
FERMT2	GeneTex, GTX84507	1/1,000	Horse anti-mouse IgG (H+L) peroxidase	Vector Laboratories, PI-2000	1/50,000
Clusterin (CLU)	Abcam, ab92548	1/1,000	Goat anti-rabbit IgG (H+L) peroxidase	Vector Laboratories, PI-1000	1/5,000

Figures

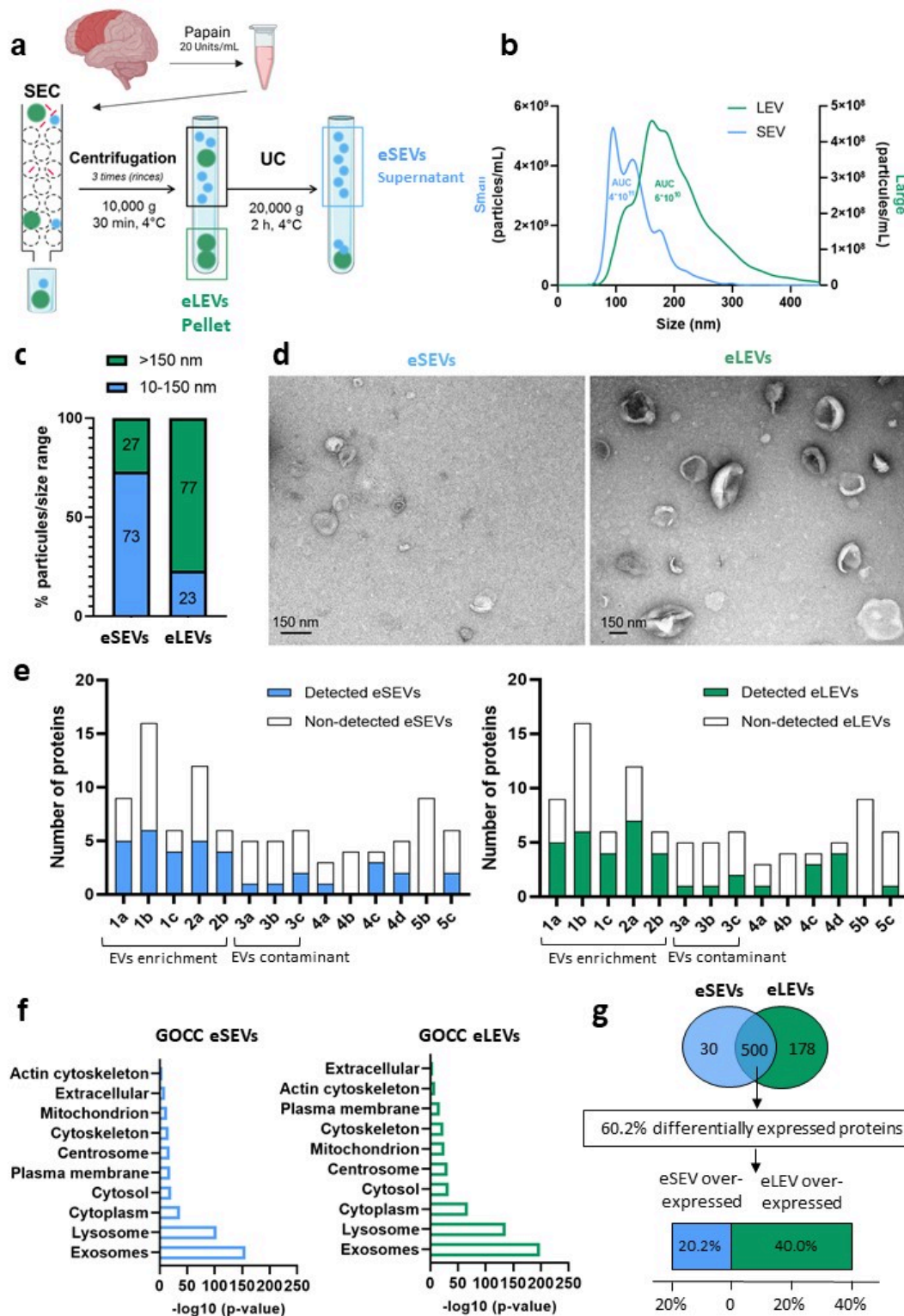


Figure 1

New size-based EVs separation protocol of BDF using papain dissociation. (a) The brain-derived fluid (BDF) is obtained by papain enzymatic dissociation as previously done for AD and CTRL brain extracts.²¹ 500 μ L of BDF were loaded on top of a sepharose SEC column and EVs were separated in PBS (500 μ L per fraction). Three consecutive 10,000 x g centrifugation steps enable separation of enriched large EVs (eLEVs: >150 nm), an additional centrifugation at 20,000 x g enables enriched small EVs (eSEVs: 10-150

nm) isolation. **(b)** Size distribution of eSEVs and eLEVs was determined by NTA analysis. **(c)** eSEVs and eLEVs separation enrichment was calculated from NTA and indicate a 73% and 77% enrichment in eSEVs and eLEVs fraction, respectively. **(d)** eSEVs and eLEVs morphology was visualized by electron microscopy. Scale bar = 150 μ m. **(e)** Vertical bar graph corresponding to the number of proteins detected in eSEVs and eLEVs as recommended by the MISEV2023 guidelines after mass spectrometry-based proteomic analysis.³ MISEV2023 categories; 1a: Multi-pass transmembrane proteins associated with plasma membrane and/or endosomes; 1b: Single-pass transmembrane proteins associated with plasma membrane and/or endosomes; 1c: GPI-or lipid-anchored proteins associated with plasma membrane and/or endosomes; 2a: Cytosolic proteins with lipid or membrane protein-binding ability; 2b: Cytosolic proteins with promiscuous incorporation into EVs; 3a: Lipoproteins; 3b: Protein and protein/nucleic acid aggregates; 3c: Exomere or supermere-enriched components; 4a: Nucleus; 4b: Mitochondria; 4c: Secretory pathway: Endoplasmic reticulum, Golgi apparatus; 4d: Autophagosomes, cytoskeleton; 5b: Cytokines and growth factors; 5c: Adhesion and extracellular matrix proteins. **(f)** Horizontal barplot of $-\log(p\text{-value})$ from LFQ intensities obtained for 10 selected gene ontology cellular component (GOCC) terms after quantitative proteomic analysis of eSEVs (left) and eLEVs (right). **(g)** Venn diagram (top) indicating unique proteins for both eSEVs and eLEVs as well as 500 common proteins of which 60.2% were found differentially expressed. A boxplot represents the repartition of differentially expressed proteins between eLEVs and eSEVs (bottom). For a to g, eSEVs are represented in blue and eLEVs in green.

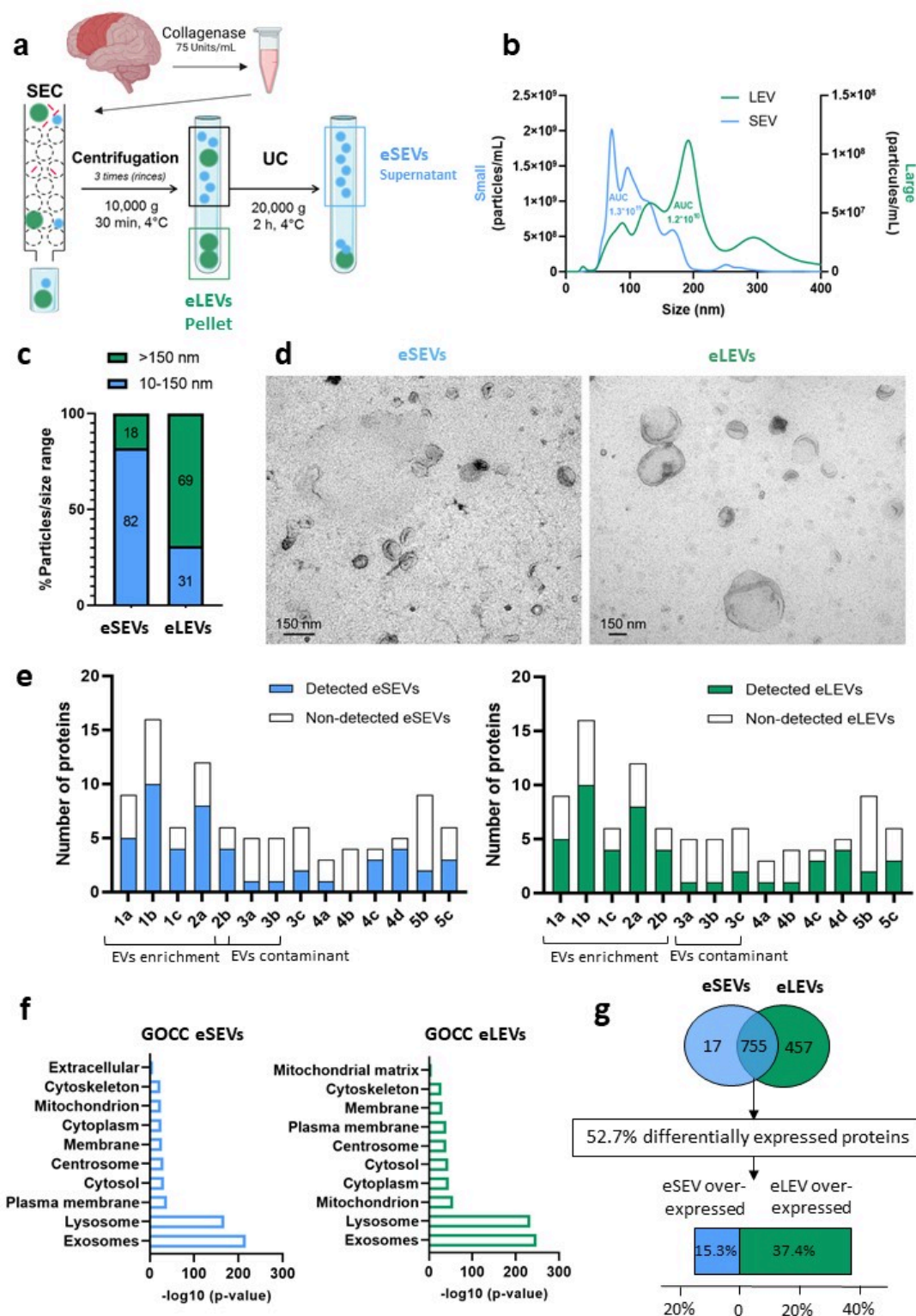


Figure 2

New size-based EVs separation protocol of BDF using collagenase type 3 dissociation. **(a)** The BDF was obtained by collagenase type 3 enzymatic dissociation following the protocol as described by Vella and collaborators for AD and CTRL brain extracts.³⁴ 500 μ L of BDF were loaded on top of a sepharose SEC column. Three consecutive 10,000 x g centrifugation steps enable separation eLEVs, an additional centrifugation at 20,000 x g enabled eSEVs isolation. **(b)** Size distribution of eSEVs and eLEVs was

determined by NTA. **(c)** eSEVs and eLEVs separation enrichment was calculated from NTA and indicated an 82% size enrichment for eSEVs and 69% for eLEVs. **(d)** eSEVs and eLEVs morphology was visualized by electron microscopy. Scale bar = 150 μ m. **(e)** Vertical bar graph corresponding to the number of proteins detected in eSEVs and eLEVs as recommended by the MISEV2023 guidelines after mass spectrometry-based proteomic analysis.³ MISEV2023 categories; 1a: Multi-pass transmembrane proteins associated with plasma membrane and/or endosomes; 1b: Single-pass transmembrane proteins associated with plasma membrane and/or endosomes; 1c: GPI-or lipid-anchored proteins associated with plasma membrane and/or endosomes; 2a: Cytosolic proteins with lipid or membrane protein-binding ability; 2b: Cytosolic proteins with promiscuous incorporation into EVs; 3a: Lipoproteins; 3b: Protein and protein/nucleic acid aggregates; 3c: Exomere or supermere-enriched components; 4a: Nucleus; 4b: Mitochondria; 4c: Secretory pathway: Endoplasmic reticulum, Golgi apparatus; 4d: Autophagosomes, cytoskeleton; 5b: Cytokines and growth factors; 5c: Adhesion and extracellular matrix proteins. **(f)** Horizontal barplot of $-\log(p\text{-value})$ from LFQ intensities obtained for 10 selected GOCC terms after quantitative proteomic analysis of eSEVs (left) and eLEVs (right). **(g)** Venn diagram (top) indicating unique proteins for both eSEVs and eLEVs and 755 common proteins of which 52.7% were found differentially expressed. A boxplot represents the repartition of differentially expressed proteins between eSEVs and eLEVs (bottom). For a to g, eSEVs are represented in blue and eLEVs in green.

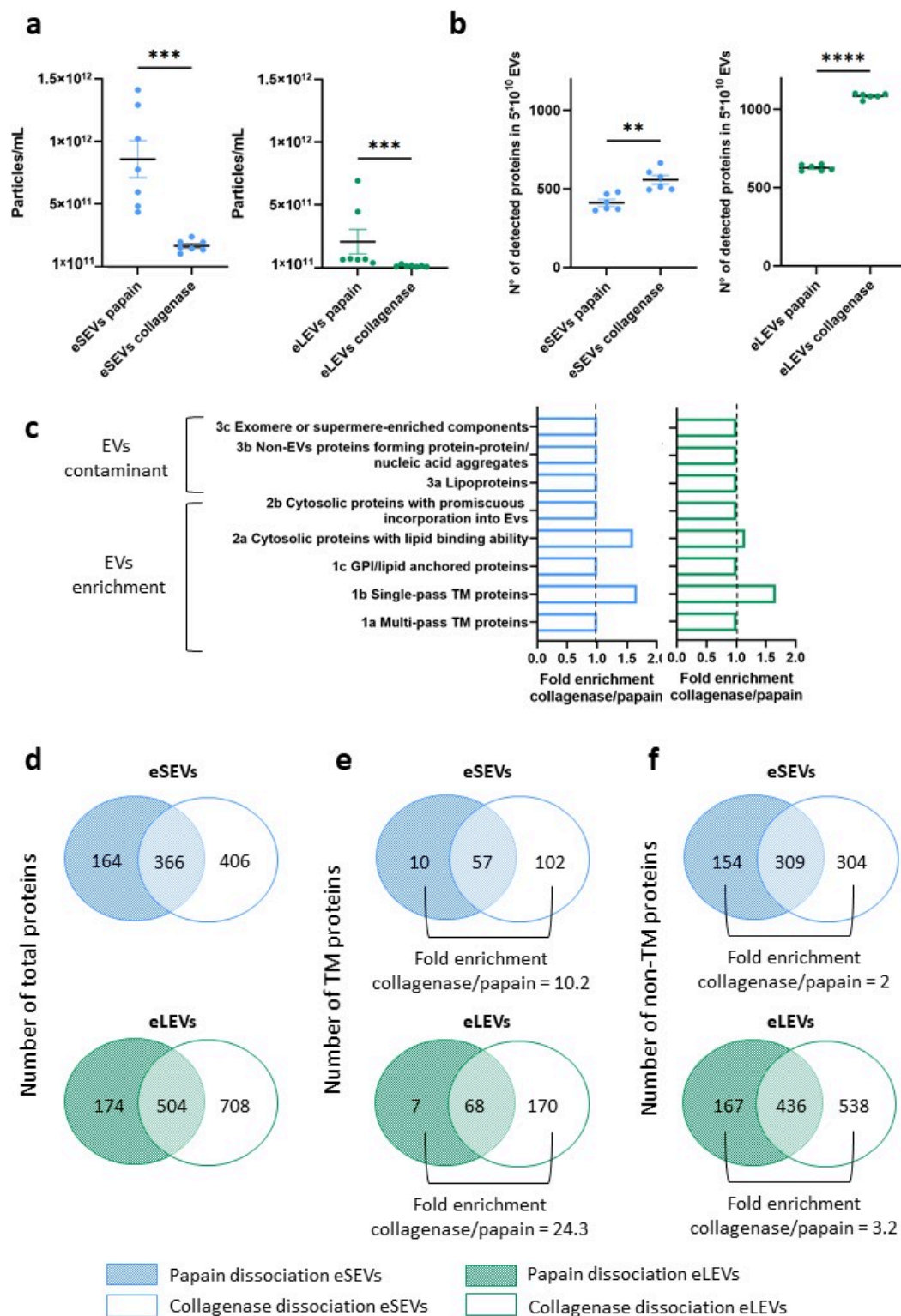


Figure 3

Comparison of enzymatic brain dissociation protocols for downstream eSEVs and eLEVs analysis. (a) EVs (eSEVs and eLEVs) concentration for both papain and collagenase was obtained by NTA measurements. Concentrations are expressed as particles/mL. For eSEVs use of unpaired t-test, parametric comparison between papain and collagenase. For eLEVs use of Mann-Whitney test, non-parametric comparison between papain and collagenase. *** $p < 0.001$. (b) Dotplot comparing the

number of proteins detected by mass spectrometry for 5×10^{10} EVs. For eSEVs and eLEVs use of unpaired t-test, parametric comparison between papain and collagenase. $**p < 0.01$, $****p < 0.0001$. Collagenase allows a better downstream protein identification. **(c)** Horizontal barplots indicating the protein fold enrichment of collagenase compared to papain according to the MISEV2023 guidelines. This enrichment revealed a higher presence in single-pass TM proteins and proteins known to be detected in EVs, while maintaining equal contaminants levels. This indicated a higher quality of both eSEVs and eLEVs using collagenase 3 as dissociation enzyme. **(d-f)** Venn diagrams to compare the number of total proteins **(d)**, TM proteins **(e)** and non-TM proteins **(f)** identified by mass spectrometry for eSEVs and eLEVs purified from papain (shaded) and collagenase (transparent). This quantitative protein comparison indicated that more TM and non-TM proteins can be identified by mass spectrometry using collagenase brain dissociation. For a to f, eSEVs are represented in blue and eLEVs in green.

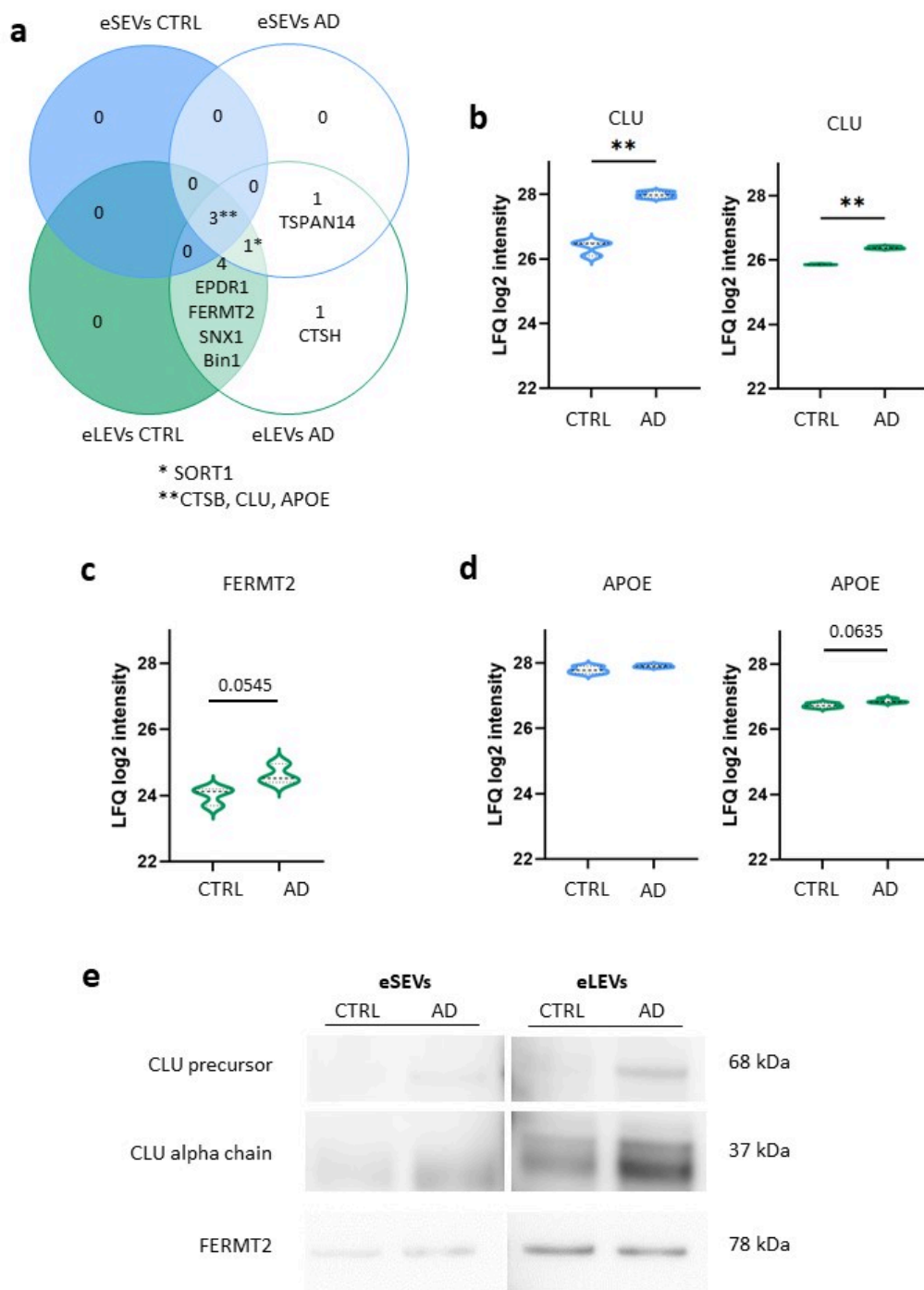


Figure 4

Differential expression profiles of GWAS genes in AD collagenase-derived eSEVs and eLEVs. (a) Venn diagram representing the GWAS associated proteins found in eSEVs and eLEVs. This analysis was done by crossing our protein data of AD and CTRL eLEVs and eSEVs with the protein list of GWAS genes.²⁷ (b-d) Statistical analysis represented in violin plots to compare a few of the GWAS genes: CLU (b), FERMT2 (c) and APOE (d) identified inside eLEVs and eSEVs between AD and CTRL. For CLU use of Mann-

Whitney, non-parametric test comparing AD to CTRL. For FERMT2 and APOE use of unpaired t-test, parametric test comparing AD to CTRL. P-values were calculated on LFQ log2 intensities. ****p < 0.01.** **(e)** Western blots of CLU and FERMT2 of eSEVs and eLEVs of CTRL and AD. For a to d, eSEVs are represented in blue and eLEVs in green.

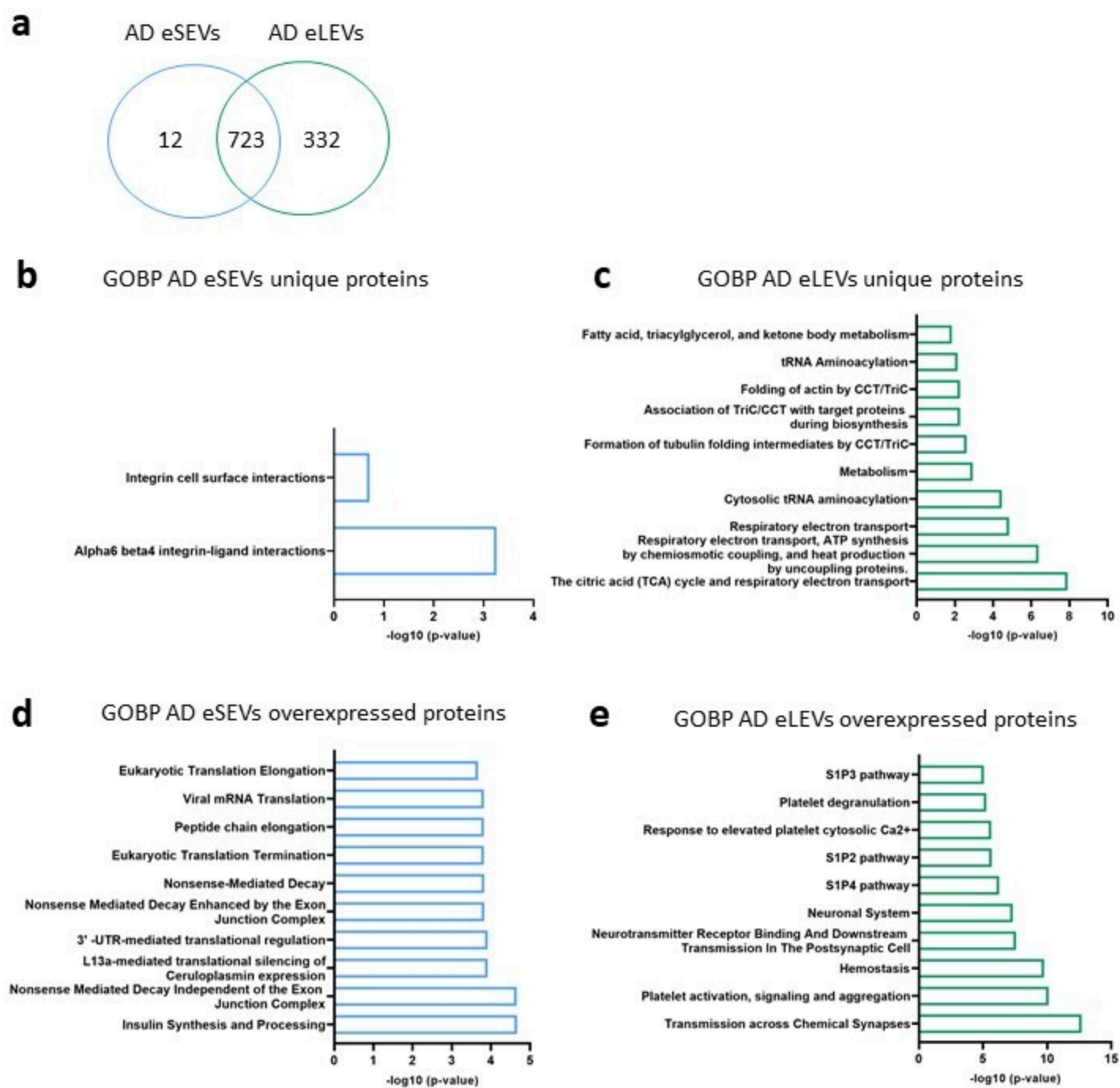


Figure 5

Proteomic profiling of AD-derived eSEVs and eLEVs. **(a)** Venn diagram indicating the number of unique and shared proteins between AD eSEVs and AD eLEVs. This indicated 12 and 332 unique proteins

respectively for AD eSEVs and AD eLEVs. GOBP was done on these unique proteins and are represented as horizontal bar graphs with the $-\log_{10}(\text{p-value})$ for at most 10 GOBP (top). **(b-c)**GOBP of AD eSEVs unique proteins indicate implication in the integrin signaling (b), while AD eLEVs unique proteins are related to the respiratory electron transport (c). **(d)** 139 from the 723 common proteins were found overexpressed in AD eSEVs with GOBP revealing a contribution to the translation processes. **(e)** 336 proteins were found overexpressed in AD eLEVs of which GOBP analysis revealed numerous pathways related to platelet activation and brain immunity. These results suggest that there is an AD EVs sub-population protein fingerprint related to specific functional implications. For a to e, eSEVs are represented in blue and eLEVs in green.

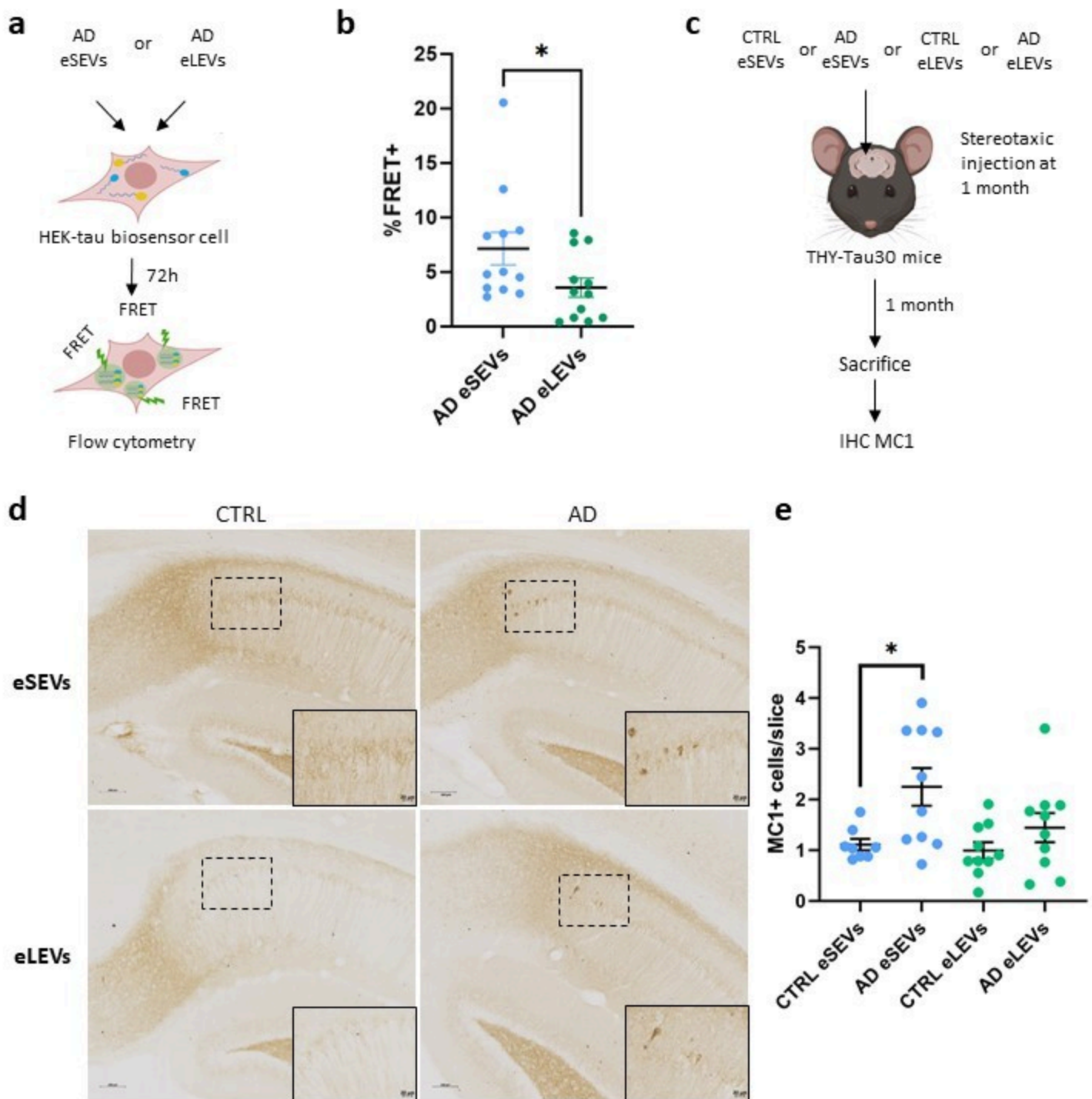


Figure 6

***In vitro* and *in vivo* study of the tau seeding capacity of eSEVs and eLEVs.** (a) Schematic representation of the HEK-tau FRET biosensor cell model used to study *in vitro* seeding capacity of AD eSEVs compared to AD eLEVs. Briefly, HEK-tau biosensor cells were plated and 24 h later freshly prepared AD eSEVs or eLEVs were lipofected onto the cells. Finally, after 72h the cells were collected and fixed followed by flow cytometry analysis to quantify the percentage of FRET-positive cells. (b) Dotplot indicating the

percentage of FRET-positive cells for AD eSEVs compared to AD eLEVs, revealing a significantly higher seeding capacity of AD eSEVs. Use of Mann-Whitney, non-parametric test comparing AD eSEVs and AD eLEVs. * $p < 0.05$. **(c)** Schematic representation of the bilateral stereotaxic injection of CTRL or AD eSEVs or eLEVs (1.6×10^9) into the hippocampus of one-month-old THY-Tau30 transgenic mice. Four weeks post-injections the mice were sacrificed, after which immunohistochemistry was done using the tau conformational antibody MC1 on brain sections. **(d)** Representative images of the immunostaining of MC1 are shown of the hippocampus (injection site) of THY-Tau30 transgenic mice brain sections. Scale bar: 100 μm . Zoomed images scale bar: 20 μm . **(e)** Dotplot representing the number of MC1 positive cells per brain slice for CTRL and AD eSEVs and eLEVs. Blinded quantification indicates a significant increase of tau seeding capacity of AD eSEVs. Ordinary one-way ANOVA, parametric comparison of all four groups. * $p < 0.05$, $n=10$ for CTRL eLEVs, AD eSEVs and AD eLEVs; $n=8$ for CTRL eSEVs.

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